## STUDIES ON FACTORS AFFECTING AXONEME ASSEMBLY AND STABILITY IN TETRAHYMENA THERMOPHILA

by

#### DRASHTI DAVE

(Under the Direction of JACEK GAERTIG)

#### ABSTRACT

Intraflagellar transport (IFT) involves the transport of ciliary precursors on oligomeric protein complexes (A, B) attached to microtubule motors (kinesin-2 and cytoplasmic dynein-1b) for ciliary assembly and maintenance. The assembly of the three axonemal segments (proximal, middle, distal) and the regulation of the different components of the IFT pathway are poorly understood. Moreover, the form in which tubulin is transported into cilia is not known. This work explores the mechanism of IFT, with respect to its regulation and assembly of distinct axonemal segments.

I studied the mechanism of suppression in a suppressor of IFT52, a complex B protein. Partial suppressors (IFT52 $\Delta$ sm) are conditional and assemble cilia under: either lower temperature or hypoxia. I showed a novel intragenic mechanism of suppression that occurs in two steps. First by native RNAi-mediated genome rearrangements for deleting micronucleus-specific sequences during conjugation of two heterokaryons carrying micronuclear copies of *neo2*-disrupted *IFT52*. Next the flanking sequences controlling *neo* expression are processed as multiple artificial introns, thereby restoring the translational frame. *Chlamydomonas* mutants of IFT46 (a complex B protein) are

partially suppressed under hypoxia, suggesting a conserved link between oxygen and IFT-regulation.

The detergent-soluble fraction of cilia from IFT52 $\Delta$ sm cells was analyzed by twodimensional gels, which showed an increase in the concentration of a single protein spot, identified as tubulin folding cofactor B (TCB). TCB is one of the five cofactors in the tubulin folding pathway that generates  $\alpha\beta$ -tubulin dimers from monomers. The presence of TCB in the cilium indicated a potential ciliary role of the components of tubulin folding factors in ciliogenesis, such as the transport of tubulin. We show that a knockout of *TCB1* in *Tetrahymena* produces a lethal phenotype associated with a general loss of microtubules. Folding  $\alpha$ -tubulin by Tcb1p is its primary role.

I explored the functions of DYF-1 (an IFT-associated protein), in axoneme assembly. In *C. elegans*, Osm-3 kinesin-2 assembles the distal-most segment of the axoneme (with outer singlets). DYF-1 is required for Osm-3 function and was proposed as a motor activator or adaptor. *Tetrahymena* cells lacking DYF-1 have non-functional cilia with a variety of axonemal defects. Zebrafish *dyf-1* mutants assemble non-functional cilia lacking polyglutamylation, an important post-translational tubulin modification. *Tetrahymena* cells lacking Dyf1p have hyperglutamylated short axonemes. Thus, DYF-1 functions in axoneme assembly and/or stability but the phenotypes from loss of DYF-1 are organism-specific.

# INDEX WORDS: IFT52, Tubulin Folding Cofactor B, hypoxia, DYF-1, polyglutamylation

# STUDIES ON FACTORS AFFECTING AXONEME ASSEMBLY AND STABILITY IN TETRAHYMENA THERMOPHILA

by

### DRASHTI DAVE

BS, University of Maryland Baltimore County, 2002

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial

Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

© 2009

### DRASHTI DAVE

All Rights Reserved

# STUDIES ON FACTORS AFFECTING AXONEME ASSEMBLY AND STABILITY IN TETRAHYMENA THERMOPHILA

by

### DRASHTI DAVE

Major Professor: JACEK GAERTIG

Committee:

HAINI CAI BORIS STRIEPEN WIN SALE

Electronic Version Approved:

Maureen Grasso Dean of the Graduate School The University of Georgia December 2009

### DEDICATION

This dissertation is dedicated to my parents Dr. Rajesh Dave and Dr. Mrs. Laila Dave for their unconditional support, encouragement and love.

#### ACKNOWLEDGEMENTS

My career as a graduate student would have been impossible without the support of my major professor, Jacek. He has been a constant source of encouragement through the years. Though we did not always see eye-to-eye on research methods, he has shown remarkable patience with my ways and has taught me a lot over the years. The most valuable lesson I learnt from him is persistence. His scientific expertise and clarity of thought guided me through many-a-blind alley. Above all I would like to thank him for keeping me grounded and for keeping me in good humor.

I owe a special debt of gratitude to Dorota for her excellent technical guidance and friendship, which has helped me through the toughest of times. She taught me everything from how to hold a pipette to how to buy the right type of shoes. Her strength gave me strength when I was going through the "bad" days with my experiments. I wish her the best for her future.

I would also like to thank all members of the Gaertig lab for making my time here a fun and fulfilling experience. I want to extend a special thanks to Shilpa for being the life of our lab and keeping us laughing and smiling through it all. She made a great roommate during the last few, very stressful and very trying days of my graduate life. I want to acknowledge Krishna, who has been so helpful and kind to me. I want to thank Swati for her support. Thanks to all of them, I will never forget our daily lunch routine, which was at times the highlight of my day. I want to acknowledge my committee members, Dr. Haini Cai and Dr. Boris Striepen for their suggestions, support and encouragement. I want to extend a special thanks to Dr. Win Sale for his excellent discussions and for travelling all the way to Athens.

I want to thank my parents without whom I would be nothing today. Their hard work and sacrifice has been paramount in bringing me to this place. They are a constant source of inspiration and I hope to achieve their level of success both professionally and personally. I would also like to thank my brother who never understood why I was doing this advanced degree, and explaining the "why" to him helped me answer that questions for myself and kept me going through tough times. My career in this country would not be possible without the love and support of my uncle and aunt, Mahendra and Harshada Parekh. I am deeply indebted to them for being my surrogate parents and opening their hearts and home for me.

My professional career would not have been nearly as much fun had it not been for my friends Meena, Haritha, Fred, Topher. Their presence in my life has been a constant source of fun and intellectual stimulation. This has served to enrich my life and helped me through my degree. My friends Kamlesh and Amruta have been a very helpful through my career and especially through the last two years of the degree. I would also like to thank Kris Miller, my TA coordinator for her support and friendship.

Lastly I would like to thank Cartic for his unconditional support. He was a constant source of inspiration for me to finish my PhD.

vi

### TABLE OF CONTENTS

Page	;
ACKNOWLEDGEMENTS	7
LIST OF TABLES	i
LIST OF FIGURES ix	ŗ
CHAPTER	
1 INTRODUCTION1	
2 REVIEW OF LITERATURE	)
3 TUBULIN FOLDING COFACTOR B IS ESSENTIAL IN	
TETRAHYMENA THERMOPHILA60	)
4 UNUSUAL INTRAGENIC SUPPRESSION OF AN INSERTIONAL <i>IFT52</i>	
MUTATION IN TETRAHYMENA THERMOPHILA OCCURS IN TWO	
STEPS: BY DNA ELIMINATION AND SPLICING OF ARTIFICIAL	
INTRONS101	
5 DYF-1 IS REQUIRED FOR ASSEMBLY OF THE AXONEME IN	
TETRAHYMENA122	)
6 CONCLUSIONS	)

### LIST OF TABLES

Page

<b>Table 3.1:</b> TCB1 $\Delta$ can be rescued using different C-terminal tagged	
versions of TCB1	
Table 5.1: Quantification of TEM images of longitudinal sections	
through basal bodies (%)	165
Table 5.2: Frequency of the various structural defects on cross sections	of locomotory
DYF1Δ axonemes	

### LIST OF FIGURES

Figure 2.1: The model for intraflagellar transport (IFT) pathway consists of six steps55
Figure 2.2: The ciliary axoneme consists of three different segments, proximal, middle
and distal
Figure 2.3: A schematic illustrating the construction of knockout heterokaryons in
<i>Tetrahymena thermophila</i> 59
Figure 3.1: The tubulin folding pathway involves two chaperonin complexes (Prefoldin
and CCT) and five cofactors (A-E) to yield assembly competent tubulin heterodimers91
Figure 3.2: IFT52 suppressor mutants assemble short, sparse cilia
Figure 3.3: TCB1 knockout is lethal in Tetrahymena
Figure 3.4: Localization of Tcb1p using different tags
Figure 3.5: Tcb1p is a cell body protein
Figure 4.1: Two subsequent sequence deletions lead to intragenic suppression of an
insertional <i>IFT52</i> mutation117
Figure 4.S1: Sequence of genomic DNA reconstructed for 4 independent
IFT52 $\Delta$ sm/mov suppressor strains shows a deletion within the <i>neo2</i> gene cassette119
<b>Figure 4.S2:</b> The cDNA sequence of IFT52Δsm/mov has 43 extra amino acids from
remaining <i>neo2</i> cassette
Figure 5.1: Tetrahymena has a DYF-1 ortholog. 150
Figure 5.2: Dyf1p is required for axoneme assembly

Figure 5.3: Loss of function of <i>DYF1</i> results in shortened, disorganized or missing	
axonemes	154
Figure 5.4: Loss of function of <i>DYF1</i> severely disorganizes the axoneme	156
Figure 5.5: GFP-DYF1p localizes to axonemes and basal bodies and axoneme remna	ints
in DYF1A cells are hyperglutamylated	158

Figure 5.6: Dyf1p, like IFT52p is a component of the detergent-soluble	
ciliary matrix	.160
<b>Figure 5.S1:</b> In DYF1 $\Delta$ cells, the <i>neo3</i> cassette is integrated at the expected position	
within the DYF1 locus	.162
Figure 5.S2: An extended cell cycle cannot explain hyperglutamylation of axonemal	
microtubules	.164

### CHAPTER 1

### INTRODUCTION

Cilia, both motile and non-motile are well-conserved organelles. Motile cilia are used for cell locomotion as well as for moving fluid over the surfaces of cells. For example, motile cilia are critical for the proper functioning of mammalian lungs, brain ventricles and sperm. Non-motile cilia perform important sensory functions. Thus, cilia in photoreceptor cells have tips filled with photosensitive components. Furthermore, almost very cell type in the mammalian body has a single sensory non-motile cilium called the primary cilium. The primary cilia are enriched in components of signaling pathways and could function as antennas for receiving and transducing external signals. The significance of non-motile cilia was discovered in the mouse model for polycystic kidney disease. The mutant mouse had an insertion in a gene required for ciliary assembly and failure to assemble kidney cilia led to polycystic kidneys. After this discovery, several other human diseases have been connected to both types of cilia (Chapter 2).

With a few exceptions, cilia are assembled by a mechanism called intraflagellar transport or IFT. During IFT, ciliary precursors are ferried into and out of the cilium as cargo attached to oligomeric IFT complexes on microtubules motors. The motors used for IFT are members of the kinesin-2 family for anterograde transport into the cilium and IFT-dynein (DHC1b) for retrograde transport back into the cell body. The motors move two oligomeric IFT complexes, A and B that are believed to act as platforms for cargo attachment. The composition of both complexes was discovered in a unicellular alga, *Chlamydomonas*. Each complex is made up of a different set of proteins that are named

after their molecular weights. The polycystic kidney disease model mouse had a mutation in IFT88, one of the IFT complex B proteins. According to the current model of IFT, all the involved components localize at the base of cilia and then assemble into the two complexes, followed by attachment of the anterograde and retrograde motors. Upon cargo recognition and loading onto the IFT complexes, the motors begin their movement into and out of cilia. Regulation of the IFT pathway, cargo recognition by IFT complexes, motor switching at the distal ends of cilia for retrograde transport, rearrangement of the complexes and loading/unloading of various ciliary proteins are some of the aspects that are currently not well understood.

The central component of the cilium is the axoneme, which is a composed of nine outer doublet microtubules. Most motile cilia have two inner singlet microtubules (central pair) as well. The axoneme is made of several hundred different proteins that form microtubules and large protein complexes such as radial spokes, dynein arms, nexin links, and terminal caps. Tubulin, the subunit of microtubules, is the most abundant component of the axoneme. However, little is known about how tubulin is assembled into the highly ordered axonemal microtubules. Moreover, the form in which tubulin is transported into the cilium, is not yet known. It may be transported as dimers, short oligomers or monomers that get folded within the cilium. This thesis explores the tubulinrelated processes during IFT.

In the chapter 3, we explore a potential connection between an IFT complex B component and tubulin. In chapter 4, we uncover the molecular nature of an unusual suppression of an *IFT52* mutant, IFT52 $\Delta$ sm. It was previously shown in *Tetrahymena* that Ift52p, an IFT complex B protein is essential for ciliogenesis. We started with an

insertion that carries a selectable marker gene, (neo2), in the IFT52 gene in the micronucleus, the germinal nucleus of ciliates. The disruption in *IFT52* coding region caused by *neo2* results in cells that do not assemble cilia and are consequently immotile. During the course of this study partial suppressors (IFT52 $\Delta$ sm) were isolated, that could only assemble cilia under low oxygen conditions. IFT52∆sm suppressions occur during conjugation, when cells develop a new macronucleus (somatic nucleus that controls the phenotype) from the zygotic micronucleus. We discovered a novel mechanism in which the suppression arises due to changes in the disrupted IFT52 locus that occur in two stages. First at the genomic level during the development of the macronucleus following conjugation, the *neo* gene of bacterial origin, in the gene disruption cassette undergoes deletion most likely via the native RNAi-dependent pathway of excision of micronucleus-specific sequences from the developing macronucleus. In the second step of suppression, at the mRNA level, the Tetrahymena-specific sequences left after the excision of the neo gene are processed as artificial introns and the reading frame is restored. There is one other case where a mutant in another complex B protein, IFT46, had a suppressor that assembled cilia only under low oxygen. Taken together, these two results indicate that IFT might have a common mechanism of regulation.

The potential connection between IFT and tubulin was revealed by a proteomic analysis of cilia obtained from IFT52 $\Delta$ sm suppressors. These cilia had an unexpected increase in amount of tubulin folding cofactor B (TCB), in the soluble fraction of their cilia. TCB is an important component of the tubulin folding pathway that includes two chaperonins and five folding cofactors. Thus, we had preliminary evidence that connected the IFT pathway to the tubulin folding pathway and we used it as a means to

learn more about the transport of tubulin (Chapter 3). We present data to show that tubulin folding cofactor B is essential in *Tetrahymena* and we confirm its main function in the folding of tubulin.

Two anterograde IFT motors belonging to the kinesin-2 family assemble the sensory cilia in C. elegans such that both function together to build most of the cilium (proximal and middle segments), but only one motor (homodimeric Osm-3) functions in the distal segment that have singlet microtubules. The function of this motor in the distal segment depends on a novel IFT protein, DYF-1. Mutant worms affected in this protein assemble the proximal and middle segments but not the distal segments of their cilia. The homolog of DYF-1 in zebrafish is Fleer and a mutation in *fleer* results in failure to assemble normally functioning cilia, which in turn leads to several morphological defects consistent with ciliary malfunction including hydrocephaly and randomization of internal organs. Moreover, mutant zebrafish have decreased ciliary polyglutamylation, a tubulin post-translational modification, suggesting that DYF-1 activity regulates tubulin posttranslational modifications. The effect of DYF-1 on a tubulin post-translational implies that DYF-1 might be indirectly important in the regulation of ciliary assembly or stability of axonemal microtubules. In the *Tetrahymena* genome, there are homologs for the homodimeric kinesin called Kin5, DYF-1/Fleer named Dyf1p and the enzymes responsible for tubulin polyglutamylation. We evaluated the function of DYF-1 in *Tetrahymena* to explore further the potential links between IFT, tubulin and axoneme assembly (Chapter 5). We discovered that in *Tetrahymena*, assembly of the entire cilium and not just the singlet microtubules at the distal ends depends on Dyf1p. The phenotype of *Tetrahymena* mutants lacking DYF-1 is consistent with a failure of ciliogenesis during

the later stages of IFT or during assembly of the axoneme once the required components are delivered by IFT. We suggest that DYF-1 is required to stabilize the axoneme during early stages of its assembly.

#### CHAPTER 2

### **REVIEW OF LITERATURE**

#### **Microtubules: Functions, Structure and Assembly**

Microtubules are important cytoskeletal elements made up of tubulin that are found in every eukaryotic cell. Microtubules are the main structural component of a wide variety of structures including the mitotic spindles, centrioles, neuronal microtubule bundles and axonemes of cilia. These structures perform essential functions such as maintaining cell shape, cell division, intra-cellular transport, cell polarity and cell motility. Microtubules are also required as a scaffold to position organelles belonging to the endo-membrane compartment (e.g. ER and Golgi networks and endosomes) correctly within the cell (Cole and Lippincott-Schwartz 1995). In long nerve cell projections, axons, membrane-bound vesicles destined for the synapse are transported along microtubule tracks by microtubule-dependant motor proteins (Brown 2003). The correct segregation of chromosomes during cell division is accomplished by the spindle apparatus, which is a microtubule-rich structure and consequently is the target of several anti-cancer drugs (Nogales 2000; Howard and Hyman 2003).

A microtubule is made up of protofilaments, linear arrays of dimers of  $\alpha$ - and  $\beta$ tubulin, arranged wall-to-wall such that they form a hollow cylinder (Desai and Mitchison 1997). The diameter of microtubules is variable and depends on the number of protofilaments. Most microtubules have 13 protofilaments, however, in some cases like the touch-receptor neurons of C. elegans, microtubules have 15 protofilaments (Savage, Hamelin et al. 1989). Additionally, there are compound fused-wall microtubules where

individual tubules have 13 and 11 protofilaments. These specialized structures are found in cilia (doublet) and centrioles (triplet) as described below. Protofilaments are polymers of  $\alpha\beta$ -tubulin heterodimers arranged in a head-to-tail manner with  $\beta$ -tubulin as the head, thus giving polarity to the entire structure (Desai and Mitchison 1997). Purified tubulin dimers can assemble into microtubules in an *in vitro* reaction, when suspended above the minimal tubulin concentration called the critical concentration and in the presence of GTP (Desai and Mitchison 1997). However, in vivo, with a few exceptions, protofilaments (and microtubules) are nucleated at specific locations called the microtubule organizing centers (MTOC). Most often, the MTOCs include basal bodies or centrosomes, where the minus ends of the protofilaments are embedded (Nogales 2000). Gamma tubulin is a highly conserved member of the tubulin family that is present at MTOCs (Nogales 2000). Unlike  $\alpha$ - and  $\beta$ -tubulin,  $\gamma$ -tubulin does not incorporate into the microtubule but assembles into a ring complex with other conserved proteins, at the minus ends of the microtubules to nucleate  $\alpha$ - and  $\beta$ -tubulin dimers (Wiese and Zheng 2006). While both  $\alpha$ - and  $\beta$ -tubulin monomers bind GTP, the GTP on  $\alpha$ -tubulin is nonhydrolyzable or exchangeable whereas the GTP on the β-tubulin undergoes hydrolysis once the dimer has been incorporated into a microtubule (Nogales 2000). Assembly of new dimers into microtubules occurs preferentially at the plus ends (ends that have a terminal  $\beta$ -tubulin). The inherent polarity of microtubules establishes the polarity of the entire cell, which is very important in control of the cell-shape, position of organelles as well as communication between cells.

Most microtubules are highly dynamic as their ends undergo frequent transitions between assembly and disassembly, the phenomenon known as "dynamic instability"

(Mitchison and Kirschner 1984; Nogales 2000). The status of the GTP/GDP bound to  $\beta$ tubulin regulates the dynamic instability. According to the GTP-cap model at the growing or stable plus end, the dimers have a GTP bound  $\beta$ -tubulin. This GTP undergoes hydrolysis some time after subunit incorporation. Thus, the body of the microtubule has subunits with GDP-β-tubulin, which makes the microtubule potentially unstable due to internal strain. The microtubule end becomes unstable and rapidly depolymerizes by peeling off protofilaments when the rate of subunit addition cannot maintain a GTP-cap at the plus end. Under these conditions, the presence of GDP-β-tubulin at the microtubule end makes the microtubule unstable and initiates a phase of rapid depolymerization aided by the internal polymer strain (reviewed in (Desai and Mitchison 1997; Nogales 2000)). The transition between polymerization and depolymerization phase is called "catastrophe" (Desai and Mitchison 1997). The GTP-dimers at the plus ends of polymerizing microtubules have a "straight" configuration, however when that GTP undergoes hydrolysis, the dimers exhibit a "curved" followed by outward peeling of the protofilaments before the entire microtubule structure collapses (Tran, Joshi et al. 1997).

*In vivo*, there are various microtubule-associated proteins (MAPs) that bind to microtubules and perform diverse function such as promoting assembly, providing stability and promoting catastrophe. Tau, MAP2 and MAP4 are examples of proteins that decorate the walls of microtubules and promote filament stability and polymerization (Drewes, Ebneth et al. 1998). XMAP215 is a MAP that binds to microtubule plus ends and promotes polymerization (Brouhard, Stear et al. 2008). Stathmin is a MAP that destabilizes microtubules possibly in two ways, by sequestering of tubulin dimers and promoting catastrophes at plus ends of microtubules (Belmont and Mitchison 1996;

Jourdain, Curmi et al. 1997; Cassimeris 2002). Certain motor-like proteins such as kinesin-13 bind to the plus ends of microtubule and promote catastrophes (Walczak, Gan et al. 2002).

Another way of regulating the properties of is by post-translational modifications of the tubulin incorporated into the microtubules. The lumen of microtubules undergoes acetylation (at lysine-40 on  $\alpha$ -tubulin) while the C-terminal tail domains on the microtubule surface undergo detyrosination, polyglycylation and polyglutamylation (Nogales 2000). Most of these modifications accumulate on microtubules, providing marks that distinguish a subset of long-lived microtubules from the more dynamic ones. These highly modified microtubules provide preferential routes for transport by motor proteins (Reed, Cai et al. 2006). For example, Kinesin-1, a microtubule motor that plays a very important role in transport of vesicles in neurons, and in vitro assays shows that the motor binds more efficiently to acetylated microtubules as compared to non-acetylated microtubules lacking the modifiable lysine-40 (Reed, Cai et al. 2006). Polyglycylation is usually found on ciliary (axonemal) microtubules where it is important for the assembly and length regulation of axonemes. Knockdown of a glycylation enzyme (TTLL3) in zebrafish results in mutants that have shorter and fewer cilia (Wloga, Webster et al. 2009). Polyglutamylation occurs on several types of microtubules such as those forming the mitotic spindles, basal bodies and axoneme (Gaertig and Wloga 2008). Perturbing the levels of polyglutamylation in vivo has severe consequences. For example, injection of antibodies that recognize that polyglutamylated tubulin into HeLa cells causes centriole disassembly, while hyper-glutamylation of ciliary microtubules results in destabilization

of axonemes in *Tetrahymena* (Bobinnec, Khodjakov et al. 1998; Wloga, Dave et al. 2009).

The rate of tubulin monomer folding and dimerization are another means of regulating microtubules. Disturbing any component of the tubulin folding pathway leads to perturbation in the levels of monomeric and dimeric tubulin, which can have severe consequences on the cell including cell-cycle arrest and lethality (Nogales 2000). In yeast cells, overexpression of  $\beta$ -tubulin causes cells to die rapidly, while overexpression of  $\alpha$ tubulin results in slow cell death (Weinstein and Solomon 1990). Prior to assembly into protofilaments, heterodimers are generated from individually translated tubulin monomers. Tubulin monomers have many surfaces that make them susceptible to misfolding and degradation (Lopez-Fanarraga, Avila et al. 2001). Hence, assembly of individual  $\alpha$ - and  $\beta$ -tubulin monomers into dimers occurs in the cytosol through a complex folding pathway that involves two chaperonins (prefoldin and CCT complex) and five folding cofactors (A to E). Both  $\alpha$ - and  $\beta$ -tubulin monomers are first captured by prefoldin as they are being translated and are delivered to the CCT complex for further folding. Folding cofactors then capture partially folded monomers: cofactor B is specific for  $\alpha$ -tubulin and cofactor A for  $\beta$ -tubulin. Further the folding intermediates interact with cofactors E ( $\alpha$ -tubulin) and D ( $\beta$ -tubulin) and in the presence of cofactor C, the two monomers come together and finally upon GTP hydrolysis,  $\alpha\beta$ -tubulin heterodimers are released (Grynberg, Jaroszewski et al. 2003). Tubulin folding cofactors are also involved in regulating ratios of the individual monomers and thereby play an important role in microtubule dynamics of the cell (Nogales 2000). The tubulin folding pathway and the proteins involved are better explained in Chapter 3. Chapter 3 focuses on a potential role

for tubulin folding cofactor B during axoneme assembly in the cilium, which is a microtubule-based organelle.

#### **Cilia: Structure and Composition**

Cilia and flagella are highly conserved organelles arising from the surface of almost every eukaryotic cell. The epithelial surfaces of organs like lungs and brain epyndyma as well as motile organisms like *Tetrahymena* and *Paramecium* have numerous motile cilia. Almost all mammalian cell types have a single non-motile, sensory cilium, called the primary cilium (reviewed in (Veland, Awan et al. 2009). Both cilia and flagella have the same basic structure however, flagella are several fold longer compared to cilia. To avoid confusion between the two words, the term cilia will be used to refer to cilia and eukaryotic flagella. Prokaryotic flagella are completely different in their protein composition and organization. The most abundant protein in the prokaryotic flagellum is flagellin and its rotary motion depends on a motor present at the base of the structure within the cell body (Bardy, Ng et al. 2003). Almost all eukaryotes have cilia except most fungi, higher plants and slime molds. These organisms lack cilia and associated structures; basal bodies (Marshall 2008).

The main component of cilia is a microtubule-based scaffold called the axoneme that is nucleated from a conserved centriole-like basal body (BB) and surrounded by a ciliary plasma membrane that is continuous with the non-ciliary plasma membrane. Typically, the axoneme in motile cilia is composed of 9 outer doublet microtubules surrounding 2 inner singlet microtubules also known as the central pair (9+2 axoneme). With a few exceptions, axonemes of non-motile cilia lack the central pair (have a 9+0

axoneme). In several cells types like the flagellate *Chlamydomonas*, the cilia are resorbed and the BB converts into centrioles that function at the spindle pole during mitosis. In differentiating mammalian cells, one of the two centrioles of the centrosome migrates towards the cell membrane, converts into a basal body and supports ciliogenesis. However, in ciliates BBs are specialized for nucleating axonemes and play no role in nuclear divisions (Beisson and Wright 2003).

Basal bodies (BBs) are polar, cylindrical structures with a proximal part that consists of a pinwheel structure with ninefold symmetry and nine triplet microtubules arranged at an angle (O'Toole, Giddings et al. 2003). The triplets are composed of a complete A tubule (13 protofilaments) and two incomplete tubules B and C (11 protofilaments each). In Chlamydomonas, towards the distal ends of BBs, nearer the plasma membrane, transition fibers radiate out from each triplet (O'Toole, Giddings et al. 2003). The C-tubule terminates at the distal end of the basal body and the region between the basal body and the main (middle) segment of the axoneme is called the transition zone (Culver, Meehl et al. 2009). The microtubules of the axoneme are composed of  $\alpha$ and  $\beta$ -tubulin heterodimers whereas the microtubules of the BBs also contain  $\gamma$ -,  $\epsilon$ - and  $\delta$ tubulin (O'Toole, Giddings et al. 2003). Additionally, there are several other proteins like centrin, SAS-6 and PCM-1 that are specifically associated with BBs (Nakazawa, Hiraki et al. 2007; Vladar and Stearns 2007). Recently a study using Tetrahymena BBs showed that the basal body proteome consisted of at least 355 proteins (Dammermann and Merdes 2002; Kilburn, Pearson et al. 2007; Culver, Meehl et al. 2009).

The ciliary axoneme arises from the distal part of the transition zone such that the A and B tubules of the BBs are continuous with the nine outer doublets, as shown in

Figure 2. The central pair arises from the transition zone after the outer doublets have already present and in *Tetrahymena*, one tubule starts earlier than the other (Allen 1969). The assembly of the central pair microtubules, and not the outer doublets, is dependent on  $\gamma$ -tubulin probably because doublets are in principle extensions of the BB triples while central microtubules originate from a central structure above the basal body where  $\gamma$ tubulin is located (McKean, Baines et al. 2003). The region that includes the distal end of the transition zone and beginning of the nine outer and two central tubules is called the proximal segment of the axoneme. Following the proximal segment is the middle segment, the main part of the axoneme which includes a complete A tubule and an incomplete B tubule and in the case of 9+2 cilia, the central pair is also present. Towards the distal end, the cilium tapers in diameter where the B tubule is terminated and the axoneme now continues consisting of only the A tubules and the central pair (Snow, Ou et al. 2004). Near the distal tip, the B tubules of outer doublets terminate at more proximal position (Sale and Satir 1977). Thus the distal segment is composed of singlet central and outer microtubules (that lack the B tubules). The ends of microtubules terminate in protein complexes that attach microtubules to the plasma membrane. The tip region is the least studied part of the axoneme and contains elaborate capping structure, which includes a central microtubule plate and a ball that attaches the axoneme to the membrane (Dentler 1980). The tip region is likely very important in cilia since it may play a role in several functions including stabilizing the membrane-microtubule attachment, promoting assembly of microtubules and serving as scaffold for the switching of the intraflagellar transport motors and unloading of the cargo carried by the motors (described below) (Sloboda 2005). An example of a protein that is localized to

tips is EB1, yet its precise function here is not known. In the cell body of mammalian cells EB1 is detected at the plus ends of cytoplasmic microtubules where it promotes microtubule assembly in conjunction with another protein, APC (Nakamura, Zhou et al. 2001). An antibody against EB1 detected the protein at the basal bodies and at the ciliary tips in *Chlamydomonas* (Pedersen, Geimer et al. 2003). Moreover, EB1 is required for assembly of primary cilia in fibroblasts as its knockdown by siRNA resulted in cells with very short cilia or no cilia (Schroder, Schneider et al. 2007). However, its precise function at ciliary tips is unknown

In addition to tubulin, there are several hundred of other proteins in the cilia. Several groups have used different techniques to characterize the ciliary proteome resulting in a list of more than 1000 proteins that are either directly found in ciliary samples or predicted to function in cilia based on phylogenomic approaches (Li, Gerdes et al. 2004; Pazour, Agrin et al. 2005; Gherman, Davis et al. 2006). The proteome consists proteins conferring stability to the axoneme, proteins needed for the motility of the axoneme, membrane-associated proteins and soluble proteins. Among those proteins that are essential for motility are the inner and outer dynein arm complexes, radial spoke proteins and the central pair apparatus. The outer and inner dynein arms are multi-subunit complexes and appear as tri-lobed and bi-lobed structures (arms) respectively, that are attached to the A tubule of outer doublet (Nicastro, Schwartz et al. 2006). Some of the subunits of the dynein arm complexes are ATPases, which generate force that causes relative sliding of the doublet microtubules against each other in groups of two (Satir and Christensen 2007). The radial spoke proteins as well as the inner singlet microtubules are essential for the regulation of the motile forces produced by dynein arms (Porter and Sale

2000; Smith and Yang 2004). Radial spokes are multi-subunit complexes as well, that contact each outer doublet microtubule on one side through a thin stalk and the projections of the central pair on the other through a globular head (Smith and Yang 2004). Apart from proteins needed for motility and sliding, the axoneme has structures strongly associated with tubulin, such as tektins (A, B and C), nexins and ribbon proteins (Sp77 and Sp83) that are probably involved in assembly and stability of axonemal structure (Linck 1976; Sui and Downing 2006). An isoform of tektin, Tektin-t, detected in mice sperm flagella is implicated in proper attachment or assembly of inner dynein arms to the outer doublets as a knockout of this protein causes impaired sperm motility (Tanaka, Iguchi et al. 2004).

The detergent-soluble part of the cilium, called the membrane and matrix fraction that surrounds the axoneme contains a very wide variety of proteins. The ciliary membrane is continuous with the cell membrane, yet it is enriched in signaling molecules that are less abundant elsewhere on the surface. For example, several receptor molecules, like the somatostatin receptor 3 serotonin receptor 5-HT(6) and the TRPV channels are detected in neuronal cilia (Handel, Schulz et al. 1999; Brailov, Bancila et al. 2000; Qin, Burnette et al. 2005). Kidney primary cilia have high levels of polycystin-2, a Ca<sup>+2</sup> cation channel, and abnormal function of this protein is linked to polycystic kidney disease (Gonzalez-Perrett, Kim et al. 2001; Pazour, San Agustin et al. 2002). There are certain evolutionary advantages for the cells in localizing signaling molecules to cilia. The area inside a cilium is smaller than that of the entire cell, which makes the downstream transmission of signals from a receptor upon ligand binding easier. Also, a ligand may

recognize a receptor molecule more easily if it were present on a cilium that projects outward from the cell (Marshall and Nonaka 2006).

So far there is no evidence of protein translational machinery like ribosomes in cilia. Hence, all ciliary components have to be transported into the cilium. The mechanism of this transport is discussed below.

#### **Intraflagellar Transport**

In the past decade we have experienced a revolution in our understanding of the ciliary assembly and diseases associated with cilia due to the discovery of intraflagellar transport (IFT) (Figure 2.1). The mechanism that carries ciliary components into and out of the cilium is called intraflagellar transport (IFT). IFT discovered as a movement of particles inside paralyzed flagella of live *Chlamydomonas* cells more than a decade ago (Kozminski, Johnson et al. 1993). These particles, called IFT complexes were also observed in electron micrographs of fixed flagella as multipartite, electron-dense complexes between the axoneme and the flagellar membrane (Kozminski, Johnson et al. 1993). The IFT particles were subsequently purified by comparing the membrane plus matrix fractions isolated from flagella of wildtype and the *fla-10* temperature-sensitive mutant cells, which cannot assemble cilia at restrictive temperature (Piperno and Mead 1997; Cole, Diener et al. 1998). On a density sucrose gradient, IFT particles cosedimented at 16-17S, but increasing ionic strength separates them into two complexes, A and B, of which A still sedimented at 16S and B broke down into three smaller fragments (Cole 2003). Hence, the granules or IFT particles seen by Kozminski and colleagues are comprised of two multimeric complexes, A and B. Subsequently, the motor proteins that

are responsible for transport into the cilium (kinesin-II) and out (cytoplasmic dynein 1b) were also discovered ((Walther, Vashishtha et al. 1994; Kozminski, Beech et al. 1995; Pazour, Dickert et al. 1999)and reviewed in (Scholey 2003)). IFT is thus the bidirectional movement of microtubule motors carrying oligomeric complexes along axonemal microtubules (Figure 2.1).

### **IFT Motors**

IFT particles are transported into the cilium by members of the kinesin-2 family (anterograde transport), while the particles are recycled back into the cell body with the help of cytoplasmic dynein, DHC1b (retrograde transport) (reviewed in (Scholey 2003)).

The retrograde motor, DHC1b, is a complex of four subunits, a heavy chain, a light intermediate chain, an intermediate chain and a light chain (Rajagopalan, Subramanian et al. 2009). *Chlamydomonas* cells with a mutation in the heavy chain of DHC1b, have short cilia with bulbous tips that are filled with electron dense material, similar to IFT particles (Pazour, Dickert et al. 1999). In these mutants, the anterograde IFT is detectable and carries the IFT particles into cilia but there is no mechanism to recycle them back into the cell body. The light chain of DHC1b (LC8) in *Chlamydomonas* is required for retrograde transport as well, since LC8 mutants exhibit the same retrograde defects of accumulating IFT particles at the flagellar tips (Pazour, Wilkerson et al. 1998). The knockout of the light intermediate chain in mice (mD2LIC) is lethal and mutants lack nodal cilia, as seen by scanning electron microscopy (Rana, Barbera et al. 2004). The sensory neurons of *C. elegans* have chemosensory non-motile cilia. A mutation in the DHC1b light intermediate chain produces animals that have

abnormal osmotic avoidance and chemotaxis associated with bulbous sensory cilia with lack of retrograde IFT (Schafer, Haycraft et al. 2003). Unexpectedly, *Tetrahymena* cells with knockouts of two of retrograde motor components (the heavy chain, DYH2 and the light intermediate chain, D2LIC) have intact, motile cilia (Rajagopalan, Subramanian et al. 2009). The *DYH2*-knockout and *D2LIC*-knockout cells had slightly shorter mean cilia (~3.14 µm) and compared to wildtype (~4.17 µm) cells, indicating that either DHC1b complex is not the only retrograde IFT motor or that retrograde IFT is not essential cilia assembly in this organism (Rajagopalan, Subramanian et al. 2009). The *Tetrahymena* genome contains about 25 different dynein heavy genes and it is likely that the DYH2 function is redundant, even though there is just one gene encoding the heavy chain (Wilkes, Watson et al. 2008). Such a redundancy is also seen in *Tetrahymena* mutants of kinesin-II, the anterograde motor as described below.

Kinesin-II complex was first identified as the anterograde motor responsible for IFT in the temperature sensitive *fla10 Chlamydomonas* mutant flagella. These mutants did not reassemble flagella at restrictive temperature after deflagellation; the average number of IFT particles moving in the anterograde direction decreased over time and finally stopped (Walther, Vashishtha et al. 1994; Kozminski, Beech et al. 1995). The FLA10 kinesin is 95 kDa motor subunit of a heterotrimeric complex of kinesin-II, originally purified from sea urchin eggs (Scholey 1996; Scholey 2003). Heterotrimeric kinesin-II, belongs to the kinesin-2 family and consists of two motor subunits (85 kDa and 95 kDa) that form a dimer and bind to an accessory subunit KAP1 (Scholey 2003). Heterotrimeric kinesin-II is the canonical anterograde IFT motor protein that co-immunoprecipitates with IFT88 in mammalian photoreceptor cells and testis and is

essential for ciliary assembly in several cell types including *Tetrahymena*,

*Chlamydomonas* and mouse nodal cells (Kozminski, Beech et al. 1995; Brown, Marsala et al. 1999; Takeda, Yonekawa et al. 1999; Baker, Freeman et al. 2003). Knockout of KIF3A (85 kDa kinesin-2 subunit) in mouse is lethal due to severe developmental defects, presumably arising from the lack of functional primary cilia during embryonic development (Takeda, Yonekawa et al. 1999). In the photoreceptor cells of mice, inducible KIF3A knockout results in cell death seen by light microscopy and mislocalization of opsin, as detected by an antibody ((Marszalek, Liu et al. 2000) and reviewed in (Insinna and Besharse 2008)). KIF3B, the 95 kDa subunit of heterotrimeric kinesin-II, when knocked out in mice also leads to lethality and the embryos exhibit randomization of left-right asymmetry due to non-functional nodal cilia whose motility determines sidedness of the organism (Nonaka, Tanaka et al. 1998). In *Tetrahymena*, knocking out two partly redundant kinesin-2 genes results in cells that have no cilia and fail to complete cytokinesis, since ciliary motility is important for the final scission of daughter cells (Brown, Hardin et al. 1999; Brown, Marsala et al. 1999).

