INVIVO SIGNIFICANCE OF TUBULIN GLUTAMYLATION GENERATED BY TTLL6E-LIGASES

by

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(Under the Direction of Jacek Gaertig)

ABSTRACT

Glutamylation is one of the most conserved post-translational modifications of α- and β- tubulin and involves addition of variable number of glutamate units as a polypeptide side-chain. Glutamylation is present on the surface of microtubules and is in a position to strongly interact with a large number of microtubule effectors such as motors, severing factors, and microtubule end-depolymerizers. Glutamhylation is present in cilia, mitotic spindle, centrioles and basal bodies. To understand the role of glutamylation in cilia, our research group has used the ciliate model system *Tetrahymena thermophila*. Recently, the catalytic subunits of tubulin glutamylases were identified as conserved enzymes related to tubulin tyrosine ligase, now known as TTLL proteins. *Tetrahymena* has 50 TTLLs and among these, the TTLL6 group of proteins are my focus of research. *Tetrahymena* has 6 paralogs of TTLL6 type - *TTLL6A, TTLL6B, TTLL6C, TTLL6D, TTLL6E,* and *TTLL6F*. In the previous studies it was shown that Ttll6Ap is a β-tubulin preferring, elongating enzyme that mainly localizes to cilia and overexpression of this protein leads to ciliary paralysis. As all of the 6 TTLL6 type E-ligases are paralogs, we suspected functional redundancy among them. We created *Tetrahymena* strains lacking these genes to study the significance of glutamylation contributed by them. We show that TTLL6 type E-ligases

mainly catalyze the elongation of glutamate side chains on β-tubulin and that glutamylation contributed by Ttll6Ap and Ttll6Fp, is required for normal ciliary motility. Mutants lacking these E-ligases swim and multiply slowly, exhibit lack of ciliary reversal, altered ciliary waveform and lower beat frequency. We also show that combined glutamylation by Ttll6Ap, Ttll6Bp, Ttll6Dp, Ttll6Ep and Ttll6Fp, at low dose are required for maintaining normal ciliary lengths. Strains lacking these proteins swim and multiply even more slowly as compared to the double mutants and their phagocytosis is impaired. Interestingly, these cilia of these mutants have significantly shorter lengths as compared to wild-type cilia. Together, in the light of our results, we show that glutamylation is required for the restraining activity of inner-dynein arms in ciliary motility and that, in dose-dependent manner, tubulin glutamylation regulates processes involved in regulating lengths of cilia.

INDEX WORDS: Microtubule, Cilia, Polyglutamylation, E-ligase, Dynein, TTLL6

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DEDICATION

I would like to dedicate this dissertation to my parents Prof. Maruti Suryavanshi and Lata Suryavanshi and my brother Rahul, as without their unconditional support, motivation and love, I would not have been where I am today. I am so grateful to my father for inculcating and nurturing my interest and curiosity in biological sciences. It is solely their dream and tremendous hard work that has facilitated me to visage the challenging task of Ph.D with perseverance. I would like to also dedicate this thesis to my husband, Dr. Pushkarraj, for his love and support.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Microtubules (MTs) are polymers of α and β-tubulin that are required to perform many crucial functions inside a cell. With the help of other proteins (called microtubule-associated proteins or MAPs), MTs perform various important functions in a cell such as division of the cell and its nucleus, organization of intracellular structures, and cell motility. However, it is not well understood how a simple MT consisting of dimerized α and β-tubulin can perform such varied functions. The fully functional MTs are formed in multiple steps, which involve synthesis of α- and β-tubulin polypeptides, folding and dimerization of tubulin, nucleation, polymerization, deposition of post-translational modifications (PTMs) and binding of MAPs. PTMs on tubulin play an important role in imparting functional diversity to MTs. Recently, several studies have started exposing the true potential of tubulin PTMs in their ability to affect properties, assembly and maintenance of MTs. Although a considerable amount of research has been done on these modifications, their exact structural role and mechanisms of their influence on MTs yet remains elusive.

Highly conserved α- and β-tubulin proteins undergo various PTMs such as lysine acetylation, phosphorylation, detyrosination, glutamylation and glycylation. Modifications such as acetylation and phosphorylation occur on a large number of proteins while glutamylation and glycylation occur on relatively few proteins, with tubulin being the best known substrate. Most of the PTMs occur on the C-terminal tail domains of α and β-tubulin in a variety of combinations. For example, glutamylation and glycylation can occur on α- as well as on β-tubulin, involving

addition of either one or multiple residues, some tubulin being heavily glutamylated or glycylated or containing both modifications.

I have used the ciliate *Tetrahymena thermophila* to study PTMs of tubulin as this organism has several experimental advantages to offer. *Tetrahymena* is easy to grow to high density and on a cheap medium with a generation time of only 3 hours. Routine gene manipulations (including gene knockouts) can be carried out that involves incorporating exogenous DNA into *Tetrahymena* genome by homologous recombination. Above all, *Tetrahymena* assembles 18 distinct types of MTs, including conserved types that form cilia, mitotic spindles, and basal bodies. The diversity of *Tetrahymena* MTs is reflected in their function as well as structure. *Tetrahymena* makes use of most of them if not all tubulin PTMs, including glutamylation, for imparting diversity to all the MTs.

Tubulin glutamylation is a highly conserved and reversible PTM that adds variable number of glutamate residues as a side-chain to the primary polypeptide (Edde et al., 1990). Tubulin glutamylation is a major modification in the mammalian brain (Audebert et al., 1993) and regulates activity of KIF1 kinesin in neuronal cells (Ikegami et al., 2007) as well as growth of MAP2 containing neurites in neuronal-like PC12 cells (Ikegami et al., 2006). Ciliary axonemes are also enriched with tubulin glutamylation where this modification may be important in ciliary motility (Gagnon et al., 1996; Ikegami et al., 2007) and cilia assembly and maintenance (Dave et al., 2009; Pathak et al., 2007). Recently, Janke and colleagues have identified the enzymes responsible for glutamylation. Tubulin glutamic acid ligases (E-ligases) belong to a family of amino acid ligases that also includes TTL (Janke et al., 2005). All these enzymes have a catalytic TTL-like domain and are therefore designated as TTL-like or TTLL proteins. *Tetrahymena* has a large number of these proteins as compared to other eukaryotes. Out of the 54 TTLL genes in

Tetrahymena, 34 belong to conserved clades and 20 belong to ciliate-specific clades. My research focuses on TTLL6 group of proteins in *Tetrahymena thermophila* and their role in the assembly and motility of cilia. This group consists of 6 paralogous proteins namely, *TTLL6A, TTLL6B, TTLL6C, TTLL6D, TTLL6E* and *TTLL6F*. The TTLL6 family of proteins catalyze the E-ligase or glutamylase activity on tubulin. My doctoral research focuses on the *in vivo* function of TTLL6 group of proteins in *Tetrahymena* by eliminating them from the cell, one by one or in various combinations. I show that tubulin glutamylation generated by TTLL6 proteins regulates the motility of cilia (via inner dynein arms) and affects the length of cilia while reducing the rate of intraflagellar transport.

This dissertation is organized into 5 chapters. Chapter 2 reviews the pertinent literature about MTs, various tubulin PTMs, functions and properties of cilia and different types of dynein. In Chapter 3 and 4, I have analyzed mutant phenotypes of all TTLL6 proteins by employing single and multiple knockout strategies to uncover the underlying mechanism of polyglutamylation. Chapter 3 explains the novel findings that polyglutamylation is essential in motility of cilia via regulation of axonemal dynein activity. Chapter 4 focuses on the significance of TTLL6 type E-ligases in maintaining ciliary lengths. In Chapter 5, I have discussed the importance of the findings of my doctoral research and proposed future experiments to enhance our understanding regarding the subject of PTMs.

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CHAPTER 2

REVIEW OF LITERATURE

Microtubules: Structure, Properties and Functions

One of the major cytoskeletal elements of all eukaryotic cells are microtubules (MTs). MTs are intracellular filamentous structures that function not unlike cellular "highways", "conveyor belts" and internal frameworks. MTs perform a variety of important functions such as imparting shape to a cell, establishing cell polarity, assembly of complex cytoskeletal structures (such as the mitotic spindle, basal bodies, centrioles, axonemes of cilia), intracellular transport, organization and positioning of membrane-bounded organelles, cell and nuclear division and cell motility. The basic unit of a MT is heterodimer of α- and β-tubulin. Each tubulin has a mass of ~55 kDa and about ~450 amino acid residues, and the two subunits are about 41% identical in sequence (Wade, 2009). FtsZ is a prokaryotic tubulin-like protein that shares 33% sequence identity with either α- or β-tubulin (Erickson, 1997). FtsZ is considered related to an ancestor protein of tubulin and is ubiquitously found in eubacteria, some archaebacteria, chloroplasts and mitochondria and plays a role in bacterial and organellar division (reviewed in (Goehring and Beckwith, 2005)). Within a typical MT, tubulin dimers non-covalently associate in a head-to-tail manner to form longitudinal protofilaments, which laterally interact to form a 24 nm-wide hollow tube of variable length. *In vivo*, MTs are commonly made of 13 protofilaments. However there are exceptions, such as the 15 protofilament MTs in the touch receptor neurons in *Ceanorhabditis elegans* (Chalfie and Thomson, 1982; Savage et al., 1989). In crayfish, sperm

contains 14, 15 or 16 protofilament MTs and the nerve cord has 12 protofilaments MTs (Burton et al., 1975). MTs with 13 to 16 protofilaments are present in the dividing macronucleus of ciliate *Paramaecium tetraurelia* (Eichenlaub-Ritter and Tucker, 1984). Furthermore, in most centrioles (and structurally similar basal bodies) and axonemes of cilia, there are compound MTs, doublets and triplets, that form by lateral fusion. Basal bodies and centrioles are typically made of 9 triplet MTs (A+B+C tubules) while axonemes have 9 doublets (A+B). The A-tubule of doublet or triplet MT is complete and contains 13 protofilaments, while the B and C-tubules (in case of basal bodies and centrioles) are incomplete tubules containing 10 protofilaments (Sui and Downing, 2006). The arrangement of the tubules is such that the B-tubule is fused to the side of A-tubule and the C-tubule is attached to the B-tubule (Figure 2.1).

Structure of Microtubules

Although the first observation of MTs dates back to 1950s (Manton and Clarke, 1952, Fawcett and Porter, 1954), the understanding of their composition came with biochemical studies that identified tubulin as the basic component of MTs (Weisenberg et al., 1968). The 3D structure of tubulin was revealed in late 90s when Nogales and colleagues employed electron crystallography to develop an atomic model of α/β-tubulin heterodimer of 3.7 Å resolution followed by a 20Å resolution model of a MT (Nogales et al., 1999; Nogales et al., 1998). The 3-D structures of α- and β-tubulin are similar as each subunit can be divided into 3 domains: the N-terminal nucleotide binding domain, the intermediate paclitaxel binding domain and the C-terminal domain that is located on the outer side of MT and interacts with MT-associated proteins (MAPs) (Figure 2.2). The N-terminal domain consists of alternate β strands (S1-S6) and helices (H1-H6). Loops T1-T6 (numbers corresponding to α-tubulin) connect the end of

each β strand to the beginning of the next helix and to the N terminal end of another core helix H7, thus forming a nucleotide-binding pocket. The intermediate domain is formed by helices H8-H10 and β strands S7-S10. The C-terminal region contains two anti-parallel helices H11 and H12. This atomic model lacks the last 10 residues of α-tubulin and last 18 residues of β-tubulin (Nogales et al., 1998) that form the carboxy terminal tails (CTT) of tubulin. The CTTs are highly acidic (Sackett, 1995) and are subjected to many post translational modifications (described below). The CTT sequences are more variable than the rest of the tubulin amino acid sequence, however, some sequence conservation in CTTs is apparent. For example, parts of CTT sequences are conserved among tubulin isotypes in vertebrates and may form the 'isotype-defining region' of tubulin. Thus, specific tubulin isotypes in animals may help to adapt MTs to diverse functions (Sullivan and Cleveland, 1986). Although, CTTs are essential for activity of α- and β-tubulin (Duan and Gorovsky, 2002), interestingly, their sequences are interchangeable between the α- and β-subunit. In *Tetrahymena*, a CTT must be present on both α- or β-tubulin to support viability, but its exact sequence is not important as long as it is a CTT. Thus, an α-tubulin with a CTT sequence of β-tubulin and a β-tubulin with a CTT sequence of α-tubulin support viability (Duan and Gorovsky, 2002). While *Tetrahymena* mutants lacking glutamic acids that undergo post-translational modifications on β-tubulin CTT are not viable, the lethality can be rescued by expressing an α-tubulin containing β-tubulin CTT (Duan and Gorovsky, 2002). This indicates that there is a degree of function-sharing between CTTs of α- and β-tubulin subunits. This could be a result of flexibility of CTTs and potential for their interactions across the dimer (Luchko et al., 2008).

GTP binding sites

There are two GTP binding sites in the α/β-tubulin dimer: the N site on α-tubulin and the E site on β-tubulin. The N site is non-exchangeable probably because it is buried between the two subunits of the dimer (Spiegelman et al., 1977). On the other hand, the GTP bound to the E site on β-tubulin can be exchanged and hydrolyzed as the E site is partially exposed on the outer surface of the dimer, in particular at the MT end (Mitchison, 1993). The orientation of MT protofilaments is such that the plus end is capped by a crown of β-tubulin subunits while the minus end is capped by a ring of α-tubulin (Nogales et al., 1999). The resulting polarity of MTs has important consequences because each end has different properties. A major difference between the properties of the two ends is in the rate of polymerization and depolymerization as the plus end has a higher polymerization/depolymerization rate and thus is known as a more dynamic end (reviewed in (Sept, 2007)). The tubulin dimer concentration plays an important role in regulation of polymerization MT (see below). The concentration of tubulin subunits at which the depolymerization rate of MT equals its polymerization rate, is called the critical concentration or Cc. Below Cc, the MT end does not grow and can undergo depolymerization. When concentration of tubulin subunits is above Cc, polymerization of MT occurs and the rate of polymerization exceeds that of depolymerization until the point when the free dimer concentration reaches Cc (Mitchison and Kirschner, 1984). *In vitro* polymerization studies have revealed that the plus end has a lower Cc as compared to that of the minus end, which means that polymerization at plus end will occur even if free tubulin concentration is too low for the minus end polymerization (Panda et al., 1996). In addition, above Cc, the rate of polymerization at plus end is ten times faster than that at minus ends (Wade, 2009). Recent studies have shown that MT

assembly can, at least under certain conditions, occur by addition of packets containing multiple tubulin dimers (Kerssemakers et al., 2006). In addition, certain proteins can catalyze MT polymerization. Some proteins accumulate on growing ends of MTs and are known as +TIP proteins. They interact with MTs and thus regulate their dynamics of MTs with cells cortex, mitotic kinetochores or different cellular organelles. XMAP215 is a processive polymerase that catalyzes MT plus end assembly and was first identified in *Xenopus* eggs (Brouhard et al., 2008; Gard and Kirschner, 1987). This MAP strongly increases the rate of polymerization of tubulin dimers at the plus ends. XMAP215 also increases the rate of depolymerization and decreases frequency of rescue, thereby increasing the tubulin turnover rate (Vasquez et al., 1994).

When MTs grow at the plus end by addition of new subunits, these are typically loaded with GTP at the E-site. Shortly after their addition to the MT lattice, GTP at the E-site gets hydrolyzed. As long as there is a layer of dimers that have GTP on the E site (so called GTP cap) at the end of the MT, the MT is stable, can polymerize, and protofilament ends have a straight conformation (reviewed in (Nogales and Wang, 2006)). When the rate of addition of new dimers is exceeded by the rate of GTP hydrolysis, the terminal β-subunits contain GDP (and thus lack a GTP cap) and change their conformation while destabilizing the MT end. During depolymerization of MTs, peeling of protofilaments occurs and GDP-tubulin starts forming curved protofilaments or ring polymers that are unable to polymerize into MTs due to changes in the conformation of α-β tubulin dimer (Muller-Reichert et al., 1998; Nogales and Wang, 2006; Nogales et al., 2003). In the GDP, the tubulin dimer has an internal tilt with a 12^{o} angle as compared to the GTP loaded dimer, and the increased curvature destabilizes the MT lattice. Upon GDP exchange for GTP, the tubulin subunit is straightened with an angle of 5^{o} allowing it now to repolymerize making both longitudinal and lateral interactions (Nogales et al., 2003).