Certain members of the kinesin-2 family form homodimers, including KIF17 in mammals and its homolog, Osm-3 in *C. elegans*. KIF17 is responsible for targeting the CNG channels to olfactory cilia in mammals but not for ciliary assembly, as cilia were present in cells expressing a dominant-negative version of KIF17 but CNGA2:YGP did not localize to cilia (Jenkins, Hurd et al. 2006). KIF17 is also expressed in vertebrate photoreceptor cells and a knockdown of this protein in zebrafish results in mislocalization of the photoreceptor pigments, rhodopsin (Insinna, Pathak et al. 2008). In *C. elegans* both homodimeric Osm-3 and heterotrimeric kinesin-II are required for the anterograde IFT

during the assembly of sensory cilia (Snow, Ou et al. 2004). Experiments measuring rates of movement of IFT particles using GFP-tagged IFT52 (Osm-6) showed that the two motors work together in the assembly of the middle segment of cilia (containing outer doublet microtubules with both A and B tubules), but the distal segments (containing the A tubule singlets) are built by Osm-3 kinesin alone (Snow, Ou et al. 2004) (Figure 2.2). Osm-3 kinesin moves with a velocity of 1.3  $\mu$ m/sec while kinesin-II moves with a velocity of 0.5  $\mu$ m/sec. In the middle segment, IFT particles move with an intermediate velocity of 0.7  $\mu$ m/sec which likely reflects cooperating kinesin-II and Osm-3 motors, while the speed of IFT particles in the distal segment is 1.3  $\mu$ m/sec consistent with Osm-3 acting as a sole anterograde motor (Snow, Ou et al. 2004).

Coordination between the two anterograde IFT motors in *C. elegans* depends on the function of at least three proteins, DYF-1, BBS7 and BBS8 that are proposed to act as adaptors that attach these motors to IFT complexes (Ou, Blacque et al. 2005). In *C. elegans*, a mutation in DYF-1 results in cilia that have no A tubule extensions and IFT particles move along the middle with a velocity of 0.5  $\mu$ m/sec, while in deficiencies in BBS-7 or BBS-8, result in IFT particles moving with a velocity of 1.3  $\mu$ m/sec along the entire cilium (Ou, Blacque et al. 2005). This implicates DYF-1 as an adaptor protein for the Osm-3 kinesin and the BBS7/8 proteins for kinesin-II. A zebrafish mutant of DYF-1 has fewer, short cilia with ultrastructural defects in the middle segment and lower levels of tubulin polyglutamylation (a tubulin post-translational modification), which led to the hypothesis that DYF-1 is an adaptor for the enzyme (E-ligase) responsible for the polymodification (Pathak, Obara et al. 2007). The lack of A tubule extensions in *C. elegans* cilia and the ultrastructural defects in zebrafish cilia may be explained if DYF-1 has a structural role in cilia in providing stability to the axoneme. DYF-1 may be required in stability of the IFT particles or it may act as an adaptor between the anterograde motor and the polyglutamylating enzyme. To address these issues, we studied the role of Dyf1p in *Tetrahymena* as described in Chapter 5.

Other kinesin families have anterograde functions in certain cell types, presumably to aid in cargo recognition. For example, a member of the kinesin-3 family KLP6 is implicated in the correct localization of polycystin-2 to the cilia in male-specific sensory neurons of C. elegans, since a GFP-tagged version of polycystin-2 was mislocalized to several neurons in *klp6*-mutants (Peden and Barr 2005).

#### **IFT complexes**

The two IFT complexes are composed of different proteins (IFT proteins) that are unique to each complex. The first study on IFT proteins showed that complex A is made up of 4 IFT proteins and has a molecular weight of 550 kDa whereas complex B, made up of 11 IFT proteins has a molecular weight of 750 kDa (Cole, Diener et al. 1998). Further investigations have found a few more IFT proteins (e.g. IFT25), the sequences of which are highly conserved among organisms that use IFT to build their cilia (Wang, Fan et al. 2009).

IFT complex A is made up of the following proteins: IFT144, IFT140, IFT139, IFT122A, IFT122B and IFT43. A screen for *Chlamydomonas* mutants that had bulges in their flagella revealed four temperature-sensitive mutants with defective (slower) velocities of retrograde transport as well as reduced frequency of IFT at the restrictive temperature (Piperno, Siuda et al. 1998). Sucrose density gradients of cilia from these

four mutants grown at the restrictive temperature, showed that they had a lower concentration of the IFT complex A proteins, which led to the hypothesis that complex A is involved in retrograde IFT (Piperno, Siuda et al. 1998; Scholey 2003). Another study in Tetrahymena has also implicated IFT complex A in the retrograde transport. A knockout of IFT122A produces cells that swim and assemble slightly abnormal shorter cilia, but the cilia accumulate complex B proteins (IFT88) at the ciliary tips (Tsao and Gorovsky 2008). IFT140 mutants in *C. elegans* have almost normal cilia with slightly larger diameters and are filled with electron dense material (Perkins, Hedgecock et al. 1986; Scholey 2003). It is intriguing that in invertebrates, the highly conserved proteins of complex A are not as important for cilia assembly as those of complex B, which may mean that the retrograde transport itself is not essential, at least in some organisms. Consistently, while the knockdowns of IFT complex B proteins in zebrafish produced severely defective cilia, a knockdown of IFT140 produces very mild phenotypes and mutants have normal cilia (Tsujikawa and Malicki 2004). On the other hand, IFT122-null mice are lethal due to several defects arising from absent or mal-formed cilia during early development, such as defects in left-right asymmetry, enlarged heart and exencephaly (Cortellino, Wang et al. 2009). Mice with abnormal IFT139 have fewer primary cilia than wildtype mice and these cilia accumulate IFT particles at their tips, seen through scanning electron microscopy (Tran, Haycraft et al. 2008). Abnormal IFT139 manifests into abnormal development like delayed eye and brain development (Tran, Haycraft et al. 2008). These differences might be due to the importance of primary and motile cilia in vertebrates during development that are involved in diverse processes like hedgehog

signaling, generation of nodal flow for left-right patterning and movement of cerebrospinal fluid (Shiratori and Hamada 2006; Veland, Awan et al. 2009).

IFT144, IFT140, IFT122A and IFT122B contain WD repeat domains at the Nterminus, which are sites for protein-protein interactions, whereas IFT139 contains several TPR domains that facilitate binding of multiple proteins at once (Cole 2003). How these different domains come together to form complex A and bind to motor proteins or bind to cargo is not yet understood.

Complex B is composed of eleven subunits: IFT172, IFT88, IFT81, IFT80, IFT74/72, IFT52, IFT57/55, IFT46, IFT27, IFT25 and IFT20 (Scholey 2003; Krock and Perkins 2008). Unlike complex A, several studies have been conducted on complex B proteins and have found that complex B proteins are essential for ciliogenesis (see below). The organization of complex B has been partially uncovered. Sucrose density gradients under high salt conditions revealed a tetrameric core complex made up of two IFT81 and two IFT74/72 subunits that form a scaffold for the attachment of four other IFT proteins; IFT46, IFT52, IFT 88 and IFT27 (Lucker, Behal et al. 2005). The remaining IFT proteins, IFT20, IFT57, IFT80 and IFT172, are possibly bound loosely to the outer surface of the core, since these were lost after incubation with high salt (Lucker, Behal et al. 2005).

Many studies indicate that mutations in IFT complex B proteins dramatically shortened cilia or lead to a complete failure of assembly of both motile and non-motile cilia that are often associated with severe organismal phenotypes reflecting diverse functions of cilia. IFT88 was characterized in *Chlamydomonas* and genome searches revealed its homology to the Tg737-gene product (polaris) in hypomorphic mutant mice

with polycystic kidneys (Pazour, Dickert et al. 2000). This result was key in highlighting the importance of cilia in vertebrate systems. Adult Tg737 mice had increased cell number in liver and bile ducts, retinal degeneration, hydrocephaly and defects in limb development; all phenotypes that can be explained by defective cilia in the kidney epithelia, epyndymal epithelia or limb bud mesenchymal cells, respectively (Pazour, Dickert et al. 2000; Lehman, Michaud et al. 2008). Gene knockouts of *IFT52* and *IFT172* in *Tetrahymena* lead to loss of cilia causing cell paralysis as well as a failure to complete cytokinesis, which is associated with cell paralysis (Brown, Fine et al. 2003; Tsao and Gorovsky 2008). As in most cases, defects in IFT complex B proteins leads to a general failure in cilia assembly. Thus, it is difficult to study specific roles for individual IFT B proteins. Accordingly, with a few exceptions, the specific roles of these proteins are poorly understood. However, a few connections between IFT components are cargoes have been made. Most notably, these studies suggest that IFT components participate in the transport of membranes destined to cilia. For example, in ciliated mammalian cells, IFT20 was detected at the basal bodies and cilia, and more interestingly as a Golgiassociated protein (Follit, Tuft et al. 2006). Under a mild knockdown of IFT20 by siRNA in rat kidney NRK cells, primary cilia assembled, but the levels of polycystin-2 on ciliary membrane were greatly reduced, implicating IFT20 in transport of membrane proteins to cilia (Follit, Tuft et al. 2006), possibly on vesicles derived from the Golgi. Strengthening this argument, a Golgi-membrane protein, GMAP210/TRIP11, is implicated in anchoring/attaching IFT20 to the Golgi apparatus as cells with a mutation in GMAP210/TRIP11 have mislocalized IFT20 (Follit, San Agustin et al. 2008). A recent study showed that IFT172, containing seven WD repeats, physically interacts with the
EB1 (detected at ciliary tips), thus leading to the hypothesis that IFT172 is involved in the switching of anterograde to retrograde motors at the tip (Pedersen, Geimer et al. 2003). Another study provided additional support for this hypothesis by showing that a truncated version of IFT172 in Tetrahymena can support ciliogenesis but accumulates at the tip and along with IFT88 (complex B protein) instead of returning back to the cell body by retrograde transport (Tsao and Gorovsky 2008). In Chlamydomonas, a null mutant of IFT46 has short and immotile flagella, but a partial intragenic suppressor of the IFT46 null mutation has normal length cilia with all ciliary components, except the outer dynein arms (Hou, Qin et al. 2007). Western blot analysis on whole cells extracts indicated that the outer dynein arms were present in the cell body and their absence in the flagellar extracts indicates that they were simply not transported by IFT, pointing to a specific role for full length IFT46 in transport of outer dynein arms (Hou, Qin et al. 2007). The simplest explanation is that the full length IFT46 has a binding surface for an outer dynein arm complex (or binds to an cargo adapter that in turn binds to outer dynein arm complex).

According to the current model for IFT, the anterograde motors attach to IFT particles with attached cargo and carry the complexes into cilia, while the retrograde motor carries the particles back into the cell body (Rosenbaum and Witman 2002) (Figure 2.1). Few studies indicate specific functions for individual IFT proteins in the transport of specialized cargo. For example, the photoreceptor cells of mutant IFT57 mutant zebrafish inefficiently assemble connecting cilia but maintain normal levels of the all the other IFT complex B proteins (Krock and Perkins 2008). However, coimmunoprecipitation using IFT88 showed increased levels of the kinesin-II motor in

*IFT57* mutant cells, possibly because the motor cannot dissociate from the IFT particles. This implies that IFT57 is specifically involved in this dissociation (Krock and Perkins 2008). A physical interaction between IFT20 and the anterograde motor, kinesin-II (KIF3B), shown by yeast-two hybrid assays, points to a role for the IFT protein in linking the motor to complex B (Baker, Freeman et al. 2003).

Apart from being a platform that carries material into and out of cilia; IFT proteins might have other functions in the cell. IFT27, an IFT complex B protein is unusual because its activity also involved in the cell cycle in *Chlamydomonas*. RNAi knockdown of IFT27 shows abnormal cell division phenotypes such as two sets of flagella, large cell size or two nuclei in one cell, in addition to flagellar defects (Qin, Wang et al. 2007). Another complex B protein, IF52 is only associated with IFT as seen by the RNAi knockdown of IFT52, which resulted in defective flagella (Qin, Wang et al. 2007). Additional studies indicate a connection between cilia and the cell cycle. IFT88 was detected by antibodies, at the basal bodies and cilia in ciliated mammalian cells during quiescence (Follit, Xu et al. 2009). Interestingly, IFT88 was also found associated with centrosomes, seen by immunofluorescence as well as by biochemical methods, during the proliferation phase in both ciliated and non-ciliated cells (Robert, Margall-Ducos et al. 2007). Depletion of IFT88 resulted in increased cell proliferation, again indicating a role for IFT in cell-cycle regulation (Robert, Margall-Ducos et al. 2007). Mammalian T-cells respond to external cues of antigen-presenting cells by reorganizing their MTOCs towards the site of the antigen, followed by trafficking of signaling molecules that eventually form complexes at the plasma membrane (Martin-Cofreces, Robles-Valero et al. 2008). The signaling molecules are recycled between the plasma

membrane and the cell body. In these non-ciliated T-cells, an anti-IFT20 antibody detected IFT20 at the MTOC as well as at Golgi apparatus, where its knockdown decreased the number of complexes formed at the plasma membrane, upon interaction with an antigen-presenting cell (Finetti, Paccani et al. 2009). This result shows that, in addition to a role in trafficking during ciliogenesis, IFT proteins might function in intracellular trafficking in non-ciliary contexts, at least in certain cell types (Finetti, Paccani et al. 2009).

IFT proteins have been implicated as key components in cell signaling and may likely be involved in relaying signals between the external and internal environments of the cell, since they constantly move from the cell body to the ciliary tips and back. For example, fertilization in *Chlamydomonas* involves flagellar adhesion resulting in a signal cascade that leads to fusion of the two gametes. IFT particles are part of the signal cascade pathway because gametic fusion does not occur in their absence even though flagellar adhesion can occur, as shown by immunoblots of flagella from kinesin-II mutants (Wang, Pan et al. 2006). This indicates that in some cases, IFT particles or some IFT proteins in the complexes may be directly participating in signaling (reviewed in (Scholey and Anderson 2006)).

With a few exceptions, the regulatory mechanisms for IFT are poorly understood. For example, the expression of IFT-encoding genes is co-regulated. In *C. elegans,* a transcription factor called DAF-19 and is expressed in ciliated neurons as indicated by its GFP-tagging (Swoboda, Adler et al. 2000). Target sites of DAF-19 are found in the gene promoters of a large number of ciliary genes including those encoding several IFT complex B components such as *IFT172*, *IFT52* and *IFT80* (Swoboda, Adler et al. 2000).

RFX-type transcription factors are involved in the assembly of cilia in *Drosophila* and the null mutants lacking these proteins have disorganized sensory cilia (Dubruille, Laurencon et al. 2002). In mammals, mutated RFX3 results in laterality defects caused by shortened nodal cilia, indicating the importance of RFX3 in ciliary assembly (Bonnafe, Touka et al. 2004). Ubiquitination of ciliary proteins is another possible regulatory mechanism that potentially could be important in the formation of cargo that returns to the cell body via retrograde IFT. Thus, the amount of ubiquitinated ciliary proteins increases prior to naturally occurring (during mating) or chemically induced complete flagellar disassembly in *Chlamydomonas* (Huang, Diener et al. 2009). Additionally, the authors show that ubiquitination increases in cilia of anterograde and retrograde IFT mutants, indicating that IFT is needed for removal of recycled proteins (Huang, Diener et al. 2009).

External control of IFT might be important in regulating events such as cilia resorption before entry into the cell cycle, signal cascades during development or in ciliary length regulation (Quarmby and Parker 2005). For example, FGF signaling is important during embryogenesis and a receptor for the FGF ligands, FGFR1 is present on ciliated epithelia in several vertebrate cells during development and cells with aberrant function of the FGFR1 have short cilia and laterality defects (Neugebauer, Amack et al. 2009). In zebrafish, when the FGFR1 receptor is knocked down by RNAi, the level of IFT88 transcript decreases, which explains the short lengths of cilia and also implicates FGF signaling in IFT and cilia regulation (Neugebauer, Amack et al. 2009).

A possibility of oxygen being an external, or even internal, cue for the regulation of IFT came from the studies of two different IFT complex B proteins. As discussed

above, null mutants of *ift46* in Chlamydomonas has very short, paralyzed flagella but a partially suppressed strain is able to assemble normal length flagella (Hou, Qin et al. 2007). The partially suppressed strain of IFT46 appeared spontaneously in a culture that had been left unaerated (Hou, Qin et al. 2007). A similar result was previously described for a partially suppressed strain of IFT52 null mutant in *Tetrahymena*. The partial suppressors of IFT52 in *Tetrahymena* assemble cilia in hypoxia and low temperature conditions (Brown, Fine et al. 2003). This points to oxygen being a common factor in the regulation of IFT and to study this further we studied the mechanism of suppression in *Tetrahymena* as described in details in chapter 5. IFT52, which is essential for ciliogenesis in *Tetrahymena* and *Chlamydomonas*, localizes to the basal body region and can be seen as puncti along cilia (Deane, Cole et al. 2001; Brown, Fine et al. 2003).

## **IFT Cargo**

One of the major problems in the understanding of IFT is how the IFT complexes associate with their "cargo". Ciliary precursors are transported into the cilium from the cell body during assembly and brought back for recycling and further maintenance. Potential cargo proteins for IFT include, tubulin, radial spoke proteins, membrane precursors, membrane proteins like polycystins and other signaling molecules and dynein arm complexes. Many components of cilia are pre-assembled into large complexes in the cell body and this form transported by IFT inside cilia. One of the best studied examples are the outer dynein arms (ODA) that are partially assembled in the cell body and must be transported as such during ciliary assembly (Fowkes and Mitchell 1998; Ahmed, Gao et al. 2008; Omran, Kobayashi et al. 2008). A gene called *kintoun/PF13* is required for the

pre-assembly of the ODA complexes in the cell body (Omran, Kobayashi et al. 2008). Three ODA subunits (heavy, light and light-intermediate chains) co-immunoprecipitate as a complex in wildtype Chlamydomonas cells, but do not in the PF13 mutants (Omran, Kobayashi et al. 2008). IFT46, a complex B protein specifically transports the outer dynein arm complex in *Chlamydomonas* as seen by the absence of one of the outer dynein arm subunits, ODA16 in the *ift46* mutants (Hou, Qin et al. 2007). This model was strengthened when the two proteins, ODA16 and IFT46 were found to physically interact in a yeast two hybrid analysis and by in vitro coimmunoprecipitation assay (Ahmed, Gao et al. 2008). Similar to the dynein arm complex, radial spoke proteins are also preassembled in a complex in the cell body, then transported into flagella by IFT, which also brings back the complex after disassembly, as seen by density sucrose gradients (Qin, Diener et al. 2004). Radial spoke protein RSP-1 co-immunoprecipitated using antibodies against IFT proteins (IFT74 and IFT52) indicating a direct interaction between IFT cargo and IFT proteins (Qin, Diener et al. 2004).

The ciliary membrane is continuous with the cell membrane, yet the membrane proteins that localize to cilia are different from those destined for the cell membrane. Both membrane precursors and proteins must be transported into cilia and it is likely that IFT components is involved in this process. As mentioned earlier, IFT20 associates with the Golgi complex and a mild knockdown of IFT20 depletes polycystin-2 channels from the plasma membrane of primary cilia on kidney cells, implying that IFT20 might play a role in targeting membrane proteins destined for cilia (Follit, Tuft et al. 2006). Based on co-immunoprecipitation and immunofluorescence assays, a complex of at least 7 BBS proteins accumulates at the basal body and enters the primary cilium in cultured

mammalian cells (Nachury, Loktev et al. 2007). The complex is called BBSome and it physically interacts with Rabin8, a guanosyl exchange factor for Rab8, which is a small GTPase that enters into the primary cilium only in its GTP-bound state (Nachury, Loktev et al. 2007). A mutant version of Rab8 that is permanently bound to GTP promotes ciliary membrane growth, as seen by increase in ciliary and axonemal lengths (Nachury, Loktev et al. 2007). Elipsa, a novel protein recently discovered in zebrafish may be part of the IFT complexes, since it interacts with several IFT proteins like (IFT20, IFT57 and IFT172) in an affinity purification assay, while its *C. elegans* homolog, DYF-11 undergoes IFT with the velocity of known IFT particles (Omori, Zhao et al. 2008). In a GST-pull down assay Elipsa also interacts with Rabaptin5, a known regulator of endocytosis and Rabaptin5 in turn interacts with Rab8, known to promote ciliary membrane growth (Nachury 2008; Omori, Zhao et al. 2008). These data indicate that there is a link between membrane transport into the cilium and IFT.

The form in which tubulin is transported into the cilium is currently not known. Early studies on different species of sea urchin embryos indicated that in the detergentsoluble fractions of cilia i.e. the membrane + matrix fractions of cilia had almost half the ciliary tubulin, seen in immunoblots using a variety of antibodies. It was hypothesized that this detergent-soluble tubulin is destined for incorporation into the axoneme, most likely by the IFT complexes, as IFT proteins are also in the membrane + matrix fraction (Stephens 1991; Stephens 1994; Cole, Diener et al. 1998). Tubulin folding cofactor B was detected in the ciliary fraction of a partial suppressor of IFT52 knockout in *Tetrahymena* (unpublished data). This result connects the tubulin folding pathway to the IFT pathway, both of which are important in ciliary assembly. This connection might be

especially important in studying tubulin transport into cilia. The functional analysis on cofactor B is presented in chapter 3.

Despite evidence that the IFT complexes are required for the transport of various ciliary cargo, the means with which the IFT particles recognize and bind different types of cargo is not known. Some possibilities in cargo recognition are; ciliary-targeting signals, specific cargo for individual IFT proteins, different motor proteins, a variety of adaptor proteins and a ciliary gate complex at the transition zone between the basal body and the cilium. In the previous section we discussed the use of different anterograde IFT motors and their functions in different cell types. There is also some evidence for a potential ciliary targeting signal. The N- terminal region of polycystin-2, a Ca<sup>+2</sup> channel, is required for its localization to cilia, since a HA-tagged version of the protein lacking this portion could be detected in the cell body but not in the cilia of mammalian cell lines (Geng, Okuhara et al. 2006). The study showed that another homologous Ca<sup>+2</sup> channel, which is normally localized to the cell body, could be detected in the cilia when its Nterminal region was replaced with that of polycystin-2 (Geng, Okuhara et al. 2006). The targeting of the CNG channels to olfactory cilia depends on co-expression of at least two of its three subunits, CNGA2, CNGA4 and CNGB1b. The CNGB1b subunit has a putative ciliary localization signal, RVxP that is common between CNGB1b and polycystin-2, and this signal is necessary for the targeting of the CNG complex to cilia (Jenkins, Hurd et al. 2006). Substitution mutant versions of the RVxP domain in CNGB1b could not be detected in the cilia even though they could form a complex with CNGA2 (co-immunoprecipitation assay) (Jenkins, Hurd et al. 2006).

## **Ciliopathies: Ciliary diseases**

Mutations in proteins that are components of cilia, or are responsible for ciliary assembly, lead to defective ciliary function, resulting in abnormal cells or diseased states, collectively called ciliopathies. Ciliopathies cause symptoms that generally overlap but are not identical because not all cilia have the same properties (Marshall 2008). Primary ciliary dyskinesia (PCD) generally results from defective motile cilia that are defective in the machinery responsible for causing ciliary motility, such as inner and outer dynein arms, as seen in the ultrastructure of cilia from patients (Bisgrove and Yost 2006). Mutations in an axonemal dynein heavy chain, DNAH5 and an inner arm dynein, DNAI1 results in PCD (Pennarun, Escudier et al. 1999; Olbrich, Haffner et al. 2002). The motility of cilia on the lung epithelia that is important in moving mucous along the surface and preventing infections is defective in PCD patients causing them to suffer from chronic respiratory infections (Afzelius 2004). Male infertility results from immotile or poorly swimming sperm cells. In some cases, patients develop hydrocephaly due to abnormal motility of ependymal cilia, which is required for moving cerebrospinal fluid along the brain ventricles (Afzelius 2004). Mutation in a gene called *kintoun/PF13* leads to a variety of PCD symptoms such as male infertility, situs inversus and hydrocephaly, since the product of this gene is required for the preassembly of axonemal dyneins in the cell body prior to transport in the cilia (Omran, Kobayashi et al. 2008). In another study, mice with a mutated protein, hydin, develop hydrocephaly at birth, resulting in lethality (Davy and Robinson 2003). The Chlamydomonas homolog of hydin was discovered to be a central pair component, by immunoelectron microscopy, and mutants had defective flagellar motility (Lechtreck and Witman 2007; Lechtreck,

Delmotte et al. 2008). In mammals, the cilia at the embryonic node have a unique type of rotational motility that is important in breaking the symmetry of the embryo and causing left-right asymmetry of internal organs (Nonaka, Tanaka et al. 1998). Several patients suffering from PCD have randomization of internal organs and sometimes the left-right asymmetry of organs is completely opposite, *situs inversus totalis* or Kartagener syndrome (Bisgrove and Yost 2006).

Defects in the immotile, primary cilia can result in diseases with very different phenotypes, like polycystic kidney disease (PKD), nephronophthisis (NPHP), retinal degeneration and skeletal defects (Lehman, Michaud et al. 2008). The most commonly discussed disease phenotype is cystic kidneys caused by a number of factors. The mechanosensory function of renal primary cilia (non-motile, sensory) depends on polycystins 1 and 2 and mutations in either of the proteins leads to cystic kidneys (Nauli, Alenghat et al. 2003; Bisgrove and Yost 2006). Yet another family of ciliary proteins called the nephrocystins (nephrocystins 1-6) is detected in a variety of cilia and basal bodies of primary cilia but their function is not known (Hildebrandt and Zhou 2007). This family of proteins is linked to NPHP, an autosomal recessive form of PKD with a range of different phenotypes such as retinitis pigmentosa and liver ataxia (Hildebrandt and Zhou 2007). Bardet-Biedl syndrome (BBS) is a multigenic disorder characterized by several phenotypes including mental retardation, retinal degeneration, kidney defects, polydactyly and obesity (Gerdes, Davis et al. 2009). Recent work has shown that BBS symptoms are caused by ciliary and basal body dysfunction due to mutant BBS proteins, as a knockdown in BBS1 and BBS5 results in lack of cilia in mammalian cells (Nachury 2008). Retinitis pigmentosa is a type of retinal degeneration that slowly leads to complete

blindness (Afzelius 2004). One cause of retinal degeneration is lack of photopigment molecules in the outer rod and cone cells due to the absence of photoreceptor cilia or defective transport across them (Marszalek, Liu et al. 2000; Afzelius 2004; Nishimura, Fath et al. 2004; Insinna and Besharse 2008).

Mutation in a protein that is common to both motile and non-motile cilia, like members of the IFT pathway, leads to more diverse phenotypes. The ORPK mouse is a very well studied example of a mutant in the IFT pathway, namely IFT88 (a complex B IFT protein) that is essential for the assembly of cilia (Pazour, Dickert et al. 2000; Cole 2003). In the ORPK mouse, the *ift88* allele has a transgene insertion that partially disrupts the expression of the gene resulting in cilia that are short with abnormal motility but not completely absent (Lehman, Michaud et al. 2008). Some of the common phenotypes described for the ORPK mouse are cystic kidneys, skeletal defects retinal degeneration and hydrocephaly, which result from defective motile and non-motile cilia (Lehman, Michaud et al. 2008). Oral-facial-digital Type I syndrome is caused by mutations in OFD1, which produces a protein detected at the centrosome and basal bodies. Mouse and zebrafish ofd1 mutants have a wide variety of defects such as cystic kidneys, digital malformations due to defective non-motile primary cilia and left-right asymmetry due to defective motile primary cilia (Romio, Wright et al. 2003; Ferrante, Zullo et al. 2006; Ferrante, Romio et al. 2009).

## Tetrahymena as a model organism

*Tetrahymena thermophila* is a good model organism for the study of number of factors influencing ciliary assembly and maintenance. The cell is covered with

approximately 500 cilia (G1 phase) and assembles about 17 different types of microtubules (Gaertig 2000). *Tetrahymena* has a completely sequenced genome of about 104 MB, with several proteins showing high level of homology to those in other organisms (Eisen, Coyne et al. 2006). *Tetrahymena* is a free-living protist that is easy to maintain in the lab and has well established genetic and biochemical methods. The most advantageous aspect of this organism is that it almost exclusively shows homologous recombination, thus making it ideal to target DNA to a particular locus for studying expression of tagged or mutated genes as well as gene knockouts (Eisen, Coyne et al. 2006). The process of introducing foreign DNA into the genome is done with relatively high efficiency using biolistic bombardment of gold particles coated with DNA (Hai and Gorovsky 1997). Another key advantage of the organism is that it has a large number of cilia, which can be isolated and fractionated by well-developed methods for biochemical analyses.

*Tetrahymena* have two non-equivalent nuclei, the somatic macronucleus (Mac) and the germline micronucleus (Mic) (Yao and Chao 2005). The polyploid Mac is transcriptionally active during the vegetative cycle of the cell, thus the phenotype of the cells is dictated by the macronuclear genome. During the normal vegetative cycle, the Mic divides mitotically whereas the Mac divides amitotically and the chromosomes are randomly segregated in the daughter cells. During conjugation of two cells of different mating types, the Mics undergo meiosis and exchange a haploid nucleus with the partner (Figure 3). The haploid Mics fuse and form a new diploid Mic containing genes from both partners. The new diploid Mics undergo two rounds of mitosis producing four equal products, out of which one is retained as the Mic, one becomes the Mac and the

remaining two are destroyed. Also, the older, parental Macs are degraded. Thus, new information is transmitted down to the new Macs that take over once the vegetative cycle begins.

The process of conjugation in *Tetrahymena* involves initial pairing of nutritionally starved cells with different mating types followed by several steps of nuclear divisions and DNA rearrangement (Yao and Chao 2005). Once the two cells are fused and form a conjugal junction, the Mics undergo meiosis to produce four haploid nuclei, three of which are randomly destroyed. The remaining haploid nucleus undergoes mitosis and produces two identical haploid pronuclei, once of which is exchanged with the partner through the junction. To obtain germline-gene knockouts; mating pairs are transformed by gene disruption cassettes during this exchange of haploid pronuclei and the DNA is targeted to the pronuclei. Once transformed, pairs typically stay together until they complete conjugation, after which they separate as ex-conjugants and the vegetative cycle begins. Since new Macs are formed from new Mics, previously transcriptionally silent genes in the Mic can now be brought to expression. This feature is very useful in making germline knockouts of genes essential for viability because it gives us a means to maintain lethal knockouts. A schematic describing the process of generating knockouts is shown in Figure 2.3. The knockouts of both, TCB1 (Tubulin folding Cofactor B) and *IFT52* were made using this technique, as described in Chapters 3 and 4 respectively.

Another technique commonly used is the generation of somatic knockouts of nonessential genes. In this method, the knockout cassette is targeted to the locus of interest in the Mac. Since the Mac divides by random segregation of chromosomes (phenotypic assortment), it is possible to assort cells under increasing selective pressure to a point

where nearly all copies of the native gene are replaced with the newly introduced DNA. This technique is quicker than obtaining germline knockouts, however, as mentioned earlier knockouts of essential genes cannot be made. *DYF1* knockout was a somatic knockout, as described in Chapter 5.

The use of inducible gene expression is very widely used in *Tetrahymena*. The cadmium-inducible (*MTT1*) and the copper-inducible (*MTT2*) metallothionein gene products normally negate the toxic effects of these two metals in the cell. They are both non-essential genes. In *Tetrahymena*, the two  $\beta$ -tubulin genes, *BTU1* and *BTU2* are redundant. This gives us the basis to introduce a recombinant gene fused with a tag under the metal-inducible promoter in the *BTU1* locus. The expression of Ift52p-GFP and GFP-Dyf1p as well as several versions of tagged Tcb1p were made using this method. Adding the chloride of either metal can induce transcription of tagged genes and the localization of the gene products can be observed. In the same way, the tagged genes can be targeted into the native locus and localization profiles can be observed under native or wildtype conditions. Knockouts can be rescued in the non-native *BTU1* locus or in the native locus, as was done for *IFT52*, *DYF1* and *TCB1*.

## References

Afzelius, B. A. (2004). "Cilia-related diseases." J Pathol 204(4): 470-7.

Ahmed, N. T., C. Gao, et al. (2008). "ODA16 aids axonemal outer row dynein assembly through an interaction with the intraflagellar transport machinery." <u>J Cell Biol</u> 183(2): 313-22.

- Allen, R. D. (1969). "The morphogenesis of basal bodies and accessory structures of the cortex of the ciliated protozoan Tetrahymena pyriformis." <u>J Cell Biol</u> 40(3): 716-33.
- Baker, S. A., K. Freeman, et al. (2003). "IFT20 links kinesin II with a mammalian intraflagellar transport complex that is conserved in motile flagella and sensory cilia." <u>J Biol Chem</u> 278(36): 34211-8.
- Bardy, S. L., S. Y. Ng, et al. (2003). "Prokaryotic motility structures." <u>Microbiology</u> **149**(Pt 2): 295-304.
- Beisson, J. and M. Wright (2003). "Basal body/centriole assembly and continuity." <u>Curr</u> <u>Opin Cell Biol</u> **15**(1): 96-104.
- Belmont, L. D. and T. J. Mitchison (1996). "Identification of a protein that interacts with tubulin dimers and increases the catastrophe rate of microtubules." <u>Cell</u> 84(4): 623-31.
- Bisgrove, B. W. and H. J. Yost (2006). "The roles of cilia in developmental disorders and disease." <u>Development</u> 133(21): 4131-43.
- Bobinnec, Y., A. Khodjakov, et al. (1998). "Centriole disassembly in vivo and its effect on centrosome structure and function in vertebrate cells." <u>J Cell Biol</u> 143(6): 1575-89.
- Bonnafe, E., M. Touka, et al. (2004). "The transcription factor RFX3 directs nodal cilium development and left-right asymmetry specification." <u>Mol Cell Biol</u> 24(10): 4417-27.
- Brailov, I., M. Bancila, et al. (2000). "Localization of 5-HT(6) receptors at the plasma membrane of neuronal cilia in the rat brain." <u>Brain Res</u> 872(1-2): 271-5.

- Brouhard, G. J., J. H. Stear, et al. (2008). "XMAP215 is a processive microtubule polymerase." <u>Cell</u> **132**(1): 79-88.
- Brown, A. (2003). "Axonal transport of membranous and nonmembranous cargoes: a unified perspective." J Cell Biol **160**(6): 817-21.
- Brown, J. M., N. A. Fine, et al. (2003). "Hypoxia regulates assembly of cilia in suppressors of Tetrahymena lacking an intraflagellar transport subunit gene." <u>Mol</u> <u>Biol Cell</u> 14(8): 3192-207.
- Brown, J. M., C. Hardin, et al. (1999). "Rotokinesis, a novel phenomenon of cell locomotion-assisted cytokinesis in the ciliate Tetrahymena thermophila." <u>Cell</u> <u>Biol Int</u> 23(12): 841-8.
- Brown, J. M., C. Marsala, et al. (1999). "Kinesin-II is preferentially targeted to assembling cilia and is required for ciliogenesis and normal cytokinesis in Tetrahymena." <u>Mol Biol Cell</u> 10(10): 3081-96.
- Cassimeris, L. (2002). "The oncoprotein 18/stathmin family of microtubule destabilizers." <u>Curr Opin Cell Biol</u> **14**(1): 18-24.
- Cole, D. G. (2003). "The intraflagellar transport machinery of Chlamydomonas reinhardtii." <u>Traffic</u> **4**(7): 435-42.
- Cole, D. G., D. R. Diener, et al. (1998). "Chlamydomonas kinesin-II-dependent intraflagellar transport (IFT): IFT particles contain proteins required for ciliary assembly in Caenorhabditis elegans sensory neurons." <u>J Cell Biol</u> 141(4): 993-1008.
- Cole, N. B. and J. Lippincott-Schwartz (1995). "Organization of organelles and membrane traffic by microtubules." <u>Curr Opin Cell Biol</u> 7(1): 55-64.

- Cortellino, S., C. Wang, et al. (2009). "Defective ciliogenesis, embryonic lethality and severe impairment of the Sonic Hedgehog pathway caused by inactivation of the mouse complex A intraflagellar transport gene Ift122/Wdr10, partially overlapping with the DNA repair gene Med1/Mbd4." <u>Dev Biol</u> 325(1): 225-37.
- Culver, B. P., J. B. Meehl, et al. (2009). "The two SAS-6 homologs in Tetrahymena thermophila have distinct functions in basal body assembly." <u>Mol Biol Cell</u> 20(6): 1865-77.
- Dammermann, A. and A. Merdes (2002). "Assembly of centrosomal proteins and microtubule organization depends on PCM-1." J Cell Biol **159**(2): 255-66.
- Davy, B. E. and M. L. Robinson (2003). "Congenital hydrocephalus in hy3 mice is caused by a frameshift mutation in Hydin, a large novel gene." <u>Hum Mol Genet</u> 12(10): 1163-70.
- Deane, J. A., D. G. Cole, et al. (2001). "Localization of intraflagellar transport protein IFT52 identifies basal body transitional fibers as the docking site for IFT particles." <u>Curr Biol</u> 11(20): 1586-90.
- Dentler, W. L. (1980). "Structures linking the tips of ciliary and flagellar microtubules to the membrane." <u>J Cell Sci</u> **42**: 207-20.
- Desai, A. and T. J. Mitchison (1997). "Microtubule polymerization dynamics." <u>Annu Rev</u> <u>Cell Dev Biol</u> **13**: 83-117.
- Drewes, G., A. Ebneth, et al. (1998). "MAPs, MARKs and microtubule dynamics." <u>Trends Biochem Sci</u> 23(8): 307-11.
- Dubruille, R., A. Laurencon, et al. (2002). "Drosophila regulatory factor X is necessary for ciliated sensory neuron differentiation." <u>Development</u> **129**(23): 5487-98.