Dynamic instability of microtubules

In vitro, dynamic instability of MTs defines the situation when plus ends of MTs randomly alternate between growing and shrinking states depending, presumably, on the changes in the size of the GTP cap (Brun et al., 2009; Mitchison and Kirschner, 1984). This phenomenon is common *in vivo* and occurs at the free dimer concentrations slightly above Cc. A transition to rapid depolymerization is known as a catastrophe, whereas a transition to polymerization is called rescue (Walker et al., 1988). Thus, the dynamic history of the polymer can be quantitatively described using the rates of polymerization and depolymerization and frequencies of catastrophes and rescues. These transitions are believed to be determined by fluctuations in the size of the GTP cap. It has not been clear however how a rapidly depolymerizing MT end that presumably has lost its GTP cap can undergo a rescue. A recent study shows that some tubulin dimers that are located within the central portions of the MT retain GTP on the E-site along their addition to the lattice (Dimitrov et al., 2008). These "islands" of trapped GTP-tubulin are proposed to promote rescue events whenever MT depolymerization exposes an internal GTP-tubulin island at the end.

In vivo, the dynamic instability is regulated by non-tubulin proteins. The minus end of MTs is usually bound and protected from depolymerization by an MTOC, from which the MT grows out. Selectively, plus ends of MTs can be captured by specific proteins that control the stability of MTs. For example, in a non-polarized mammalian tissue cultured cell there is continuous growth and shrinkage of MTs from a centrosome in all directions that appear random. When the cell polarizes, for example, as a response to tissue wounding, a subset of MTs comes in contact with a region of the cell cortex near the wound and undergoes selective stabilization (suppression of catastrophes). This selective stabilization of MTs leads to redistribution of MTs

and helps the cell become polarized (Euteneuer and Schliwa, 1992; Gundersen and Bulinski, 1988). In wounded fibroblasts, so called +TIP proteins such as EB1-APC are recruited to the plus ends of MTs by activated GTPase Rho and its effector mDia protein (Wen et al., 2004). MTs stabilized by +TIP proteins undergo post translational modifications (PTMs) such as detyrosination. It is also possible that PTMs themselves stabilize MTs. Effects of proteins and PTMs on MTs are discussed in the section below (see Post translational modifications).

Nucleation of microtubules

In vivo, MT growth is initiated by nucleation events that often, but not always, occur at a discrete structure known as the MT organizing center (MTOC). *In vivo*, MTOCs catalyze the polymerization events when free dimer concentration is lower than the Cc (for polymerization of pure tubulin). Thus, the formation of functionally diverse MTs to large extent depends on the location and activity of MTOCs within the cell.

As the number of MTOCs *in vivo* is limited, many MTs originate from a limited number of locations, with minus ends attached to MTOCs. γ-tubulin, a protein related to α- and β-tubulin, plays an important role in promoting the MT nucleation as it assembles into a ring-like structure of the size of a MT diameter, with other associated proteins and operates as template for growth of MT (Geissler et al., 1996; Joshi et al., 1992; Moritz et al., 1995; Oakley et al., 1990; Zheng et al., 1995). In almost all animal cells, centrosomes (composed of two centrioles) operate as MTOCs. Another type of MTOC is the basal body, a plasma membrane-bound centriole that gives rise to a cilium. Centrosomes are composed of two centrioles, perpendicular to each other, and surrounded by the pericentriolar material. Typically, in an animal cell, prior to mitosis, the centrosome duplicates and each centrosome moves to opposite sides of nucleus. During mitosis, MTs emanate from the centrosomes, forming the mitotic spindle poles.

However, MTs do not always require the presence of an MTOC for their growth. Cells of higher plants lack any obvious MTOC and form interphase MT arrays and the mitotic spindle (Paradez et al., 2006). Furthermore, mammalian oocytes during meiosis lack centrosomes (Szollosi et al., 1972). Even cells that have a centrosome, can produce some MTs in an acentrosomal manner. A recent study showed that in a polarizing neuron, that extends an axon, MTs that support the axon nucleation assemble even in the absence of centrosomes after laser irradiation (Michael Stiess, Science 2010).

However, most MTs form at the MTOCs. In particular, axonemes of cilia require a basal body to form and most cytoplasmic MTs in animal cells originate at the centrosome. MTOCs that have centrioles (basal bodies and centrosomes) themselves have MTs, usually 9 triplets. The centrioles form by self-assembly from soluble proteins. Usually a new centriole appears near a pre-exisiting centriole. For example, new basal bodies form near an old basal body. However, centrioles can also form *de novo*. The centriole assembly is activated by a rise in activity of a kinase called SAK (Rodrigues-Martins et al., 2007). This kinase phosphorylates a protein called SAS6 that promotes the formation of a central ring structure (Kitagawa et al., 2009). Next, the spokes assemble and the rest of the centrioles, including triplet MTs form at the ends of spokes of the cartwheel (Figure 2.3). So called alternative tubulins play crucial roles in the formation of triplets, most likely by acting as nucleating structures specific to B- and C-tubules. In addition to γ-tubulin, ζ, δ and η- tubulin that share 28-35% identity with α and β-tubulin, participate in the nucleation of triplet MTs. Depletion of γ-tubulin in *Paramecium* inhibits duplication of basal bodies (Ruiz et al., 1999). In *Tetrahymena*, γ-tubulin is not only required for duplication of basal bodies but is also essential for the maintenance of pre-existing basal bodies (Shang et al., 2002). δ-tubulin was first identified in *Chlamydomonas* through genetic studies (Dutcher and Trabuco,

1998). *Chlamydomonas* cells having a mutation of δ-tubulin have fewer flagella. In these mutants, the basal bodies are composed of doublet instead of triplet MTs (Garreau de Loubresse et al., 2001). RNAi-based depletion of δ-tubulin in *Paramecium* showed a similar loss of the C-tubule of the basal body triplets Also, in some basal bodies, triplet MTs were reduced to singlet or no MTs at all (Garreau de Loubresse et al., 2001). This data suggests that C-tubule is required for anchoring, development and positioning of basal body, but not for basal body duplication and the lack of C-tubule does not affect the structure of ciliary MTs, as the C-tubule does not extend into the axoneme. ε-tubulin is less conserved as compared to γ-tubulin. Using genomic approaches, ε-tubulin was discovered in mammals (Chang and Stearns, 2000) and *Trypanosoma* (Vaughan et al., 2000). In mammals, ε-tubulin localizes to centrosomes and its localization is cell-cycle dependent (Lange et al., 2000; Lange and Gull, 1995, 1996). In *Paramecium*, ε-tubulin is required for the basal body duplication and RNAi depletion of ε-tubulin in *Paramecium* resulted in missing B and C-tubules in the proximal part of basal bodies and C-tubules missing in distal part of basal bodies (Dupuis-Williams et al., 2002). Temperature sensitive mutations in η-tubulin in *Paramaecium*, cause death of cells after a few cell divisions due to inhibition of duplication of basal bodies (Ruiz et al., 2000). Most likely, the alternative tubulins act together as a nucleating mechanism to give rise to triplet MTs.

Sources of functional diversity of microtubules

The nucleation of MTs by a variety of alternative tubulins alone, can explain only some functional adaptation of MTs, but not all. But often MTs that originate from the same MTOC are distinct. For example, doublets in the axoneme arise as outgrowths of the A- and B-tubule of the basal body. But all doublets within an axoneme are not identical. For example, certain structures

are associated with only specific doublet MTs and not all doublets slide when the cilium bends. Inner dynein arm composition is different on doublets 1 and 9 (Bui et al., 2009). B-tubule of doublets 1, 5 and 6 in *Chlamydomonas* flagella show morphological specializations such as a beak-like structure (Bui et al., 2009; Hoops and Witman, 1983). These doublets could give the axoneme its inherent structural polarity. Also, the two central MTs in the axoneme are not identical. In squid central pair MTs, the C1-tubule contains single row of 18 nm long sheath of projections with axial periodicity of 16 nm while C2-tubule displays several rows of 9 nm globular sheath of proteins with axial periodicity of 32 nm. C1 tubule contains electron-dense material filled lumen while C2 tubule lumen is hollow (Linck et al., 1981). Subsets of MTs originating from the same centrosome can be more stable while other MTs are dynamic. For example, among the cytoplasmic MTs, majority of the MTs are dynamic as they are required for various activities involved in the morphogenesis of a cell while 10%-20% of the MTs are stable (Schulze et al., 1987) .

Thus, additional mechanisms must exist that act post-nucleation to specify the function of an individual MT or its segment. We can think of three mechanisms that could generate the functional diversity of MTs: a) tubulin isotypes, (b) non-tubulin MAPs and (c) post-translational modifications of either tubulin or MAPs. The role of tubulin diversity and and specifically the functions of its post-translational modifications is discussed further below.

Tubulin isotypes

One possible answerfor the diversity of MTs, is that the cell generates MTs using tubulin subunits with differences at the primary sequence level. Many cell types express multiple isotypes of α- and β-tubulin. The last 15 or so amino acids in the CTT of tubulin are more variable as compared to the rest of the tubulin sequence and represent the isotypic region of

tubulin in vertebrates that is conserved between lineages. Cleveland and colleagues described diverse tubulin multigene families in various organisms (Sanchez et al., 1980). Within an organism there can be several isotypes of α- and β-tubulin whose expression is tissue or developmental stage dependent. Multiple isotypes are found in higher plants, animals and in some protists (Luduena, 1993).

The patterns of isotype expression can be quite distinct in a single organism. In mice, the levels of α4A, α3 and α4 isotypes increase during postnatal development while α2 and α7 levels do not change (Lewis et al., 1985; Villasante et al., 1986). The α-1 and α-2 isotypes are highly expressed in brain and lungs, α-3 is expressed mainly in testes, α-4 in the heart and muscles and α-6 is quantitatively minor and present in multiple organs (Luduena, 1998). In *Plasmodium falciparum*, a single β gene is expressed in both the sexual and asexual stages but there are 2 α genes, one of which is constitutively expressed and the other that is expressed only during male gametogenesis (Delves et al., 1990; Kooij et al., 2005).

Some isotypes may be involved in adaptability to changes in environment. For example, *Arabidopsis thaliana* has 9 β-tubulin isotypes. Transcriptional studies of β-tubulin genes showed that when leaves of this plant are exposed to low temperature, there is a decrease in the mRNA levels of only a subset of the β-tubulin isotypes (Chu et al., 1993; Snustad et al., 1992). The existence of tubulin isotypes may be attributed to their multiple functions. On the other hand, some isotypes could be functionally equivalent but their specific patterns of expression could reflect the evolution of regulatory non-coding sequences. The idea that different isotypes have different functions is known as the multi-tubulin hypothesis (Fulton and Simpson, 1976).

The best documented case of isotype specialization is that of *Drosophila melanogaster*. *Drosophila* expresses several β-tubulin isotypes, out of which β2 is found in post mitotic germ cells of testis and is involved in the assembly of sperm axonemes. β1 is expressed in specific subset of cells during developmental stages such as in embryo, pupa and in ovarian follicle cells, but not in testis (Kaltschmidt et al., 1991). When the axoneme specific β2-tubulin coding region was replaced with that of the β1 isotype in the postmitotic germ cells of *Drosophila*, the β1 isotype supported the formation of non-axonemal MTs, but within the axoneme, β1 failed to support the assembly of central MTs (forming abnormal 9+0 instead of 9+2 axonemes) and the male flies were sterile (Hoyle and Raff, 1990). When the CTT of β1 was replaced with the CTT of β2 , normal 9+2 axonemes formed indicating that the determinant for the formation of the central pair lies in the CTT of β2 (Nielsen et al., 2001). Further studies have revealed the exact functional difference between β2 and β1 resides in the CTT, more precisely in the sequence of 2 amino acids that are part of the so-called axoneme motif. A double substitution within the axoneme motif allowed β1 tubulin to support the assembly of central MTs (Nielsen et al., 2001; Raff et al., 2000). Interestingly, the substitutions are upstream of the cluster of glutamic acid residues that are likely to be subject to post-translational modifications known as polymodifications (polyglutamylation and polyglycylation, see below) (Gaertig, 2008). Thus, some isotypic differences may underlie the ability of these isotypes to be modified post-translationally. Although this case agrees with the multi-tubulin hypothesis, contradictorily, there are many cases of tubulin isotypes that are sufficient for participating in the assembly of various MT structures. For example, a single α-tubulin operates throughout the spermatogenesis stage in *Drosophila* (Kemphues et al., 1982).

Post translational modifications

There is a wide range of mostly reversible post-translational modifications (PTMs) on tubulin subunits that have been proposed to enable MTs to perform variety of functions simultaneously in different locations within a cell. Both tubulin and MAPs are known to undergo PTMs. Here I will focus on PTMs that affect tubulin, as this is the main topic of my thesis. Among the known tubulin PTMs is lysine acetylation, glycylation and glutamylation, detyrosination and Δ2 modification, phosphorylation (reviewed in (Gaertig, 2008; Ikegami and Setou, 2010; Verhey and Gaertig, 2007)), palmitoylation (Caron, 1997; Zambito and Wolff, 1997), lysine methylation (Iwabata et al., 2005), glycosylation (Cicchillitti et al., 2008; Hino et al., 2003), glutamic acid methylation (Xiao et al., 2010), ubiquitination (Huang et al., 2009; Ren et al., 2003), arginylation (Wong et al., 2007) and sumoylation (Rosas-Acosta et al., 2005). Most of these modifications affect the carboxy-terminal tails (CTT) of α/β-tubulins, which are located on the outside surface of the MTs, where they are well positioned to influence interactions with MAPs. Acetylation, on the other hand, occurs on lysine 40 in the N-terminal region of α-tubulin inside the lumen of a MT (Nogales 1999). Not all tubulin PTMs act on tubulin within the MT. For example, phosphorylation of β tubulin on Ser172 occurs on the free dimer and blocks polymerization (Fourest-Lieuvin et al., 2006). Lately, an expansion in the research of PTMs has occurred to enlighten us about their differential distribution in various organisms, cells and tissues. Yet, extensive studies on these PTMs need to be executed as many of their functions remain elusive.

PTMs could either change the inherent properties of MTs (such as their dynamics) or act indirectly by regulating MAPs. Recently Verhey and Gaertig have noticed parallels between the PTMs affecting tubulin and those affecting histones and propose that a biochemical ‘tubulin

code' could exist on MTs that is analogous to the 'histone code' on chromatin (Verhey and Gaertig, 2007). In both chromatin and MTs, specific subsets of polymers undergo PTMs that enable them to be functionally distinguishable from the rest of the polymers. For example, in chromatin, transcriptional activation of a subset of genes, is associated with acetylation of lysine residues on core histones in specific regions (Kouzarides, 2007). Similarly, in order to adapt to local conditions, such as following a tissue wound, a subpopulation of MTs is acetylated and detyrosinated (Khawaja et al., 1988). Also, in both tubulin and histones most of the PTMs occur on the tail domains, located on the surface of polymers. However, there is an important difference between the "histone code" and "tubulin code". The pattern of histone modifications can be directly passed between the old and new chromatin during DNA replication (Groth et al., 2007). In the case of tubulin, while some of the new microtubular organelles are formed in the vicinity of older organelles (centrioles and basal bodies), the pattern of PTMs on an older organelle is not copied onto the new organelle. Rather, the newly formed MT undergoes maturation during which the pattern of PTMs changes. Thus, in MTs, the PTMs distinguish between new and old organelles, which could be functionally important (Iftode et al., 2000; Lechtreck and Geimer, 2000; Sharma et al., 2007).

Carboxy terminal tails (CTTs) of tubulin

I will now discuss what is known about the function of CTTs as these domains are subjected to most known PTMs including glutamylation, the subject of my thesis. CTTs of α/β subunits are located on the exposed side of MTs and are very acidic due to presence of many glutamates or, less frequently, aspartate residues. (Carpenter et al., 2006). A genetic removal of CTT of either α/β subunit is lethal in *Tetrahymena* (Duan and Gorovsky, 2002). It is likely that negatively charged CTTs, through their electrostatic interactions, regulate the rate of

polymerization and depolymerization of MTs (Bhattacharyya et al., 1985). The CTT of β-tubulin may be important for tubulin dimerization as well as for association of MTs with MAPs. The β2 isotype in *Drosophila* is required for normal assembly of 9+2 axonemes in sperm flagella. With a mutated β2- lacking the CTT, supported the assembly of non-motile axonemes that lack central microtubules (9+0). With an increased dose of truncated β2-tubulin, the defects were more severe in the assembled axoneme (Fackenthal et al., 1995; Popodi et al., 2005).