- Eisen, J. A., R. S. Coyne, et al. (2006). "Macronuclear genome sequence of the ciliate Tetrahymena thermophila, a model eukaryote." <u>PLoS Biol</u> **4**(9): e286.
- Ferrante, M. I., L. Romio, et al. (2009). "Convergent extension movements and ciliary function are mediated by ofd1, a zebrafish orthologue of the human oral-facialdigital type 1 syndrome gene." <u>Hum Mol Genet</u> 18(2): 289-303.
- Ferrante, M. I., A. Zullo, et al. (2006). "Oral-facial-digital type I protein is required for primary cilia formation and left-right axis specification." <u>Nat Genet</u> 38(1): 112-7.
- Finetti, F., S. R. Paccani, et al. (2009). "Intraflagellar transport is required for polarized recycling of the TCR/CD3 complex to the immune synapse." <u>Nat Cell Biol</u> 11(11): 1332-9.
- Follit, J. A., J. T. San Agustin, et al. (2008). "The Golgin GMAP210/TRIP11 anchors IFT20 to the Golgi complex." <u>PLoS Genet</u> 4(12): e1000315.
- Follit, J. A., R. A. Tuft, et al. (2006). "The intraflagellar transport protein IFT20 is associated with the Golgi complex and is required for cilia assembly." <u>Mol Biol</u> <u>Cell</u> 17(9): 3781-92.
- Fowkes, M. E. and D. R. Mitchell (1998). "The role of preassembled cytoplasmic complexes in assembly of flagellar dynein subunits." <u>Mol Biol Cell</u> 9(9): 2337-47.
- Gaertig, J. (2000). "Molecular mechanisms of microtubular organelle assembly in Tetrahymena." J Eukaryot Microbiol **47**(3): 185-90.
- Gaertig, J. and D. Wloga (2008). "Ciliary tubulin and its post-translational modifications." <u>Curr Top Dev Biol</u> **85**: 83-113.
- Geng, L., D. Okuhara, et al. (2006). "Polycystin-2 traffics to cilia independently of polycystin-1 by using an N-terminal RVxP motif." <u>J Cell Sci</u> 119(Pt 7): 1383-95.

- Gerdes, J. M., E. E. Davis, et al. (2009). "The vertebrate primary cilium in development, homeostasis, and disease." <u>Cell</u> **137**(1): 32-45.
- Gherman, A., E. E. Davis, et al. (2006). "The ciliary proteome database: an integrated community resource for the genetic and functional dissection of cilia." <u>Nat Genet</u> 38(9): 961-2.
- Gonzalez-Perrett, S., K. Kim, et al. (2001). "Polycystin-2, the protein mutated in autosomal dominant polycystic kidney disease (ADPKD), is a Ca2+-permeable nonselective cation channel." <u>Proc Natl Acad Sci U S A</u> 98(3): 1182-7.
- Grynberg, M., L. Jaroszewski, et al. (2003). "Domain analysis of the tubulin cofactor system: a model for tubulin folding and dimerization." <u>BMC Bioinformatics</u> 4: 46.
- Hai, B. and M. A. Gorovsky (1997). "Germ-line knockout heterokaryons of an essential alpha-tubulin gene enable high-frequency gene replacement and a test of gene transfer from somatic to germ-line nuclei in Tetrahymena thermophila." <u>Proc Natl Acad Sci U S A</u> 94(4): 1310-5.
- Handel, M., S. Schulz, et al. (1999). "Selective targeting of somatostatin receptor 3 to neuronal cilia." <u>Neuroscience</u> 89(3): 909-26.
- Hildebrandt, F. and W. Zhou (2007). "Nephronophthisis-associated ciliopathies." <u>J Am</u> <u>Soc Nephrol</u> **18**(6): 1855-71.
- Hou, Y., H. Qin, et al. (2007). "Functional analysis of an individual IFT protein: IFT46 is required for transport of outer dynein arms into flagella." <u>J Cell Biol</u> 176(5): 653-65.

- Howard, J. and A. A. Hyman (2003). "Dynamics and mechanics of the microtubule plus end." <u>Nature</u> **422**(6933): 753-8.
- Huang, K., D. R. Diener, et al. (2009). "The ubiquitin conjugation system is involved in the disassembly of cilia and flagella." <u>J Cell Biol</u> 186(4): 601-13.
- Insinna, C., N. Pathak, et al. (2008). "The homodimeric kinesin, Kif17, is essential for vertebrate photoreceptor sensory outer segment development." <u>Dev Biol</u> 316(1): 160-70.
- Jenkins, P. M., T. W. Hurd, et al. (2006). "Ciliary targeting of olfactory CNG channels requires the CNGB1b subunit and the kinesin-2 motor protein, KIF17." <u>Curr Biol</u> 16(12): 1211-6.
- Jourdain, L., P. Curmi, et al. (1997). "Stathmin: a tubulin-sequestering protein which forms a ternary T2S complex with two tubulin molecules." <u>Biochemistry</u> **36**(36): 10817-21.
- Kilburn, C. L., C. G. Pearson, et al. (2007). "New Tetrahymena basal body protein components identify basal body domain structure." <u>J Cell Biol</u> 178(6): 905-12.
- Kozminski, K. G., P. L. Beech, et al. (1995). "The Chlamydomonas kinesin-like protein FLA10 is involved in motility associated with the flagellar membrane." <u>J Cell</u>
  <u>Biol</u> 131(6 Pt 1): 1517-27.
- Kozminski, K. G., K. A. Johnson, et al. (1993). "A motility in the eukaryotic flagellum unrelated to flagellar beating." <u>Proc Natl Acad Sci U S A</u> **90**(12): 5519-23.
- Krock, B. L. and B. D. Perkins (2008). "The intraflagellar transport protein IFT57 is required for cilia maintenance and regulates IFT-particle-kinesin-II dissociation in vertebrate photoreceptors." <u>J Cell Sci</u> 121(Pt 11): 1907-15.

- Lechtreck, K. F., P. Delmotte, et al. (2008). "Mutations in Hydin impair ciliary motility in mice." J Cell Biol **180**(3): 633-43.
- Lehman, J. M., E. J. Michaud, et al. (2008). "The Oak Ridge Polycystic Kidney mouse: modeling ciliopathies of mice and men." <u>Dev Dyn</u> **237**(8): 1960-71.
- Lopez-Fanarraga, M., J. Avila, et al. (2001). "Review: postchaperonin tubulin folding cofactors and their role in microtubule dynamics." <u>J Struct Biol</u> **135**(2): 219-29.
- Lucker, B. F., R. H. Behal, et al. (2005). "Characterization of the Intraflagellar Transport Complex B Core: DIRECT INTERACTION OF THE IFT81 AND IFT74/72 SUBUNITS." J. Biol. Chem. 280(30): 27688-27696.
- Marshall, W. F. (2008). "Basal bodies platforms for building cilia." <u>Curr Top Dev Biol</u>85: 1-22.
- Marshall, W. F. (2008). "The cell biological basis of ciliary disease." <u>J Cell Biol</u> **180**(1): 17-21.
- Marshall, W. F. and S. Nonaka (2006). "Cilia: tuning in to the cell's antenna." <u>Curr Biol</u> **16**(15): R604-14.
- Marszalek, J. R., X. Liu, et al. (2000). "Genetic evidence for selective transport of opsin and arrestin by kinesin-II in mammalian photoreceptors." <u>Cell</u> **102**(2): 175-87.
- Martin-Cofreces, N. B., J. Robles-Valero, et al. (2008). "MTOC translocation modulates IS formation and controls sustained T cell signaling." <u>J Cell Biol</u> **182**(5): 951-62.
- McKean, P. G., A. Baines, et al. (2003). "Gamma-tubulin functions in the nucleation of a discrete subset of microtubules in the eukaryotic flagellum." <u>Curr Biol</u> 13(7): 598-602.

- Nachury, M. V. (2008). "Tandem affinity purification of the BBSome, a critical regulator of Rab8 in ciliogenesis." <u>Methods Enzymol</u> **439**: 501-13.
- Nachury, M. V., A. V. Loktev, et al. (2007). "A core complex of BBS proteins cooperates with the GTPase Rab8 to promote ciliary membrane biogenesis." <u>Cell</u> **129**(6): 1201-13.
- Nakamura, M., X. Z. Zhou, et al. (2001). "Critical role for the EB1 and APC interaction in the regulation of microtubule polymerization." <u>Curr Biol</u> **11**(13): 1062-7.
- Nauli, S. M., F. J. Alenghat, et al. (2003). "Polycystins 1 and 2 mediate mechanosensation in the primary cilium of kidney cells." <u>Nat Genet</u> 33(2): 129-37.
- Neugebauer, J. M., J. D. Amack, et al. (2009). "FGF signalling during embryo development regulates cilia length in diverse epithelia." <u>Nature</u> **458**(7238): 651-4.
- Nicastro, D., C. Schwartz, et al. (2006). "The molecular architecture of axonemes revealed by cryoelectron tomography." <u>Science</u> **313**(5789): 944-8.
- Nishimura, D. Y., M. Fath, et al. (2004). "Bbs2-null mice have neurosensory deficits, a defect in social dominance, and retinopathy associated with mislocalization of rhodopsin." <u>Proc Natl Acad Sci U S A</u> **101**(47): 16588-93.
- Nogales, E. (2000). "Structural insights into microtubule function." <u>Annu Rev Biochem</u> 69: 277-302.
- Nonaka, S., Y. Tanaka, et al. (1998). "Randomization of left-right asymmetry due to loss of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein." <u>Cell</u> 95(6): 829-37.

- O'Toole, E. T., T. H. Giddings, et al. (2003). "Three-dimensional organization of basal bodies from wild-type and delta-tubulin deletion strains of Chlamydomonas reinhardtii." <u>Mol Biol Cell</u> **14**(7): 2999-3012.
- Olbrich, H., K. Haffner, et al. (2002). "Mutations in DNAH5 cause primary ciliary dyskinesia and randomization of left-right asymmetry." <u>Nat Genet</u> **30**(2): 143-4.
- Omori, Y., C. Zhao, et al. (2008). "Elipsa is an early determinant of ciliogenesis that links the IFT particle to membrane-associated small GTPase Rab8." <u>Nat Cell Biol</u> **10**(4): 437-44.
- Omran, H., D. Kobayashi, et al. (2008). "Ktu/PF13 is required for cytoplasmic preassembly of axonemal dyneins." <u>Nature</u> **456**(7222): 611-6.
- Ou, G., O. E. Blacque, et al. (2005). "Functional coordination of intraflagellar transport motors." <u>Nature</u> 436(7050): 583-7.
- Pathak, N., T. Obara, et al. (2007). "The zebrafish fleer gene encodes an essential regulator of cilia tubulin polyglutamylation." <u>Mol Biol Cell</u> **18**(11): 4353-64.
- Pazour, G. J., B. L. Dickert, et al. (2000). "Chlamydomonas IFT88 and its mouse homologue, polycystic kidney disease gene tg737, are required for assembly of cilia and flagella." <u>J Cell Biol</u> 151(3): 709-18.
- Pazour, G. J., B. L. Dickert, et al. (1999). "The DHC1b (DHC2) isoform of cytoplasmic dynein is required for flagellar assembly." <u>J Cell Biol</u> 144(3): 473-81.
- Pazour, G. J., J. T. San Agustin, et al. (2002). "Polycystin-2 localizes to kidney cilia and the ciliary level is elevated in orpk mice with polycystic kidney disease." <u>Curr</u> <u>Biol</u> 12(11): R378-80.

- Pazour, G. J., C. G. Wilkerson, et al. (1998). "A dynein light chain is essential for the retrograde particle movement of intraflagellar transport (IFT)." <u>J Cell Biol</u> 141(4): 979-92.
- Peden, E. M. and M. M. Barr (2005). "The KLP-6 kinesin is required for male mating behaviors and polycystin localization in Caenorhabditis elegans." <u>Curr Biol</u> 15(5): 394-404.
- Pedersen, L. B., S. Geimer, et al. (2003). "The Microtubule plus end-tracking protein EB1 is localized to the flagellar tip and basal bodies in Chlamydomonas reinhardtii." <u>Curr Biol</u> 13(22): 1969-74.
- Pennarun, G., E. Escudier, et al. (1999). "Loss-of-function mutations in a human gene related to Chlamydomonas reinhardtii dynein IC78 result in primary ciliary dyskinesia." <u>Am J Hum Genet</u> 65(6): 1508-19.
- Perkins, L. A., E. M. Hedgecock, et al. (1986). "Mutant sensory cilia in the nematode Caenorhabditis elegans." <u>Dev Biol</u> 117(2): 456-87.
- Piperno, G., E. Siuda, et al. (1998). "Distinct mutants of retrograde intraflagellar transport (IFT) share similar morphological and molecular defects." <u>J Cell Biol</u> 143(6): 1591-601.
- Porter, M. E. and W. S. Sale (2000). "The 9 + 2 axoneme anchors multiple inner arm dyneins and a network of kinases and phosphatases that control motility." <u>J Cell</u> <u>Biol</u> 151(5): F37-42.
- Qin, H., D. T. Burnette, et al. (2005). "Intraflagellar transport is required for the vectorial movement of TRPV channels in the ciliary membrane." <u>Curr Biol</u> **15**(18): 1695-9.

- Qin, H., D. R. Diener, et al. (2004). "Intraflagellar transport (IFT) cargo: IFT transports flagellar precursors to the tip and turnover products to the cell body." <u>J Cell Biol</u> 164(2): 255-66.
- Qin, H., Z. Wang, et al. (2007). "Intraflagellar transport protein 27 is a small G protein involved in cell-cycle control." <u>Curr Biol</u> 17(3): 193-202.
- Rajagopalan, V., A. Subramanian, et al. (2009). "Dynein-2 affects the regulation of ciliary length but is not required for ciliogenesis in Tetrahymena thermophila."
  <u>Mol Biol Cell</u> 20(2): 708-20.
- Rana, A. A., J. P. Barbera, et al. (2004). "Targeted deletion of the novel cytoplasmic dynein mD2LIC disrupts the embryonic organiser, formation of the body axes and specification of ventral cell fates." <u>Development</u> 131(20): 4999-5007.
- Reed, N. A., D. Cai, et al. (2006). "Microtubule acetylation promotes kinesin-1 binding and transport." <u>Curr Biol</u> 16(21): 2166-72.
- Robert, A., G. Margall-Ducos, et al. (2007). "The intraflagellar transport component IFT88/polaris is a centrosomal protein regulating G1-S transition in non-ciliated cells." <u>J Cell Sci</u> 120(Pt 4): 628-37.
- Romio, L., V. Wright, et al. (2003). "OFD1, the gene mutated in oral-facial-digital syndrome type 1, is expressed in the metanephros and in human embryonic renal mesenchymal cells." J Am Soc Nephrol 14(3): 680-9.
- Rosenbaum, J. L. and G. B. Witman (2002). "Intraflagellar transport." <u>Nat Rev Mol Cell</u> <u>Biol</u> **3**(11): 813-25.
- Sale, W. S. and P. Satir (1977). "The termination of the central microtubules from the cilia of Tetrahymena pyriformis." <u>Cell Biol Int Rep</u> 1(1): 45-9.

- Satir, P. and S. T. Christensen (2007). "Overview of structure and function of mammalian cilia." <u>Annu Rev Physiol</u> 69: 377-400.
- Savage, C., M. Hamelin, et al. (1989). "mec-7 is a beta-tubulin gene required for the production of 15-protofilament microtubules in Caenorhabditis elegans." <u>Genes</u> <u>Dev</u> 3(6): 870-81.
- Schafer, J. C., C. J. Haycraft, et al. (2003). "XBX-1 encodes a dynein light intermediate chain required for retrograde intraflagellar transport and cilia assembly in Caenorhabditis elegans." <u>Mol Biol Cell</u> 14(5): 2057-70.
- Scholey, J. M. (1996). "Kinesin-II, a membrane traffic motor in axons, axonemes, and spindles." <u>J Cell Biol</u> 133(1): 1-4.
- Scholey, J. M. (2003). "Intraflagellar transport." Annu Rev Cell Dev Biol 19: 423-43.
- Schroder, J. M., L. Schneider, et al. (2007). "EB1 is required for primary cilia assembly in fibroblasts." <u>Curr Biol</u> 17(13): 1134-9.
- Shiratori, H. and H. Hamada (2006). "The left-right axis in the mouse: from origin to morphology." <u>Development</u> 133(11): 2095-104.
- Sloboda, R. D. (2005). "Intraflagellar transport and the flagellar tip complex." <u>J Cell</u> <u>Biochem</u> 94(2): 266-72.
- Smith, E. F. and P. Yang (2004). "The radial spokes and central apparatus: mechanochemical transducers that regulate flagellar motility." <u>Cell Motil Cytoskeleton</u> 57(1): 8-17.
- Snow, J. J., G. Ou, et al. (2004). "Two anterograde intraflagellar transport motors cooperate to build sensory cilia on C. elegans neurons." <u>Nat Cell Biol</u> 6(11): 1109-13.

- Stephens, R. E. (1991). "Tubulin in sea urchin embryonic cilia: characterization of the membrane-periaxonemal matrix." <u>J Cell Sci</u> 100 ( Pt 3): 521-31.
- Stephens, R. E. (1994). "Tubulin and tektin in sea urchin embryonic cilia: pathways of protein incorporation during turnover and regeneration." <u>J Cell Sci</u> 107 ( Pt 2): 683-92.
- Sui, H. and K. H. Downing (2006). "Molecular architecture of axonemal microtubule doublets revealed by cryo-electron tomography." <u>Nature</u> 442(7101): 475-8.
- Swoboda, P., H. T. Adler, et al. (2000). "The RFX-type transcription factor DAF-19 regulates sensory neuron cilium formation in C. elegans." <u>Mol Cell</u> 5(3): 411-21.
- Takeda, S., Y. Yonekawa, et al. (1999). "Left-right asymmetry and kinesin superfamily protein KIF3A: new insights in determination of laterality and mesoderm induction by kif3A-/- mice analysis." <u>J Cell Biol</u> 145(4): 825-36.
- Tanaka, H., N. Iguchi, et al. (2004). "Mice deficient in the axonemal protein Tektin-t exhibit male infertility and immotile-cilium syndrome due to impaired inner arm dynein function." <u>Mol Cell Biol</u> 24(18): 7958-64.
- Tran, P. T., P. Joshi, et al. (1997). "How tubulin subunits are lost from the shortening ends of microtubules." <u>J Struct Biol</u> 118(2): 107-18.
- Tran, P. V., C. J. Haycraft, et al. (2008). "THM1 negatively modulates mouse sonic hedgehog signal transduction and affects retrograde intraflagellar transport in cilia." <u>Nat Genet</u> 40(4): 403-10.
- Tsao, C. C. and M. A. Gorovsky (2008). "Different effects of Tetrahymena IFT172 domains on anterograde and retrograde intraflagellar transport." <u>Mol Biol Cell</u> 19(4): 1450-61.

- Tsao, C. C. and M. A. Gorovsky (2008). "Tetrahymena IFT122A is not essential for cilia assembly but plays a role in returning IFT proteins from the ciliary tip to the cell body." J Cell Sci 121(Pt 4): 428-36.
- Tsujikawa, M. and J. Malicki (2004). "Intraflagellar transport genes are essential for differentiation and survival of vertebrate sensory neurons." <u>Neuron</u> **42**(5): 703-16.
- Veland, I. R., A. Awan, et al. (2009). "Primary cilia and signaling pathways in mammalian development, health and disease." <u>Nephron Physiol</u> 111(3): p39-53.
- Walczak, C. E., E. C. Gan, et al. (2002). "The microtubule-destabilizing kinesin XKCM1 is required for chromosome positioning during spindle assembly." <u>Curr Biol</u> 12(21): 1885-9.
- Walther, Z., M. Vashishtha, et al. (1994). "The Chlamydomonas FLA10 gene encodes a novel kinesin-homologous protein." <u>J Cell Biol</u> 126(1): 175-88.
- Wang, Q., J. Pan, et al. (2006). "Intraflagellar transport particles participate directly in cilium-generated signaling in Chlamydomonas." <u>Cell</u> 125(3): 549-62.
- Wang, Z., Z. C. Fan, et al. (2009). "Intraflagellar transport (IFT) protein IFT25 is a phosphoprotein component of IFT complex B and physically interacts with IFT27 in Chlamydomonas." <u>PLoS ONE</u> 4(5): e5384.
- Weinstein, B. and F. Solomon (1990). "Phenotypic consequences of tubulin overproduction in Saccharomyces cerevisiae: differences between alpha-tubulin and beta-tubulin." <u>Mol Cell Biol</u> **10**(10): 5295-304.
- Wiese, C. and Y. Zheng (2006). "Microtubule nucleation: gamma-tubulin and beyond." J <u>Cell Sci</u> **119**(Pt 20): 4143-53.

- Wilkes, D. E., H. E. Watson, et al. (2008). "Twenty-five dyneins in Tetrahymena: A reexamination of the multidynein hypothesis." <u>Cell Motil Cytoskeleton</u> 65(4): 342-51.
- Wloga, D., D. Dave, et al. (2009). "Hyperglutamylation of tubulin can either stabilize or destabilize microtubules in the same cell." <u>Eukaryot Cell</u>.
- Wloga, D., D. M. Webster, et al. (2009). "TTLL3 Is a tubulin glycine ligase that regulates the assembly of cilia." <u>Dev Cell</u> **16**(6): 867-76.

## **Figure legends**

**Figure 2.1:** The model for intraflagellar transport (IFT) pathway consists of six steps. 1. Ciliary precursors, IFT motors and IFT proteins assemble at the base of the cilium. IFT complexes A and B are formed, cargo is loaded onto the complexes and motor proteins are attached to the complexes. 2. Anterograde IFT motor, kinesin-II moves along axonemal microtubules and carries cargo from the cell body to the ciliary tip. 3. At the ciliary tips, cargo is unloaded and the IFT particles undergo rearrangement. 4. The motor is switched to the retrograde IFT motor, cytoplasmic dynein-2 moves along axonemal microtubules and returns the turnover products back into the cell body. 6. IFT components and cargo dissociate.

# The Intraflagellar transport pathway



Adapted from Pedersen L.B. et al. 2008 Dev Dyn;Aug;237(8):1993-2006

**Figure 2.2:** The ciliary axoneme consists of three different segments, proximal, middle and distal. The proximal segment consists of the distal part of the basal body and the transition zone. The middle segment consists of the nine outer doublet microtubules and two inner singlet microtubules. The distal segment consists of nine outer singlet microtubules and two inner singlet microtubules. The ciliary tip has two inner singlet microtubules and variable number outer singlet microtubules as they terminate at the tip. The images to the left are TEM cross-sections of *Tetrahymena* axonemes.



**Figure 2.3:** A schematic illustrating the construction of knockout heterokaryons in *Tetrahymena thermophila.* 1-8. Heterozygous transformants are constructed by biolistic bombardment of mating cells. 9. Heterozygous transformants that have lost the drug resistance cassette from the macronucleus are obtained by phenotypic assortment. 10-15. A cross of assorted transformant with a star strain yields heterokaryons that carry wildtype alleles of the disrupted locus in the macronucleus and are homozygous for the disrupted locus in the micronucleus. 16-18. A standard conjugation between two heterokaryons of different mating types produces progeny that are homozygous for the disrupted allele in both the micro- and the macronucleus.



## CHAPTER 3

## TUBULIN FOLDING COFACTOR B IS ESSENTIAL IN TETRAHYMENA THERMOPHILA

## Background

Individual tubulin monomers are transcribed in the cell prior to nucleation into microtubules. The folding and assembly of individual  $\alpha$ - and  $\beta$ -tubulin monomers into dimers occurs in the cytosol through a complex folding pathway. In addition, elements of the folding pathway or closely related proteins directly regulate microtubules. As microtubules are essential, disturbing components of the tubulin folding pathway often leads to perturbation in the levels of monomeric and dimeric tubulin, which can have severe consequences on the cell including cell-cycle arrest and lethality (Nogales 2000).

Monomeric  $\alpha$ - and  $\beta$ -tubulin are folded in at least three stages that involve distinct sets of folding factors, 1) prefoldin, 2) the CCT chaperonin complex and 3) five tubulin-specific cofactors (A-E) (Figure 3.1) (Grynberg, Jaroszewski et al. 2003). Prefoldin and CCT are oligomeric chaperonin complexes, which partially fold both  $\alpha$ and  $\beta$ -tubulin. Of the five folding cofactors, A and D act on  $\beta$ -tubulin whereas B and E act on  $\alpha$ -tubulin. The interaction of cofactors D, E and C is responsible for bringing the two monomers together and generating assembly competent tubulin dimers (Lopez-Fanarraga, Avila et al. 2001).

Prefoldin is a heterohexameric complex that is involved in the folding of several proteins, including  $\alpha$ -,  $\beta$ - and  $\gamma$ -tubulin, and actin (Vainberg, Lewis et al. 1998). Eukaryotic prefoldin is made up of two different types of peptides ( $\alpha$ ,  $\beta$ ) (Gu, Deng et al.
2008). There are two  $\alpha$ -type subunits and four  $\beta$ -type subunits that form a 90kDa complex, which has a six coiled-coil domains projecting from a central barrel-shaped core (Hartl and Hayer-Hartl 2002). The prefold in complex binds to  $\alpha$ - and  $\beta$ -tubulin polypeptides as they emerge from translation of the tubulin mRNAs. As shown by *in* vitro translation assays, after the translation of about 250 amino acids, prefoldin binds to the nascent β-tubulin (Hansen, Cowan et al. 1999). This co-translational association of prefoldin with tubulin continues until the entire tubulin mRNA transcript is translated. The unfolded, non-native protein is then transferred to a group II chaperonin complex, Chaperonin Containing TCP-1 (CCT), which is a large 800-900 kDa complex (Seixas, Casalou et al. 2003). In addition to tubulin, CCT participates in the folding of several other proteins including actin, cofilin, heavy meromyosin subunit HMM and the VonHippel-Lindau tumor suppressor protein (Valpuesta, Martin-Benito et al. 2002). The CCT complex is composed of eight subunits, arranged in two back-to-back rings such that they form a cavity (Seixas, Casalou et al. 2003). Unfolded tubulin binds to the apical domain of the CCT chaperonin and enters the cavity, after which the CCT subunits bind ATP and undergo large conformational changes (Valpuesta, Martin-Benito et al. 2002). The tip of the apical domain closes the entire structure and traps the unfolded protein, thus isolating it from the cytosol, which mediates folding into a quasi-native form (Valpuesta, Martin-Benito et al. 2002).

Once the tubulin monomers are partially folded by the CCT complex, they are released from the CCT cavity to a set of five folding cofactors. Cofactor B (TCB) binds to  $\alpha$ -tubulin while  $\beta$ -tubulin is captured by cofactor A (TCA). A partially folded  $\alpha$ tubulin is transferred from TCB to cofactor E (TCE) and similarly,  $\beta$ -tubulin is

transferred from TCA to cofactor D (TCD). Next, TCE- $\alpha$ -tubulin forms a complex with TCD- $\beta$ -tubulin and cofactor C (TCC), which brings the monomers together. The three cofactors act as GTP-activating proteins and mediate the hydrolysis of the GTP bound to  $\beta$ -tubulin that is associated with the release of an  $\alpha\beta$ -tubulin heterodimer (Beghin, Galmarini et al. 2007). The released dimers have GDP-loaded  $\beta$ -tubulin, but an exchange back to GTP occurs before the dimers can be incorporated into microtubules (Nogales 2000; Stephan, Vaughan et al. 2007).

In addition to their function in folding of tubulin dimers, some components of the above mentioned complexes are implicated in additional functions that could be distinct from the tubulin folding reactions that occur before dimers are assembled into microtubules. More precisely, components of CCT and cofactors have been associated with microtubules including those present in cell projections. The CCT $\alpha$  subunit is present at the ends of axons in developing neurites and associates with un-polymerized, completely folded actin monomers, where it could be involved in the transport of actin to the remote areas of the cell or in actin polymerization (Roobol, Holmes et al. 1995).  $CCT\alpha$  is also associated with the centrosomes of various mammalian cell lines where it is proposed to participate in nucleation of microtubules (Brown, Doxsey et al. 1996). TCD is enriched at the centrosome and its depletion results in mitotic defects without affecting the levels of dimeric tubulin, suggesting that TCD has a centrosomal function in mitosis (Cunningham and Kahn 2008). In addition, some lineages have proteins closely related to CCT or cofactor subunits that have microtubule-associated functions. For example, the retinitis pigmentosa gene 2 (RP2) has a domain that is related to TCC and mutations in RP2 cause retinitis pigmentosa, a disease characterized by degeneration of photoreceptor

cells (Bartolini, Bhamidipati et al. 2002). RP2 localizes to the basal body in *T. brucei* and its downregulation through RNAi disrupts the structure of axonemal microtubules, pointing to a role in ciliary assembly and/or maintenance (Stephan, Vaughan et al. 2007). A ciliopathy called the Bardet-Biedl Syndrome (BBS) is a developmental disorder resulting from abnormal functioning of cilia and/or basal bodies, caused by mutations in one or more of the 12 known BBS genes (Mykytyn and Sheffield 2004). BBS6 protein shares domain homology with CCT $\alpha$ , one of the subunits of the CCT chaperonin (Kim, Ou et al. 2005). In mammalian cells, an anti-BB6 antibody detected BBS6 at the centrosome and basal body region as well as at the mid-body in dividing cells, where its RNAi-mediated silencing resulted in defective cytokinesis due to incomplete abscission of the bridge between the two dividing cells (Kim, Ou et al. 2005).

TCB was the fifth cofactor to be discovered as an activity that increases the efficiency of heterodimer formation from monomeric tubulin in an in vitro folding reaction (Tian, Lewis et al. 1997). TCB is well conserved in all organisms including ciliated and non-ciliated species.

There are three functional domains in TCB namely, the N-terminal ubiquitin-like domain (Ubl), the central coiled-coil domain and the C-terminal CAP-Gly domain (Radcliffe and Toda 2000). The 3-dimensional structure of the C-terminal domain of TCB was determined by X-ray crystallography, which showed the presence of a well conserved microtubule-binding domain, the CAP-gly domain (Radcliffe and Toda 2000; Li, Finley et al. 2002). NMR spectroscopy determined that there was a ubiquitin-like (Ubl) domain at the N-terminus of TCB (Lytle, Peterson et al. 2004) (Grynberg, Jaroszewski et al. 2003). The central region of the protein contains a coiled-coil domain

required for the stability of cofactor B as well as for efficient folding of  $\alpha$ -tubulin (Radcliffe and Toda 2000). Interestingly, the other  $\alpha$ -tubulin cofactor, TCE, also contains the same two terminal domains as TCB, except in a reverse order such that the CAP-gly domain is at the N-terminal and the ubiquitin-like domain is at the C-terminal (Grynberg, Jaroszewski et al. 2003). It has also been postulated that the two  $\alpha$ -tubulin cofactors interact with each other via their N-terminal Ubl domains (Kortazar, Fanarraga et al. 2007).

Most of the data on the functional analysis of domains of TCB have been performed in fission yeast, where this protein is required for viability. The Ubl domain and the central coiled-coil regions are sufficient for the function of TCB as shown in fission yeast, where a construct lacking the C-terminal CAP-gly domain was able to rescue the lethality of the TCB gene knockout (Radcliffe and Toda 2000). It has been hypothesized that the Ubl domain is not involved in interactions with  $\alpha$ -tubulin and TCE (Lytle, Peterson et al. 2004; Kortazar, Fanarraga et al. 2007). The Ubl domain also affects the stability of TCB by regulating its degradation. Gigaxonin mediates the degradation of TCB via the ubiquitin-proteasome pathway (Wang, Ding et al. 2005). The kelch repeats present near the C-terminal end of gigaxonin physically interact with the N-terminal Ubl domain of TCB, and mediate the poly-ubiquitination of TCB, which in turn leads to its degradation by the proteasome (Wang, Ding et al. 2005). A deficiency in the gigaxoninmediated degradation leads to accumulation of TCB, which promotes microtubule disassembly, likely by lowering the levels of soluble tubulin below the critical concentration (Cc) for assembly (Wang, Ding et al. 2005). The N-terminal and the central coiled coil domains contain important amino acids that are phosphorylated by the p-21

activated kinase 1 (Pak1) (Vadlamudi, Barnes et al. 2005). The study shows that in mammalian cells, endogenous TCB is enriched at the newly formed microtubules and a knockdown of either Pak1 or TCB leads to decreased microtubule polymerization, suggesting that the phosphorylation of TCB by Pak1 is required for the formation of microtubules (Vadlamudi, Barnes et al. 2005). TCB also undergoes nitration in vivo. While phosphorylation of TCB by Pak1stimulates microtubule assembly, nitration on key tyrosine residues, also near the N-terminus, retards microtubule growth (Rayala, Martin et al. 2007). Inducing nitration of tagged TCB with a nitric oxide donor in mammalian cells resulted in a reduction of the signal for newly polymerized microtubules as indicated by an antibody against tyrosinated- $\alpha$ -tubulin (Rayala, Martin et al. 2007). Overexpression of TCB in HeLa cells leads to microtubule depolymerization, and the depolymerization effect increases when TCE is co-expressed (Kortazar, Fanarraga et al. 2007). The study proposed a model where the two cofactors interact with each other through their Ubl domains sequester  $\alpha$ -tubulin from the tubulin heterodimers and guide it for refolding or degradation (Kortazar, Fanarraga et al. 2007). These data indicate a connection between TCB and microtubule dynamics/regulation.

TCB could also play a role in early neuronal differentiation as it is present at the very ends of the growth cones in neuroblast cells, a region characterized by rapid changes in the microtubule and actin cytoskeletons (Dent and Kalil 2001; Lopez-Fanarraga, Carranza et al. 2007). Thus, cofactor B might be negatively regulating axonal growth since its overexpression leads to neuronal degeneration and knockdown by siRNA results in longer axons (Lopez-Fanarraga, Carranza et al. 2007). Hence, it can be concluded that the Ubl and the CAP-gly termini of cofactor B are involved in functions other than their

known, canonical roles and that apart from folding  $\alpha$ -tubulin, cofactor B is also involved in regulating microtubule dynamics.

#### Results

#### TCB of *Tetrahymena* is enriched in cilia of an IFT mutant

An unexpected connection between ciliary assembly and the tubulin folding pathway was revealed by our study of suppressor mutants of the *IFT52* knockout in *Tetrahymena*. IFT52 is a well-conserved protein that is involved in the intraflagellar transport (IFT) pathway required for the assembly and maintenance of most cilia (Deane, Cole et al. 2001). The IFT complexes A and B are oligomeric platforms whose components were first isolated/purified from *Chlamydomonas* flagella using density sucrose gradients (Cole, Diener et al. 1998). Complex A is composed of at least six IFT proteins and is implicated in retrograde transport, while complex B has at least eleven IFT proteins and primarily affects the anterograde transport into the cilium (Cole 2003).

IFT52, a complex B protein has been studied in a variety of organisms, such as *Chlamydomonas*, *C. elegans* and *Tetrahymena*. A knockout of *IFT52* gene in *Tetrahymena* resulted in non-motile cells due to their inability to assemble cilia (Brown, Fine et al. 2003). *Tetrahymena* cells use ciliary motility to complete cytokinesis by a mechanism called rotokinesis (Brown, Hardin et al. 1999). In the absence of an external mechanical force (such as flask shaking), many *IFT52* knockout cells (IFT52 $\Delta$ ) are unable to complete cytokinesis and hence frequently formed multi-nucleated cells called 'monsters'. Surprisingly, in some cultures of *IFT52* knockout cells, slowly moving cells

appeared (Brown, Fine et al. 2003). These IFT52 $\Delta$ sm suppressors have sparse, short and partially functional cilia due to which the cells are motile and able to complete cytokinesis (Figure 3.2C). Originally, eight IFT52 $\Delta$ sm suppressors were studied and among them one strain evolved towards a more complete suppression and was named IFT52 $\Delta$ mov (Figure 3.2D) (Brown, Fine et al. 2003). All these suppressions occurred spontaneously (without any mutagen treatment) upon mating of *IFT52* heterokaryons and at the surprisingly high frequency of 3%. The IFT52 $\Delta$ sm suppressors are able to assemble short cilia either under hypoxia (either by growing cells at high density or by removing oxygen) or at a low (room) temperature, whereas the IFT52 $\Delta$ mov suppression phenotype is non-conditional (Brown, Fine et al. 2003). The first level suppression (IFT52 $\Delta$  to IFT52 $\Delta$ sm) is intragenic and results in expression of a mutated Ift52p protein, as explained in more detail in Chapter 4.

Here we explore further the biochemical consequences of IFT52 $\Delta$  suppression by comparing the protein composition of cilia between suppressor and wildtype cells using two-dimensional protein gels. The IFT52 $\Delta$ sm suppression phenotype is expressed at specific conditions, namely in hypoxia and at the lower temperature. The suppression could be associated with changes in the components involved in signal transduction. Thus, we analyzed the protein composition of suppressor cilia, with particular emphasis on the membrane + matrix (detergent-soluble) fraction that contains components associated with the plasma membrane. Short cilia from both the IFT52 $\Delta$ sm (grown under hypoxia and low temperature) and IFT52 $\Delta$ mov (grown under standard conditions) cells (~3x10<sup>8</sup> cells) were isolated by the calcium shock method (adapted from (Rosenbaum and Carlson 1969)). Cilia were fractionated using a detergent (NP-40) into an insoluble

fraction containing axonemal microtubules and attached proteins, and a soluble membrane plus matrix fraction. Samples were loaded for two-dimensional gel analysis followed by silver staining. The two-dimensional gel analysis of the soluble fraction of cilia from both suppressors revealed a dramatic increase in the intensity of a single spot (Figure 3.4A). A piece of the gel containing the protein spot was cut out and subjected to trypsin digestion followed by HPLC separation and Edman degradation of several peptides. Peptide sequences obtained after Edman degradation were used in a BLAST search in the NCBI database and four out of the five peptides identified the same protein sequence, encoded by the TTHERM\_00691610 gene of *Tetrahymena thermophila*. The identity of this protein was determined as *Tetrahymena* tubulin folding cofactor B (Tcb1p).

There is evidence of other members of the tubulin folding pathway in cilia. For example, polyclonal antibodies detected the CCT subunits  $\alpha$ ,  $\delta$ ,  $\varepsilon$  and  $\eta$  in the cilia of *Tetrahymena pyriformis*, suggesting a role in guiding tubulin for building axonemes (Seixas, Casalou et al. 2003). CCT $\alpha$  was also detected in sea urchin and rabbit tracheal cilia by an anti-CCT $\alpha$  antibody, while CCT $\varepsilon$  was identified by mass spectrometry of flagellar proteins of *Chlamydomonas* (Stephens and Lemieux 1999; Pazour, Agrin et al. 2005). From most of the studies performed so far, the main function of TCB is to fold  $\alpha$ tubulin. Additionally, it is expected that the folding reactions occur in the cytoplasm, where most of the protein translational machinery is present. However, recently Lopez-Fanarraga and colleagues have used a TCB antibody to detect TCB expression in several areas of the developing neo-natal brain and TCB was highly concentrated in the epithelial (epyndymal) cells lining the ventricles of adult brains (Lopez-Fanarraga, Carranza et al.