The α- and β-tubulin heterodimer, undergoes several types of post-translational modifications (PTMs) that mostly occur after MT assembly. Polyglutamylation and polyglycylation are two of the polymodifications that occur on α and β-tubulin. Both of these polymodifications occur on the glutamic acid (E) amino acid of CTT of tubulin heterodimers (PTMs explained in the section below). Mutating 3 of the Es on CTT of β tubulin (namely $\beta DDDE_{440}$) was lethal in *Tetrahymena* due to arrested cytokinesis. This mutation also affected the assembly and maintenance of cilia. The newly formed cilia were very short and lacked central pair as well as the B-tubule of outer doublets (Thazhath et al., 2002). All the Es on the α-subunit are dispensable as replacement of Es with Ds led to drastic reduction in levels of glutamylation as well as glycylation but did not affect the survival of cells, indicating that polymodifications on α-tubulin are not essential in *Tetrahymena* (Wloga et al., 2008; Xia et al., 2000). In *Tetrahymena*, functions of α/β subunit tails are interchangeable, as changing the sequence of α subunit CTT to that of β subunit CTT in mutants with all nine Es of β subunit CTT replaced with Ds, rescued their lethal phenotype (Duan and Gorovsky, 2002). All the above observations indicate that precise location of the glutamic residues in the CTT are not as important as their mere presence. Thus, if the function of CTTs is mediated by MAPs, then these

MAPs do not distinguish between the α- and β-tubulin subunit. This is a surprising observation in the light of what is known about MAPs. For example, kinesin is known to bind strongly to β-tubulin and not to α- tubulin (Hoenger et al., 2000).

My research work, explained in Chapter 3, shows that activity of dynein arms in cilia is affected by modifications on the CTT of β-tubulin and α–tubulin (Kubo et al., 2010; Suryavanshi et al., 2010).

Post translational modifications of tubulin:

In this section I will focus on PTMs that affect the CTTs. I will also discuss a non-CTT modification, K40 acetylation on α-tubulin, as this PTM appears to affect motors, a subject closely relevant to observations made here for tubulin glutamylation.

Acetylation of K40 on α-tubulin

Acetylation was first identified on α- tubulin in *Chlamydomonas* flagellar MTs (L'Hernault and Rosenbaum, 1983), and was later found to occur at the N-terminal region of α-tubulin on the lysine residue at position 40 (L'Hernault and Rosenbaum, 1985; LeDizet and Piperno, 1987) inside the lumen of MTs (Nogales et al., 1999). The peculiar luminal location of acetylation may enable it to interact with objects, presumably proteins known to reside inside the MT lumen (Garvalov et al., 2006; Nicastro et al., 2006; Sui and Downing, 2006). This modification is mostly found on stable MT structures such as axonemal MTs as well as on a subset of cytoplasmic MTs that are long-lived (LeDizet and Piperno, 1991). In the neuron, acetylation is enriched in MTs in the axon as compared to those in the dendrites (Ahmad et al., 1993; Baas and Black, 1990; Witte et al., 2008). Several studies suggest the significance of acetylated tubulin in neurodegenerative diseases such as Parkinsons' disease, Huntington's disease

and Alzheimers (Dompierre et al., 2007; Outeiro et al., 2007; Suzuki et al., 2007). HDAC6 (histone deacetylase 6) and Sir2 have been recently identified as the deacetylating enzymes, that remove acetyl group from K40 *in vivo* as well as *in vitro* ((Hubbert et al., 2002; North et al., 2003). HDAC6 belongs to class II of HDAC family and deacetylates a number of proteins in addition to a-tubulin: HSP90, HSP70, and cortactin (Kovacs et al., 2005; Luxton and Gundersen, 2007; Valenzuela-Fernandez et al., 2008). SIRT2 (silent information regulator 2 protein) is another α-tubulin deacetylase, belonging to class III of HDAC family. In comparison to acetylase histone H3 peptide, SIRT2 prefers acetylated tubulin, (North et al., 2003). SIRT2 over-expression affects acetylation levels of neuronal α-tubulin (Pandithage et al., 2008). In mammalian cells, SirT2 can regulate several other proteins besides tubulin (Jin et al., 2008; Lynn et al., 2008). SIRT2 has been demonstrated to regulate cell processes such as gene expression, genome stability, mitosis, nutrient metabolism, aging, mitochondrial function and cell motility (Harting and Knoll, 2010).

K40 acetylation is dispensable in *Tetrahymena* as replacing the lysine residue with arginine does not affect its major cellular activities such as motility or multiplication rate (Gaertig et al., 1995). In *Chlamydomonas*, the substitution of K to A or R (up to 80% of total α tubulin) caused no obvious change in the phenotype (Kozminski et al., 1993a). In *C elegans* the majority of MTs have 11 protofilaments, some of the neuronal MTs have 13 protofilaments and specific neurons known as touch receptor neurons (TRNs) have MTs with an unusually large diameter due to the presence of15 protofilaments. These TRN MTs are composed of K40 containing MEC-12 α-tubulin and MEC-7 β-tubulin. MEC-12 is the only isotype that has K40 and consequently, acetylated MTs are mostly restricted to TRNs (Fukushige et al., 1999). The *C. elegans* mutants lacking MEC-7 or MEC-12 have defective TRNs as the 15 protofilaments fail to

assemble (Fukushige et al., 1999). Unexpectedly, it was possible to rescue the *mec-12* mutants by expressing a MEC-12 with a K40Q substitution (Fukushige et al., 1999), indicating K40 acetylation is not critical for the function of TRNs. However, worms expressing either K40R or K40Q NMEC0-12 have reduced touch response, indicating that K40 acetylation is important for the function of TRN but without this PTM TRNs form and are partially responsive (S. Akella et al., submitted).

Within neurons, acetylated MTs are non-uniformly distributed. In cultured hippocampal neurons, axons are more enriched in acetylated MTs as compared to dendrites. A quantitative study indicates that acetylated tubulin is highest in axons of polarized neurons and future axons of unpolarized neurons compared with other neurites (Witte et al., 2008). Even before the morphogenetic differentiation of neurons begins, the selective translocation of kinesin-1 is the earliest marker of axon formation (Jacobson et al., 2006). In polarized neurons motor proteins kinesin-1 preferentially enters highly acetylated axons of polarized neurons (Jacobson et al., 2006; Nakata and Hirokawa, 2003). Even in cultured fibroblasts, kinesin-1preferes highly acetylated MTs (Cai et al., 2009; Dunn et al., 2008). These data suggest that MT stabilization imparted by acetylation maybe an important factor in polarization of neurons. However, according to the studies by Hammond and Verhey, acetylation of K-40 in α-tubulin is not enough to drive the selective localization of kinesin-1 to axons (Hammond et al., 2010). According to them, multiple PTMs of tubulin, together influence the specific localization of kinesin-1 into axons. Thus, acetylation appears not to be important for polarization but may still be important for maintenance of axon/dendrite identity.

The issue of MT stabilizing effect of K40 acetylation has been controversial as it was not clear whether acetylation is the result or cause of stabilized MTs. A Study by Palazzo and

colleagues indicate that an increase in the tubulin acetylation levels caused by inhibition of HDAC6 deacetylase (discussed below) does not increase the number of stable MTs in fibroblasts, and that stabilization of MTs probably occurs by other mechanisms such as plus end capping (Infante et al., 2000). Thus the already stable MTs could accumulate acetylation and detyrosination (Palazzo et al., 2003), which is consistent with other studies demonstrating that the levels of tubulin acetylation did not affect the rate of MT polymerization *in vitro* (Maruta et al., 1986) and that acetylated tubulin is detected mainly in long-lived stable MTs *in vivo* (Webster and Borisy, 1989). On the other hand, it has been shown that tubulin acetylation can affect MT dynamics, possibly in a relatively subtle way, below the level of detection in certain studies. Dynamic MTs are required in fibroblasts for remodeling and turnover of focal adhesions. During cell migration, de-adhesion at the rear of moving cells requires dynamic MTs that physically interact with adhesion junctions (Waterman-Storer et al., 2000). On increasing the levels of tubulin acetylation by inhibiting the activity of HADC6, focal adhesion capacity of cells that is required for rapid cell migration was severely affected due to change in dynamics of MTs (Tran et al., 2007).