2007). In cultured, murine epyndymal cells cofactor B was observed at the basal bodies and as spots along the cilia. The authors of this study investigate a further role for cofactor B in regulating microtubule dynamics in the extensions of cells, namely in developing neuronal axons (Lopez-Fanarraga, Carranza et al. 2007). These data support a possibility that TCB functions inside cell projections, and specifically inside cilia. On the other side, our observations suggest that there is a link between TCB and IFT pathway.

Although other folding related chaperones have been localized to cilia, their precise cilia-specific functions have not been discovered. Importantly, the form in which tubulin is transported into the cilium is not known. It is possible that the entire tubulin folding pathway is present inside the cilium and it is responsible for maintaining the heterodimers prior to their incorporation into the axoneme. Another possibility is that the chaperones and folding proteins that are involved in folding other (non-tubulin) ciliary components. Since it was upregulated in an IFT mutant, Tcb1p could be involved in the transport of tubulin into the cilium via the IFT pathway. Indeed, TCE has been implicated in the routing of tubulin that is made in the cell body into the axons of spinal motors neurons. In cell cultures of motor neurons, incorporation of new GFP-tagged  $\alpha$ -tubulin at the distal ends of the axons is impaired while its levels in the cell body are near wildtype levels during RNAi of TCE (Schaefer, Schmalbruch et al. 2007). Yet another possibility is that TCB is involved in the turnover of tubulin that is already incorporated into the axoneme, based on the depolymerizing effects when over-expressed (Kortazar, Fanarraga et al. 2007). The IFT52 suppressor and cofactor B result pointed to an as yet unknown connection between the tubulin folding and IFT pathways and hence we pursued the function of Tcb1p and its precise role in cilia.

#### Tubulin folding cofactor B (Tcb1p) is essential in Tetrahymena

The requirement for the different folding cofactors is species-specific. Except for cofactor A, all the others are required for survival in *S. pombe* (Lopez-Fanarraga, Avila et al. 2001). Strikingly, none of the cofactors are essential for viability in *S. cerevisiae* (Radcliffe, Hirata et al. 1999; Lopez-Fanarraga, Avila et al. 2001). In *Arabidopsis* and in certain mammalian cell lines (MC-7 and HeLa cells) TCA is required for viability (Steinborn, Maulbetsch et al. 2002; Nolasco, Bellido et al. 2005). Interesting, in *Arabidopsis*, the other four cofactors (B-E) are not essential for viability (Steinborn, Maulbetsch et al. 2002). Mutations in TCE cause progressive motor neuronopathy in mice due to which they die within 4 to 6 week after birth (Martin, Jaubert et al. 2002).

The 2D protein gel (Figure 3.4A) detected Tcb1p in cilia, organelles that are not essential for survival in *Tetrahymena*. Thus a knockout of the gene might result in a viable mutant with abnormal cilia if it is not required for life. It was therefore reasonable to test the function of Tcb1p by eliminating its gene using DNA homologous recombination. To determine the *in vivo* significance of Tcb1p in *Tetrahymena*, we constructed *TCB1* gene knockout heterokaryons, strains that are homozygous for the *neo3*-disrupted allele in the micronucleus, but have wildtype macronuclei. Upon conjugation, a new macronuclear genome forms that is derived from the zygotic micronucleus and the disruption phenotype is expressed. The two *TCB1* knockout heterokaryons were starved, mixed for conjugation and pairs were isolated into drops of media for further observation. As a control, two wildtype strains of different mating types were also mixed for conjugation and isolated into drops. Out of the 48 pairs that were

isolated, approximately 14 drops had cells that appeared to be phenotypically wildtype, in that they swam as vigorously as the wildtype control cells. Such a result is not uncommon and is generally observed as a result of abortive conjugation where conjugants do not develop new macronuclei and fail to express the micronucleus-based genome.

Yet, majority of the drops (34) contained cells that failed to give rise to vigorous clones and were assumed to represent TCB1 knockout progeny (TCB1D). The knockout cells were moving slowly, underwent an average of 2 cell divisions and became misshaped and paralyzed (Figure 3.3A). In approximately 56 hrs post-heterokaryon mixing

(phm), virtually all of the TCB1D cells completely stop moving, become round and die (n = 447, few drops had more than 8 cells, range between 2 and 16 cells). Immunofluorescence assay of knockout cells at approximately 44 phm using anti- $\alpha$ -tubulin antibody revealed that the tubulin cytoskeleton was highly abnormal (Figure 3.3B). The TCB1 $\Delta$  cells showed decreased levels of  $\alpha$ -tubulin signal when compared to wildtype cells and they had lost almost all cell body microtubules. A similar result was observed in the strain of *S. pombe* lacking TCB, where cells lost their shape, had disorganized cytoskeletons and eventually died (Radcliffe, Hirata et al. 1999). Loss of cell body microtubules indicated that in *Tetrahymena* as in other organisms, the main role of Tcb1p is in folding  $\alpha$ -tubulin. Disruption in the folding leads to compromised microtubule function which causes cell death very fast and thus we were not able to study a separate ciliary or non-folding functions of Tcb1p.

#### Rescue of *TCB1* knockout using tagged versions of Tcb1p

To further verify that the lethal phenotype described in the previous section is specific to TCB1 knockout, we rescued mating TCB1 knockout heterokaryon by introduction of tagged versions of Tcb1p. A variety of fragments encoding tagged versions of *TCB1* were constructed to target the tag to either a non-native or native locus within the *Tetrahymena* genome. The non-native locus used for expression was a  $\beta$ tubulin gene (BTU1) and the tagged form of TCB1 was under the control of the cadmiuminducible metallothionein promoter (MTT1). Biolistic transformation with plasmids carrying fragments encoding Tcb1p with three different C-terminal tags, namely GFP, HA and 3xHA were used for rescue lethality of mating TCB1 knockout heterokaryons, while control (mock) transformations without any DNA were performed as a control (Figure 3.4 and 3.5). The rescue efficiencies for the tags expressed in the non-native (*BTU1*) as well as the native loci are in Table 3.1. Thus, all forms of the tagged protein were able to rescue the lethal phenotype, confirming that the lethal phenotype observed is specific to disruption in *TCB1* and that Tcb1p is indeed an essential protein and that none of the tags interferes with the normal folding and functioning of Tcb1p. The rescues resulted in viable and motile cells where the only functional version of Tcb1p available to the cell was the tagged version of the protein, expressed either from a non-native or an endogenous locus.

Cells rescued with a *TCB1-GFP* construct expressed in the *BTU1* locus showed a diffused, cell body localization of Tcb1p-GFP without an obvious presence in cilia, even when its expression was induced by cadmium (data not shown). Tcb1p-HA expressed in a similar manner also had a diffused cytoplasmic signal. Immunofluorescence signal was

detected using a monoclonal anti-HA antibody, yet the signal in cells expressing the tag was only slightly above the background level seen in control wildtype cells (data not shown). This pattern of localization of tagged TCB is also found in S. pombe, S. *cerevisiae* and in mammalian cells in which overexpression leads to loss of microtubules (Radcliffe and Toda 2000; Kortazar, Fanarraga et al. 2007). Immunofluorescence assays with anti-HA antibody for cells rescued with TCB1-3xHA from the non-native locus indicated a strong ciliary signal, which was present in the absence as well as in the presence of cadmium (Figure 3.4B). Upon three hours of induction of the *MTT1* promoter by cadmium, the signal from cilia was brighter by several folds compared to un-induced or wildtype cells. Even in un-induced rescued transformants, the Tcb1p-3HA signal was seen in cilia, which was above the non-specific signal observed in wildtype cells. This is not completely unexpected as the MTT1 promoter is known to be "leaky" that is it has a basal level of expression without cadmium (Brown, Fine et al. 2003). Despite the strong overexpression of all three tagged versions of Tcb1p, the cells were viable, motile and did not have compromised microtubules. This is a distinct different between other models (S. *pombe* and various mammalian cells) and *Tetrahymena*, where the over-expression of Tcb1p is lethal and has a negative effect on the ability of the cell to grow and divide (Radcliffe, Hirata et al. 1999; Wang, Ding et al. 2005; Kortazar, Fanarraga et al. 2007; Lopez-Fanarraga, Carranza et al. 2007).

The above studies show that under certain condition, Tcb1p localizes to cilia. There is a limitation of this data however, in that even without cadmium induction, the tagged Tcb1p could be present at excessive levels and thus the ciliary localization could be an artifact. Thus it was important to see whether Tcb1p is a ciliary protein when

expressed in its native locus under its own promoter and regulatory sequences. HA and 3xHA tagged versions of *TCB1* were introduced at the native locus by rescue of mating knockout heterokaryons as before and transformants were selected based on their viability. Immunofluorescence with anti-HA antibodies for both HA and 3HA tags in the native locus showed a general, diffused cytoplasmic signal for Tcb1p and no obvious signal in cilia (data not shown). Again, the signal in cells expressing the tag was only slightly above that in control wildtype cells, indicating non-specific binding of the antibody. It is possible that Tcb1p localizes to assembling cilia, especially since other studies have shown TCB co-localization with newly formed microtubules as well as at the very tips of neuronal growth cones (Vadlamudi, Barnes et al. 2005; Lopez-Fanarraga, Carranza et al. 2007). To increase the proportion of assembling cilia, cells were deciliated using the standard pH shock protocol and stained at various time points during cilia regeneration with anti-HA antibody. Typically, in *Tetrahymena* cilia fully regenerate in 3 hours. After 60 minutes of cilia regeneration, Tcb1p-HA was observed within cilia, especially at the ciliary tips (Figure 3.4C). The ciliary signal was above the non-specific signal seen in wildtype cells. Unfortunately, this result was not reproducible and the ciliary signal was not detected in the subsequent experiments.

We performed western blots on the ciliary and cell body fractions of cells rescued with *TCB1-HA* and used an anti-HA antibody to distinguish between the ciliary and cell body pools of this protein. Three types of fractions were analyzed, whole cells, cell bodies and cilia. As expected a strong band of the right size was seen in the whole cell and cell body fractions but a very faint band was observed in the lane corresponding to cilia (Figure 3.5A). The faint Tcb1p-HA signal from cilia might be a result of

contamination during the process of harvesting cilia. We used an anti-polyglycylation antibody that in addition to tubulin recognizes a 94kDa cell body specific (ER-targeted) protein as a control for cell body contamination and observed that longer exposures revealed a faint 94kDa band in the lanes containing ciliary fractions (data not shown). We repeated the experiment with more a stringent protocol for isolation of cilia. Unfortunately, in the new western blots, the previously visible strong signal for Tcb1p-HA from the whole cell and cell body fractions was no longer visible (data not shown). The 3xHA tagged version of Tcb1p was able to localize the protein to cilia under nonnative conditions. However, under native conditions the signal was diffused and cytoplasmic. Also, the protein was not seen at ciliary tips in regenerating cilia (data not shown).

Thus, were not able to obtain convincing evidence that Tcb1p localizes to cilia in *Tetrahymena* cells that have a functional IFT pathway.

#### Analysis of Tcb1p in IFT5∆sm cells

The first indication that cofactor B is a ciliary protein came from the study of IFT52 suppressor mutants (IFT52 $\Delta$ sm). It is possible that Tcb1p is present in wildtype cilia at very low levels and accumulates in cilia in the IFT52 $\Delta$ sm cells. Alternatively, Tcb1p could be a non-ciliary protein and could relocate to cilia in response to an IFT deficiency. To determine whether Tcb1p accumulates in cilia of an IFT52 mutant strain, we targeted *TCB1-GFP* to the *TCB1* locus within IFT52 $\Delta$ sm and wildtype cells. The construct also carried a linked drug resistance cassette such that the transformants could be selected based on paromomycin resistance. The *IFT52* knockout heterokaryons carry a

*neo2* resistance marker that confers paromomycin resistance upon conjugation. The IFT52 $\Delta$ sm-Tcb1p-GFP and the WT-Tcb1p-GFP transformants were grown under paromomycin selective pressure to promote complete replacement of all wildtype *TCB1* with the *TCB1-GFP* version by phenotypic assortment (Merriam and Bruns 1988). Both types of transformants were grown at conditions that allow for expression of the suppressor phenotype in IFT52 $\Delta$ sm cells and formation of cilia: without shaking at high cell density (low oxygen) and at room temperature.

The two-dimensional gels of ciliary membrane-matrix fractions of IFT52 $\Delta$ sm and IFT52 $\Delta$ mov suppressors showed a dramatic increase in the levels of Tcb1p as compared to a similar fraction of wildtype cilia (Figure 3.5B). Yet, GFP signal on western blots of IFT52 $\Delta$ sm-Tcb1p-GFP cells did not show such an increase in the signal in the cell body or whole cell extracts when compared to WT-Tcb1p-GFP cells. In fact there was no signal in the ciliary fraction, even after loading 10x the amount of ciliary protein.

Next, we imaged the GFP-signal from IFT52Δsm-Tcb1p-GFP and WT-Tcb1p-GFP cells. Contrary to the initial 2D gel data, GFP signal from IFT52Δsm-Tcb1p-GFP cells was minimal, even in the cell body. There was no marked increase in the IFT52Δsm-Tcb1p-GFP cells compared to WT-Tcb1p-GFP cells. Also, Tcb1p-GFP could not be detected in the cilia in both types of transformants, including cells with regenerating cilia (data not shown).

## Discussion

An important and unanswered question in cilia biology is in what form in tubulin is transported into and inside cilia. An apparent upregulation of TCB in the IFT52 conditional mutant cilia has presented an apparent connection between the IFT and tubulin maturation pathways, suggesting that TCB participates in tubulin transport. This idea was pursued to find either the form of tubulin destined for cilia or a novel, ciliary function for Tcb1p. Thus, we pursued studies on the significance and localization of Tcb1p, to uncover its potential ciliary functions.

A single study detected TCB near the basal bodies and in cilia of the epyndymal cells that form the lining of brain ventricles (Lopez-Fanarraga, Carranza et al. 2007). The authors speculated that TCB might play an important role in the growth and maintenance of axonemal microtubules, but did not further expand on any specific function (Lopez-Fanarraga, Carranza et al. 2007). For functional analysis of the protein, specifically in the context of cilia we generated knockout of *TCB1* in *Tetrahymena*. We show that TCB is an essential protein in *Tetrahymena* and that its activity is required for assembly or maintenance of cell body microtubules. Given that Tcb1p is essential in *Tetrahymena*, studying potential ciliary functions of TCB in this model is problematic. For example, there are fewer cilia in cells that are progeny of TCB1 knockout heterokaryons (Figure 3.3) and the remaining cilia seem shorter, but these and any additional defects could be attributed to inability to fold and dimerize tubulin cilia-destined tubulin in the cell body. Thus, the Tcb1p knockout is not informative regarding the ciliary functions of TCB in *Tetrahymena*.

However, we have used the TCB1 knockout heterokaryons to express tagged versions of Tcb1p and determine whether this protein localizes to cilia. While we detected tagged Tcb1p in cilia when the protein was overexpressed, we could not reproducibly detect Tcb1p in cilia when expressed in the native locus under its own promoter. Furthermore, using both microscopic techniques and fractionation combined with western blotting, we could not detect an obvious accumulation of tagged Tcb1p in cilia in the IFT52 $\Delta$ sm cells grown under suppressing conditions. Thus, we failed to confirm the initial 2D gel data showing increased levels of Tcb1p in the soluble fraction of cilia. It is possible that during isolation of cilia for the 2D gels there was contamination from the cytosol in the sample. Alternatively, the tags used for rescue and subsequent immunolocalization interfered with the correct folding of the protein or affected its localization. Hence, despite using several tags and fixation techniques we were unable to localize Tcb1p to cilia under conditions of native expression. Thus, either Tcb1p is exclusively a cell body protein in Tetrahymena, or it is present in cilia at the levels below the detection that its primary role is in the tubulin folding pathway.

## **Materials and Methods**

#### Strains and Cultures

*Tetrahymena thermophila* strains were grown at 30°C (IFT52∆sm and its rescue were grown at room temperature) with shaking, in either SPP medium (Gorovsky, Yao et al. 1975) with an antibiotic-antimycotic mixture (Invitrogen, Carlsbad, CA). Wildtype

strains CU428 and CU522 were obtained from the *Tetrahymena* Stock Center, (Cornell University, Ithaca, NY).

#### Disruption of TCB1

The coding region of *TCB1* (TTHERM\_00691610) was identified in the *Tetrahymena* Genome Database by blast searches using the protein sequence obtained after sequencing. Using *Tetrahymena* genomic DNA as a template, two non-overlapping fragments of *TCB1* were amplified using the following primer pairs:

5'-AGCAATCGAATACAACATCCTA-3', 5'-GTGTCTAACCCCCAAATATCATA-3' and 5'-GATAAAGTGGAAGTTGGTGATT-3', 5'-ACATTCATCTCCACATTTCCAA-3'

Using restriction site sequences that were incorporated near the 5' ends of the above primers and the two fragments were subcloned into pTvec-Neo3 (Shang, Song et al. 2002), so that the *neo3* gene was positioned in a reverse transcriptional orientation. The targeting fragments were designed to flank almost the entire gene (~0.7 kb) of *TCB1*. The resulting targeting fragment containing *TCB1-neo3* was separated from the rest of the plasmid using SacI and ApaI. CU428 and B2086 cells at 2 x  $10^5$  cells/ml were starved at 30°C for 20 hours in 10 mM Tris-HCl buffer pH 7.5, mixed for conjugation and subjected to macronuclear biolistic transformation (Cassidy-Hanley, Bowen et al. 1997). The knockout cells were obtained by several crosses as described in (Sharma, Bryant et al. 2007).

## Tagging of Tcb1p

The coding sequence of *TCB1* was amplified using primers:

5'-TGCACTCAGTTTAAGCCCT-3' and 5'-ATTTAGTTGAAGATGAAGATGAAGTT-3' The resulting fragment was digested using HindIII and MluI followed by ligation into pMTT1-Nrk2-GFP for C-terminal tagging with GFP (Wloga, Camba et al. 2006). Nrk2 was replaced in pMTT1-GFP-Nrk2-N-terminal by HA tag using MluI and BgIII and the GFP was then replaced by TCB1 (coding region) using HindIII and MluI to get pMTT1-TCB1-HA tag (Wloga, Camba et al. 2006). To get pMTT1-TCB1-3XHA tag, 3HA was amplified from pCpfSub2-3XHA (gift from) using primers: 5'-

GATTACCCTTACGATGTTC-3' and 5'-GATTACGCTTGAGGTTACCACGCGT-3'.

For inserting HA tag in the native locus, a fragment containing the 5'UTR along with the coding sequence of *TCB1* was amplified using primers: 5'-

GTGAATTTTTCTATTTATTGTGC-3' and 5'- ATTTAGTTGAAGATGAAGATGAAGTT-3' and subcloned into pGEM-TEasy vector (Promega Corp., Madison, WI). The TCB1-HA sequence was amplified from pMTT1-TCB1-HA using primer: 5'-

TGCACTCAGTTTAAGCCCT-3' and an HA tag-specific primer: 5'-

ATCAGATCTTCAAGCATAATCAGGAACATCATAAGGATAACGCGTGGT-3' and the product was also subcloned into pGEM-TEasy vector. The insert (TCB1-HA) was digested from its vector and cloned into the vector containing the 5'UTR and coding sequence of TCB1, using EcoRV and NsiI. A fragment containing the 3'UTR of *TCB1* was amplified using primers: 5'-AAACAGCATAATTTATTTATCCAACA-3' and 5'-GGTAAATGAGGAACCGCGT-3'. The fragment (3'UTR) was cloned into the vector containing 5'UTR, coding region of *TCB1* and the HA tag in frame, using BgIII and SalI. For transforming *Tetrahymena*, pTCB1-HA-Native was digested using NcoI and SalI. The 3HA tag was amplified from pCpfSub2-3XHA (gift from) using primers: 5'-GATTACCCTTACGATGTTC-3' and 5'-GATTACGCTTGAGGTTACCACGCGT-3' and subcloned into the pTCB-HA-Native vector using MluI and BgIII. For transforming *Tetrahymena*, pTCB1-3XHA-Native was digested using HincII and SacII.

### Rescue of TCB1-knockout

*TCB1* knockout heterokaryons cells (at 2 x  $10^5$  cells/ml) were starved overnight at room temperature, mixed and incubated at 30°C for 20 hours for conjugation and biolistically transformed as described above, suspended in SPP medium with 1.5 µg/ml CdCl<sub>2</sub>, plated in 96-well and incubated at 30°C for 3 days. Transformants were identified based on their viability and cell motility. As a negative control, a mock biolistic transformation was performed without plasmid DNA.

*Tagging of Tcb1p with GFP (native locus) and transformation of IFT52Δsm cells* A fragment containing the 3'UTR of *TCB1* was amplified using primers: 5'-TTTATCCAACACATATTAATTATC-3' and 5'-CTTTTAAATTAGTGATCCAGTTA-3' and subcloned into pKin13C-GFP-Native (K. Vasudevan and J. Gaertig, unpublished data) using ClaI and SacI. A fragment containing the 5'UTR and the coding region of *TCB1* was amplified using primers: 5'-GTGAATTTTCTATTTATTGTGC-3' and 5'-ATTTAGTTGAAGATGATGAAGTT-3' and cloned into pKin13C-GFP-Native with 3'UTR of *TCB1* using SacII and MluI. For transforming *Tetrahymena*, pTCB1-GFP-Native was digested using AatII (a site on both sides of the targeting fragment). For transformation: IFT52 $\Delta$ sm and wildtype cells (each 7.5 x 10<sup>6</sup> cells) were grown in SPP at room temperature without shaking. The cells were transformed biolistically and suspended in SPP with 1.5 µg/ml CdCl<sub>2</sub> and 90 µg/ml paromomycin for selection and incubated at room temperature for 3 days. The transformants were grown on increasing paromomycin concentrations from 90 µg/ml to 300 µg/ml for replacement of endogenous gene with the GFP-tagged version.

#### Microscopy

For immunofluorescence with 12G10 monoclonal anti-α-tubulin antibody (1:50 dilution) and SG polyclonal anti-tubulin antibodies, ~100 *Tetrahymena* cells were simultaneously fixed and permeabilized with a mixture (1:1) of 2% paraformaldehyde and 0.5% Triton-X-100 in PHEM buffer. For staining with monoclonal anti-HA antibody (Sigma-Aldrich Inc., St. Louis, MO) or polyclonal anti-GFP antibody (Abcam Inc., Cambridge, MA), ~100 *Tetrahymena* cells were fixed with 2% paraformaldehyde for 10 seconds and permealiblized with 0.5% Triton-X-100 in PHEM buffer. Fixed cells were air-dried at 30°C and processed for immunofluorescence labeling as described in (Gaertig, Cruz et al. 1995). The secondary antibody used was either goat-anti-mouse or goat-anti-rabbit FITC conjugate (1:200, Zymed). Cells were observed under a Leica TCS SP confocal microscope.

## Western blots

*Tetrahymena* cells were grown in SPP, washed with Tris-HCl buffer pH 7.5 and suspended in Tris-HCl (pH 7.5)-10mM CaCl<sub>2</sub> with protease inhibitors and processed for cilia isolation as described in (Wloga, Rogowski et al. 2008). Total proteins from  $6 \times 10^5$  whole cells as well as cell bodies and cilia from  $6 \times 10^5$  cells were used per lane and blots were prepared as described in (Wloga, Rogowski et al. 2008) with following antibodies: monoclonal anti-HA antibody (1:1000) and polyclonal anti-GFP (1:6000).

## Two-dimensional gels

Cilia were isolated by the calcium-shock method (adapted from (Rosenbaum and Carlson 1969)).  $\sim 3 \times 10^8$  cells grown in SPP were concentrated into cold 50 ml conical tubes and suspended in 10 ml of ice-cold solution A (10 mM EDTA and 50 mM sodium acetate, pH 5.0). After 30 seconds, ice-cold water was added and cells were gently mixed and incubated for 1 min. 0.5 ml of 0.2 M CaCl<sub>2</sub> was added, cells were gently mixed, incubated for 4 min and centrifuged at 1500 g for 5 min at 4°C. The pellet contains cell bodies. The supernatant containing cilia was centrifuged at 11,000 g for 15 min at 4°C to pellet cilia.

## References

Bartolini, F., A. Bhamidipati, et al. (2002). "Functional overlap between retinitis pigmentosa 2 protein and the tubulin-specific chaperone cofactor C." J Biol Chem 277(17): 14629-34.

- Beghin, A., C. M. Galmarini, et al. (2007). "Tubulin folding pathways: implication in the regulation of microtubule dynamics." <u>Curr Cancer Drug Targets</u> 7(8): 697-703.
- Brown, C. R., S. J. Doxsey, et al. (1996). "Molecular chaperones and the centrosome. A role for TCP-1 in microtubule nucleation." J Biol Chem 271(2): 824-32.
- Brown, J. M., N. A. Fine, et al. (2003). "Hypoxia regulates assembly of cilia in suppressors of Tetrahymena lacking an intraflagellar transport subunit gene." <u>Mol</u> <u>Biol Cell</u> 14(8): 3192-207.
- Brown, J. M., C. Hardin, et al. (1999). "Rotokinesis, a novel phenomenon of cell locomotion-assisted cytokinesis in the ciliate Tetrahymena thermophila." <u>Cell</u> <u>Biol Int</u> 23(12): 841-8.
- Cassidy-Hanley, D., J. Bowen, et al. (1997). "Germline and somatic transformation of mating Tetrahymena thermophila by particle bombardment." <u>Genetics</u> 146(1): 135-47.
- Cole, D. G. (2003). "The intraflagellar transport machinery of Chlamydomonas reinhardtii." <u>Traffic</u> **4**(7): 435-42.
- Cole, D. G., D. R. Diener, et al. (1998). "Chlamydomonas kinesin-II-dependent intraflagellar transport (IFT): IFT particles contain proteins required for ciliary assembly in Caenorhabditis elegans sensory neurons." <u>J Cell Biol</u> 141(4): 993-1008.
- Cunningham, L. A. and R. A. Kahn (2008). "Cofactor D functions as a centrosomal protein and is required for the recruitment of the gamma-tubulin ring complex at centrosomes and organization of the mitotic spindle." J Biol Chem 283(11): 7155-65.

- Deane, J. A., D. G. Cole, et al. (2001). "Localization of intraflagellar transport protein IFT52 identifies basal body transitional fibers as the docking site for IFT particles." <u>Curr Biol</u> 11(20): 1586-90.
- Dent, E. W. and K. Kalil (2001). "Axon branching requires interactions between dynamic microtubules and actin filaments." J Neurosci 21(24): 9757-69.
- Gaertig, J., M. A. Cruz, et al. (1995). "Acetylation of lysine 40 in alpha-tubulin is not essential in Tetrahymena thermophila." J Cell Biol **129**(5): 1301-10.
- Gorovsky, M. A., M. C. Yao, et al. (1975). "Isolation of micro- and macronuclei of Tetrahymena pyriformis." <u>Methods Cell Biol</u> **9**(0): 311-27.
- Grynberg, M., L. Jaroszewski, et al. (2003). "Domain analysis of the tubulin cofactor system: a model for tubulin folding and dimerization." <u>BMC Bioinformatics</u> 4: 46.
- Gu, Y., Z. Deng, et al. (2008). "Prefoldin 6 is required for normal microtubule dynamics and organization in Arabidopsis." <u>Proc Natl Acad Sci U S A</u> 105(46): 18064-9.
- Hansen, W. J., N. J. Cowan, et al. (1999). "Prefoldin-nascent chain complexes in the folding of cytoskeletal proteins." <u>J Cell Biol</u> 145(2): 265-77.
- Hartl, F. U. and M. Hayer-Hartl (2002). "Molecular chaperones in the cytosol: from nascent chain to folded protein." <u>Science</u> 295(5561): 1852-8.
- Kim, J. C., Y. Y. Ou, et al. (2005). "MKKS/BBS6, a divergent chaperonin-like protein linked to the obesity disorder Bardet-Biedl syndrome, is a novel centrosomal component required for cytokinesis." <u>J Cell Sci</u> 118(Pt 5): 1007-20.
- Kortazar, D., M. L. Fanarraga, et al. (2007). "Role of cofactors B (TBCB) and E (TBCE) in tubulin heterodimer dissociation." <u>Exp Cell Res</u> **313**(3): 425-36.

- Li, S., J. Finley, et al. (2002). "Crystal structure of the cytoskeleton-associated protein glycine-rich (CAP-Gly) domain." J Biol Chem 277(50): 48596-601.
- Lopez-Fanarraga, M., J. Avila, et al. (2001). "Review: postchaperonin tubulin folding cofactors and their role in microtubule dynamics." <u>J Struct Biol</u> **135**(2): 219-29.
- Lopez-Fanarraga, M., G. Carranza, et al. (2007). "Tubulin cofactor B plays a role in the neuronal growth cone." <u>J Neurochem</u> 100(6): 1680-7.
- Lytle, B. L., F. C. Peterson, et al. (2004). "Solution structure of a ubiquitin-like domain from tubulin-binding cofactor B." J Biol Chem **279**(45): 46787-93.
- Martin, N., J. Jaubert, et al. (2002). "A missense mutation in Tbce causes progressive motor neuronopathy in mice." <u>Nat Genet</u> **32**(3): 443-7.
- Merriam, E. V. and P. J. Bruns (1988). "Phenotypic assortment in Tetrahymena thermophila: assortment kinetics of antibiotic-resistance markers, tsA, death, and the highly amplified rDNA locus." <u>Genetics</u> **120**(2): 389-95.
- Mykytyn, K. and V. C. Sheffield (2004). "Establishing a connection between cilia and Bardet-Biedl Syndrome." <u>Trends Mol Med</u> **10**(3): 106-9.
- Nogales, E. (2000). "Structural insights into microtubule function." <u>Annu Rev Biochem</u> **69**: 277-302.
- Nolasco, S., J. Bellido, et al. (2005). "Tubulin cofactor A gene silencing in mammalian cells induces changes in microtubule cytoskeleton, cell cycle arrest and cell death." <u>FEBS Lett</u> **579**(17): 3515-24.
- Pazour, G. J., N. Agrin, et al. (2005). "Proteomic analysis of a eukaryotic cilium." <u>J Cell</u> <u>Biol</u> **170**(1): 103-13.

- Radcliffe, P. A., D. Hirata, et al. (1999). "Functional dissection and hierarchy of tubulinfolding cofactor homologues in fission yeast." <u>Mol Biol Cell</u> 10(9): 2987-3001.
- Radcliffe, P. A. and T. Toda (2000). "Characterisation of fission yeast alp11 mutants defines three functional domains within tubulin-folding cofactor B." <u>Mol Gen</u> <u>Genet</u> 263(5): 752-60.
- Rayala, S. K., E. Martin, et al. (2007). "Dynamic interplay between nitration and phosphorylation of tubulin cofactor B in the control of microtubule dynamics."
  <u>Proc Natl Acad Sci U S A</u> 104(49): 19470-5.
- Roobol, A., F. E. Holmes, et al. (1995). "Cytoplasmic chaperonin complexes enter neurites developing in vitro and differ in subunit composition within single cells."
  <u>J Cell Sci</u> 108 ( Pt 4): 1477-88.
- Rosenbaum, J. L. and K. Carlson (1969). "Cilia regeneration in Tetrahymena and its inhibition by colchicine." <u>J Cell Biol</u> **40**(2): 415-25.
- Schaefer, M. K., H. Schmalbruch, et al. (2007). "Progressive motor neuronopathy: a critical role of the tubulin chaperone TBCE in axonal tubulin routing from the Golgi apparatus." <u>J Neurosci</u> 27(33): 8779-89.
- Seixas, C., C. Casalou, et al. (2003). "Subunits of the chaperonin CCT are associated with Tetrahymena microtubule structures and are involved in cilia biogenesis." <u>Exp</u> <u>Cell Res</u> 290(2): 303-21.
- Shang, Y., X. Song, et al. (2002). "A robust inducible-repressible promoter greatly facilitates gene knockouts, conditional expression, and overexpression of homologous and heterologous genes in Tetrahymena thermophila." <u>Proc Natl</u> <u>Acad Sci U S A</u> 99(6): 3734-9.

- Sharma, N., J. Bryant, et al. (2007). "Katanin regulates dynamics of microtubules and biogenesis of motile cilia." <u>J Cell Biol</u> 178(6): 1065-79.
- Steinborn, K., C. Maulbetsch, et al. (2002). "The Arabidopsis PILZ group genes encode tubulin-folding cofactor orthologs required for cell division but not cell growth." <u>Genes Dev</u> 16(8): 959-71.
- Stephan, A., S. Vaughan, et al. (2007). "An essential quality control mechanism at the eukaryotic basal body prior to intraflagellar transport." <u>Traffic</u> 8(10): 1323-30.
- Stephens, R. E. and N. A. Lemieux (1999). "Molecular chaperones in cilia and flagella: implications for protein turnover." <u>Cell Motil Cytoskeleton</u> 44(4): 274-83.
- Tian, G., S. A. Lewis, et al. (1997). "Tubulin subunits exist in an activated conformational state generated and maintained by protein cofactors." <u>J Cell Biol</u> 138(4): 821-32.
- Vadlamudi, R. K., C. J. Barnes, et al. (2005). "p21-activated kinase 1 regulates microtubule dynamics by phosphorylating tubulin cofactor B." <u>Mol Cell Biol</u> 25(9): 3726-36.
- Vainberg, I. E., S. A. Lewis, et al. (1998). "Prefoldin, a chaperone that delivers unfolded proteins to cytosolic chaperonin." <u>Cell</u> **93**(5): 863-73.
- Valpuesta, J. M., J. Martin-Benito, et al. (2002). "Structure and function of a protein folding machine: the eukaryotic cytosolic chaperonin CCT." <u>FEBS Lett</u> 529(1): 11-6.
- Wang, W., J. Ding, et al. (2005). "Gigaxonin interacts with tubulin folding cofactor B and controls its degradation through the ubiquitin-proteasome pathway." <u>Curr Biol</u> 15(22): 2050-5.

- Wloga, D., A. Camba, et al. (2006). "Members of the NIMA-related kinase family promote disassembly of cilia by multiple mechanisms." <u>Mol Biol Cell</u> 17(6): 2799-810.
- Wloga, D., K. Rogowski, et al. (2008). "Glutamylation on alpha-tubulin is not essential but affects the assembly and functions of a subset of microtubules in Tetrahymena thermophila." <u>Eukaryot Cell</u> 7(8): 1362-72.

# **Figure legends**

**Figure 3.1:** The tubulin folding pathway involves two chaperonin complexes (Prefoldin and CCT) and five cofactors (A-E) to yield assembly competent tubulin heterodimers.



**Figure 3.2:** IFT52 suppressor mutants assemble short, sparse cilia. A-D. *Tetrahymena* cells stained with anti- $\alpha$ -tubulin antibodies with A – Wildtype, B – IFT52 $\Delta$ , C – IFT52 $\Delta$ sm and D – IFT52mov. The image was adapted from (Brown, Fine et al. 2003)



Adapted from Brown J.M. et al. 2003 Mol Biol Cell;14(8):3192-207

**Figure 3.3:** *TCB1* knockout is lethal in *Tetrahymena*. A. Growth curve of *TCB1* knockout cells (TCB1-KO) compared to wildtype cells (WT). B. Anti- $\alpha$ -tubulin antibody (12G10) staining of wildtype and TCB1 knockout (TCB $\Delta$ 1) cells. Note: Scale bar indicates 10 µm.



**Figure 3.4:** Localization of Tcb1p using different tags. A. 2D gel of the soluble fraction of cilia from IFT52 $\Delta$ mov cells (by Yan Gao). Note: Tcb1p is indicated by \*. B. Anti-HA antibody was used to stain wildtype and cells expressing Tcb1p-3HA from a non-native locus, under induced (+Cd) and non-induced (-Cd) conditions. C. Anti-HA antibody was used to stain wildtype and cells expressing Tcb1p-HA from the endogenous locus after 60 minutes of cilia regeneration. Note: Scale bar indicates 10 µm.


**Figure 3.5:** Tcb1p is a cell body protein. A. Western blot using anti-HA-antibody of different fractions of wildtype cells and cells expressing Tcb1-HA (WCE – whole cell extracts, CB – cell bodies, 1Xcilia – cilia from the same number of cells used to load in lanes with WCE and CB). Note: Tcb1p-HA and its degradation products are indicated by arrows. The Tcb1p-HA band is indicated by a box across the lanes. B. Western blot using anti-GFP antibody of different fractions of IFT52sm-Tcb1p-GFP, WT-Tcb1p-GFP and wildtype cells (WCE – whole cell extracts, CB – cell bodies). 2x and 10x cilia from the same number of cells used to load in lanes with WCE and CB. Note: The Tcb1p-GFP band is indicated by a box across the lanes.



Table 3.1: TCB1 $\Delta$  can be rescued using different C-terminal tagged versions of

### TCB1

	Tag	*DNA	*No DNA/Mock	Efficiency (%)
Non-native	GFP	72/490	5/490	~ 15
locus	HA	82/490	5/490	~ 17
	3xHA	4/490	0/490	~ 0.4 (2/490)**
Native locus	HA	17/490	5/490	~ 0.4 (2/490)**
	3xHA	3/490	3/490	~ 0.4 (2/490)**

Efficiency is calculated as per the number of real transformants that showed the tagged gene in the correct locus upon PCR analysis.

\* Values given are: number of wells with live cells/ total number of wells. In cases where false transformants were obtained in mock transformation, the presence of tagged-*TCB1* was verified in all transformants by PCR.

\*\* Numbers in parentheses refer to real transformants that were verified after PCR analysis.

Note: For GFP tag in the non-native locus, 2 transformants were verified using PCR and for HA tag in the non-native locus, one transformant was verified using PCR.

### CHAPTER 4

## UNUSUAL INTRAGENIC SUPPRESSION OF AN INSERTIONAL *IFT52* MUTATION IN TETRAHYMENA THERMOPHILA OCCURS IN TWO STEPS: BY DNA ELIMINATION AND SPLICING OF ARTIFICIAL INTRONS

### Abstract

Intraflagellar transport (IFT) is a motility pathway that occurs inside cilia and is required for the assembly and maintenance of most cilia. IFT involves a microtubule motor-driven transport of ciliary precursors that are associated with oligomeric protein complexes (A and B). Ift52p, an IFT complex B protein, is essential for cilia assembly, in diverse organisms including *Tetrahymena thermophila*. We showed previously that *Tetrahymena* cells carrying insertion of a selectable marker gene, *neo2*, into the coding region of the *IFT52* in the micronucleus, undergo frequent phenotypic suppressions and partially regain ability to assemble cilia under certain conditions, namely at lower temperature and in hypoxia (Brown, Fine et al. 2003). Here we show that the suppressions are intragenic and occur when the new macronucleus develops from the micronucleus during conjugation. First, most of the *neo* coding sequence is deleted; presumably by the RNAimediated genome rearrangement pathway that deletes micronucleus-specific sequences. Next, the flanking sequences that control the *neo2* gene expression are processed as multiple introns, as a result of which, the translational frame of IFT52 is restored. Our observations combined with those made recently in Chlamydomonas (Hou, Qin et al. 2007) argue that oxygen plays a conserved role in regulation of either assembly or function of IFT complex B.