In non-neuronal cells, over-expression of HDAC6 in murine fibroblasts decreases the levels of acetylated α-tubulin and increases chemotactic motility, suggesting that acetylated MTs inhibit cell motility (Hubbert et al., 2002). In neuronal cells, inhibition of HDAC6 by tubacin, a small synthetic molecule drug, caused a decrease in cell motility, delocalized p58, MAP that binds Golgi proteins to MTs (Haggarty et al., 2003), increases transport of kinesin-1 cargo protein JIP1 to neurite tips (Bulinski, 2007; Reed et al., 2006) and stimulated the rate of transport of vesicles containing brain-derived neurotrophic factor (BDNF) (Dompierre et al., 2007).

When HDAC6 in embryonic stem cells was disrupted, there was an increase in levels of acetylated MTs; however, there was no change in the rate of cell proliferation or differentiation (Zhang et al., 2003). In cultured mammalian cells, depletion of HDAC6 increased the levels of K40 acetylation. This led to mild stabilization of MTs, as they showed increased resistance to drug-induced depolymerization (Matsuyama et al., 2002) and decreased dynamicity (Tran et al., 2007). However, surprisingly, when HDAC6 was deleted in mice, except for mild defects in bone structure and immune response, they developed normally. Although the K40 acetylation levels were high during spermatogenesis, no defects were detected in the sperm development (Zhang et al., 2008).

Detyrosination

Almost all α-tubulins, except for murine α4A and α8 and fungal tubulins, have a C-terminal Y residue. This Y in many tubulins is subjected to proteolytic cleavage known as detyrosination. The removal of Y residue is mediated by an α-tubulin specific carboxypeptidase (Arce and Barra, 1983). Nna1/CCP1, may be the long sought-after carboxypeptidase as mice lacking this protein have lower levels of detyrosinated tubulin (deTyr-T) and higher levels of unmodified tyrosinated tubulin in the mitral cells olfactory bulb (Kalinina et al., 2007). Purkinje cell degeneration *(pcd)* mice display ataxia in 3-4 weeks of age due to postnatal degeneration of Purkinje cells. The *Pcd* mice have mutations in Nna1/CCP1.

Detyrosination preferentially occurs on polymerized tubulin whereas tyrosination takes place on soluble tubulin (Kumar and Flavin, 1981). Tyrosinated α-tubulin dimerizes with α-tubulin prior to assembly into MTs (Gundersen et al., 1987). As the MT grows older, the Y residue from α-tubulin is removed to expose the penultimate E residue at carboxy terminus

(detyrosinated tubulin) (Gundersen et al., 1987). After MT depolymerization, detyrosinated α-tubulin can be restored to the unmodified state by Y ligation. The enzyme tubulin tyrosine ligase (TTL) catalyzes the addition of Y residue to α-tubulin and has a binding site on β-tubulin (Wehland and Weber, 1987a). Just like acetylation, detyrosination tends to occur on long-living, stable MTs whereas MTs containing mainly unmodified tyrosinated tubulin are more dynamic MTs (Khawaja et al., 1988; Paturle-Lafanechere et al., 1994). Taxol-stabilized MTs are rapidly detyrosinated in cells that usually have low levels of this modification (Wehland and Weber, 1987b), suggesting that detyrosination simply accumulates on more stable MTs. However new studies indicate that detyrosination itself, can to some extent, stabilize MTs. Kinesin-13 is a motor-like protein that is not a true motor but a MT-depolymerizing protein (Peris et al., 2009). Recent *in vitro* studies have shown that detyrosination in fibroblasts imparts stability to MTs by inhibiting end-depolymerizing activity of kinesin-13 (MCAK). Thus, detyrosination of subset of MTs may give rise to a pool of disassembly-resistant MTs (Peris et al., 2009). In regulating entry of kinesin-1 into axons, tubulin detyrosination appears to play a key role. *In vitro,* kinesin-1 binds more strongly to detyrosinated as compared to tyrosinated MTs (Konishi and Setou, 2009; Liao and Gundersen, 1998).

TTL-null mice with lower levels of tyrosinated MTs exhibit defects in the formation of central nervous system and die during embryonic development (Erck et al., 2005). Detyrosination appears to strongly also influence interaction of kinesin-1 with MTs and affect transport cargoes containing intermediate filament vimentin by this motor (Dunn et al., 2008; Kreitzer et al., 1999). Cultured TTL-null neurons show defective neurite growth and axonal differentiation (Erck et al., 2005). Also, TTL-null cells show normal localization of MAP1B, MAP2, EB1 and dynein but have mislocalized CLIP170, a +TIP, which can be linked to the abnormal development of

nervous system (Akhmanova and Steinmetz, 2008; Galjart, 2005; Jaworski et al., 2008). During development of neurons, proper organization of actin and MT cytoskeleton is required for axon outgrowth and pathfinding. Recent studies show that TTL null mice show disturbed localization of actin and MT cytoskeleton, deregulation of small GTPase activity as well as abnormal neuronal morphogenesis with defective organizing and path finding ability of growth cones (Marcos et al., 2009).

The detyrosinated and acetylated tubulin are often seen on the same MTs but their pattern and time of occurrence appear different (Sasse and Gull, 1988). Chang and colleagues showed that, on inhibition of detyrosination using 3-nitrotyrosine in myoblasts, myogenesis was prevented without affecting the levels of acetylation indicating the lack of close coupling between the two tubulin PTMs (Chang et al., 2002). Within neurons, growth cones at the end of the axon have largely unmodified MTs that are dynamic. These unstable distal ends of axonal are highly tyrosinated, whereas its stable proximal end is enriched with detyrosination and acetylation (Ahmad et al., 1993; Baas and Black, 1990).

Within an axoneme, distribution of detyrosinated tubulin is uneven as in flagella *Chlamydomonas*, the central pair of MTs are enriched with tyrosinated tubulin while outer doublets have high levels of both tyrosinated and detyrosinated isoforms. Within the doublets, the B-tubule has higher levels of detyrosinated tubulin - as compared to the A-tubule. Because the B-tubule serves as a track for ciliary dynein, the primary role of tubulin detyrosination in cilia could be to regulate the motor activity of ciliary dynein. The indication that detyrosination influences flagellar beating comes from the fact that it accumulates on regenerated, beating flagella as opposed to tyrosination which localizes in newly regenerating flagellar tubulin (Johnson, 1998).

Glycylation

Glycylation is an evolutionarily conserved PTM. It is a process that involves addition of variable number of glycyl residues to γ carboxyl group of conserved glutamic acid residues on CTT of α or β-tubulin (Bre et al., 1998; Vinh et al., 1999). The modification was discovered in *Paramecium* by Adoutte and colleagues in 1985, as epitopes detected on ciliary tubulin but not on cytoplasmic tubulin in various species (Adoutte et al., 1985). The modification accumulates on older, already assembled ciliary MTs (Adoutte et al., 1991). This modification was determined to be glycylation by mass spectrometry of ciliary tubulin in *Paramaecium* (Redeker et al., 1994), and was also detected in *Giardia* (Weber et al., 1997) and mammals (Rudiger et al., 1995). Glycylation is mainly found in ciliated and flagellated cells (Bre et al., 1996), except that some species such as *Plasmodium* and *Trypanosoma* have cilia but lack apparently lack glycylation (Fennell et al., 2008; Schneider et al., 1997). Thus, glycylation despite its conservation, is not essential for either the assembly of motility or sensory functions of cilia. When ATP reactivated sea urchin sperm flagella were treated with anti-glycylated tubulin antibodies, their motility was inhibited, hinting at the role for this PTM in regulation of ciliary dynein (Bre et al., 1996). However glycylation is also found in non-motile cilia such as primary cilia in mammalian cells, suggesting that glycylation could play a role in the assembly of cilia rather than in their motility (Davenport et al., 2007; Ong and Wheatley, 2003).

Glycylation occurs in two steps known as initiation and elongation. Initiation is the ligation of the first glycyl unit to form monoglycylated tubulin (Kann et al., 1998) whereas elongation involves addition of subsequent glycyl residues to form polyglycated tubulin. Elongation can involve addition of up to 34 glycyl residues per tubulin (Redeker et al., 1994). Newly forming cilia are enriched with monoglycylated tubulin (Iftode et al., 2000); in matured

cilia there is a decrease in monoglycylation and an increase in polyglycylation (Sharma et al., 2007) presumably by delayed chain elongation. As for the patterns of glycylation within a ciliary axoneme they are quite variable. Using antibodies TAP952 and AXO 49 that are directed toward monoglycylated and polyglycylated tubulin , respectively, it was found that in *Paramaecium* there seem to be equal amounts of this modification on outer doublets and central pair (Pechart et al., 1999). In sea urchin, mammalian sperms (Kann et al., 1998) and *Tetrahymena* (Wloga et al., 2009) cilia higher levels of glycylation is found on outer doublets (Kann et al., 1998; Pechart et al., 1999; Wloga et al., 2009). Since the outer doublets may have a higher level of glycylation, it is possible that this PTM regulates the intraflagellar transport (IFT) as IFT mainly occurs on the B-tubule of the outer doublet (Kozminski, 1995; Pigino et al., 2009). A mutation of polyglycylation/polyglutamylaton sites on CTT of β-tubulin led to accumulation of electron dense material that resembled IFT complexes near the outer doublets of cilia (Redeker et al., 2005). However, on the contrary, *Drosophila* axonemes of sperm flagella are enriched with glycylation (Bressac 1995) and yet assemble without IFT, indicating that the function of glycylation other than IFT (Han et al., 2003). Glycylation on B-tubule is consistent with the possibility of this modification being involved in function of ciliary motility or both IFT and ciliary motility.

Very recent studies have shown that TTLL3 is the chain initiating G-ligase enzyme. TTLL3-null *Tetrahymena* and TTLL3-depleted *Zebrafish* cilia have severely reduced levels of glycylation with shorter or completely lost of cilia, respectively (Rogowski et al., 2009; Wloga et al., 2009) split references to match with pieces of data. Mice have TTLL3 (and paralog TTLL8) as initiating and TTLL10 as elongating G-ligases, whereas in *Drosophila*, homologues of TTLL3 catalyze initiation as well as elongation, and there is no TTLL10 homologue in the genome.

RNAi based knockdown of TTLL3 G-ligase in *Drosophila* led to significant reduction in the levels of glycylation and was associated with abnormal sperm maturation, male sterility and lethality of early embryos. Although the sperm axoneme assembles, under reduced G-ligase activity the assembly is incomplete and arrests at an early stage (Rogowski et al., 2009). However, the results of RNAi experiments need to be confirmed by mutational studies in TTLL3 genes in *Drosophila*. One problem in interpretation is that there appears to be a large number of proteins that undergo TTLL3-dependent glycylation in *Drosophila* (Rogowski et al., 2009) and thus it is not clear to what extent TTLL3 significance is mediated by its activity on tubulin. In humans, sperm tubulin undergoes only monoglycylation whereas the murine sperm has both mono- and polyglycylation (Bre et al., 1996). The likely cause for the absence of elongated side chains in human sperm tubulin is the substitution of 2 amino acids in the human G-ligase elongase enzyme, TTLL10 (see below). Non-human primates have elongated side chains and active TTLL10, suggesting that the that loss of polyglycylation was a significant event in the evolution of humans (Rogowski et al., 2009). It also means that a minimal glycylation (just initiation) is sufficient to perform the basic PTM function in the assembly and motility of cilia (Rogowski et al., 2009). This is consistent with a hypothesis that the main function of glycylation is to compete with glutamylation on shared modification sites as a single glycine ligated to a glutamic acid is sufficient to block competing glutamylation (see below).

Glutamylation

Glutamylation was discovered in 1990 on mammalian brain tubulin using mass spectrometry (Edde et al., 1990) and is one of the most conserved tubulin modifications (Rogowski et al., 2009; Wloga et al., 2008). It is an apparently reversible PTM (Audebert et al., 1993) that adds variable number of glutamate residues as a side-chain to the primary peptide of

proteins. The first glutamate is linked by an amide bond between its α-amino group to the γ-carboxylic group of the modified glutamate residue in the primary peptide chain of the protein (Janke et al., 2005; Mukai et al., 2009; Wloga et al., 2008). Additional units are linked by standard isopeptidic bonds, thus, leading to the formation of a linear side chain of glutamate residues (Janke et al., 2005; Suryavanshi et al., 2010; Wloga et al., 2008)**.** High levels of this modification are found on basal bodies/centrioles, axonemes, cytoplasmic MTs, mitotic spindle and neuronal microtubules (Kann et al., 2003; Lechtreck and Geimer, 2000; Plessmann et al., 2004; Wolff et al., 1992). Cytoplasmic MTs have low level of glutamylation during interphase, but the mitotic spindle MTs are enriched with (Bobinnec et al., 1998a; Bobinnec et al., 1998b; Regnard et al., 1999). Neuronal tubulin has glutamyl side chains of 1 to 6 glutamate residues while ciliary axonemes have longer side chains (Audebert et al., 1994; Million et al., 1999). In contrast to glycylation, this modification has apparent maximal levels early during the axoneme assembly before other modifications such as acetylation, detyrosination and glycylation peak off, indicating a role in assembly of axonemes (Lechtreck and Geimer, 2000; Sharma et al., 2007). While the levels of glutamylation may decrease in mature axonemes, the PTM is still present and plays a crucial role in regulation of ciliary motility; this is a major finding of my thesis (see Chapter 3).

The diplomonad *Giardia lamblia,* representing an early branch of eukaryotes, also has tubulin polyglutamylation, indicating the evolutionary conservation of this modification. This modification maybe even present in prokaryotes as there is a prokaryotic TTLL that is related to TTLL4, an E-ligase (Iyer et al., 2009). However, this modification seems to have disappeared from some eukaryotes such as fungi, which also lack basal bodies and cilia. It is not clear whether higher plants have this PTM. Immunofluorescence studies suggest that this is the case (Wloga et

al., 2008) but higher plants lack enzymes -TTLLs that are known to catalyze glutamic acid ligation (see below). If plants lack glutamylation, it would appear that the PTM co-evolves with cilia and centrioles. Importantly, there is conservation of the main glutamylation sites in organisms ranging from *Trypanosoma* to mammals (Plessmann and Weber, 1997). Tubulin glutamylation can be detected using antibodies GT335 (Wolff et al., 1992) and polyE (van Dijk et al., 2007) that are directed towards number of side chains and length of side chains, respectively. While in all species the PTM is enriched in axonemes and centrioles/basal bodies, the patterns of glutamylation vary considerably across species. Sea urchin sperm cilia seem to show approximately equal distribution of this modification on central pair and outer doublets (Hoyle et al., 2008) whereas *Chlamydomonas* appears to have higher amount of glutamylation on doublets than central pair (Lechtreck and Geimer, 2000). However, these antibody labeling patterns may not be precise as these antibodies may not detect glutamate side chains of all lengths and at all sites. Also, side chains of adjacent CTT could potentially exhibit steric inhibition and block antibodies from reaching its epitopes. Thus, there is a possibility that tubulin glutmaylation levels maybe under represented (Gaertig, 2008). In *Drosophila,* central pair and singlet MTs seem to be enriched with glutamylation (Hoyle et al., 2008). In *Tetrahymena*, glutamylation is enriched on outer doublets, especially on the B-tubule (Suryavanshi et al., 2010) as explained in Chapter 3.

Polyglutamylation could play an important role in ciliary and flagellar motility (Gagnon et al., 1996; Million et al., 1999), assembly and maintenance of axoneme (Dave et al., 2009; Pathak et al., 2007) and centrosomes (Abal et al., 2005; Bobinnec et al., 1998a). *In vitro* binding assays suggest that glutamylation could act as a regulator of various MAPs and molecular motors like kinesin and dynein (Bonnet et al., 2001; Boucher et al., 1994; Ikegami et al., 2007). Studies have shown that polyglutamylation is a major tubulin modification in the mammalian brain, and it

accumulates in cultured neurons and *in vivo* during brain development (Audebert et al., 1993; Audebert et al., 1994). Interestingly, the patterns of glutamylation of α and β-tubulin are distinct during brain development, as β-tubulin polyglutamylation levels increase during maturation of neurons while the levels of polyglutamylated α-subunit reach a plateau at birth (Ikegami et al., 2007). Not only the timing, but also the distribution within the neuron is subunit-specific as α-tubulin glutamylation is enriched in axons and β-tubulin glutamylation levels are high in the soma and dendrites (Ikegami et al., 2006). Tubulin polyglutamylation also regulates motor proteins as of mice lacking PGs1, an α-tubulin E-ligase, have decreased levels of α-tubulin glutamylation levels and eurites of these mice have reduced levels of KIF1 a kinesin required for synaptic transmission (Ikegami et al., 2007). The substrate of polyglutamylation is not just tubulin but the PTM also occurs on nucleosome assembly proteins (NAPs) (Regnard et al., 2000).