### Introduction

Intraflagellar transport (IFT) is a conserved motility pathway that was first observed inside *Chlamydomonas* flagella (Kozminski, Johnson et al. 1993). IFT involves bi-directional movements of protein aggregates from the cell body to the distal tips of cilia (anterograde IFT) and back into the cell body (retrograde IFT). Kinesin-2 motors carry out the anterograde IFT, whereas cytoplasmic dynein1b is responsible for the retrograde IFT (Scholey 2003). These motors move IFT particles composed of complexes A and B. The IFT particles are proposed to act as cargo-binding platforms that carry axonemal and membrane precursors required for the assembly and maintenance of cilia (Cole 2003). IFT complex B, implicated mainly in anterograde IFT, is made of at least 11 proteins (Cole, Diener et al. 1998). In many organisms, including protists like *Chlamydomonas* and *Trypanosoma* and animals, deficiencies in anterograde IFT components lead to loss of ability to assemble and maintain cilia (Brown, Marsala et al. 1999; Pazour, Dickert et al. 1999; Pazour, Dickert et al. 2000; Haycraft, Swoboda et al. 2001; Beales, Bland et al. 2007; Absalon, Blisnick et al. 2008).

IFT52 is a complex B component that was initially identified in *Chlamydomonas* flagella and is well conserved among ciliated species (Cole, Diener et al. 1998). Cells of several organisms including *Tetrahymena*, *Chlamydomonas* and *C. elegans* fail assemble cilia without a functional IFT52 protein (Deane, Cole et al. 2001; Brown, Fine et al. 2003). A gene knockout of *IFT52* in *Tetrahymena* results in loss of cilia, causing cell paralysis that is associated with an arrest at cytokinesis, a secondary consequence of lack

of ciliary motility in this protist (Brown, Hardin et al. 1999). Unexpectedly, in some cultures of *IFT52*-null *Tetrahymena* (IFT52 $\Delta$ ), cells appeared that had regained partial ability to assemble cilia, as a result of apparent spontaneous suppressions that occurred at an unusually high frequency. The suppressor cells (IFT52 $\Delta$ sm) assemble cilia under specific conditions: either at a lower temperature or under hypoxia (Brown, Fine et al. 2003). We were intrigued by the conditional nature of the *IFT52* suppressors phenotype, because recently in Chlamydomonas reinhardtii, a mutation in a different IFT complex B protein, IFT46, was shown to confer a similar hypoxia-dependent assembly of cilia (Hou, Qin et al. 2007). Thus, there could be a conserved relationship between the anterograde IFT pathway and oxygen. Since little is known about how IFT is regulated, and whether or how IFT is influenced by extracellular signals, here we investigate the mechanism of the IFT52 gene knockout suppressions further. We show that the IFT52 $\Delta$ sm suppressions are intragenic, and as a result, the suppressed cells most likely express a mutated Ift52p protein. Furthermore, we document a novel mechanism of genetic suppression in Tetrahymena that is based on genomic DNA rearrangement associated with macronuclear development and splicing of artificial introns. Taken together with the observations made in Chlamydomonas, our observations argue that there is a conserved link between oxygen-dependent pathways and IFT.

### **Results and Discussion**

*Tetrahymena* cells have two nuclei, a transcriptionally silent germline micronucleus (Mic) and a transcriptionally active, somatic macronucleus (Mac) (Yao and Chao 2005). Earlier, we disrupted the *IFT52* gene by insertion of a gene knockout cassette (*neo2*) within the coding region (Figure 4.1D). To this end, we constructed gene knockout heterokaryon strains that were homozygous in their Mics for the disrupted allele but had wild-type alleles in their Macs (Cassidy-Hanley, Bowen et al. 1997; Brown, Fine et al. 2003). When two such heterokaryons with different mating types undergo conjugation, they produce progeny cells in which the Mic and Mac have only the disrupted copies of *IFT52*. As described earlier, the majority of progeny cells produced by matings between *IFT52* gene knockout heterokaryons are paralyzed and lacked cilia (except for occasional assembly of short 9+0 non-motile axoneme remnants). These IFT52 $\Delta$  cells often do not complete cytokinesis due to inability to rupture the cytoplasmic bridge and form large multinucleated cells (Brown, Fine et al. 2003). The IFT52∆ cells can be maintained in shaken culture, which provides mechanical force required for the scission of the cytoplasmic bridge during cytokinesis (Brown, Hardin et al. 1999).

When *IFT52* knockout heterokaryons undergo conjugation, 3% of the progeny cells regain partial ability to assemble motile cilia, due to apparent spontaneous genetic suppressions. The suppressed cells, IFT52 $\Delta$ sm, assemble 9+2 cilia that are 30% the length of wild-type cilia. Additionally, the suppressions are conditional; the IFT52 $\Delta$ sm cells retain a non-suppressed phenotype at the standard temperature (30°C) when grown at low cell density, but express a suppressed phenotype either when grown at a lower (room) temperature or when grown at the standard temperature but at high cell density.

We showed earlier that the high density effect is based on generation of pericellular hypoxia (Brown, Fine et al. 2003). In a single event, an apparent secondary suppression in a clone of the IFT52 $\Delta$ sm cells led to the appearance of faster moving cells called IFT52 $\Delta$ mov. IFT52 $\Delta$ mov cells are capable of assembling cilia that are ~70% of wild-type length and the assembly is not temperature- or hypoxia-dependent (Brown, Fine et al. 2003).

The high frequency of the IFT52∆sm suppressions and the fact that suppressions required a mating, suggested that the mechanism of suppression is based on processes that occurs inside the developing macronucleus. Mating *Tetrahymena* cells undergo a series of nuclear events that culminate in the replacement of the existing macronucleus with a new macronucleus that develops by differentiation of a zygotic micronucleus (Coyne, Chalker et al. 1996). When the new macronucleus develops, Mic-specific sequences accounting for about 15% of the genome are removed by a pathway that involves an RNAi-dependent mechanism of sequence recognition and degradation (Yao and Chao 2005). According to a recent model, double stranded scan RNAs (scnRNAs) are produced by transcription of the Mic genome. Next, only those scnRNAs that have no corresponding DNA sequence in the pre-existing Mac enter the newly developing Mac, where they mediate deletion of foreign DNA segments (Yao and Chao 2005). Not only the Mic-specific native sequences but also foreign sequences that are experimentally inserted into the Mic undergo RNAi-mediated deletion during macronuclear development. Specifically, Yao and colleagues showed that the *neo2* sequence, a gene disruption cassette inserted into multiple loci undergoes RNAi-mediated deletion during macronuclear development (Yao, Fuller et al. 2003). Thus, we analyzed the organization

of the *IFT52* locus in the IFT52 $\Delta$  and suppressed cells, to determine whether the disruption cassette that was originally inserted, *neo2*, undergoes developmental rearrangement that could explain the phenotypic suppression.

The IFT52 gene knockout in the micronucleus was based on insertion of the neo2 cassette into exon 4 in a reverse transcriptional orientation as compared to the reading frame of targeted locus (Figure 4.1D) (Gaertig, Gu et al. 1994). The neo2 cassette consists of the neomycin phosphotransferase coding region (*neo*) placed between fragments of the *HHF4* (histone H4) gene promoter and the *BTU2* ( $\beta$ -tubulin) transcription terminator (Gaertig, Gu et al. 1994). The HHF4 and BTU2 segments are native to *Tetrahymena*, while the *neo2* coding region encodes a neomycin phosphotransferase of bacterial origin and thus is a non-native sequence (Gaertig, Gu et al. 1994). We isolated total genomic DNA from wildtype, IFT52 $\Delta$ , IFT52 $\Delta$ sm and IFT52∆mov cells and amplified a region across the *neo2* insertion site in *IFT52* using primers that recognize sequences within exons 3 and 4 (Figure 4.1D). Amplification of genomic DNA of wild-type cells produced a fragment of expected size of 1.3 kb. The same primers used with genomic DNA of IFT52 $\Delta$  (non-suppressed) cells, produced a larger fragment of  $\sim 2.7$  kb consistent with insertion of *neo2* (1.4 kb) (Figure 4.1A). Strikingly, the same primers produced a smaller fragment (~1.9 kb) with genomic DNA of both types of suppressor strains (mov and sm), suggesting that the suppressions are associated with deletions around the *neo2* insertion site. We sequenced the amplified fragments from three independent IFT52∆sm suppressor clones and a single IFT52∆mov clone and found that their nucleotide sequences are identical. Fragments from both types of suppressors lack a large portion of the neo coding sequence (~0.8 kb) with deletion

sites at exactly the same positions, while the non-coding sequences of *neo2* cassette, the *HHF4* (histone H4) gene promoter and *BTU2* transcriptional terminator (both of *Tetrahymena* origin) remain largely intact (Figure 4.1D). Specifically, the deletions occurred between the nucleotide at position +45 in the *neo* coding sequence and the fifth nucleotide downstream of the stop codon within the *BTU2* 3' UTR region (Figure 4.S1). Thus, in the IFT52 suppressor cells, most of the foreign DNA sequence of *neo2* is deleted while the native sequences (*HHF4* and *BTU2*), despite their presence in the non-native context, are retained (Figure 4.S1). These observations are consistent with earlier reports on deletions of *neo2* sequences during macronuclear development (Yao, Fuller et al. 2003; Liu, Song et al. 2005).

Despite the observed *neo* sequence deletions, this event does explain the mechanism of phenotypic suppression because the sequence of the residual *neo* cassette composed of *HHF4* and *BTU2* sequences (~0.7 kb) has stop codons in all forward translational frames. Thus, an Ift52p translated from the predicted mRNA containing the residual *neo* sequence would be severely truncated; lacking 5 out of a total of 8 exons that all contain highly conserved sequences (Figure 4.S1). Nevertheless, it is apparent that suppressions correlate with deletions of the *neo* coding sequence.

To establish whether the genomic DNA deletion within the disrupted IFT52 locus is sufficient for suppression, we introduced a fragment of the rearranged *IFT52* genomic locus (amplified from IFT52 $\Delta$ mov genomic DNA; Figure 4.1D) that includes the residual *neo2* cassette, into non-motile IFT52 $\Delta$  cells by biolistic bombardment. After a few days of incubation at room temperature the bombarded populations were inspected for motile cells. We obtained cells that regained motility and such cells did not appear within mock-

transformed IFT52 $\Delta$  cells. The efficiency of transformation was very low; only two wells had live, motile cells amongst a total of 480 wells after biolistic transformation. PCR amplification of the genomic DNA confirmed that the targeting fragment replaced the corresponding region of the disrupted locus (results not shown). The rescued cells showed the phenotype of IFT52 $\Delta$ sm cells, that is, a hypoxia-dependent and temperaturedependant suppression (Figure 4.1C and results not shown). IFT52 $\Delta$ sm cells arise each time *IFT52* heterokaryons undergo conjugation, but IFT52 $\Delta$ mov cells were initially isolated from IFT52 $\Delta$ sm as a separate and unique event. These data indicate that there might be another mutation in IFT52 $\Delta$ mov cells outside the region being analyzed, which enables the cells to assemble cilia independent of the temperature and oxygen conditions.

It is likely that despite the presence of multiple stop codons, the rearranged *IFT52* gene containing a residual *neo* sequence produces a partly functional Ift52p. Thus, either an extremely truncated Ift52p is sufficient for conditional cilia assembly or an additional mechanism restores the translational reading frame across the residual *neo2* cassette.

To determine the predicted sequence of translated Ift52p in the suppressed cells, we amplified the region that encompasses the *neo2* insertion site from total cDNA obtained using mRNA of IFT52 $\Delta$ sm cells (Figure 4.1D). For a fully spliced wild-type *IFT52* mRNA, the amplified fragment was expected to be ~0.3 kb. For an mRNA in the IFT52 $\Delta$ sm background that contains a residual *neo2* cassette, the expected size of cDNA fragment was 0.9 kb. However, the size of the amplified product from the IFT52 $\Delta$ sm suppressor cDNA was ~0.4 kb, indicating that a further deletion occurs at the level of mRNA processing (Figure 4.1B). Sequencing of the amplified cDNA fragment of the IFT52 $\Delta$ sm origin revealed that ~0.8 kb of the residual *neo2* cassette sequence was absent

in the predicted mRNA. A comparison of cDNA with genomic DNA from the suppressors showed that most of the residual *neo2* sequence, which consisted of *HHF4* and *BTU2* sequences, is removed as 3 artificial introns. The intron junctions have sequences that are consistent with the natural intron junctions used by *Tetrahymena* (Jaillon, Bouhouche et al. 2008); Figure 4.S1). Moreover, processing of the residual *neo2* sequence as artificial introns restored the translational frame across the site of *neo2* insertion (Figure 4.S2). As a result, the predicted Ift52 protein that is likely expressed in the suppressor cells has 43 additional amino acids but lacks 7 original amino acids as a the *neo2* cloning procedure (Figure 4.1D and Figure 4.S2). Either the presence of these extra amino acids or the absence of the 7 endogenous amino acids in Ift52p-sm/mov (or a combination of both) results in the intragenic suppression.

To conclude, we show that suppression of the *IFT52* insertional mutation is intragenic and requires two steps: 1) a large portion of the *neo2* cassette, mainly comprising of the *neo* gene of bacterial origin is deleted by RNAi-mediated mechanisms that are active during macronuclear development and 2) portions of the remaining ATrich sequence of *Tetrahymena* (*HHF4* and *BTU2*) origin are processed during mRNA splicing. The first step, where the non-native segment of *neo2* undergoes deletion from the genomic DNA almost certainly occurs via the RNAi-mediated developmental genome rearrangement pathway. This form of genomic DNA deletion is thought to have evolved as a means of genome surveillance that eliminates foreign transposon-type DNA sequences from the sequence of the transcriptionally active Mac (Yao and Chao 2005). The majority (97%) of progeny of *IFT52* heterokaryon progeny have a non-suppressed

phenotype. Most likely, in these cells, the deletions of *neo2* do not occur, or occur on an insufficient number of chromosomes to achieve a threshold for phenotypic effects (there are 45 copies of each chromosome in the G1 macronucleus). An earlier study showed that the efficiency with which foreign DNA is deleted by the RNAi-dependent pathway is locus-specific (Liu, Song et al. 2005). In 4 independent suppressors we detected a genomic deletion at precisely identical positions. Previous studies showed variability in the deletion sites (Yao, Fuller et al. 2003; Liu, Song et al. 2005). It is likely that other deletions do occur in the disrupted *IFT52* locus but do not lead to a processed genomic region, in which splicing restores the translational frame of *IFT52*.

In *Chlamydomonas* reinhardtii, an insertional mutation of another complex B protein, IFT46 that results in loss of ability to assemble cilia, also underwent a spontaneous conditional suppression in hypoxia (Hou, Qin et al. 2007). The IFT46 suppression could be associated with expression of the C-terminal domain of IFT46. Both studies allow for a generalization that partial function of mutated IFT complex B components is restored by hypoxic conditions. Hou and colleagues showed that in the suppressed *Chlamydomonas* IFT46 mutant cilia, there are assembled IFT B complexes. It is likely that suppressed IFT52∆sm *Tetrahymena* cells also regain ability to assemble IFT complex B. It is tempting to speculate a role for oxygen in the complex B assembly. Hou and colleagues proposed that IFT complex B undergoes folding by a chaperone whose levels increase under hypoxia. Taken with our observations, both studies argue that the oxygen-dependent IFT complex B assembly is a conserved mechanism.

### **Materials and Methods**

For maintenance, IFT52Δ, IFT52Δsm and IFT52Δmov cells were grown at the room temperature in MEPP medium (Orias and Rasmussen 1976) with an antibioticantimycotic mixture (Invitrogen, Carlsbad, CA).

Isolation of total genomic DNA was done as described (Dave, Wloga et al. Accepted 2009). The genomic region across exons 3 and 4 was amplified using the following primers: 5'-ATGCCCTCAAATAAT-3' and 5'-TAGAGTTGGTTTAGATTT-3'. The resulting fragments were cloned into pGEM-T-vector (Promega Corp, Madison, WI) and sequenced.

To determine whether a genomic fragment of IFT52 of suppressor origin is sufficient to confer suppression of the IFT52 $\Delta$  phenotype (lack of cilia), IFT52 $\Delta$  cells were biolistically bombarded with a genomic fragment of IFT52 $\Delta$ mov origin, separated from the pGEM-T-vector plasmid with NcoI and SalI digestion. Bombarded cells were grown at the room temperature and transformants were identified based on recovery of cell motility (Cassidy-Hanley, Bowen et al. 1997).

To prepare total mRNA, cells were grown to a concentration of  $2 \times 10^5$  cells/ml in MEPP medium (Gorovsky, Yao et al. 1975), washed with 10 mM Tris-HCl buffer pH 7.5 and used for total RNA extraction with TRI-reagent (MRC Inc, Cincinnati, OH) as per protocol.

Total cDNA was prepared using the SMART IV-forward and CDS III-reverse primers from the RT-PCR kit (Clontech Inc, Mountainview, CA).

### Acknowledgements

This work was supported by a grant from the National Science Foundation (MBC-033965) to J.G.

### References

- Absalon, S., T. Blisnick, et al. (2008). "Intraflagellar transport and functional analysis of genes required for flagellum formation in trypanosomes." <u>Mol Biol Cell</u> 19(3): 929-44.
- Beales, P. L., E. Bland, et al. (2007). "IFT80, which encodes a conserved intraflagellar transport protein, is mutated in Jeune asphyxiating thoracic dystrophy." <u>Nat Genet</u> 39(6): 727-9.
- Brown, J. M., N. A. Fine, et al. (2003). "Hypoxia regulates assembly of cilia in suppressors of Tetrahymena lacking an intraflagellar transport subunit gene." <u>Mol</u> <u>Biol Cell</u> 14(8): 3192-207.
- Brown, J. M., C. Hardin, et al. (1999). "Rotokinesis, a novel phenomenon of cell locomotion-assisted cytokinesis in the ciliate Tetrahymena thermophila." <u>Cell</u> <u>Biol Int</u> 23(12): 841-8.

- Brown, J. M., C. Marsala, et al. (1999). "Kinesin-II is preferentially targeted to assembling cilia and is required for ciliogenesis and normal cytokinesis in Tetrahymena." <u>Mol Biol Cell</u> 10(10): 3081-96.
- Cassidy-Hanley, D., J. Bowen, et al. (1997). "Germline and somatic transformation of mating Tetrahymena thermophila by particle bombardment." <u>Genetics</u> 146(1): 135-47.
- Cole, D. G. (2003). "The intraflagellar transport machinery of Chlamydomonas reinhardtii." <u>Traffic</u> **4**(7): 435-42.
- Cole, D. G., D. R. Diener, et al. (1998). "Chlamydomonas kinesin-II-dependent intraflagellar transport (IFT): IFT particles contain proteins required for ciliary assembly in Caenorhabditis elegans sensory neurons." <u>J Cell Biol</u> 141(4): 993-1008.
- Coyne, R. S., D. L. Chalker, et al. (1996). "Genome downsizing during ciliate development: nuclear division of labor through chromosome restructuring." <u>Annu Rev Genet</u> 30: 557-78.
- Dave, D., D. Wloga, et al. (Accepted 2009). "Manipulating ciliary protein-encoding genes in Tetrahymena." <u>Methods Cell Biol</u>.
- Deane, J. A., D. G. Cole, et al. (2001). "Localization of intraflagellar transport protein IFT52 identifies basal body transitional fibers as the docking site for IFT particles." <u>Curr Biol</u> 11(20): 1586-90.
- Gaertig, J., L. Gu, et al. (1994). "High frequency vector-mediated transformation and gene replacement in Tetrahymena." <u>Nucleic Acids Res</u> **22**(24): 5391-8.

- Gorovsky, M. A., M. C. Yao, et al. (1975). "Isolation of micro- and macronuclei of Tetrahymena pyriformis." <u>Methods Cell Biol</u> **9**(0): 311-27.
- Haycraft, C. J., P. Swoboda, et al. (2001). "The C. elegans homolog of the murine cystic kidney disease gene Tg737 functions in a ciliogenic pathway and is disrupted in osm-5 mutant worms." <u>Development</u> 128(9): 1493-505.
- Hou, Y., H. Qin, et al. (2007). "Functional analysis of an individual IFT protein: IFT46 is required for transport of outer dynein arms into flagella." <u>J Cell Biol</u> 176(5): 653-65.
- Jaillon, O., K. Bouhouche, et al. (2008). "Translational control of intron splicing in eukaryotes." <u>Nature</u> 451(7176): 359-62.
- Kozminski, K. G., K. A. Johnson, et al. (1993). "A motility in the eukaryotic flagellum unrelated to flagellar beating." <u>Proc Natl Acad Sci U S A</u> **90**(12): 5519-23.
- Liu, Y., X. Song, et al. (2005). "Elimination of foreign DNA during somatic differentiation in Tetrahymena thermophila shows position effect and is dosage dependent." <u>Eukaryot Cell</u> 4(2): 421-31.
- Orias, E. and L. Rasmussen (1976). "Dual capacity for nutrient uptake in Tetrahymena.
  IV. Growth without food vacuoles and its implications." <u>Exp Cell Res</u> 102(1): 127-37.
- Pazour, G. J., B. L. Dickert, et al. (2000). "Chlamydomonas IFT88 and its mouse homologue, polycystic kidney disease gene tg737, are required for assembly of cilia and flagella." <u>J Cell Biol</u> 151(3): 709-18.
- Pazour, G. J., B. L. Dickert, et al. (1999). "The DHC1b (DHC2) isoform of cytoplasmic dynein is required for flagellar assembly." <u>J Cell Biol</u> 144(3): 473-81.

Scholey, J. M. (2003). "Intraflagellar transport." Annu Rev Cell Dev Biol 19: 423-43.

- Yao, M. C. and J. L. Chao (2005). "RNA-guided DNA deletion in Tetrahymena: an RNAi-based mechanism for programmed genome rearrangements." <u>Annu Rev</u> <u>Genet</u> 39: 537-59.
- Yao, M. C., P. Fuller, et al. (2003). "Programmed DNA deletion as an RNA-guided system of genome defense." <u>Science</u> 300(5625): 1581-4.

### **Figure legends**

Figure 4.1: Two subsequent sequence deletions lead to intragenic suppression of an insertional IFT52 mutation. A. Results of PCR amplifications of the genomic region of IFT52 locus with primers corresponding to sequences to sites in exons 3 and 4 in wildtype (IFT52), gene knockout (IFT52 $\Delta$ ) and the suppressor (IFT52 $\Delta$ sm/mov) cells. Amplified fragments were separated on an agarose gel. An asterisk marks an apparent non-specific amplification product. Image by Gautham Pandiyan. B. PCR amplifications of total cDNA obtained from mRNA using wildtype (*IFT52*), knockout (*IFT52A*) and suppressors (*IFT52* $\Delta$ *sm/mov*) cells using the same primers as in panel A. The amplification products were separated on an agarose gel. C. IFT52 $\Delta$  cells rescued with *IFT52* $\Delta$ *sm/mov* DNA and wild-type cells both at a concentration of  $3 \times 10^{5}$  were diluted down to different concentrations (1x to 100x) on a 96-well microtiter plate, and incubated at 30°C. After 12 hours of incubation, the number of motile cells (cells showing detectable displacement and lacking cytokinesis defects) was determined. D. Schematic diagram detailing the *IFT52* locus in wildtype (*IFT52*), knockout (*IFT52* $\Delta$ ) and the suppressors ( $IFT52\Delta sm/mov$ ) cells and the cDNA in the suppressors ( $IFT52\Delta sm/mov$ ) *processed mRNA*) cells. F and R represent primers used for the various PCR reactions.



Figure 4.S1: Sequence of genomic DNA reconstructed for 4 independent

IFT52 $\Delta$ sm/mov suppressor strains shows a deletion within the *neo2* gene cassette. Segments of DNA that correspond to distinct parts of *neo2* are color coded (*BTU2*, *HHF4* and *neo1*) cassette are shown in different colors. The sequence corresponds to the region between exons 3 and 4. Natural intron junctions are marked as grey boxes. Artificial intron junctions are marked as open boxes. Note: Within the residual neo cassette left after genomic deletion, there is a stop codon (red \*) in every translational frame.

### IFT52Asm/mov genomic DNA sequence

### IFT52 sequence (position 583 from start)

ATGCCCTCAAATAATACTTAGAAAGTGGTGGTGGTCTTGAGTTTGAGCTCGAAGGTGGTGGTGGTGATAAgtaattaacataaaaacaattctaagaatgcctttctattttctatata M P S N N T Q K V V V V S Q F \* A L K V V V I S N Q H K N N S K N A F L F P Y I C P Q I I L R K M W S C L S F E L \* R M W S Q V I N I K T I L R M P Y F S I Q A L K Q Y L E S G G R V L V L S S E G G G H K Q L T Q K Q F Q E C L S I F L Y N 

Y P K M P H Q N F K I N S N L S L E I S K Y K K A R M N E Q M K I Y K Q I D K I L K C L I K I S K L I Q I L V Q R L A N I K R R G \* M N K \* K F I N R L I K L F Q N A S L K F Q N Q F K S Q F R D Q Q I Q K G E D E \* I N E N L Q I D \* Q

aaataaggagaatattttaagcaacgcetcgtacatttatttatetttaagttaattetaaaacttattatttatttattagagaaaataaattcaaattettaactaaatttaaacaaattQ G E Y F K Q R L V H L F I P K L I L K L I I L F R E N K P K F L T K F K Q I N K E N I L S N A S Y I Y L S L S Q F Q N L L F Y L E K I N S N S Q L N L N K I I R R I F Q A T P R T F I Y L Q V N S K T Y Y F I Q R K Q I Q I L N Q I Q T N N

M S Q N S L N F T K Q F I E I L \* K Q F H Y Q G I \* K F I F K N I L I Q I Q Q C H K I V \* I L L N N L S K F F E N N F I I K E S E N L F L K I F Q Y K F N V I K Q F E F Y Q I I Y R N S L K T I S L S R N L K I Y F Q K Y F N I N L I Q

agcttaactgattcaaatagatttgaaaatgttataaaaaagtttgaattcactaatatttgtcaaagtctttgagaacaattcattatttttttccaaggaatctgaaaatt S L T D S N R F G K C Y K K V \* I S L N N L S K F F E N N F I I F F P R N L K I A Q L I Q I D L E S V I K K F E F H Q I I C Q S S L R T I S L F F P Q G I \* K L L N \* F K Q I N K V L Q K S L N F T K Q F V K V L \* E Q F H Y F F S K E S E N Q

### BTU2 sequence(blue)

N I I I G F A D K F L R A N M Y V K R N F F F Q K V K K N N \* H K I Y I Q M S K I L L L D L Q T N F Q E L T C M \* R G I F F F R K L K K I I D I K Y I Y K \* V K Y Y Y W I C R Q I F K S Q H V C E E E F F F L E S Q K K Q L T Q N I Y T N E L K

#### neo sequence(pink) HHF4 sequence(green)

### IFT52 sequence (position 1758 from start)

 
 P
 T
 Q
 R
 N
 K
 T
 P
 L
 V
 K
 Y
 K
 R
 \*
 I
 L
 K
 G
 \*
 R
 \*
 I
 L
 K
 G
 T
 N
 L
 N
 V
 I
 G
 K
 R
 T
 I
 L
 N
 P
 P
 P
 L
 D
 N
 I
 L
 N
 I
 R
 N
 I
 L
 N
 I
 C
 N
 I
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 D
 Q

**Figure 4.S2:** The cDNA sequence of IFT52 $\Delta$ sm/mov has 43 extra amino acids from remaining *neo2* cassette. The remaining *neo2* cassette consisting of *BTU2* and *HHF4 Tetrahymena* sequences have been processed as artificial introns. The sequence corresponds to the region between exons 3 and 4.

TAT ANA GAN ACA TAT ANG TGC GGC AGA NAT ANA GAN GAT ATC ACA TAT GAT AGA CTA ANG ATG GCT TCT TTG GTC ATA TTT TTT TGC CCC KCGRNK E D I TYDRL K M A E T s L. V т C ANA GAN ATG TIT ACC ANG GAN GAN TIC GAT GCC CTC ANA TAN TAC TIA GAN AGT GGT GGT GGT GGT GTC TIA GTT TIG AGC TCT GAN GGT GGT TKEE F DALKOYLESG GR s G ggt cat ang bat cgc aca bat ata bat ttc ttc ttg gbb tab tat ggt atc agt atc bat bac gbt tgt gtc gtt agb act gca ttt tgg G т N N F L E O Y I S I N D C A BTU2 cDNA(blue) neo cDNA(pink) HHF4 cDNA (green) get gea tit tit eac gge egg aga act ige gig eaa tee ate eaa get ige eat itit ig eeg aas aat tat eea ate aga ate agt ett A A F F H G R R T C V Q S I Q A C H F S P K N Y P I R I S L IFT52 cDNA(black) tot aga gat toa gat ttt gat got toa ata aga taG GAG ACT TAT GTT CAT TCT GGT ATT TTA AAT GAA GAA GTA ACA AGA GTA GCA AAT DSDFDASIR QET Y v H S GILNE SR E v GGT TTA CCT ANA GAA ACA ANG AGA CCC CAA AAT ACA TTC TTG TAA AAC GTT ATT GGA AAG GAT GAT GAA GAA GAT GAA TAT TAA AAG GAA G L P K E T K R P Q N T F L Q N V I G K D D E E D E Y Q K E CAA TCT AGA GTT GGT TTA GAT TTT GTT TAT GCC TTT GGT GCT ACC TTG ACT GTT TAA TAA CCT GCA CAC GCT ATT TTA GGT TCT GGT CCT QSR V G L D F V Y A F G A T L T V QQPAHAIL G S G CTT TCT TAC CCT TCT AAT AGA CCA GTT TCT GCT ATC GTC TAA ACT AAA AAT GAT AGA CTT GCA GTT ATT GGT TCA TTC GAA ATG TTT L S Y P S N R P V S A I V Q T K N N G R L A V I G S F EMB ACA GAT GAA TAT TTT GAC AAT GAA GAT AAC TCC AAG ATT TTT GAT TTC TTT ATA AAA TAT TTG CTC ACA AAT GAG TGC GAA TTT GAA TTT D N E D N s K I F DF і к т т D E Y F F LL N E C E AGT CCT AAA GAA CCT GAT GTT GAA TAC TTC AAG GTT CCT GAT ATT GCT GAA TTA GCT GAT AAC CTC AAG AGT TGC TTA CAA GAA AGT GAC S P K E P D V E Y F K V P D I A E L A D N L K S C L Q E S D CCA TTA CCA TTT GAT AGC AAG CAA TTA TTT ATG ACA GAC TTG TTT AAG TAT GAT GTA GAT TTA GTT CCA GAA GCT GTA AAA TTG TAT GAA F D S K O L F M T D L F K Y D V DL P E A v T. P v K T. ACT CTT GGA GTA AAG CAC GAT CCT CTT GCT CTT ATA GTT CCT TAA TTC GAA ACT CCA CTC CTT GGA CTT GTT TCA GCT GTT TTC CCA CCT v KHDPLALI VPQFETP LLG L V Τ. G S A V D ATT TTA AMA GAM TTA GCT CCT CCA AGT TTA GAM TTG TTT GAT TTA GAT GAT GAM TTT GCT TCA GAM AMA GTA AGA CTG GCC TAM CTC ACA KELAPP S L E L F D L D D E F A S EKV RLAQ ANT ANA TEC ANC ANC ANC GAT TTA GAT TAT TAC ATT ANA GAN TCA GET GAT ATC TTE GET GTA ACA GAT ANA GTT ANG ANC ANA CAT GAT NKCNNNDLDYYIKESGDILGVTDK K N A K A I L R Y V L E E L I N F K K L N N

ATG AGT GGA GAA TAG AAA ATT ATT GTC TTC AAC GCT TCA AAG AAG GAG GCT GGT AAC CCT AGT ACA AAT ATT AAA AAG ATT ATC AAG AAA M S G E Q K I I V F N A S K K E A G N P S T N I K K I I K K

IFT52∆sm/mov cDNA sequence (470 codons)

IFT52 cDNA(black)

### CHAPTER 5

### DYF-1 IS REQUIRED FOR ASSEMBLY OF THE AXONEME IN TETRAHYMENA

### Abstract

In most cilia, the axoneme can be subdivided into three segments: proximal (the transition zone), middle (with outer doublet microtubules) and distal (with singlet extensions of outer doublet microtubules). How the functionally distinct segments of the axoneme are assembled and maintained is not well understood. DYF-1 is a highly conserved ciliary protein containing tetratricopeptide repeats. In *Caenorhabditis elegans,* DYF-1 is specifically needed for assembly of the distal segment (Ou, Blacque et al. 2005). We show that *Tetrahymena* cells lacking an ortholog of DYF-1, Dyf1p, can assemble only extremely short axoneme remnants that have structural defects of diverse nature, including the absence of central pair and outer doublet microtubules and incomplete or absent B tubules on the outer microtubules. Thus, in *Tetrahymena*, DYF-1 is needed for either the assembly or stability of the entire axoneme. Our observations support the conserved function for DYF-1 in axoneme assembly or stability, but also show that the consequences of loss of DYF-1 on axoneme segments are organism-specific.

### Introduction

Cilia are microtubule-rich cellular extensions that arise from basal bodies near the surface of most eukaryotic cell types. Defective cilia cause a wide variety of diseases including the polycystic kidney disease, primary ciliary dyskinesia and retinal degeneration (Badano, Mitsuma et al. 2006). A typical motile cilium has a microtubulebased framework, the axoneme, which contains 9 outer (mostly doublet) microtubules and 2 central (singlet) microtubules. In most cilia, the axoneme can be subdivided into three segments: proximal (transition zone), middle (containing outer doublet microtubules), and distal (containing singlet extensions of peripheral microtubules). The outer doublet microtubules of the middle segment have a complete tubule A made of 13 protofilaments and an incomplete tubule B made of 11 protofilaments that is fused to the wall of the A tubule (Nicastro, Schwartz et al. 2006; Sui and Downing 2006). The outer microtubules in the distal segment lack the B tubule (Sale and Satir 1976; Mesland, Hoffman et al. 1980). The distal segment also lacks dynein arms and radial spokes and its microtubules are terminated by caps that are associated with the plasma membrane at the tips of cilia (Sale and Satir 1977; Dentler 1980). The distal segments are characterized by high level of microtubule turnover, which could play a role in the regulation of length of cilia (Marshall and Rosenbaum 2001).

The mechanisms that establish the segmental subdivision of the axoneme are not well understood. Studies in *C. elegans* indicate that the distal segment is assembled using a mechanism that differs from the one utilized in the middle and proximal segment (Signor, Wedaman et al. 1999). In most cell types, ciliogenesis is dependent on the intraflagellar transport (IFT) pathway: a bi-directional motility of protein aggregates,

known as IFT particles, which occurs along outer microtubules (Kozminski, Johnson et al. 1993; Kozminski, Beech et al. 1995; Piperno and Mead 1997; Cole, Diener et al. 1998). IFT particles are believed to provide platforms for transport of axonemal precursors (Qin, Burnette et al. 2005; Hou, Qin et al. 2007). Kinesin-2 motors are responsible for carrying out the anterograde component of IFT; delivering cargo from the cell body to the tips of cilia (Walther, Vashistha et al. 1994; Kozminski, Beech et al. 1995), whereas cytoplasmic dynein DHC1b is responsible for the retrograde IFT (Pazour, Dickert et al. 1999; Porter, Bower et al. 1999; Signor, Wedaman et al. 1999). Importantly, in the well studied amphid cilia of *C. elegans*, two distinct kinesin-2 complexes are involved in the anterograde IFT and differ in movement velocity: the "slow" heterotrimeric kinesin-II and the "fast" homodimeric OSM-3 kinesin (Signor, Wedaman et al. 1999). While kinesin-II and OSM-3 work redundantly to assemble the middle segment; OSM-3 alone functions in the assembly of the distal segment (Snow, Ou et al. 2004; Ou, Blacque et al. 2005).

In *C. elegans*, DYF-1 is specifically required for the distal segment assembly (Ou, Blacque et al. 2005). In the DYF-1 mutant, the rate of IFT in the remaining middle segment is reduced to the level of the slow kinesin-II, suggesting that the Osm3 complex is non-functional, and kinesin-II functions alone in the middle segment. Thus, DYF-1 could either activate OSM-3 kinesin or could dock OSM-3 to IFT particles (Ou, Blacque et al. 2005; Evans, Snow et al. 2006).

However, a recent study in zebrafish has led to a different model for DYF-1 function. Zebrafish embryos that are homozygous for a loss-of-function of *fleer*, an ortholog of DYF-1, have shortened olfactory and pronephric cilia and ultrastructural

defects in the axonemes. In the middle segment, the *fleer* axonemes have B tubules that are disconnected from the A tubule, indicating that DYF-1 functions in the middle segment, and could play a role in the stability of doublet microtubule (Pathak, Obara et al. 2007). Earlier, a similar mutant phenotype was reported in *Tetrahymena*, for a mutation in the C-terminal tail domain of  $\beta$ -tubulin, at the glutamic acid residues that are used by posttranslational polymodifications (glycylation and glutamylation) (Redeker, Levilliers et al. 2005). Glycylation (Redeker, Levilliers et al. 1994) and glutamylation (Eddé, Rossier et al. 1990) are conserved polymeric post-translational modifications that affect tubulin and are highly enriched on microtubules of axonemes and centrioles (reviewed in (Gaertig and Wloga 2008)). Other studies have indicated that tubulin glutamylation contributes to the assembly and stability of axonemes and centrioles (Bobinnec, Khodjakov et al. 1998; Campbell, Waymire et al. 2002). The *fleer* mutant zebrafish cilia have reduced levels of glutamylated tubulin (Pathak, Obara et al. 2007). Pathak and colleagues proposed that the primary role of DYF-1/fleer is to serve as an IFT cargo adapter for a tubulin glutamic acid ligase (Janke, Rogowski et al. 2005), and that the effects of lack of function of DYF-1/fleer could be caused by deficiency in tubulin glutamylation in the axoneme. As an alternative hypothesis, the same authors propose that DYF-1 is a structural component that stabilizes the doublet microtubules in the axoneme (Pathak, Obara et al. 2007).

Here, we evaluate the significance of a DYF-1 ortholog, Dyf1p, in *Tetrahymena thermophila*. Unexpectedly, we find that *Tetrahymena* cells lacking Dyf1p either fail to assemble an axoneme or can assemble an axoneme remnant. While our observations reveal major differences in the significance of DYF-1 for segmental differentiation in

diverse models, it is clear that DYF-1 is a conserved and critical component that is required for assembly of the axoneme.

### Results

### Loss of Dyf1p results in extreme truncation of cilia and severe axonemal defects

Using the *C. elegans* protein sequence of DYF-1 (F54C1.5a), we searched gene predictions derived from the *Tetrahymena* macronuclear genome (Eisen, Coyne et al. 2006) available at the *Tetrahymena* Gene Database using pBLAST, and we identified a single gene encoding a closely related protein (TTHERM\_00313720), named *DYF1*. The predicted Dyf1p amino acid sequence is 43% identical to DYF-1 of *C. elegans*. Dyf1p is predicted to have 3 TPR domains that are located at positions similar to those in other DYF-1 proteins (Figure 5.1A). Phylogenetic analyses showed that Dyf1p is most closely related to homologs of other protists, *Paramecium tetraurelia* (GSPATT00028247001) and *Chlamydomonas reinhardtii* (FAP259) (Figure 5.1B).

Next, we eliminated all expressed copies of *DYF1* by DNA homologous recombination. Ciliates have two functionally distinct nuclei, the micronucleus and the macronucleus. Only the macronuclear genes contribute to the phenotype in vegetatively growing cells. There are about 45 copies of each protein coding gene in the G1 macronucleus (reviewed in (Turkewitz, Orias et al. 2002)). We constructed *Tetrahymena* strains with a macronuclear knockout of *DYF1* via DNA homologous recombination, using a fragment of *DYF1* interrupted by *neo3* (Shang, Song et al. 2002) (Figure 5.S1) and eliminated all macronuclear copies of *DYF1* by phenotypic assortment under drug

selection that favors retention of the *neo3* disrupted alleles (see Material and Methods). After 2 weeks of selective growth, single cells were isolated into drug free medium. Among isolates, we detected clones with all or nearly all cells that were paralyzed. Recloning of the motility-deficient cells established phenotypically stable populations of paralyzed cells that also displayed frequent arrests in cytokinesis and become multinucleated (data not shown). The cytokinesis failure is consistent with loss of ciliary motility (Brown, Marsala et al. 1999; Brown, Fine et al. 2003; Williams, Tsao et al. 2006; Tsao and Gorovsky 2008). These observations indicated that phenotypic assortment under drug selection has produced cells lacking macronuclear copies of *DYF1*. Importantly, we rescued the motility of paralyzed DYF1A cells by biolistic bombardment with a transgene encoding GFP-Dyf1p inserted into another non-essential locus (see below). Thus, we conclude that Dyf1p is required for cell motility.

A DYF1 $\Delta$  population grew with a generation time that was twice as long as that of wildtype (Figure 5.1C), at a rate similar to the rate displayed by a strain lacking Ift52p, an IFT complex B protein, in which cilia fail to assemble (Brown, Fine et al. 2003). Video recordings of live cells showed that DYF1 $\Delta$  cells were completely paralyzed (Figure 5.2A-C). In addition to locomotory functions, *Tetrahymena* cells use oral cilia for phagocytosis (Orias and Rasmussen 1976). Wildtype cells fed with India ink had an average of ~6.8 ink-filled food vacuoles per cell after 15 min of incubation in medium with ink (n = 39). Ink-filled vacuoles were not observed in DYF1 $\Delta$  cells, even after 30 min (n = 40) (data not shown). Thus, Dyf1p is needed for the function of both locomotory and oral cilia.

Immunofluorescence of DYF1 $\Delta$  cells with anti-tubulin antibodies showed that extremely short axonemes were present (Figure 5.2D, E) that resembled those observed in IFT52 $\Delta$  cells that are deficient in anterograde IFT (Figure 5.2F and (Brown, Fine et al. 2003)) or kinesin-2 (Brown, Marsala et al. 1999), except that many axoneme stubs appeared slightly longer in DYF1 $\Delta$  cells (Figure 5.2E,F).

Transmission electron microscopy (TEM) showed that basal bodies in DYF1 $\Delta$ cells had a wildtype appearance (Figure 5.3 and results not shown for cross-sections). In the longitudinal sections, most basal bodies (82%) have at least a partially assembled axoneme (Figure 5.3B-D and Table 5.1). Among the basal bodies lacking an axoneme, most have the central granule (the structure from which the central microtubules originate), but the corresponding portions of outer doublet microtubules are often either partly or completely missing (Figure 5.3E,F). Thus, the lack of Dyf1p affects the organization of microtubules within the transition zone. Many basal bodies lacking an axoneme are associated with an apparent ciliary membrane outgrowth (Figures 5.3E,F, Figures 5.4I,J and Table 5.1). In contrast, in IFT52 $\Delta$  cells, ~80% of basal bodies entirely lack an axoneme and the naked basal bodies lack membrane outgrowths (Table 5.1) and (Brown, Fine et al. 2003). In the DYF1 $\Delta$  cells, the axoneme, if present, could assemble to a variable extent (Figures 5.3B-D). Moreover, nearly all axoneme remnants of DYF1 $\Delta$ cells had an abnormal organization (Table 5.2). Frequently, DYF1 $\Delta$  axoneme crosssections lacked central pair microtubules (Figures 5.4C-H) or one or more outer microtubules (Figure 5.4E). Many DYF1 $\Delta$  axoneme cross-sections contain a mixture of outer doublets and singlets indicating instability or failure to assemble the B tubule (Figures 5.4B,D,F), or have partially truncated or opened B tubules (Figures 5.4G,H).

Thus, DYF1 $\Delta$  cells can assemble only extremely short and highly disorganized axonemes.

### Dyf1p localizes to the basal bodies and cilia

To test whether the ciliary defects observed in DYF1 $\Delta$  cells are caused by the loss of Dyf1p, we attempted to rescue the DYF1 $\Delta$  cells with a transgene encoding GFP-Dyf1p that was targeted to a non-essential BTU1 locus (Gaertig, Gao et al. 1999). Among the DYF1 $\Delta$  cells bombarded with a GFP-DYF1 transgene, motile cells appeared at the frequency of 0.007% while no motile cells were seen among the mock-transformed cells  $(n = 10^7)$ . Rescued GFP-Dyf1p cells were motile, had wildtype morphology (Figures 5.5A-A''). GFP-Dyflp localized to basal bodies and along axonemes, which resembled the localization of GFP- Ift52p (Brown, Fine et al. 2003). GFP alone that was expressed in the same locus and under the same promoter did not localize to cilia or basal bodies (Figures 5.5B-B''). The GFP-Dyf1p signal was lost almost completely when cells were extracted with a detergent prior to fixation (Figures 5.6A-A"). A similar observation was made for cells expressing GFP-IFT52p (Figures 5.6B-B"). These observations indicate that Dyf1p is not stably associated with the axoneme, and most likely is a component of the ciliary matrix. Strong overproduction of GFP- Dyf1p had no obvious effect on the length of cilia or cell motility (results not shown).

# Residual axonemes in the *DYF1* knockout cells have hyperglutamylated microtubules

A study in zebrafish showed that DYF-1/*fleer* mutants have short cilia that have reduced levels of tubulin glutamylation (Pathak, Obara et al. 2007). We evaluated DYF1 $\Delta$  cells by immunofluorescence with ID5, a monoclonal antibody (Rüdiger, Rüdiger et al. 1999) that in *Tetrahymena* is specific to polyglutamylated tubulin (Wloga, Rogowski et al. 2008). Unexpectedly, the levels of tubulin polyglutamylation were increased in the axoneme remnants of DYF1 $\Delta$  cells imaged side-by-side with wild type axonemes (Figure 5.5C). In wildtype cells, ID5 strongly labels basal bodies and weakly labeled axonemes (Figure 5.5C, cell indicated by arrow). However, in the DYF1 $\Delta$ mutants, the signal of ID5 is equally strong in the basal body and in the axoneme remnant (Figure 5.5C). In wildtype cells, short cilia that are in the course of assembly have increased levels of tubulin glutamylation as compared to full length mature cilia (Sharma, Bryant et al. 2007). We compared wildtype cells undergoing cilia regeneration (after deciliation) with DYF1 $\Delta$  cells side-by-side (Figure 5.5D). It appears that the levels of tubulin polyglutamylation in axoneme stubs in DYF1 $\Delta$  cells are higher even then in assembling wildtype axonemes. Thus, the DYF1 $\Delta$  axoneme remnants have hyperglutamylated microtubules.

Interestingly, the axoneme stubs in IFT52 $\Delta$  cells also have elevated levels of tubulin glutamylation (Figure 5.5E). Thus, tubulin hyperglutamylation could be generally associated with a failed axoneme assembly. However, it is known that some post-translational modifications of tubulin accumulate on long-lived microtubules (reviewed in (Verhey and Gaertig 2007)). Thus, an alternative explanation is that the longer cell

cycle in cells lacking functional cilia (Figure 5.1C) is associated with accumulation of post-translationally modified microtubules. However, antibodies specific to monoglycylation (TAP952) and polyglycylation (AXO49) (Bré, Redeker et al. 1998) showed no obvious differences in the levels of glycylated tubulin isoforms between wildtype and DYF1 $\Delta$  or IFT52 $\Delta$  axonemes and basal bodies (data not shown). To explore further whether a longer cell cycle is associated with increased tubulin glutamylation in axonemes, we created a knockout strain lacking a *Tetrahymena* ortholog of PF20 (TTHERM 00134890), a protein required for stability of the central pair in *Chlamydomonas reinhardtii* (Smith and Lefebvre 1997). PF20A cells were paralyzed and grew slowly, with a generation time similar to that of DYF1 $\Delta$  and IFT52 $\Delta$  cells (Figure 5.S2B). PF20 $\Delta$  cells assembled normal length axonemes. TEM showed a mixture of 9+2 and 9+0 axoneme cross-sections indicating a partial defect in the formation or stability of central microtubules (our unpublished data). A side-by-side labeling of PF20 $\Delta$  and wildtype cells showed only a slight increase in the levels of tubulin glutamylation in the basal bodies and axonemes of PF20A cells (Figures 5.S2A-A3). However, unlike in DYF1 $\Delta$  and IFT52 $\Delta$  cells, the axoneme in PF20 $\Delta$  cells maintains a lower level of glutamylation as compared to the adjacent basal body (Figures 5.S2A-A3). We conclude that hyperglutamylation of residual axonemes in IFT52 $\Delta$  and DYF1 $\Delta$  cells can not be explained solely by an increased time of exposure to tubulin modifying enzymes, and that in *Tetrahymena*, tubulin hyperglutamylation could be associated with a severe failure of axonemal assembly.

### Discussion

Studies in *C. elegans* showed that the assembly of the distal segments of axonemes that contain only A tubules of outer microtubules, requires the homodimeric OSM-3 kinesin motor and DYF-1. It has been proposed that DYF-1 activates OSM-3 or links IFT particles to OSM-3 (Snow, Ou et al. 2004; Ou, Blacque et al. 2005; Evans, Snow et al. 2006). *Tetrahymena* has the distal portions of cilia with prominent singlet extensions (Sale and Satir 1976; Suprenant and Dentler 1988). Moreover, the genome of Tetrahymena encodes orthologs of motor subunits of OSM-3 kinesin-2, including Kin5p, a motor that localizes to cilia (Awan, Bernstein et al. 2004), and as we show here, Tetrahymena has a DYF1 ortholog, Dyf1p. To our surprise, knocking out DYF1 in Tetrahymena resulted in a complete or nearly complete loss of the axoneme, indicating that in *Tetrahymena*, DYF-1 is required for either the assembly or stability all axoneme segments. Studies in zebrafish and Trypanosoma support our observations, indicating a broader role for DYF-1 in the axoneme assembly or stability. Cilia in the zebrafish dyf-*1/fleer* mutant embryos, in addition to reduced length, showed defects in the structure of the outer doublets in the middle segment (Pathak, Obara et al. 2007). Consistently, in *Tetrahymena* cells lacking DYF-1, the B tubule is often either completely absent or detached from the A tubule. Moreover, in cilia of both *Tetrahymena* and in zebrafish deficient in DYF-1, central microtubules fail to assemble (see Figure 7B in (Pathak, Obara et al. 2007)). Recently, RNAi-based depletion of a DYF-1 related protein of Trypanosoma has led to loss of flagella, consistent with a role of DYF-1 for the entire axoneme (Absalon, Blisnick et al. 2008).
At first, our, zebrafish (Pathak, Obara et al. 2007) and the *Trypanosoma* data (Absalon, Blisnick et al. 2008) appear to contradict the C. elegans studies (Ou, Blacque et al. 2005; Evans, Snow et al. 2006) showing a specific requirement of DYF-1 for the distal segment assembly. However, it is possible that the different outcomes of DYF1 deficiencies in diverse models reflect structural and functional differences between cilia in these models. In particular, cilia in *C. elegans* are quite unique due to their lack of motility, absence of central microtubules and unusual arrangement of outer microtubules (Inglis, Ou et al. 2007). Also, it is possible that in *Tetrahymena*, zebrafish and Trypanosoma, DYF-1 is also specifically involved in the assembly of the distal segment, but a failure to develop the distal segment leads to a catastrophic lack of stability of the entire axoneme. For example, a failure to assemble the distal segment could prevent the assembly of caps, structures that connect the ends of outer and central pair microtubules to the ciliary membrane (Dentler 1980). This hypothesis could be tested in the future, if a temperature-sensitive mutation in Dyf1p can be identified, to destabilize the activity of Dyf1p in an already assembled cilium. The availability of DYF-1 knockout *Tetrahymena* cells will allow for systematic mutagenesis of Dyf1p based on functional rescue.

Pathak and colleagues observed a decrease in the levels of tubulin glutamylation in the *dyf-1/fleer* mutant axonemes (Pathak, Obara et al. 2007). According one of their models, DYF-1/fleer acts as a cargo adaptor that links IFT particles to enzymes required for glutamylation of tubulin (Pathak, Obara et al. 2007). However, we show that the residual axonemes in DYF1 $\Delta$  *Tetrahymena* cells have elevated levels of tubulin polyglutamylation. We also show that in *Tetrahymena*, an extreme truncation of the axoneme is generally associated with tubulin hyperglutamylation. It is possible that in

*Tetrahymena*, in the absence of Dyf1p, tubulin glutamic acid ligase enzymes accumulate in the axoneme remnant. An alternative explanation of the zebrafish DYF-1 knockdown effect on tubulin polyglutamylation can also be considered (Pathak, Obara et al. 2007). In axonemes of several organisms, tubulin glutamylation is concentrated on the B tubule of outer doublets (Fouquet, Edde et al. 1994; Multigner, Pignot-Paintrand et al. 1996; Lechtreck and Geimer 2000). If in zebrafish, loss of DYF-1 primarily destabilizes the B tubules, and the corresponding A tubules remain stable, this itself could lead to a selective loss of glutamylated tubulin from the axoneme.

In *Tetrahymena*, the consequences of loss of Dyf1p resemble the phenotype of loss of function of anterograde IFT, including deficiencies in the complex B proteins: IFT52 (Brown, Fine et al. 2003) and IFT172 (Tsao and Gorovsky 2008), and a deficiency in kinesin-2 (Brown, Marsala et al. 1999). In contrast, deficiencies in the retrograde IFT do not lead to a loss of the axonemes in *Tetrahymena* (Tsao and Gorovsky 2008) (Rajagopalan, Subramanian et al. 2009). These data taken together suggest that Dyf1p is associated with the anterograde IFT. This suggestion is supported by our observations that Dyf1p is present primarily in the detergent-soluble fraction of cilia (Figure 5.6). However, in contrast to the anterograde IFT *Tetrahymena* mutants, in DYF1 $\Delta$  cells the axonemes assemble to some extent. Moreover, some axoneme stubs in DYF1 $\Delta$  cells have a central pair, while central microtubules fail to assemble in anterograde IFT deficient cells (Morris and Scholey 1997; Gaertig, Gao et al. 1999; Brown, Fine et al. 2003). In fact, the common feature of DYF-1 deficiencies in C. elegans, Tetrahymena, and zebrafish is that axonemal microtubules in all these models assemble to some extent. Thus, DYF-1 is probably not required for transport of axonemal precursor tubulin.

Instead, DYF-1 could serve as an anterograde IFT adapter for cargo that is needed to stabilize the assembling axoneme.

Interestingly, 20% of basal bodies in *Tetrahymena* DYF1 $\Delta$  cells lacked an axoneme but had a "bubble" of an apparent ciliary plasma membrane. It is possible that under Dyf1p deficiency, the axoneme can initially assemble but breaks down with a delayed resorption of the corresponding ciliary membrane. Alternatively, the ciliary membrane can expand without a corresponding assembly of the axoneme. A similar phenotype was observed for several IFT complex B deficiencies in Trypanosoma (Absalon, Blisnick et al. 2008). There is growing evidence that the delivery of precursor membranes destined to cilia is coupled to IFT. For example, multiple proteins that are either components of IFT particles or interact with IFT, bind to ciliary membranes or membrane-associated proteins, including Rab8, a small G-protein that interacts with the BBSome complex (Nachury 2008), and Elipsa that directly interacts with IFT20 and indirectly with Rab8 (Omori, Zhao et al. 2008). Moreover, IFT20 (an IFT complex B protein) localizes to the Golgi network and is required for targeting polycystin-2 channels to the ciliary membranes (Follit, Tuft et al. 2006). Thus, we confirm earlier observations (Absalon, Blisnick et al. 2008) that during IFT, the axoneme assembly and ciliary membrane expansion can be uncoupled under certain conditions.

#### **Materials and Methods**

### Strains and cultures

*Tetrahymena thermophila* strains were grown at 30°C with shaking, in either SPP (Gorovsky, Yao et al. 1975) or MEPP medium (Orias and Rasmussen 1976) with an antibiotic-antimycotic mixture (Invitrogen, Carlsbad, CA). Wildtype strains CU428 and CU522 were obtained from the *Tetrahymena* Stock Center, (Cornell University, Ithaca, NY).

#### *Phylogentic studies*

Sequences of DYF-1 homologs were obtained from the NCBI databases. Gene accession numbers are listed in the legend of Figure 5.1. Sequences were aligned with ClustalX 1.82 (Jeanmougin, Thompson et al. 1998) and corrected manually in SEAVIEW (Galtier, Gouy et al. 1996). A neighbor-joining tree was calculated using the Phylip package (SEQBOOT, PROTDIST, NEIGHBOR, CONSENSE, DRAWGRAM) (Felsenstein 1997).

### Disruption of DYF1

The coding region of *DYF1* (TTHERM\_00313720) was identified in the *Tetrahymena* Genome Database by BLAST searches using the *C. elegans* DYF-1 sequence. Using *Tetrahymena* genomic DNA as a template, two non-overlapping fragments of *DYF1* were amplified using the following primer pairs:

5'ATAGGGCCCGTTTAGAGATACCAGAATTT-3', 5'-TTTCCCGGGCTTGATTGGCTTCATTTTT-3'and 5'-

## CCCACTAGTGCGTTTTGATTCTTTTTG G-3', 5'-

## TTTGCGGCCGCGGTATCAGTGTTAATCTTT-3'

Using restriction site sequences that were incorporated near the 5' ends of the above primers and the two fragments were subcloned into pTvec-Neo3, (Shang, Song et al. 2002) so that the *neo3* gene was positioned in an opposite transcriptional orientation. The targeting fragments were designed to flank the first four exons ( $\sim 1.6$  kb) of DYF1. The targeting fragment was separated from the rest of the plasmid using ApaI and NotI. CU428 Tetrahymena cells were grown to 2 x 10<sup>5</sup> cells/ml and were starved at 30°C for 20 hr in 10 mM Tris-HCl buffer pH 7.5, and subjected to macronuclear biolistic transformation (Cassidy-Hanley, Bowen et al. 1997). Bombarded cells were incubated at  $30^{\circ}$ C for 2 hr in SPP with 2 µg/ml CdCl<sub>2</sub> followed by selection in SPP medium with 100  $\mu$ g/ml paromomycin and 2  $\mu$ g/ml CdCl<sub>2</sub> at 30°C for 3 days. Several transformant clones were phenotypically assorted for a complete elimination of wildtype copies of DYF1 by growing under increasing selective pressure of paromomycin (up to 500  $\mu$ g/ml) and 1 µg/ml CdCl<sub>2</sub> during 14 days. Single cell isolations were made into drops of a drug-free MEPP medium. Immotile cells appeared in 31% of drops (n = 96). The immotile cells were re-isolated twice. Upon prolonged growth (20 generations) in a drug-free medium, no reversion to a motile phenotype was observed, indicating that almost all native copies of *DYF1* were lost due to phenotypic assortment.

The *Tetrahymena* gene (TTHERM\_00134890) encoding a protein orthologous to PF20 of *Chlamydomonas reinhardtii* (Smith and Lefebvre 1997) was eliminated from the macronucleus using a similar strategy. The following primers were used to amplify targeting fragments for PF20 that were subcloned on the sides of the *neo2* cassette: 5'-

# TTATAGAGCTCGCAACGGGTTACAAGACT-3', 5'-ATATTGGATCCTGGCTTTTTATCTTCCTTAG-3', 5-TAATTGGATCCCGAAGATAAAGTAGAAGACG-3' and 5'-AATTACTCGAGATATCATTTATCCTTGCTTCTA-3'.

## Phenotypic studies

To measure the rate of phagocytosis, cells were fed with 0.2% India ink in SPP for 15 min at 30°C, and fixed with 2% paraformaldehyde. To measure the rate of cell locomotion, paths of moving cells were recorded using a Nikon TMS microscope and measured using NIH Image 1.62. To determine the growth rate, cells (at the initial concentration of 10<sup>4</sup> cells/ml) were grown in 25 ml of MEPP at 30°C and counted every 3 hr.

## GFP tagging of DYF1

The predicted coding sequence of *DYF1* was amplified using primers: 5'-AAA ACG CGT C ATG AAG CCA ATC AAG TAG ATT-3', 5'-TTT GGA TCC TAA TTT TAT CAA TTT CTT AAC TTG-3'. The resulting fragment was digested using MluI and BamHI, and ligated into pMTT1-GFP for N-terminal tagging with GFP. The *DYF1* knockout cells were starved overnight in 10 mM Tris-HCl pH 7.5, at room temperature and biolistically transformed as described above, suspended in MEPP medium with 1.5 µg/ml CdCl<sub>2</sub> and incubated at 30°C for 3 days. Transformant clones were identified based on the recovery of cell motility. As a negative control, a mock biolistic transformation was performed without plasmid DNA.

#### Microscopy

For immunofluorescence with 12G10 anti- $\alpha$ -tubulin monoclonal antibody (1:50 dilution) and SG polyclonal anti-tubulin antibodies (1:100), ~100 Tetrahymena cells were simultaneously fixed and permeabilized with 2% paraformaldehyde and 0.5% Triton-X-100 in PHEM buffer. For staining with ID5 monoclonal antibody (Rüdiger, Rüdiger et al. 1999) that in *Tetrahymena* is specific for polyglutamylated tubulin (Wloga, Rogowski et al. 2008)(1:40 dilution) ~100 Tetrahymena cells was isolated on coverslips and permeabilized with 0.5% Triton-X-100 in PHEM buffer for 45 seconds and fixed with 2% paraformaldehyde in the same buffer. Cells fixed with either method were air-dried at 30°C and processed for immunofluorescence labeling as described in (Gaertig, Cruz et al. 1995). The secondary antibody used was either goat-anti-mouse or goat-anti-rabbit FITC conjugate (1:200, Zymed). For direct detection of GFP and testing detergent solubility of GFP fusion proteins, cells expressing either GFP-Dyf1p or IFT52p-GFP or GFP alone were prepared for microscopic observations using 3 methods as follows (all fixation/permeabilization were solutions based on the PHEM buffer). Cells ( $\sim 1 \times 10^{5}$ ) were washed with 10 mM Tris pH 7.5 and the pellet was combined with 300 ml of 1) 300 ml of 0.5% Triton-X-100, after 3 min washed with PHEM buffer (1 ml) and fixed with 2% paraformaldehyde (300 ml) for 30 min; 2) 300 ml of 2% paraformaldehyde for 30 min; 3) 300 ml of 0.25% Triton-X-100 and 1% paraformaldehyde for 30 min. All samples were washed with phosphate buffered saline and observed as explained above. For TEM, cells were processed as described in (Jerka-Dziadosz, Strzyewska-Jowko et al. 2001).

#### Acknowledgements

This work was supported by National Science Foundation grant MBC-033965 to JG. We acknowledge excellent assistance with TEM by Mary Ard (College of Veterinary Medicine, UGA). We thank Winfield Sale (Emory University) for helpful suggestions. We also thank Martin A. Gorovsky (University of Rochester) for SG antibodies, Joseph Frankel (University of Iowa) for the 12G10 antibody (available from the Developmental Studies Hybridoma Bank), Klaus Weber (Max Planck Institute, Goettingen) for the ID5 antibody and Marie-Helene Bré and Nicolette Levilliers (Université Paris-Sud, Orsay) for TAP952 and AXO49 antibodies.

#### References

- Absalon, S., T. Blisnick, et al. (2008). "Intraflagellar transport and functional analysis of genes required for flagellum formation in Trypanosomes." <u>Mol. Biol. Cell.</u> 19: 929-944.
- Awan, A., M. Bernstein, et al. (2004). "Cloning and characterization of Kin5, a novel Tetrahymena ciliary kinesin II." <u>Cell Motil Cytoskeleton</u> 58(1): 1-9.
- Badano, J. L., N. Mitsuma, et al. (2006). "The ciliopathies: an emerging class of human genetic disorders." <u>Annu Rev Genomics Hum Genet</u> 7: 125-48.
- Bobinnec, Y., A. Khodjakov, et al. (1998). "Centriole disassembly *in vivo* and its effect on centrosome structure and function in vertebrate cells." <u>J. Cell Biol.</u> 143: 1575-1589.

- Bré, M. H., V. Redeker, et al. (1998). "Tubulin polyglycylation: differential posttranslational modification of dynamic cytoplasmic and stable axonemal microtubules in *Paramecium*." <u>Mol. Biol. Cell</u> **9**: 2655-2665.
- Brown, J. M., N. A. Fine, et al. (2003). "Hypoxia regulates assembly of cilia in suppressors of Tetrahymena lacking an intraflagellar transport subunit gene." <u>Mol</u> <u>Biol Cell</u> 14(8): 3192-207.
- Brown, J. M., C. Marsala, et al. (1999). "Kinesin-II is preferentially targeted to assembling cilia and is required for ciliogenesis and normal cytokinesis in *Tetrahymena*." <u>Mol. Biol. Cell</u> 10: 3081-3096.
- Campbell, P. K., K. G. Waymire, et al. (2002). "Mutation of a novel gene results in abnormal development of spermatid flagella, loss of intermale aggression and reduced body fat in mice." <u>Genetics</u> 162(1): 307-20.
- Cassidy-Hanley, D., J. Bowen, et al. (1997). "Germline and somatic transformation of mating *Tetrahymena thermophila* by particle bombardment." <u>Genetics</u> 146: 135-147.
- Cole, D. G., D. R. Diener, et al. (1998). "*Chlamydomonas* kinesin-II-dependent intraflagellar transport (IFT):
- IFT particles contain proteins required for ciliary assembly in

Caenorhabditis elegans sensory neurons." J. Cell Biol. 18: 993-1008.

- Dave, D., D. Wloga, et al. (2009). "DYF-1 Is required for assembly of the axoneme in Tetrahymena thermophila." <u>Eukaryot Cell</u> **8**(9): 1397-406.
- Dentler, W. L. (1980). "Structures linking the tips of ciliary and flagellar microtubules to the membrane." J Cell Sci 42: 207-20.

- Eddé, B., J. Rossier, et al. (1990). "Posttranslational glutamylation of α-tubulin." <u>Science</u> 247: 83-85.
- Eisen, J. A., R. S. Coyne, et al. (2006). "Macronuclear Genome Sequence of the Ciliate Tetrahymena thermophila, a Model Eukaryote." <u>PLoS Biol</u> **4**(9).
- Evans, J. E., J. J. Snow, et al. (2006). "Functional modulation of IFT kinesins extends the sensory repertoire of ciliated neurons in Caenorhabditis elegans." <u>J Cell Biol</u> 172(5): 663-9.
- Felsenstein, J. (1997). "An alternating least squares approach to inferring phylogenies from pairwise distances." <u>Syst. Biol.</u> **46**(1): 101-11.
- Follit, J. A., R. A. Tuft, et al. (2006). "The intraflagellar transport protein IFT20 is associated with the Golgi complex and is required for cilia assembly." <u>Mol Biol</u> <u>Cell</u> 17(9): 3781-92.
- Fouquet, J. P., B. Edde, et al. (1994). "Differential distribution of glutamylated tubulin during spermatogenesis in mammalian testis." <u>Cell Motil.Cytoskeleton</u>. **27**: 49-58.
- Gaertig, J., M. A. Cruz, et al. (1995). "Acetylation of lysine 40 in alpha-tubulin is not essential in *Tetrahymena thermophila*." J.Cell Biol. **129**: 1301-1310.
- Gaertig, J., Y. Gao, et al. (1999). "Surface display of a parasite antigen in the ciliate *Tetrahymena thermophila*." <u>Nat. Biotech.</u> **17**: 462-465.
- Gaertig, J. and D. Wloga (2008). Ciliary Tubulin and Its Post-translational Modifications. <u>Ciliary Function in Mammalian Development</u>. B. K. Yoder, Elsevier Inc. 85: 83-109.

- Galtier, N., M. Gouy, et al. (1996). "SEAVIEW and PHYLO\_WIN: two graphic tools for sequence alignment and molecular phylogeny." <u>Comput Appl Biosci</u> 12(6): 543-8.
- Gorovsky, M. A., M.-C. Yao, et al. (1975). "Isolation of micro- and macronuclei of Tetrahymena pyriformis." <u>Methods Cell Biol.</u> **IX**: 311-327.
- Hou, Y., H. Qin, et al. (2007). "Functional analysis of an individual IFT protein: IFT46 is required for transport of outer dynein arms into flagella." <u>J Cell Biol</u> 176(5): 653-65.
- Inglis, P. N., G. Ou, et al. (2007). "The sensory cilia of Caenorhabditis elegans." <u>WormBook</u>: 1-22.
- Janke, C., K. Rogowski, et al. (2005). "Tubulin polyglutamylase enzymes are members of the TTL domain protein family." <u>Science</u> 308: 1758-1762.
- Jeanmougin, F., J. D. Thompson, et al. (1998). "Multiple sequence alignment with Clustal X." <u>Trends Biochem Sci</u> 23(10): 403-5.
- Jerka-Dziadosz, M., I. Strzyewska-Jowko, et al. (2001). "The dynamics of filamentous structures in the apical band, oral crescent, fission line and the postoral meridional filament in *Tetrahymena thermophila* revealed by the monoclonal antibody 12G9." <u>Protist</u> 152(1): 53-67.
- Kozminski, K. G., P. L. Beech, et al. (1995). "The *Chlamydomonas* kinesin-like protein FLA10 is involved in motility associated with the flagellar membrane." <u>J. Cell</u>
   <u>Biol.</u> 131: 1517-1527.
- Kozminski, K. G., K. A. Johnson, et al. (1993). "A motility in the eukaryotic flagellum unrelated to flagellar beating." <u>Proc. Natl. Acad. Sci. USA</u> **90**: 5519-5523.

- Lechtreck, K.-F. and S. Geimer (2000). "Distributon of polyglutamylated tubulin in the flagellar apparatus of green flagellates." <u>Cell Motility Cytoskeleton</u> **47**: 219-235.
- Marshall, W. F. and J. L. Rosenbaum (2001). "Intraflagellar transport balances continuous turnover of outer doublet microtubules: implications for flagellar length control." <u>J Cell Biol.</u> 155(3): 405-414.
- Mesland, D. A., J. L. Hoffman, et al. (1980). "Flagellar tip activation stimulated by membrane adhesions in Chlamydomonas gametes." <u>J Cell Biol</u> 84(3): 599-617.
- Morris, R. L. and J. M. Scholey (1997). "Heterotrimeric kinesin-II is required for the assembly of motile 9+2 ciliary axonemes on sea urchin embryos." <u>J. Cell Biol.</u>
  138: 1009-1022.
- Multigner, L., I. Pignot-Paintrand, et al. (1996). "The A and B tubules of the outer doublets of sea urchin sperm axonemes are composed of different tubulin variants." <u>Biochemistry</u> 35: 10862-10871.
- Nachury, M. V. (2008). "Tandem affinity purification of the BBSome, a critical regulator of Rab8 in ciliogenesis." <u>Methods Enzymol</u> **439**: 501-13.
- Nicastro, D., C. Schwartz, et al. (2006). "The molecular architecture of axonemes revealed by cryoelectron tomography." <u>Science</u> **313**(5789): 944-8.
- Omori, Y., C. Zhao, et al. (2008). "Elipsa is an early determinant of ciliogenesis that links the IFT particle to membrane-associated small GTPase Rab8." <u>Nat Cell Biol</u> **10**(4): 437-44.
- Orias, E. and L. Rasmussen (1976). "Dual capacity for nutrient uptake in tetrahymena. IV. growth without food vacuoles." <u>Exp. Cell Res.</u> **102**: 127-137.

- Ou, G., O. E. Blacque, et al. (2005). "Functional coordination of intraflagellar transport motors." <u>Nature</u> 436(7050): 583-7.
- Pathak, N., T. Obara, et al. (2007). "The Zebrafish fleer gene encodes an essential regulator of cilia tubulin polyglutamylation." <u>Mol Biol Cell</u> **18**(11): 4353-64.
- Pazour, G. J., B. L. Dickert, et al. (1999). "The DHC1b (DHC2) isoform of cytoplasmic dynein is required for flagelar assembly." <u>J.Cell.Biol</u> 144(3): 473-481.
- Piperno, G. and K. Mead (1997). "Transport of a novel complex in the cytoplasmic matrix of *Chlamydomonas* flagella." <u>Proc. Natl. Acad. Sci. USA</u> 94: 4457-4462.
- Porter, M. E., R. Bower, et al. (1999). "Cytoplasmic dynein heavy chain 1b is required for flagellar assembly in Chlamydomonas." <u>Mol. Biol. Cell</u> **10**: 693-712.
- Qin, H., D. T. Burnette, et al. (2005). "Intraflagellar transport is required for the vectorial movement of TRPV channels in the ciliary membrane." <u>Curr Biol</u> 15(18): 1695-9.
- Rajagopalan, V., A. Subramanian, et al. (2009). "Dynein-2 affects the regulation of ciliary length but is not required for ciliogenesis in Tetrahymena thermophila."
   <u>Mol Biol Cell</u> 20(2): 708-20.
- Redeker, V., N. Levilliers, et al. (1994). "Polyglycylation of tubulin: a post-translational modification in axonemal microtubules." <u>Science</u> 266: 1688-1691.
- Redeker, V., N. Levilliers, et al. (2005). "Mutations of tubulin glycylation sites reveal cross-talk between the C termini of alpha- and beta-tubulin and affect the ciliary matrix in *Tetrahymena*." J Biol Chem 280(1): 596-606.
- Rüdiger, A. H., M. Rüdiger, et al. (1999). "Monoclonal antibody ID5: epitope characterization and minimal requirements for the recognition of polyglutamylated alpha- and beta-tubulin." <u>Eur J Cell Biol</u> 78(1): 15-20.

- Sale, W. and P. Satir (1976). "Splayed Tetrahymena cilia. A system for analyzing sliding and
- axonemal spoke arrangements." J. Cell Biol. 71: 589-565.
- Sale, W. S. and P. Satir (1977). "The termination of the central microtubules from the cilia of Tetrahymena pyriformis." <u>Cell Biol Inter</u> 1(1): 45-49.
- Shang, Y., X. Song, et al. (2002). "A robust inducible-repressible promoter greatly facilitates gene knockouts, conditional expression, and overexpression of homologous and heterologous genes in Tetrahymenathermophila." <u>Proc Natl</u> <u>Acad Sci</u> 99: 3734-3739.
- Sharma, N., J. Bryant, et al. (2007). "Katanin regulates dynamics of microtubules and biogenesis of motile cilia." <u>J Cell Biol</u> 178(6): 1065-79.
- Signor, D., K. P. Wedaman, et al. (1999). "Role of a class DHC1b dynein in retrograde trasnport of IFT motors and IFT raft particles along cilia but not dentrites, in chemosensory neurons of living *Caenorhabditis elegans*." <u>J.Cell.Biol</u> 147(3): 519-530.
- Signor, D., K. P. Wedaman, et al. (1999). "Two heteromeric kinesin complexes in chemosensory neurons and sensory cilia of Caenorhabditis elegans." <u>Mo. Biol.</u> <u>Cell</u> 10: 345-360.
- Smith, E. F. and P. A. Lefebvre (1997). "PF20 gene product contains WD repeats and localizes to the intermicrotubule bridges in Chlamydomonas flagella." <u>Mol Biol</u> <u>Cell</u> 8(3): 455-67.

- Snow, J. J., G. Ou, et al. (2004). "Two anterograde intraflagellar transport motors cooperate to build sensory cilia on C. elegans neurons." <u>Nat Cell Biol</u> 6(11): 1109-13.
- Sui, H. and K. H. Downing (2006). "Molecular architecture of axonemal microtubule doublets revealed by cryo-electron tomography." <u>Nature</u> 442(7101): 475-8.
- Suprenant, K. A. and W. L. Dentler (1988). "Release of intact microtubule-capping structures from Tetrahymena cilia." J.Cell Biol. 107: 2259-2269.
- Tsao, C. C. and M. A. Gorovsky (2008). "Different effects of tetrahymena IFT172 domains on anterograde and retrograde intraflagellar transport." <u>Mol Biol Cell</u> 19(4): 1450-61.
- Tsao, C. C. and M. A. Gorovsky (2008). "Tetrahymena IFT122A is not essential for cilia assembly but plays a role in returning IFT proteins from the ciliary tip to the cell body." <u>J Cell Sci</u> 121(Pt 4): 428-36.
- Turkewitz, A. P., E. Orias, et al. (2002). "Functional genomics: the coming of age for *Tetrahymena thermophila*." <u>Trends in Genetics</u> 18: 35-40.

Verhey, K. J. and J. Gaertig (2007). "The tubulin code." Cell Cycle 6(17): 2152-60.

- Walther, Z., M. Vashistha, et al. (1994). "The *Chlamydomonas FLA10* gene encodes a novel kinesin-homologous protein." <u>J.Cell Biol.</u> 126: 175-188.
- Williams, N. E., C. C. Tsao, et al. (2006). "The actin gene ACT1 is required for phagocytosis, motility, and cell separation of Tetrahymena thermophila."
   <u>Eukaryot Cell</u> 5(3): 555-67.
- Wloga, D., A. Camba, et al. (2006). "Members of the Nima-related kinase family prmote disassembly of cilia by multiple mechanisms "<u>Mol. Biol. Cell</u> 17: 2799-2810.

Wloga, D., K. Rogowski, et al. (2008). "Glutamylation on α-tubulin is not essential but affects the assembly and functions of a subset of microtubules in *Tetrahymena*." <u>Eukaryot Cell</u> 7: 1362-1372.

## **Figure Legends**

**Figure 5.1**. *Tetrahymena* has a DYF-1 ortholog. A. A schematic representation of DYF-1 protein sequences with TPR domains marked by grey boxes. B. An unrooted phylogenetic tree of DYF-1 proteins. The tree was calculated by a neighbor-joining method. The nodes at branches indicate bootstrap values >50%. Abbreviations: Tt, *Tetrahymena thermophila* (TTHERM\_00313720); Pt, *Paramecium tetraurelia* (XP\_001424610.1); Cr, *Chlamydomonas reinhardtii* (XP\_001692406.1); Li, *Leishmania infantum* (XP\_001466521.1); Tb, *Trypanosoma brucei* (XP\_844139.1); Tc, *Trypanosoma cruzi* (XP\_818837.1); Gl, *Giardia lambila* (XP\_001706451.1); Dm, *Drosophila melanogaster* (CG5142-PB); Ag, *Anopheles gambiae* (XP\_312036.2); Ce, *Caenorhabditis elegans* (F54C1.5a); Am, *Apis mellifera* (XP\_397369.2); Sp, *Stronglyocentrotus purpuratus* (XP\_794254.2); Dr, *Danio rerio* (NP\_001098119.2); Xl, *Xenopus laevis*; Hs, *Homo sapiens* (NP\_689730.2); Gg, *Gallus gallus* (XP\_426574.2). C. Growth curves for DYF1Δ and IFT52Δ strains grown at 30°C in MEPP medium.



**Figure 5.2**. Dyf1p is required for axoneme assembly. A-C. Video images of live wild type cells recorded for 5 sec (A) and DYF1 $\Delta$  recorded for 5 sec (B) and 15 sec (C). The paths of motile wildtype cells are marked with arrows. Note that DYF1 $\Delta$  cells do not show motility even during 15 sec. D-F. Wild type (D), DYF1 $\Delta$  (E) and IFT52 $\Delta$  (F) cells stained with anti-tubulin antibodies. Bars represent 10 µm.



**Figure 5.3**. Loss of function of *DYF1* results in shortened, disorganized or missing axonemes. Longitudinal TEM sections of wildtype cilia (A) and DYF1 $\Delta$  cilia (B-F). Bars represent 0.2  $\mu$ m.



Figure 5.4. Loss of function of *DYF1* severely disorganizes the axoneme.

TEM cross-sections of wildtype (A), DYF1 $\Delta$  locomotory (B-I) and DYF1 $\Delta$  oral cilia (J).

DYF1A locomotory cilia have a wide range of defects: missing central pair microtubules

(C-J), incomplete B tubules (G, H), outer singlet microtubules (B, D, F) and displaced

outer doublet microtubules (D, E). I-J. Portions of the DYF1A somatic cortex (I) and oral

region (J), arrowheads mark membrane outgrowths. Bars represent 0.2 µm.



**Figure 5.5**. GFP-DYF1p localizes to axonemes and basal bodies and axoneme remnants in DYF1 $\Delta$  cells are hyperglutamylated. A-B''. GFP distribution in cells expressing GFP-Dyf1p (A-A'') and GFP alone (B-B''). The GFP-DYF1 (A-A'') or GFP (B-B'') transgenes are inserted into the *BTU1* locus and both operate under the *MTT1* promoter. GFP-DYF1 transgene was introduced by rescue of DYF1 $\Delta$  knockout strains while the GFP transgene was introduced as described (Wloga, Camba et al. 2006). Cells were induced to express the transgenes by incubation with 2.5 mg/ml CdCl<sub>2</sub> for 3 hr. The insets show higher magnifications of the boxed areas in A'' and B''. C-E. Growing (C) and cilia-regenerating (30 min) wild type cells were mixed with either DYF1 $\Delta$  cells (C, D) or IFT52 $\Delta$  (E) cells and labeled side-by side with the anti-polyglutamylated tubulin ID5 antibodies. Arrowheads mark wildtype cells and the insets show higher magnification of boxed areas in C-E. Bars represent 10 µm.



**Figure 5.6.** Dyf1p, like IFT52p is a component of the detergent-soluble ciliary matrix. Cells expressing GFP-Dyf1p (A, A', A''), IFT52p-GFP (B, B', B'') and GFP (C, C', C'') were fixed using three different methods. Cells shown in panels A-C were permeabilized with Triton-X-100 (3 min) and postfixed with paraformaldehyde (PFA). Cells shown in panels A'-C' were fixed with PFA and permeabilized with Triton-X-100. Cells shown in panels A''-C'' were simultaneously fixed and permeabilized in a mixture of PFA and Triton-X-100. Bar represents 10 μm.



**Figure 5.S1**. In DYF1 $\Delta$  cells, the *neo3* cassette is integrated at the expected position within the *DYF1* locus. A. A scheme showing the predicted organization of the wildtype and disrupted *DYF1* loci. The grey boxes show portions of the locus subcloned onto the targeting fragment. Arrows mark the positions of diagnostic primers used for amplification of the junction between *neo3* and the flanking region of the *DYF1* locus that is positioned outside of the targeting fragment. B. A PCR analysis of the genomic DNA of wild-type and DYF1 $\Delta$  cells using either a pair of primers marked in A or a pair of control primers designed to amplify a non-targeted *NRK1* locus. As expected, a 2.7 kb amplification product is seen in the DYF1 $\Delta$  sample but not in wild-type cells indicating that the *neo3* cassette is integrated at the desired position.



**Figure 5.S2**. An extended cell cycle cannot explain hyperglutamylation of axonemal microtubules. A-B3. Wildtype and PF20 $\Delta$  cells were stained side-by side with the monoclonal anti-polyglutamylated tubulin antibody, ID5 (A) and polyclonal anti-tubulin antibodies SG (A1). A2 shows a merged image. Wildtype cells were fed with black India ink and are identified by phase contrast (A3). B. A growth curve graph of wildtype and PF20 $\Delta$  grown in MEPP medium. Note: PF20 $\Delta$  was generated by Neeraj Sharma. Bars represent 10 µm.



**Table 5.1:** Quantification of TEM images of longitudinal sections through basal bodies

 (%)

	With an axoneme	With a membrane bubble	Naked
			basal body
DYF1 $\Delta$ (n = 28)	82.1	10.7	8.7
IFT52 $\Delta$ (n = 84)	21.4	0	78.6
Wildtype $(n = 23)$	86.95	0	13.05*

\*Note: Some basal bodies remain unciliated for most of the cell cycle in wildtype.

**Table 5.2:** Frequency of the various structural defects on cross sections of locomotory

 $DYF1\Delta \ axonemes$ 

	DYF1Δ	Wildtype
Axonemal organization	% (n = 121)	% (n = 167)
9+2 (normal)	6.6	97
9+0	19	0
Missing outer doublets, central pair present	20.6	0
Outer singlets, central pair present	11.6	3**
Outer doublets and singlets without a central pair	22.3	0
Outer doublets and singlets with a central pair	1.7	0
Membrane bubbles lacking an axoneme	18.2	0

\*\*Note: This corresponds to the distal segments that have singlet outer tubules.

# CHAPTER 6

## CONCLUSIONS

Intraflagellar transport (IFT) is the bi-directional motility of ciliary precursors into and out of the cilium. IFT depends on two oligomeric complexes (A and B) and the microtubule motors that carry the complexes on axonemal microtubules. The work described in this dissertation began with the isolation of partial suppressor strains of the *IFT52*-gene knockout in *Tetrahymena* (Brown, Fine et al. 2003). IFT52 is an IFT complex B protein. Complex B is associated with anterograde IFT, carrying cargo of ciliary precursors from the cell body into the cilium. Previous studies showed that cells lacking complex B components or the anterograde motor (kinesin-2) do not assemble cilia. For example, the movement of IFT particles into the flagella of a temperaturesensitive *Chlamydomonas* kinesin-2 mutant, *fla10*, ceases after being shifted to the restrictive temperature (Kozminski, Beech et al. 1995). Loss of other complex B proteins such as IFT88, IFT172 and IFT27 leads to extremely short and non-functional cilia (Pazour, Dickert et al. 2000; Qin, Wang et al. 2007; Gorivodsky, Mukhopadhyay et al. 2009).

Lack of sufficient levels of IFT52 in *Chlamydomonas*, *C. elegans* and zebrafish results in a failure of cilia assembly that further leads to severe phenotypes especially in zebrafish where cilia perform many diverse organismal functions (Brazelton, Amundsen et al. 2001; Deane, Cole et al. 2001; Tsujikawa and Malicki 2004). To knockout IFT52 in *Tetrahymena*, we inserted a gene disruption cassette (*neo2*) into the coding region of *IFT52*, a commonly used technique in *Tetrahymena* (Gaertig, Gu et al. 1994; Brown,

Fine et al. 2003). The *neo2* cassette comprises of the neomycin phosphotransferase (*neo*) coding region of bacterial origin that confers resistance to paromomycin (Shang, Song et al. 2002). The *neo* sequence is flanked on the 5' end with *HHF4* (histone) promoter and on the 3' end with BTU2 ( $\beta$ -tubulin) transcription terminator sequences, both from the Tetrahymena genome. Using Mendelian genetic techniques and a variety of crosses, the micronucleus (germline, where most if not all genes are not expressed during vegetative stage) of *Tetrahymena* cells is made homozygous for the gene disruption cassette, but the macronucleus (somatic, expressed) contains wildtype copies of *IFT52*. When two such cells (heterokaryons) of different mating types undergo conjugation, the disrupted IFT52 allele is brought to expression by the newly developed macronucleus. As in other organisms, most of the conjugation progeny developed the *IFT52* knockout phenotype, in that the knockout cells were strongly defective in ciliary assembly (Brown, Fine et al. 2003). However, unlike other organisms, a small percentage of the conjugation progeny underwent a partial, conditional suppression event and could assemble short cilia under conditions of lower oxygen or at lower temperature. An insertional mutation in another complex B protein, IFT46 resulted in Chlamydomonas cells that assemble very short, paralyzed flagella lacking the central pair and dynein arms (Hou, Qin et al. 2007). Interestingly, a partial suppressor of this knockout was isolated in an unaerated culture. Thus, the partial suppressor of IFT46 grew normal length flagella but only under low oxygen. The conditional nature of both IFT52 and IFT46 suppressors led to a hypothesis that oxygen might be involved in a common regulatory mechanism for the IFT pathway. We investigated this possibility by first studying the mechanism of suppression in IFT52∆sm.

The *Tetrahymena* suppressors, called IFT52∆sm arise at a relatively high frequency (3%) every time *IFT52*-knockout heterokaryons undergo conjugation, which led us to think that the suppression was most likely the result of the DNA rearrangements that occur during the formation of the new macronucleus following conjugation. As discussed in Chapter 4, the suppression was determined to be intragenic and occurred in two steps. First, the neo gene of bacterial origin was specifically deleted from the knockout cassette, while DNA segments of *Tetrahymena* origin in the *neo2* cassette remain mostly intact. The mechanism responsible for the genomic deletion is most likely the RNAi-dependent pathway that deletes micronucleus-specific genomic DNA during macronuclear development. The remaining DNA segments, comprising of *HHF4* and BTU2 sequences, was present as an insertion at the beginning of the fourth exon and had stop codons in every reading frame. Transcription of the *IFT52* locus from IFT52 $\Delta$ sm cells would lead to severely truncated mRNA transcripts lacking four out of the seven highly conserved exons. We examined the cDNA of IFT52 $\Delta$ sm cells and surprisingly found that the remaining DNA is processed as multiple introns such that the reading frame of IFT52 is restored. Others have also reported the deletion of foreign DNA from the macronucleus following conjugation (Yao, Fuller et al. 2003; Liu, Song et al. 2005). The unique feature of the IFT52 $\Delta$ sm suppression is the further processing of the leftover DNA as multiple introns restores a functional albeit mutated IFT52 protein.

The suppressed *IFT52Asm* locus has 129 extra base pairs corresponding to the leftover parts of *HHF4* and *BTU2* that are not present elsewhere in the genome. Additionally, the locus lacks 21 endogenous base pairs that were lost during the insertion of the gene disruption cassette. Either the presence of the 129 base pairs (43 codons) or
the lack of the 21 base pairs (7 codons) may result in the conditional suppression phenotype. The modified locus with 43 new codons and lacking 7 native codons confers sensitivity to oxygen since IFT52-knockout (IFT52 $\Delta$ ) cells rescued with DNA from *IFT52* $\Delta$ *sm* locus assembled cilia in hypoxic conditions. The 43 extra codons are not native to *Tetrahymena* as they come from the promoter and transcription terminator of two unrelated genes. Hence, if oxygen is plays a role in regulating the IFT pathway, then the sensitivity of the suppressors must come from the lack of the 7 endogenous codons. An experiment to rescue IFT52-knockout (IFT52 $\Delta$ ) cells with DNA lacking the 7 endogenous codons might give more information on the role they play in the native protein. Another experiment is to introduce these 7 codons into another IFT complex B protein and see if it confers sensitivity to oxygen. To learn more about the role of oxygen in the IFT pathway, IFT complex B mutants can be screened for suppressors that assemble cilia under hypoxia. The significance of this result is that it gives a potential system for the study of IFT regulation by an external factor. It also uncovers a novel mechanism of suppression in *Tetrahymena*.

The short cilia of IFT52 $\Delta$ sm cells were further analyzed for their protein content by two-dimensional gels. The detergent-extracted fraction of the cilia was enriched in one particular protein, tubulin folding cofactor B (Tcb1p). This was an unusual localization for a protein belonging to the tubulin folding pathway, since it has been assumed that tubulin folding occurs in the cell body.  $\alpha$ - and  $\beta$ -tubulin monomers are folded to form heterodimers through a conserved pathway that includes two chaperonins and five folding cofactors. Prefoldin interacts with the  $\alpha$ - and  $\beta$ -tubulin monomers as they are

being transcribed and remains attached until transcription is completed (Vainberg, Lewis et al. 1998). Next, the two monomers interact with the CCT complex and get folded into their quasi-native forms (Lopez-Fanarraga, Avila et al. 2001). From here on,  $\alpha$ -tubulin interacts with cofactors B and E while  $\beta$ -tubulin interacts with cofactors A and D, in that order. The two monomers, cofactors E, D and C come together in a complex and upon GTP hydrolysis a  $\alpha\beta$ -tubulin heterodimer is released (Lopez-Fanarraga, Avila et al. 2001).

It is still not known in what form tubulin is transported into cilia for assembly. Since Tcb1p was upregulated in an IFT mutant, we had a novel connection between the two pathways that are likely involved in tubulin transport to cilia. It is possible that tubulin is transported into cilia along with the ciliary membrane (Stephens 1991). On the other hand it may be transported in the form of short oligomers as suggested (Seixas, Casalou et al. 2003). The third possibility is that the folding of tubulin into dimers occurs inside cilia. Certain members of the tubulin folding pathway, such as the  $\alpha$ -subunit of the CCT complex, have been detected in cilia (Seixas, Casalou et al. 2003). Recently, tubulin folding cofactor B was detected in the epyndymal cilia of cultured mammalian neurons (Lopez-Fanarraga, Carranza et al. 2007). Yet another possibility is that tubulin folding components play a role after axoneme assembly, in maintaining or perhaps turning over microtubules. That Tcb1p is in detected in cilia of *Tetrahymena* IFT mutants indicates a role for the protein either in folding tubulin within cilia or a novel ciliary function.

For functional analysis of Tcb1p (Chapter 3), we generated a knockout in *Tetrahymena*. *TCB1* is essential and cells die within approximately two days of exhibiting the knockout phenotype. The phenotype includes misshaped cells with

degrading tubulin cytoskeleton. The observed lethal phenotype, while not unexpected, could not be predicted based on limited studies done in other organisms. As an example, a TCB ortholog is essential in the fission yeast but dispensable in the budding yeast (Radcliffe, Garcia et al. 2000; Lopez-Fanarraga, Avila et al. 2001). The function of TCB has not been evaluated in a ciliated model. Since cilia are not essential organelles in *Tetrahymena*, we conclude that TCB plays an essential non-ciliary role, most likely in folding  $\alpha$ -tubulin in the cell body. Moreover, this function is possibly required for folding of cilia-destined tubulin as well. The lethality made it very difficult to study a novel role for Tcb1p as its tubulin folding role is more important than its putative ciliary role. If TCB has a ciliary function, this protein should be detectable in cilia. Originally, we detected an upregulated TCB spot in purified cilia of IFT52sm cells. To explore a potential function of TCB in cilia, we undertook extensive effort to localize TCB, to determine whether this protein is indeed targeted to cilia. We used a variety of epitopetagged versions of Tcb1p to rescue the knockout. This strategy allowed us to confirm that the knockout phenotype was specific to disruption in *TCB1* and that the tagged versions were functional. Versions of tagged TCB expressed under a cadmium-inducible promoter rescued the knockout lethality and the transgenic TCB accumulated in cilia. The drawback of this study is that the inducible promoter might overexpress the transgene protein. While we have also detected TCB in cells in which the controlling promoter was not induced by exogenous cadmium, a possibility remained that even under those conditions TCB was overproduced. Thus, we attempted to rescue TCB knockouts by targeting a tagged TCB to the native locus. Unfortunately, even after using three different epitope tags and various fixation methods, we were not able to detect natively tagged

Tcb1p in the cilia. The simplest explanation for this observation is that, the detergentsoluble fraction of IFT52Asm cilia that was originally used for identification of TCB in cilia; was contaminated by proteins from the cell body. Possibly the amount of contaminating cell body content is increased in IFT52Asm cells due to the fact that cilia are shorter than wildtype. From our studies we conclude that Tcb1p Has an essential function in the cell body but its ciliary role is unclear. TCB can accumulate in cilia under certain conditions, but its native levels in cilia are below the detection limit. In the future, these studies could be followed by tagging native TCB using more sensitive probes such as codon optimized multiple fluorescent proteins.

In the second part of the thesis (Chapter 5) I explored the function of DYF-1 in cilia assembly. This protein has been proposed to function as an IFT component that interacts with complex B, but some data suggest that DYF-1 plays a role in building up the entire axoneme and might even interact with enzymes that modify tubulin. The ciliary axoneme is comprised of three distinct segments, proximal, middle and distal. The proximal segment is closest to the cell body and includes the transition zone between the basal body and the beginning of the cilium. The middle segment makes up most of the cilium and consists of nine outer doublet (A and B tubules) microtubules. The distal segment consists of nine outer singlet microtubules, which just have A tubule extensions. How the distinct parts assemble as well as their significance in a cilium is not well understood.

In a *C. elegans* mutant, *dyf-1*, the axonemes of *dyf-1* mutants have normal middle segments but lack the distal segments with A tubule extensions (Ou, Blacque et al. 2005).

This phenotype was initially observed in another C. elegans mutant that had a defect in the homodimeric kinesin (Osm-3) used exclusively for building the distal segments (Snow, Ou et al. 2004). Taken together, the two studies suggested that DYF-1 acts as an adaptor to link the IFT complex B to the Osm-3 kinesin. However, the role of DYF-1 became less clear after a study of this protein in zebrafish. A zebrafish mutant of dyf-1 called *fleer* had short, paralyzed cilia due to defective axonemes with incomplete B tubules and lack of central microtubules in the middle segments (Pathak, Obara et al. 2007). Interestingly, these zebrafish mutant axonemes also have reduced levels of an important tubulin post-translational modification called polyglutamylation (Pathak, Obara et al. 2007). The authors proposed a role for DYF-1 in transporting the enzyme responsible for tubulin polyglutamylation into cilia, a role that does not exclude its adaptor function described in C. elegans (Pathak, Obara et al. 2007). Both studies taken together suggest that DYF-1 plays an unspecified role in axoneme assembly but its restricted role in the distal segment could be specific to some organisms such as C. elegans. On the other side, the zebrafish data suggested a broader role for DYF-1 in building up or stabilizing the axoneme. We showed in the DYF-1 knockout cells in *Tetrahymena*, the axonemes were severely defective and experience several types of defects such as missing and incomplete B tubules, displaced doublets and missing central pair microtubules and outgrowth of ciliary membrane (Chapter 5). The spectrum of defects was very broad, from complete failures in axoneme assembly ("naked basal bodies") to shortened fairly normal 9+2 axonemes. Contrary, to the zebrafish data, DYF-1 knockout cells in *Tetrahymena* have hyperglutamylated residual axonemes. Thus, the potential role of DYF-1 in tubulin polyglutamylation remains unclear. Furthermore, upon

re-evaluation of the zebrafish observations, we proposed a more trivial explanation of the effects of DYF-1 deficiency on the levels of tubulin glutamylation observed in this model. It is likely that DYF-1 has a more complex role in axoneme assembly. The zebrafish result showing reduction in polyglutamylation can be explained by the fact that mutant ciliary axonemes have incomplete B tubules, and we know from previous work that this modification is primarily present on the B tubule (Fouquet, Edde et al. 1994; Lechtreck and Geimer 2000).

From our study it appears that IFT is partially functional, in that ciliary assembly does begin but the axoneme appears to collapse quickly. This result implies a structural role for Dyf1p. Moreover, we show that Dyf1p is present predominantly in the detergentsoluble fraction of cilia, like other IFT particle components. Thus, it is unlikely that the structural role of DYF-1 is based on its incorporation into the axoneme. Rather, our data suggest that DYF-1 is associated with IFT particles and functions to provide a link between IFT particles and unknown cargo that is required to stabilize the axoneme. In the future, it will be of critical importance to determine the binding partners for DYF-1. This task can be accomplished in *Tetrahymena* using standard biochemical methods and will be facilitated by tagged versions of DYF-1 that we have created. It should be explained that our data do not directly contradict the C. elegans model, since the cilia in C. elegans are different from the ones in *Tetrahymena*, in that they are non-motile. It is possible that in C. elegans the distal singlets have subtle differences compared to those in Tetrahymena and zebrafish, and are not required for stabilizing the axoneme. However, in *Tetrahymena* and zebrafish, the absence of the distal segments might cause the

collapse of the entire structure. Indeed the distal segments are a lot longer compared to the length of the whole cilium in *C. elegans*.

It is now possible to divide IFT proteins into three broad categories based on where they may be required, namely; for anterograde function, for retrograde function and for stabilizing the axoneme as it is being assembled. This last class of IFT proteins may be part of the IFT complexes that act as adaptors for cargo meant for stabilizing the axonemes. From our data, it appears that Dyf1p interacts with the IFT complexes, as it is present in the detergent-soluble fraction of cilia like other IFT proteins, however it does not co-purify with the other more canonical IFT complex B proteins (Absalon, Blisnick et al. 2008).

Interestingly, ~18% of the cilia in *DYF1* knockout cells had membrane bubbles without any axonemes. The transport of membrane precursors and membrane proteins to cilia is another issue regarding IFT. A comprehensive study on IFT proteins, including DYF-1 in *T. brucei* also showed that knocking down these proteins results in extension of the flagellar membrane without corresponding increase in the axoneme (Absalon, Blisnick et al. 2008). Taken together these two results imply that the pathways for membrane transport and IFT can be uncoupled. Another study on the BBSome complex showed uncoupling between membrane transport and IFT, when the function of a small GTPase, Rab8 is disrupted (Nachury, Loktev et al. 2007). Surprisingly, BBS7 and BBS8 that are part of the BBSome are implicated as adaptors for the heterotrimeric kinesin, kinesin-II in anterograde transport if *C. elegans* sensory cilia, which shows a connection between the BBS proteins and the IFT pathway (Ou, Blacque et al. 2005). A link between

IFT and membrane transport also comes from IFT20 since it is directly involved in transport of a membrane protein, polycystin-2 to the primary cilia of mammalian cells (Follit, San Agustin et al. 2008). Hence, it appears that only under certain conditions, membrane transport and IFT are upcoupled.

The function of Dyf1p can be studied in more details if a temperature-sensitive mutant is isolated. The effect of its depletion on ciliary assembly and maintenance can be observed slowly over time. Another key experiment is to know the precise location of Dyf1p in the axoneme using immunogold electron microscopy. Dyf1p may be part of the IFT complexes, in which case it will co-localize with other IFT proteins. *Tetrahymena* have homolog of the homodimeric kinesin called Kin5p (Awan, Bernstein et al. 2004). We do not know if *Kin5*-gene knockout results in defective assembly of distal singlets. It would be interesting to know if like Osm-3 kinesin, Kin5p is also specific to assembly of distal singlets. Moreover, the localization of Dyf1p in the background of *Kin5*-gene knockout might indicate if Dyf1p is an adaptor for this motor in *Tetrahymena*.

## References

- Absalon, S., T. Blisnick, et al. (2008). "Intraflagellar transport and functional analysis of genes required for flagellum formation in trypanosomes." <u>Mol Biol Cell</u> 19(3): 929-44.
- Absalon, S., T. Blisnick, et al. (2008). "Intraflagellar transport and functional analysis of genes required for flagellum formation in Trypanosomes." <u>Mol. Biol. Cell.</u> 19: 929-944.

Afzelius, B. A. (2004). "Cilia-related diseases." J Pathol 204(4): 470-7.

- Ahmed, N. T., C. Gao, et al. (2008). "ODA16 aids axonemal outer row dynein assembly through an interaction with the intraflagellar transport machinery." <u>J Cell Biol</u> 183(2): 313-22.
- Allen, R. D. (1969). "The morphogenesis of basal bodies and accessory structures of the cortex of the ciliated protozoan Tetrahymena pyriformis." <u>J Cell Biol</u> 40(3): 716-33.
- Awan, A., M. Bernstein, et al. (2004). "Cloning and characterization of Kin5, a novel Tetrahymena ciliary kinesin II." <u>Cell Motil Cytoskeleton</u> 58(1): 1-9.
- Badano, J. L., N. Mitsuma, et al. (2006). "The ciliopathies: an emerging class of human genetic disorders." <u>Annu Rev Genomics Hum Genet</u> 7: 125-48.
- Baker, S. A., K. Freeman, et al. (2003). "IFT20 links kinesin II with a mammalian intraflagellar transport complex that is conserved in motile flagella and sensory cilia." J Biol Chem 278(36): 34211-8.
- Bardy, S. L., S. Y. Ng, et al. (2003). "Prokaryotic motility structures." <u>Microbiology</u> **149**(Pt 2): 295-304.
- Bartolini, F., A. Bhamidipati, et al. (2002). "Functional overlap between retinitis pigmentosa 2 protein and the tubulin-specific chaperone cofactor C." J Biol Chem 277(17): 14629-34.
- Beales, P. L., E. Bland, et al. (2007). "IFT80, which encodes a conserved intraflagellar transport protein, is mutated in Jeune asphyxiating thoracic dystrophy." <u>Nat Genet</u> 39(6): 727-9.

- Beghin, A., C. M. Galmarini, et al. (2007). "Tubulin folding pathways: implication in the regulation of microtubule dynamics." <u>Curr Cancer Drug Targets</u> 7(8): 697-703.
- Beisson, J. and M. Wright (2003). "Basal body/centriole assembly and continuity." <u>Curr</u> <u>Opin Cell Biol</u> **15**(1): 96-104.
- Belmont, L. D. and T. J. Mitchison (1996). "Identification of a protein that interacts with tubulin dimers and increases the catastrophe rate of microtubules." <u>Cell</u> 84(4): 623-31.
- Bisgrove, B. W. and H. J. Yost (2006). "The roles of cilia in developmental disorders and disease." <u>Development</u> 133(21): 4131-43.
- Bobinnec, Y., A. Khodjakov, et al. (1998). "Centriole disassembly *in vivo* and its effect on centrosome structure and function in vertebrate cells." <u>J. Cell Biol.</u> 143: 1575-1589.
- Bobinnec, Y., A. Khodjakov, et al. (1998). "Centriole disassembly in vivo and its effect on centrosome structure and function in vertebrate cells." <u>J Cell Biol</u> 143(6): 1575-89.
- Bonnafe, E., M. Touka, et al. (2004). "The transcription factor RFX3 directs nodal cilium development and left-right asymmetry specification." <u>Mol Cell Biol</u> 24(10): 4417-27.
- Brailov, I., M. Bancila, et al. (2000). "Localization of 5-HT(6) receptors at the plasma membrane of neuronal cilia in the rat brain." <u>Brain Res</u> **872**(1-2): 271-5.
- Brazelton, W. J., C. D. Amundsen, et al. (2001). "The bld1 mutation identifies the Chlamydomonas osm-6 homolog as a gene required for flagellar assembly." <u>Curr</u> <u>Biol</u> 11(20): 1591-4.

- Bré, M. H., V. Redeker, et al. (1998). "Tubulin polyglycylation: differential posttranslational modification of dynamic cytoplasmic and stable axonemal microtubules in *Paramecium*." <u>Mol. Biol. Cell</u> **9**: 2655-2665.
- Brouhard, G. J., J. H. Stear, et al. (2008). "XMAP215 is a processive microtubule polymerase." <u>Cell</u> **132**(1): 79-88.
- Brown, A. (2003). "Axonal transport of membranous and nonmembranous cargoes: a unified perspective." J Cell Biol **160**(6): 817-21.
- Brown, C. R., S. J. Doxsey, et al. (1996). "Molecular chaperones and the centrosome. A role for TCP-1 in microtubule nucleation." J Biol Chem 271(2): 824-32.
- Brown, J. M., N. A. Fine, et al. (2003). "Hypoxia regulates assembly of cilia in suppressors of Tetrahymena lacking an intraflagellar transport subunit gene." <u>Mol</u> <u>Biol Cell</u> 14(8): 3192-207.
- Brown, J. M., C. Hardin, et al. (1999). "Rotokinesis, a novel phenomenon of cell locomotion-assisted cytokinesis in the ciliate Tetrahymena thermophila." <u>Cell</u>
   <u>Biol Int</u> 23(12): 841-8.
- Brown, J. M., C. Marsala, et al. (1999). "Kinesin-II is preferentially targeted to assembling cilia and is required for ciliogenesis and normal cytokinesis in Tetrahymena." <u>Mol Biol Cell</u> 10(10): 3081-96.
- Brown, J. M., C. Marsala, et al. (1999). "Kinesin-II is preferentially targeted to assembling cilia and is required for ciliogenesis and normal cytokinesis in *Tetrahymena*." <u>Mol. Biol. Cell</u> 10: 3081-3096.

- Campbell, P. K., K. G. Waymire, et al. (2002). "Mutation of a novel gene results in abnormal development of spermatid flagella, loss of intermale aggression and reduced body fat in mice." <u>Genetics</u> 162(1): 307-20.
- Cassidy-Hanley, D., J. Bowen, et al. (1997). "Germline and somatic transformation of mating *Tetrahymena thermophila* by particle bombardment." <u>Genetics</u> 146: 135-147.
- Cassidy-Hanley, D., J. Bowen, et al. (1997). "Germline and somatic transformation of mating Tetrahymena thermophila by particle bombardment." <u>Genetics</u> 146(1): 135-47.
- Cassimeris, L. (2002). "The oncoprotein 18/stathmin family of microtubule destabilizers." <u>Curr Opin Cell Biol</u> **14**(1): 18-24.
- Cole, D. G. (2003). "The intraflagellar transport machinery of Chlamydomonas reinhardtii." <u>Traffic</u> **4**(7): 435-42.
- Cole, D. G., D. R. Diener, et al. (1998). "Chlamydomonas kinesin-II-dependent intraflagellar transport (IFT):

IFT particles contain proteins required for ciliary assembly in

Caenorhabditis elegans sensory neurons." J. Cell Biol. 18: 993-1008.

- Cole, D. G., D. R. Diener, et al. (1998). "Chlamydomonas kinesin-II-dependent intraflagellar transport (IFT): IFT particles contain proteins required for ciliary assembly in Caenorhabditis elegans sensory neurons." <u>J Cell Biol</u> 141(4): 993-1008.
- Cole, N. B. and J. Lippincott-Schwartz (1995). "Organization of organelles and membrane traffic by microtubules." <u>Curr Opin Cell Biol</u> 7(1): 55-64.

- Cortellino, S., C. Wang, et al. (2009). "Defective ciliogenesis, embryonic lethality and severe impairment of the Sonic Hedgehog pathway caused by inactivation of the mouse complex A intraflagellar transport gene Ift122/Wdr10, partially overlapping with the DNA repair gene Med1/Mbd4." <u>Dev Biol</u> **325**(1): 225-37.
- Coyne, R. S., D. L. Chalker, et al. (1996). "Genome downsizing during ciliate development: nuclear division of labor through chromosome restructuring." <u>Annu Rev Genet</u> **30**: 557-78.
- Culver, B. P., J. B. Meehl, et al. (2009). "The two SAS-6 homologs in Tetrahymena thermophila have distinct functions in basal body assembly." <u>Mol Biol Cell</u> 20(6): 1865-77.
- Cunningham, L. A. and R. A. Kahn (2008). "Cofactor D functions as a centrosomal protein and is required for the recruitment of the gamma-tubulin ring complex at centrosomes and organization of the mitotic spindle." J Biol Chem 283(11): 7155-65.
- Dammermann, A. and A. Merdes (2002). "Assembly of centrosomal proteins and microtubule organization depends on PCM-1." J Cell Biol **159**(2): 255-66.
- Dave, D., D. Wloga, et al. (Accepted 2009). "Manipulating ciliary protein-encoding genes in Tetrahymena." <u>Methods Cell Biol</u>.
- Davy, B. E. and M. L. Robinson (2003). "Congenital hydrocephalus in hy3 mice is caused by a frameshift mutation in Hydin, a large novel gene." <u>Hum Mol Genet</u> 12(10): 1163-70.

- Deane, J. A., D. G. Cole, et al. (2001). "Localization of intraflagellar transport protein IFT52 identifies basal body transitional fibers as the docking site for IFT particles." <u>Curr Biol</u> 11(20): 1586-90.
- Dent, E. W. and K. Kalil (2001). "Axon branching requires interactions between dynamic microtubules and actin filaments." J Neurosci 21(24): 9757-69.
- Dentler, W. L. (1980). "Structures linking the tips of ciliary and flagellar microtubules to the membrane." <u>J Cell Sci</u> **42**: 207-20.
- Desai, A. and T. J. Mitchison (1997). "Microtubule polymerization dynamics." <u>Annu Rev</u> <u>Cell Dev Biol</u> **13**: 83-117.
- Drewes, G., A. Ebneth, et al. (1998). "MAPs, MARKs and microtubule dynamics." <u>Trends Biochem Sci</u> 23(8): 307-11.
- Dubruille, R., A. Laurencon, et al. (2002). "Drosophila regulatory factor X is necessary for ciliated sensory neuron differentiation." <u>Development</u> **129**(23): 5487-98.
- Eddé, B., J. Rossier, et al. (1990). "Posttranslational glutamylation of α-tubulin." <u>Science</u> 247: 83-85.
- Eisen, J. A., R. S. Coyne, et al. (2006). "Macronuclear Genome Sequence of the Ciliate Tetrahymena thermophila, a Model Eukaryote." <u>PLoS Biol</u> **4**(9).
- Eisen, J. A., R. S. Coyne, et al. (2006). "Macronuclear genome sequence of the ciliate Tetrahymena thermophila, a model eukaryote." <u>PLoS Biol</u> 4(9): e286.
- Evans, J. E., J. J. Snow, et al. (2006). "Functional modulation of IFT kinesins extends the sensory repertoire of ciliated neurons in Caenorhabditis elegans." <u>J Cell Biol</u> 172(5): 663-9.

- Felsenstein, J. (1997). "An alternating least squares approach to inferring phylogenies from pairwise distances." <u>Syst. Biol.</u> **46**(1): 101-11.
- Ferrante, M. I., L. Romio, et al. (2009). "Convergent extension movements and ciliary function are mediated by ofd1, a zebrafish orthologue of the human oral-facialdigital type 1 syndrome gene." <u>Hum Mol Genet</u> 18(2): 289-303.
- Ferrante, M. I., A. Zullo, et al. (2006). "Oral-facial-digital type I protein is required for primary cilia formation and left-right axis specification." <u>Nat Genet</u> 38(1): 112-7.
- Finetti, F., S. R. Paccani, et al. (2009). "Intraflagellar transport is required for polarized recycling of the TCR/CD3 complex to the immune synapse." <u>Nat Cell Biol</u> 11(11): 1332-9.
- Follit, J. A., J. T. San Agustin, et al. (2008). "The Golgin GMAP210/TRIP11 anchors IFT20 to the Golgi complex." <u>PLoS Genet</u> **4**(12): e1000315.
- Follit, J. A., R. A. Tuft, et al. (2006). "The intraflagellar transport protein IFT20 is associated with the Golgi complex and is required for cilia assembly." <u>Mol Biol</u> <u>Cell</u> 17(9): 3781-92.
- Follit, J. A., F. Xu, et al. (2009). "Characterization of mouse IFT complex B." <u>Cell Motil</u> <u>Cytoskeleton</u> **66**(8): 457-68.
- Fouquet, J. P., B. Edde, et al. (1994). "Differential distribution of glutamylated tubulin during spermatogenesis in mammalian testis." <u>Cell Motil Cytoskeleton</u> 27(1): 49-58.
- Fouquet, J. P., B. Edde, et al. (1994). "Differential distribution of glutamylated tubulin during spermatogenesis in mammalian testis." <u>Cell Motil.Cytoskeleton</u>. **27**: 49-58.

- Fowkes, M. E. and D. R. Mitchell (1998). "The role of preassembled cytoplasmic complexes in assembly of flagellar dynein subunits." <u>Mol Biol Cell</u> 9(9): 2337-47.
- Gaertig, J. (2000). "Molecular mechanisms of microtubular organelle assembly in Tetrahymena." J Eukaryot Microbiol **47**(3): 185-90.
- Gaertig, J., M. A. Cruz, et al. (1995). "Acetylation of lysine 40 in alpha-tubulin is not essential in Tetrahymena thermophila." <u>J Cell Biol</u> 129(5): 1301-10.
- Gaertig, J., M. A. Cruz, et al. (1995). "Acetylation of lysine 40 in alpha-tubulin is not essential in *Tetrahymena thermophila*." J.Cell Biol. **129**: 1301-1310.
- Gaertig, J., Y. Gao, et al. (1999). "Surface display of a parasite antigen in the ciliate *Tetrahymena thermophila*." <u>Nat. Biotech.</u> **17**: 462-465.
- Gaertig, J., L. Gu, et al. (1994). "High frequency vector-mediated transformation and gene replacement in Tetrahymena." <u>Nucleic Acids Res</u> **22**(24): 5391-8.
- Gaertig, J. and D. Wloga (2008). Ciliary Tubulin and Its Post-translational Modifications. <u>Ciliary Function in Mammalian Development</u>. B. K. Yoder, Elsevier Inc. 85: 83-109.
- Gaertig, J. and D. Wloga (2008). "Ciliary tubulin and its post-translational modifications." <u>Curr Top Dev Biol</u> **85**: 83-113.
- Galtier, N., M. Gouy, et al. (1996). "SEAVIEW and PHYLO\_WIN: two graphic tools for sequence alignment and molecular phylogeny." <u>Comput Appl Biosci</u> 12(6): 543-8.
- Geng, L., D. Okuhara, et al. (2006). "Polycystin-2 traffics to cilia independently of polycystin-1 by using an N-terminal RVxP motif." <u>J Cell Sci</u> 119(Pt 7): 1383-95.

- Gerdes, J. M., E. E. Davis, et al. (2009). "The vertebrate primary cilium in development, homeostasis, and disease." <u>Cell</u> **137**(1): 32-45.
- Gherman, A., E. E. Davis, et al. (2006). "The ciliary proteome database: an integrated community resource for the genetic and functional dissection of cilia." <u>Nat Genet</u> 38(9): 961-2.
- Gonzalez-Perrett, S., K. Kim, et al. (2001). "Polycystin-2, the protein mutated in autosomal dominant polycystic kidney disease (ADPKD), is a Ca2+-permeable nonselective cation channel." Proc Natl Acad Sci U S A **98**(3): 1182-7.
- Gorivodsky, M., M. Mukhopadhyay, et al. (2009). "Intraflagellar transport protein 172 is essential for primary cilia formation and plays a vital role in patterning the mammalian brain." <u>Dev Biol</u> **325**(1): 24-32.
- Gorovsky, M. A., M.-C. Yao, et al. (1975). "Isolation of micro- and macronuclei of Tetrahymena pyriformis." <u>Methods Cell Biol.</u> IX: 311-327.
- Gorovsky, M. A., M. C. Yao, et al. (1975). "Isolation of micro- and macronuclei of Tetrahymena pyriformis." <u>Methods Cell Biol</u> **9**(0): 311-27.
- Grynberg, M., L. Jaroszewski, et al. (2003). "Domain analysis of the tubulin cofactor system: a model for tubulin folding and dimerization." <u>BMC Bioinformatics</u> 4: 46.
- Gu, Y., Z. Deng, et al. (2008). "Prefoldin 6 is required for normal microtubule dynamics and organization in Arabidopsis." <u>Proc Natl Acad Sci U S A</u> 105(46): 18064-9.
- Hai, B. and M. A. Gorovsky (1997). "Germ-line knockout heterokaryons of an essential alpha-tubulin gene enable high-frequency gene replacement and a test of gene

transfer from somatic to germ-line nuclei in Tetrahymena thermophila." <u>Proc Natl</u> <u>Acad Sci U S A</u> 94(4): 1310-5.

- Handel, M., S. Schulz, et al. (1999). "Selective targeting of somatostatin receptor 3 to neuronal cilia." <u>Neuroscience</u> 89(3): 909-26.
- Hansen, W. J., N. J. Cowan, et al. (1999). "Prefoldin-nascent chain complexes in the folding of cytoskeletal proteins." <u>J Cell Biol</u> 145(2): 265-77.
- Hartl, F. U. and M. Hayer-Hartl (2002). "Molecular chaperones in the cytosol: from nascent chain to folded protein." <u>Science</u> 295(5561): 1852-8.
- Haycraft, C. J., P. Swoboda, et al. (2001). "The C. elegans homolog of the murine cystic kidney disease gene Tg737 functions in a ciliogenic pathway and is disrupted in osm-5 mutant worms." <u>Development</u> 128(9): 1493-505.
- Hildebrandt, F. and W. Zhou (2007). "Nephronophthisis-associated ciliopathies." <u>J Am</u> <u>Soc Nephrol</u> **18**(6): 1855-71.
- Hou, Y., H. Qin, et al. (2007). "Functional analysis of an individual IFT protein: IFT46 is required for transport of outer dynein arms into flagella." <u>J Cell Biol</u> 176(5): 653-65.
- Howard, J. and A. A. Hyman (2003). "Dynamics and mechanics of the microtubule plus end." <u>Nature</u> **422**(6933): 753-8.
- Huang, K., D. R. Diener, et al. (2009). "The ubiquitin conjugation system is involved in the disassembly of cilia and flagella." <u>J Cell Biol</u> 186(4): 601-13.
- Inglis, P. N., G. Ou, et al. (2007). "The sensory cilia of Caenorhabditis elegans." <u>WormBook</u>: 1-22.

- Insinna, C. and J. C. Besharse (2008). "Intraflagellar transport and the sensory outer segment of vertebrate photoreceptors." <u>Dev Dyn</u> **237**(8): 1982-92.
- Insinna, C., N. Pathak, et al. (2008). "The homodimeric kinesin, Kif17, is essential for vertebrate photoreceptor sensory outer segment development." <u>Dev Biol</u> 316(1): 160-70.
- Jaillon, O., K. Bouhouche, et al. (2008). "Translational control of intron splicing in eukaryotes." <u>Nature</u> 451(7176): 359-62.
- Janke, C., K. Rogowski, et al. (2005). "Tubulin polyglutamylase enzymes are members of the TTL domain protein family." <u>Science</u> 308: 1758-1762.
- Jeanmougin, F., J. D. Thompson, et al. (1998). "Multiple sequence alignment with Clustal X." <u>Trends Biochem Sci</u> **23**(10): 403-5.
- Jenkins, P. M., T. W. Hurd, et al. (2006). "Ciliary targeting of olfactory CNG channels requires the CNGB1b subunit and the kinesin-2 motor protein, KIF17." <u>Curr Biol</u> 16(12): 1211-6.
- Jerka-Dziadosz, M., I. Strzyewska-Jowko, et al. (2001). "The dynamics of filamentous structures in the apical band, oral crescent, fission line and the postoral meridional filament in *Tetrahymena thermophila* revealed by the monoclonal antibody 12G9." <u>Protist</u> 152(1): 53-67.
- Jourdain, L., P. Curmi, et al. (1997). "Stathmin: a tubulin-sequestering protein which forms a ternary T2S complex with two tubulin molecules." <u>Biochemistry</u> **36**(36): 10817-21.
- Kilburn, C. L., C. G. Pearson, et al. (2007). "New Tetrahymena basal body protein components identify basal body domain structure." <u>J Cell Biol</u> 178(6): 905-12.

- Kim, J. C., Y. Y. Ou, et al. (2005). "MKKS/BBS6, a divergent chaperonin-like protein linked to the obesity disorder Bardet-Biedl syndrome, is a novel centrosomal component required for cytokinesis." <u>J Cell Sci</u> **118**(Pt 5): 1007-20.
- Kortazar, D., M. L. Fanarraga, et al. (2007). "Role of cofactors B (TBCB) and E (TBCE) in tubulin heterodimer dissociation." <u>Exp Cell Res</u> **313**(3): 425-36.
- Kozminski, K. G., P. L. Beech, et al. (1995). "The Chlamydomonas kinesin-like protein FLA10 is involved in motility associated with the flagellar membrane." <u>J Cell</u> <u>Biol</u> 131(6 Pt 1): 1517-27.
- Kozminski, K. G., P. L. Beech, et al. (1995). "The *Chlamydomonas* kinesin-like protein FLA10 is involved in motility associated with the flagellar membrane." <u>J. Cell</u>
   <u>Biol.</u> 131: 1517-1527.
- Kozminski, K. G., K. A. Johnson, et al. (1993). "A motility in the eukaryotic flagellum unrelated to flagellar beating." <u>Proc Natl Acad Sci U S A</u> **90**(12): 5519-23.
- Kozminski, K. G., K. A. Johnson, et al. (1993). "A motility in the eukaryotic flagellum unrelated to flagellar beating." Proc. Natl. Acad. Sci. USA **90**: 5519-5523.
- Krock, B. L. and B. D. Perkins (2008). "The intraflagellar transport protein IFT57 is required for cilia maintenance and regulates IFT-particle-kinesin-II dissociation in vertebrate photoreceptors." <u>J Cell Sci</u> 121(Pt 11): 1907-15.
- Lechtreck, K.-F. and S. Geimer (2000). "Distributon of polyglutamylated tubulin in the flagellar apparatus of green flagellates." <u>Cell Motility Cytoskeleton</u> **47**: 219-235.
- Lechtreck, K. F., P. Delmotte, et al. (2008). "Mutations in Hydin impair ciliary motility in mice." J Cell Biol **180**(3): 633-43.

- Lechtreck, K. F. and S. Geimer (2000). "Distribution of polyglutamylated tubulin in the flagellar apparatus of green flagellates." <u>Cell Motil Cytoskeleton</u> **47**(3): 219-35.
- Lechtreck, K. F. and G. B. Witman (2007). "Chlamydomonas reinhardtii hydin is a central pair protein required for flagellar motility." <u>J Cell Biol</u> **176**(4): 473-82.
- Lehman, J. M., E. J. Michaud, et al. (2008). "The Oak Ridge Polycystic Kidney mouse: modeling ciliopathies of mice and men." <u>Dev Dyn</u> **237**(8): 1960-71.
- Li, J. B., J. M. Gerdes, et al. (2004). "Comparative genomics identifies a flagellar and basal body proteome that includes the BBS5 human disease gene." <u>Cell</u> 117(4): 541-52.
- Li, S., J. Finley, et al. (2002). "Crystal structure of the cytoskeleton-associated protein glycine-rich (CAP-Gly) domain." J Biol Chem **277**(50): 48596-601.
- Linck, R. W. (1976). "Flagellar doublet microtubules: fractionation of minor components and alpha-tubulin from specific regions of the A-tubule." <u>J Cell Sci</u> **20**(2): 405-39.
- Liu, Y., X. Song, et al. (2005). "Elimination of foreign DNA during somatic differentiation in Tetrahymena thermophila shows position effect and is dosage dependent." <u>Eukaryot Cell</u> 4(2): 421-31.
- Lopez-Fanarraga, M., J. Avila, et al. (2001). "Review: postchaperonin tubulin folding cofactors and their role in microtubule dynamics." <u>J Struct Biol</u> **135**(2): 219-29.
- Lopez-Fanarraga, M., G. Carranza, et al. (2007). "Tubulin cofactor B plays a role in the neuronal growth cone." J Neurochem 100(6): 1680-7.
- Lucker, B. F., R. H. Behal, et al. (2005). "Characterization of the Intraflagellar Transport Complex B Core: DIRECT INTERACTION OF THE IFT81 AND IFT74/72 SUBUNITS." J. Biol. Chem. 280(30): 27688-27696.

- Lytle, B. L., F. C. Peterson, et al. (2004). "Solution structure of a ubiquitin-like domain from tubulin-binding cofactor B." J Biol Chem **279**(45): 46787-93.
- Marshall, W. F. (2008). "Basal bodies platforms for building cilia." <u>Curr Top Dev Biol</u>85: 1-22.
- Marshall, W. F. (2008). "The cell biological basis of ciliary disease." <u>J Cell Biol</u> **180**(1): 17-21.
- Marshall, W. F. and S. Nonaka (2006). "Cilia: tuning in to the cell's antenna." <u>Curr Biol</u> **16**(15): R604-14.
- Marshall, W. F. and J. L. Rosenbaum (2001). "Intraflagellar transport balances continuous turnover of outer doublet microtubules: implications for flagellar length control." <u>J Cell Biol.</u> 155(3): 405-414.
- Marszalek, J. R., X. Liu, et al. (2000). "Genetic evidence for selective transport of opsin and arrestin by kinesin-II in mammalian photoreceptors." <u>Cell</u> **102**(2): 175-87.
- Martin, N., J. Jaubert, et al. (2002). "A missense mutation in Tbce causes progressive motor neuronopathy in mice." <u>Nat Genet</u> **32**(3): 443-7.
- Martin-Cofreces, N. B., J. Robles-Valero, et al. (2008). "MTOC translocation modulates IS formation and controls sustained T cell signaling." <u>J Cell Biol</u> **182**(5): 951-62.
- McKean, P. G., A. Baines, et al. (2003). "Gamma-tubulin functions in the nucleation of a discrete subset of microtubules in the eukaryotic flagellum." <u>Curr Biol</u> 13(7): 598-602.
- Merriam, E. V. and P. J. Bruns (1988). "Phenotypic assortment in Tetrahymena thermophila: assortment kinetics of antibiotic-resistance markers, tsA, death, and the highly amplified rDNA locus." <u>Genetics</u> **120**(2): 389-95.

- Mesland, D. A., J. L. Hoffman, et al. (1980). "Flagellar tip activation stimulated by membrane adhesions in Chlamydomonas gametes." <u>J Cell Biol</u> **84**(3): 599-617.
- Mitchison, T. and M. Kirschner (1984). "Dynamic instability of microtubule growth." <u>Nature</u> **312**(5991): 237-42.
- Morris, R. L. and J. M. Scholey (1997). "Heterotrimeric kinesin-II is required for the assembly of motile 9+2 ciliary axonemes on sea urchin embryos." <u>J. Cell Biol.</u>
  138: 1009-1022.
- Multigner, L., I. Pignot-Paintrand, et al. (1996). "The A and B tubules of the outer doublets of sea urchin sperm axonemes are composed of different tubulin variants." <u>Biochemistry</u> 35: 10862-10871.
- Mykytyn, K. and V. C. Sheffield (2004). "Establishing a connection between cilia and Bardet-Biedl Syndrome." <u>Trends Mol Med</u> **10**(3): 106-9.
- Nachury, M. V. (2008). "Tandem affinity purification of the BBSome, a critical regulator of Rab8 in ciliogenesis." <u>Methods Enzymol</u> **439**: 501-13.
- Nachury, M. V., A. V. Loktev, et al. (2007). "A core complex of BBS proteins cooperates with the GTPase Rab8 to promote ciliary membrane biogenesis." <u>Cell</u> **129**(6): 1201-13.
- Nakamura, M., X. Z. Zhou, et al. (2001). "Critical role for the EB1 and APC interaction in the regulation of microtubule polymerization." <u>Curr Biol</u> **11**(13): 1062-7.
- Nakazawa, Y., M. Hiraki, et al. (2007). "SAS-6 is a cartwheel protein that establishes the 9-fold symmetry of the centriole." <u>Curr Biol</u> **17**(24): 2169-74.

- Nauli, S. M., F. J. Alenghat, et al. (2003). "Polycystins 1 and 2 mediate mechanosensation in the primary cilium of kidney cells." <u>Nat Genet</u> 33(2): 129-37.
- Neugebauer, J. M., J. D. Amack, et al. (2009). "FGF signalling during embryo development regulates cilia length in diverse epithelia." <u>Nature</u> **458**(7238): 651-4.
- Nicastro, D., C. Schwartz, et al. (2006). "The molecular architecture of axonemes revealed by cryoelectron tomography." <u>Science</u> **313**(5789): 944-8.
- Nishimura, D. Y., M. Fath, et al. (2004). "Bbs2-null mice have neurosensory deficits, a defect in social dominance, and retinopathy associated with mislocalization of rhodopsin." <u>Proc Natl Acad Sci U S A</u> **101**(47): 16588-93.
- Nogales, E. (2000). "Structural insights into microtubule function." <u>Annu Rev Biochem</u> **69**: 277-302.
- Nolasco, S., J. Bellido, et al. (2005). "Tubulin cofactor A gene silencing in mammalian cells induces changes in microtubule cytoskeleton, cell cycle arrest and cell death." <u>FEBS Lett</u> **579**(17): 3515-24.
- Nonaka, S., Y. Tanaka, et al. (1998). "Randomization of left-right asymmetry due to loss of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein." <u>Cell</u> 95(6): 829-37.
- O'Toole, E. T., T. H. Giddings, et al. (2003). "Three-dimensional organization of basal bodies from wild-type and delta-tubulin deletion strains of Chlamydomonas reinhardtii." <u>Mol Biol Cell</u> **14**(7): 2999-3012.
- Olbrich, H., K. Haffner, et al. (2002). "Mutations in DNAH5 cause primary ciliary dyskinesia and randomization of left-right asymmetry." <u>Nat Genet</u> **30**(2): 143-4.

- Omori, Y., C. Zhao, et al. (2008). "Elipsa is an early determinant of ciliogenesis that links the IFT particle to membrane-associated small GTPase Rab8." <u>Nat Cell Biol</u> **10**(4): 437-44.
- Omran, H., D. Kobayashi, et al. (2008). "Ktu/PF13 is required for cytoplasmic preassembly of axonemal dyneins." <u>Nature</u> **456**(7222): 611-6.
- Orias, E. and L. Rasmussen (1976). "Dual capacity for nutrient uptake in tetrahymena.IV. growth without food vacuoles." <u>Exp. Cell Res.</u> 102: 127-137.
- Orias, E. and L. Rasmussen (1976). "Dual capacity for nutrient uptake in Tetrahymena.
   IV. Growth without food vacuoles and its implications." <u>Exp Cell Res</u> 102(1): 127-37.
- Ou, G., O. E. Blacque, et al. (2005). "Functional coordination of intraflagellar transport motors." <u>Nature</u> 436(7050): 583-7.
- Pathak, N., T. Obara, et al. (2007). "The Zebrafish fleer gene encodes an essential regulator of cilia tubulin polyglutamylation." <u>Mol Biol Cell</u> **18**(11): 4353-64.
- Pazour, G. J., N. Agrin, et al. (2005). "Proteomic analysis of a eukaryotic cilium." <u>J Cell</u> <u>Biol</u> **170**(1): 103-13.
- Pazour, G. J., B. L. Dickert, et al. (2000). "Chlamydomonas IFT88 and its mouse homologue, polycystic kidney disease gene tg737, are required for assembly of cilia and flagella." <u>J Cell Biol</u> 151(3): 709-18.
- Pazour, G. J., B. L. Dickert, et al. (1999). "The DHC1b (DHC2) isoform of cytoplasmic dynein is required for flagelar assembly." <u>J.Cell.Biol</u> 144(3): 473-481.
- Pazour, G. J., B. L. Dickert, et al. (1999). "The DHC1b (DHC2) isoform of cytoplasmic dynein is required for flagellar assembly." <u>J Cell Biol</u> 144(3): 473-81.

- Pazour, G. J., J. T. San Agustin, et al. (2002). "Polycystin-2 localizes to kidney cilia and the ciliary level is elevated in orpk mice with polycystic kidney disease." <u>Curr</u> <u>Biol</u> 12(11): R378-80.
- Pazour, G. J., C. G. Wilkerson, et al. (1998). "A dynein light chain is essential for the retrograde particle movement of intraflagellar transport (IFT)." J Cell Biol 141(4): 979-92.
- Peden, E. M. and M. M. Barr (2005). "The KLP-6 kinesin is required for male mating behaviors and polycystin localization in Caenorhabditis elegans." <u>Curr Biol</u> 15(5): 394-404.
- Pedersen, L. B., S. Geimer, et al. (2003). "The Microtubule plus end-tracking protein EB1 is localized to the flagellar tip and basal bodies in Chlamydomonas reinhardtii." <u>Curr Biol</u> 13(22): 1969-74.
- Pennarun, G., E. Escudier, et al. (1999). "Loss-of-function mutations in a human gene related to Chlamydomonas reinhardtii dynein IC78 result in primary ciliary dyskinesia." <u>Am J Hum Genet</u> 65(6): 1508-19.
- Perkins, L. A., E. M. Hedgecock, et al. (1986). "Mutant sensory cilia in the nematode Caenorhabditis elegans." <u>Dev Biol</u> 117(2): 456-87.
- Piperno, G. and K. Mead (1997). "Transport of a novel complex in the cytoplasmic matrix of Chlamydomonas flagella." <u>Proc Natl Acad Sci U S A</u> 94(9): 4457-62.
- Piperno, G. and K. Mead (1997). "Transport of a novel complex in the cytoplasmic matrix of *Chlamydomonas* flagella." <u>Proc. Natl. Acad. Sci. USA</u> 94: 4457-4462.

- Piperno, G., E. Siuda, et al. (1998). "Distinct mutants of retrograde intraflagellar transport (IFT) share similar morphological and molecular defects." <u>J Cell Biol</u> 143(6): 1591-601.
- Porter, M. E., R. Bower, et al. (1999). "Cytoplasmic dynein heavy chain 1b is required for flagellar assembly in Chlamydomonas." <u>Mol. Biol. Cell</u> **10**: 693-712.
- Porter, M. E. and W. S. Sale (2000). "The 9 + 2 axoneme anchors multiple inner arm dyneins and a network of kinases and phosphatases that control motility." <u>J Cell</u> <u>Biol</u> 151(5): F37-42.
- Qin, H., D. T. Burnette, et al. (2005). "Intraflagellar transport is required for the vectorial movement of TRPV channels in the ciliary membrane." <u>Curr Biol</u> **15**(18): 1695-9.
- Qin, H., D. R. Diener, et al. (2004). "Intraflagellar transport (IFT) cargo: IFT transports flagellar precursors to the tip and turnover products to the cell body." <u>J Cell Biol</u> 164(2): 255-66.
- Qin, H., Z. Wang, et al. (2007). "Intraflagellar transport protein 27 is a small G protein involved in cell-cycle control." <u>Curr Biol</u> 17(3): 193-202.
- Quarmby, L. M. and J. D. Parker (2005). "Cilia and the cell cycle?" <u>J Cell Biol</u> **169**(5): 707-10.
- Radcliffe, P. A., M. A. Garcia, et al. (2000). "The cofactor-dependent pathways for alpha- and beta-tubulins in microtubule biogenesis are functionally different in fission yeast." <u>Genetics</u> 156(1): 93-103.
- Radcliffe, P. A., D. Hirata, et al. (1999). "Functional dissection and hierarchy of tubulinfolding cofactor homologues in fission yeast." <u>Mol Biol Cell</u> **10**(9): 2987-3001.

- Radcliffe, P. A. and T. Toda (2000). "Characterisation of fission yeast alp11 mutants defines three functional domains within tubulin-folding cofactor B." <u>Mol Gen</u> <u>Genet</u> 263(5): 752-60.
- Rajagopalan, V., A. Subramanian, et al. (2009). "Dynein-2 affects the regulation of ciliary length but is not required for ciliogenesis in Tetrahymena thermophila."
   <u>Mol Biol Cell</u> 20(2): 708-20.
- Rana, A. A., J. P. Barbera, et al. (2004). "Targeted deletion of the novel cytoplasmic dynein mD2LIC disrupts the embryonic organiser, formation of the body axes and specification of ventral cell fates." <u>Development</u> 131(20): 4999-5007.
- Rayala, S. K., E. Martin, et al. (2007). "Dynamic interplay between nitration and phosphorylation of tubulin cofactor B in the control of microtubule dynamics."
  Proc Natl Acad Sci U S A 104(49): 19470-5.
- Redeker, V., N. Levilliers, et al. (1994). "Polyglycylation of tubulin: a post-translational modification in axonemal microtubules." <u>Science</u> 266: 1688-1691.
- Redeker, V., N. Levilliers, et al. (2005). "Mutations of tubulin glycylation sites reveal cross-talk between the C termini of alpha- and beta-tubulin and affect the ciliary matrix in *Tetrahymena*." J Biol Chem 280(1): 596-606.
- Reed, N. A., D. Cai, et al. (2006). "Microtubule acetylation promotes kinesin-1 binding and transport." <u>Curr Biol</u> 16(21): 2166-72.
- Robert, A., G. Margall-Ducos, et al. (2007). "The intraflagellar transport component IFT88/polaris is a centrosomal protein regulating G1-S transition in non-ciliated cells." <u>J Cell Sci</u> **120**(Pt 4): 628-37.

- Romio, L., V. Wright, et al. (2003). "OFD1, the gene mutated in oral-facial-digital syndrome type 1, is expressed in the metanephros and in human embryonic renal mesenchymal cells." J Am Soc Nephrol 14(3): 680-9.
- Roobol, A., F. E. Holmes, et al. (1995). "Cytoplasmic chaperonin complexes enter neurites developing in vitro and differ in subunit composition within single cells."
   <u>J Cell Sci</u> 108 ( Pt 4): 1477-88.
- Rosenbaum, J. L. and K. Carlson (1969). "Cilia regeneration in Tetrahymena and its inhibition by colchicine." J Cell Biol 40(2): 415-25.
- Rosenbaum, J. L. and G. B. Witman (2002). "Intraflagellar transport." <u>Nat Rev Mol Cell</u> <u>Biol</u> **3**(11): 813-25.
- Rüdiger, A. H., M. Rüdiger, et al. (1999). "Monoclonal antibody ID5: epitope characterization and minimal requirements for the recognition of polyglutamylated alpha- and beta-tubulin." <u>Eur J Cell Biol</u> 78(1): 15-20.
- Sale, W. and P. Satir (1976). "Splayed Tetrahymena cilia. A system for analyzing sliding and

axonemal spoke arrangements." J. Cell Biol. 71: 589-565.

- Sale, W. S. and P. Satir (1977). "The termination of the central microtubules from the cilia of Tetrahymena pyriformis." <u>Cell Biol Inter</u> 1(1): 45-49.
- Sale, W. S. and P. Satir (1977). "The termination of the central microtubules from the cilia of Tetrahymena pyriformis." <u>Cell Biol Int Rep</u> 1(1): 45-9.
- Satir, P. and S. T. Christensen (2007). "Overview of structure and function of mammalian cilia." <u>Annu Rev Physiol</u> 69: 377-400.

- Savage, C., M. Hamelin, et al. (1989). "mec-7 is a beta-tubulin gene required for the production of 15-protofilament microtubules in Caenorhabditis elegans." <u>Genes</u> <u>Dev</u> 3(6): 870-81.
- Schaefer, M. K., H. Schmalbruch, et al. (2007). "Progressive motor neuronopathy: a critical role of the tubulin chaperone TBCE in axonal tubulin routing from the Golgi apparatus." <u>J Neurosci</u> 27(33): 8779-89.
- Schafer, J. C., C. J. Haycraft, et al. (2003). "XBX-1 encodes a dynein light intermediate chain required for retrograde intraflagellar transport and cilia assembly in Caenorhabditis elegans." <u>Mol Biol Cell</u> 14(5): 2057-70.
- Scholey, J. M. (1996). "Kinesin-II, a membrane traffic motor in axons, axonemes, and spindles." <u>J Cell Biol</u> 133(1): 1-4.
- Scholey, J. M. (2003). "Intraflagellar transport." <u>Annu Rev Cell Dev Biol</u> 19: 423-43.
- Scholey, J. M. and K. V. Anderson (2006). "Intraflagellar transport and cilium-based signaling." <u>Cell</u> 125(3): 439-42.
- Schroder, J. M., L. Schneider, et al. (2007). "EB1 is required for primary cilia assembly in fibroblasts." <u>Curr Biol</u> **17**(13): 1134-9.
- Seixas, C., C. Casalou, et al. (2003). "Subunits of the chaperonin CCT are associated with Tetrahymena microtubule structures and are involved in cilia biogenesis." <u>Exp</u> <u>Cell Res</u> 290(2): 303-21.
- Shang, Y., X. Song, et al. (2002). "A robust inducible-repressible promoter greatly facilitates gene knockouts, conditional expression, and overexpression of homologous and heterologous genes in Tetrahymena thermophila." <u>Proc Natl</u> <u>Acad Sci U S A</u> 99(6): 3734-9.

- Shang, Y., X. Song, et al. (2002). "A robust inducible-repressible promoter greatly facilitates gene knockouts, conditional expression, and overexpression of homologous and heterologous genes in Tetrahymenathermophila." <u>Proc Natl</u> <u>Acad Sci</u> 99: 3734-3739.
- Sharma, N., J. Bryant, et al. (2007). "Katanin regulates dynamics of microtubules and biogenesis of motile cilia." <u>J Cell Biol</u> 178(6): 1065-79.
- Shiratori, H. and H. Hamada (2006). "The left-right axis in the mouse: from origin to morphology." <u>Development</u> 133(11): 2095-104.
- Signor, D., K. P. Wedaman, et al. (1999). "Role of a class DHC1b dynein in retrograde trasnport of IFT motors and IFT raft particles along cilia but not dentrites, in chemosensory neurons of living *Caenorhabditis elegans*." <u>J.Cell.Biol</u> 147(3): 519-530.
- Signor, D., K. P. Wedaman, et al. (1999). "Two heteromeric kinesin complexes in chemosensory neurons and sensory cilia of Caenorhabditis elegans." <u>Mo. Biol.</u> <u>Cell</u> 10: 345-360.
- Sloboda, R. D. (2005). "Intraflagellar transport and the flagellar tip complex." <u>J Cell</u> <u>Biochem</u> 94(2): 266-72.
- Smith, E. F. and P. A. Lefebvre (1997). "PF20 gene product contains WD repeats and localizes to the intermicrotubule bridges in Chlamydomonas flagella." <u>Mol Biol</u> <u>Cell</u> 8(3): 455-67.
- Smith, E. F. and P. Yang (2004). "The radial spokes and central apparatus: mechanochemical transducers that regulate flagellar motility." <u>Cell Motil Cytoskeleton</u> 57(1): 8-17.

- Snow, J. J., G. Ou, et al. (2004). "Two anterograde intraflagellar transport motors cooperate to build sensory cilia on C. elegans neurons." <u>Nat Cell Biol</u> 6(11): 1109-13.
- Steinborn, K., C. Maulbetsch, et al. (2002). "The Arabidopsis PILZ group genes encode tubulin-folding cofactor orthologs required for cell division but not cell growth." <u>Genes Dev</u> 16(8): 959-71.
- Stephan, A., S. Vaughan, et al. (2007). "An essential quality control mechanism at the eukaryotic basal body prior to intraflagellar transport." <u>Traffic</u> 8(10): 1323-30.
- Stephens, R. E. (1991). "Tubulin in sea urchin embryonic cilia: characterization of the membrane-periaxonemal matrix." <u>J Cell Sci</u> 100 ( Pt 3): 521-31.
- Stephens, R. E. (1994). "Tubulin and tektin in sea urchin embryonic cilia: pathways of protein incorporation during turnover and regeneration." <u>J Cell Sci</u> 107 ( Pt 2): 683-92.
- Stephens, R. E. and N. A. Lemieux (1999). "Molecular chaperones in cilia and flagella: implications for protein turnover." <u>Cell Motil Cytoskeleton</u> 44(4): 274-83.
- Sui, H. and K. H. Downing (2006). "Molecular architecture of axonemal microtubule doublets revealed by cryo-electron tomography." <u>Nature</u> 442(7101): 475-8.
- Suprenant, K. A. and W. L. Dentler (1988). "Release of intact microtubule-capping structures from Tetrahymena cilia." <u>J.Cell Biol.</u> 107: 2259-2269.
- Swoboda, P., H. T. Adler, et al. (2000). "The RFX-type transcription factor DAF-19 regulates sensory neuron cilium formation in C. elegans." <u>Mol Cell</u> **5**(3): 411-21.

- Takeda, S., Y. Yonekawa, et al. (1999). "Left-right asymmetry and kinesin superfamily protein KIF3A: new insights in determination of laterality and mesoderm induction by kif3A-/- mice analysis." J Cell Biol 145(4): 825-36.
- Tanaka, H., N. Iguchi, et al. (2004). "Mice deficient in the axonemal protein Tektin-t exhibit male infertility and immotile-cilium syndrome due to impaired inner arm dynein function." <u>Mol Cell Biol</u> 24(18): 7958-64.
- Tian, G., S. A. Lewis, et al. (1997). "Tubulin subunits exist in an activated conformational state generated and maintained by protein cofactors." <u>J Cell Biol</u> 138(4): 821-32.
- Tran, P. T., P. Joshi, et al. (1997). "How tubulin subunits are lost from the shortening ends of microtubules." <u>J Struct Biol</u> 118(2): 107-18.
- Tran, P. V., C. J. Haycraft, et al. (2008). "THM1 negatively modulates mouse sonic hedgehog signal transduction and affects retrograde intraflagellar transport in cilia." <u>Nat Genet</u> 40(4): 403-10.
- Tsao, C. C. and M. A. Gorovsky (2008). "Different effects of tetrahymena IFT172 domains on anterograde and retrograde intraflagellar transport." <u>Mol Biol Cell</u> 19(4): 1450-61.
- Tsao, C. C. and M. A. Gorovsky (2008). "Tetrahymena IFT122A is not essential for cilia assembly but plays a role in returning IFT proteins from the ciliary tip to the cell body." J Cell Sci 121(Pt 4): 428-36.
- Tsujikawa, M. and J. Malicki (2004). "Intraflagellar transport genes are essential for differentiation and survival of vertebrate sensory neurons." <u>Neuron</u> **42**(5): 703-16.

- Turkewitz, A. P., E. Orias, et al. (2002). "Functional genomics: the coming of age for *Tetrahymena thermophila*." <u>Trends in Genetics</u> 18: 35-40.
- Vadlamudi, R. K., C. J. Barnes, et al. (2005). "p21-activated kinase 1 regulates microtubule dynamics by phosphorylating tubulin cofactor B." <u>Mol Cell Biol</u> 25(9): 3726-36.
- Vainberg, I. E., S. A. Lewis, et al. (1998). "Prefoldin, a chaperone that delivers unfolded proteins to cytosolic chaperonin." <u>Cell</u> 93(5): 863-73.
- Valpuesta, J. M., J. Martin-Benito, et al. (2002). "Structure and function of a protein folding machine: the eukaryotic cytosolic chaperonin CCT." <u>FEBS Lett</u> 529(1): 11-6.
- Veland, I. R., A. Awan, et al. (2009). "Primary cilia and signaling pathways in mammalian development, health and disease." <u>Nephron Physiol</u> 111(3): p39-53.
- Verhey, K. J. and J. Gaertig (2007). "The tubulin code." Cell Cycle 6(17): 2152-60.
- Vladar, E. K. and T. Stearns (2007). "Molecular characterization of centriole assembly in ciliated epithelial cells." <u>J Cell Biol</u> 178(1): 31-42.
- Walczak, C. E., E. C. Gan, et al. (2002). "The microtubule-destabilizing kinesin XKCM1 is required for chromosome positioning during spindle assembly." <u>Curr Biol</u> 12(21): 1885-9.
- Walther, Z., M. Vashishtha, et al. (1994). "The Chlamydomonas FLA10 gene encodes a novel kinesin-homologous protein." <u>J Cell Biol</u> 126(1): 175-88.
- Walther, Z., M. Vashistha, et al. (1994). "The *Chlamydomonas FLA10* gene encodes a novel kinesin-homologous protein." <u>J.Cell Biol.</u> 126: 175-188.

- Wang, Q., J. Pan, et al. (2006). "Intraflagellar transport particles participate directly in cilium-generated signaling in Chlamydomonas." <u>Cell</u> 125(3): 549-62.
- Wang, W., J. Ding, et al. (2005). "Gigaxonin interacts with tubulin folding cofactor B and controls its degradation through the ubiquitin-proteasome pathway." <u>Curr Biol</u> 15(22): 2050-5.
- Wang, Z., Z. C. Fan, et al. (2009). "Intraflagellar transport (IFT) protein IFT25 is a phosphoprotein component of IFT complex B and physically interacts with IFT27 in Chlamydomonas." <u>PLoS ONE</u> 4(5): e5384.
- Weinstein, B. and F. Solomon (1990). "Phenotypic consequences of tubulin overproduction in Saccharomyces cerevisiae: differences between alpha-tubulin and beta-tubulin." <u>Mol Cell Biol</u> **10**(10): 5295-304.
- Wiese, C. and Y. Zheng (2006). "Microtubule nucleation: gamma-tubulin and beyond." J <u>Cell Sci</u> **119**(Pt 20): 4143-53.
- Wilkes, D. E., H. E. Watson, et al. (2008). "Twenty-five dyneins in Tetrahymena: A reexamination of the multidynein hypothesis." <u>Cell Motil Cytoskeleton</u> 65(4): 342-51.
- Williams, N. E., C. C. Tsao, et al. (2006). "The actin gene ACT1 is required for phagocytosis, motility, and cell separation of Tetrahymena thermophila." <u>Eukaryot Cell</u> 5(3): 555-67.
- Wloga, D., A. Camba, et al. (2006). "Members of the Nima-related kinase family prmote disassembly of cilia by multiple mechanisms "<u>Mol. Biol. Cell</u> 17: 2799-2810.

- Wloga, D., A. Camba, et al. (2006). "Members of the NIMA-related kinase family promote disassembly of cilia by multiple mechanisms." <u>Mol Biol Cell</u> 17(6): 2799-810.
- Wloga, D., D. Dave, et al. (2009). "Hyperglutamylation of tubulin can either stabilize or destabilize microtubules in the same cell." <u>Eukaryot Cell</u>.
- Wloga, D., K. Rogowski, et al. (2008). "Glutamylation on a-tubulin is not essential but affects the assembly and functions of a subset of microtubules in *Tetrahymena*." <u>Eukaryot Cell</u> 7: 1362-1372.
- Wloga, D., K. Rogowski, et al. (2008). "Glutamylation on alpha-tubulin is not essential but affects the assembly and functions of a subset of microtubules in Tetrahymena thermophila." <u>Eukaryot Cell</u> 7(8): 1362-72.
- Wloga, D., D. M. Webster, et al. (2009). "TTLL3 Is a tubulin glycine ligase that regulates the assembly of cilia." <u>Dev Cell</u> 16(6): 867-76.
- Yao, M. C. and J. L. Chao (2005). "RNA-guided DNA deletion in Tetrahymena: an RNAi-based mechanism for programmed genome rearrangements." Annu Rev Genet 39: 537-59.
- Yao, M. C., P. Fuller, et al. (2003). "Programmed DNA deletion as an RNA-guided system of genome defense." Science 300(5625): 1581-4.