Appropriate lengths of glutamate side chains may be regulated by deglutamylase activity of unidentified enzymes that remove glutamate residues from the distal and proximal end of glutamate side chain in a sequential manner (Audebert et al., 1993). Recent studies show the discovery of this reversible enzyme. In *C.elegans*, sensory cilia are polyglutamylated by TTLL-4. Overexpression of one of the cytosolic carboxypeptidases, *ccp-p6* in *C. elegans,* resulted in decrease in levels of GT335 signal. Loss of *CCPP-6* led to increase in GT335 signal of sensory cilia whereas double knockout of ttll4 and CCPP-6 led to loss of this enhanced GT335 signal. Also, overexpression of *CCP-5*, murine orthologue of *CCPP-6,* resulted in decrease in GT335 signal in mouse cortical neurons. Recombinant CCPP-6 also showed deglutamylase activity *in vitro* (Kimura et al., 2010).

Only recently have the enzymes that catalyze this modification have been identified by Janke et al as part of large family of TTL-like proteins (Janke et al., 2005). My research focuses on the role of E-ligases that regulate tubulin as discussed in chapter 3 and 4.

Inter-dependence of PTMs

The C-terminal tails of α and β tubulin are 'hot spots' for several PTMs and thus it is likely that multiple PTMs influence each other. In *Tetrahymena,* CTT of α-tubulin has 6 and β subunit has 9 glutamate residues (E) that are in close proximity with each other. Site directed mutagenesis studies in *Tetrahymena* have revealed the presence of 3 glycylation sites on α-tubulin and 5 glycylation sites on β-tubulin and these sites are homologous to the glycylation sites in *Paramecium* that were revealed by mass-spectrometry (Vinh et al., 1999; Xia et al., 2000). The glutamylation sites in *Tetrahymena* and *Paramecium*, have not been mapped yet, but the sites of glutamylation that are known in other organisms are either adjacent or they overlap with the sites of glycylation and these sites may undergo both type of modifications (Mary et al., 1996; Redeker et al., 2004; Vinh et al., 1999).

The close proximity of glutamylation and glycylation sites opens up the possibility of interactions among the two polymodifications. This hypothesis was tested in studies done by Redeker and colleagues (Redeker et al., 2005). The viable glycylation site *Tetrahymena* mutants - $\beta EDDD_{440}$ show greatly reduced levels of β-tubulin polyglycylation. Remarkably, these mutants also show a major reduction in α-tubulin polyglytamylation levels and hyperglycylation of α-tubulin. Thus, along with extensive interaction of the two polymodifications, these studies also showed that there is a cross talk between α- and β-tubulin subunits.

One of the possible reason for this cross-talk could be that the non-modified subunit (α-tubulin in this case) could act as an enzyme binding site (Redeker et al., 2005).

Recent studies showed that polyglutamylation and polyglycylation negatively regulate each other. In *Tetrahymena,* over-expression of G-ligase (TTLL3A), is associated with an increase in the levels of tubulin glycylation as well as a decrease in tubulin glutamylation (Wloga et al., 2009). Deletion of initiating G-ligases, TTLL3in *Tetrahymena*, resulted in strong reduction in the levels of glycylation and a 3 fold increase in tubulin glutamylation (Wloga et al., 2009). *Tetrahymena* strains lacking E-ligases, TTLL1 and TTLL9, show higher levels of axonemal tubulin glycylation along with reduction in tubulin glutamylation levels (Wloga et al., 2008). These data are consistent with glycylation and glutamylation being in competition for shared sites (glutamic acids) on tubulin. In mice lacking PGs1, a noncatalytic subunit of α-tubulin polyglutamylase TTLL1, decreased expression levels of α-tubulin tyrosination were observed (Ikegami et al., 2007). Thus polyglutamylation could influence the enzymes that detyrosinate as both these modifications act on the same CTT.

Tubulin tyrosine ligase like proteins (TTLL)

Janke et al immunoprecipitated the tubulin glutamic acid (E-ligase) enzyme complex using a monoclonal antibody (mAb206) that blocked tubulin glutamic acid ligase activity in vitro. Peptide sequence analysis of this fraction containing several polypeptides revealed PGs3, an ortholog of human TTLL1 protein (Janke et al., 2005). TTLL1 contains a domain that has 17% amino acid identity to the ATPase-dependent catalytic domain of tubulin tyrosine ligase (Trichet et al., 2000). This domain is conserved among many organisms. Several subsequent studies have revealed the existence of a large family of TTLL group of proteins- TTLL1 to TTLL13 in mammals with orthologs in many eukaryotes including invertebrates and protists (van Dijk et al.,

2008; van Dijk et al., 2007; Wloga et al., 2008). Most of these proteins catalyze either glycylation or glutamylation, initiation or elongation and show preferences for either α or β-tubulin. Several subtypes of conserved TTLLs such as TTLL12, have not been characterized and could have distinct activities that are likely also based on amino acid ligation.

Mice contain 13 TTLL proteins, namely TTLL1 through TTLL13 (Figure 2.4). Some of these proteins (TTLL1, 2, 4, 5, 6, 7, 9, 11and 13) have a core domain and an extended domain While the core domain contains sequence for ATPase activity and is suggested to be involved in amino acid ligation activity, the extended domain is suspected to determine the specificity of the amino acid that is being ligated such as glutamate or glycine (van Dijk et al., 2007).

Within the murine group of TTLL proteins, TTLL 1, 2, 4, 5, 6, 9, 11 and 13 form a closely related phylogenetic group whereas TTLL3, 8, 10 and 12 are distinct. TTLL1, TTLL2, TTLL4, TTLL5, TTLL6, TTLL9, TTLL7, TTLL11 and TTLL13 are confirmed or suspected E-ligases (Ikegami et al., 2006; Janke et al., 2005; van Dijk et al., 2007; Wloga et al., 2008). TTLL3, 8 and 10 function as tubulin G-ligases (Rogowski et al., 2009; Wloga et al., 2009) and the activity of TTLL12 is still unknown.

Among E-ligases, TTLL4 and 5 show strong initiation and 6, 11 and 13 show strong elongation activity on tubulin. *In vitro* assays demonstrated that murine TTLL7 can catalyze initiation as well as elongation reactions on new born mouse brain tubulin (Mukai et al., 2009). In *Tetrahymena thermophila*, TTLL1 and TTLL9 are α-tubulin initiating and elongating E-ligases, respectively (Wloga et al., 2008). Murine TTLL5, 6, 11 and 13 prefer α-tubulin while TTLL4 and 7 prefer β-tubulin.

Some of these proteins can act as single subunits such as Ttll6Ap, which when over-expressed in *Tetrahymena* increased the levels of tubulin glutamylation (Janke et al., 2005;

Wloga et al., 2010). But some proteins probably require other cofactors for their activity, as, over-expression of TTLL1, TTLL2 and TTLL9 in mammalian culture as well as bacteria, does not result in detectable increase of glutamylation. Expression of TTLL proteins is tissue-specific and they localize on subsets of MT with cells. When EYFP-tagged murine TTLL proteins were over-expressed in MDCK cells, TTLL4 localized to cilia, TTLL1, 9 and 7 to basal bodies and TTLL5, 6 and 7 to basal bodies as well as cilia (van Dijk et al., 2007).

Tubulin PTMs contributed by TTLLs is important in regulation of assembly, maintenance and motility of complex organelles such as cilia/flagella. A morpholino based knockdown of TTLL6 in zebrafish resulted in shortening and loss of olfactory cilia (Pathak et al., 2007). Mutation in PGs1, a non-catalytic subunit of TTLL1 in mice, led to assembly of defective sperm axoneme (Campbell et al., 2002; Regnard et al., 2003). Overexpression of Ttll6A p in *Tetrahymena* causes cessation of ciliary motility and shortening of cilia (Wloga et al., 2010). TTLL1 in mice is required for normal motility of respiratory cilia and for the assembly and maintenance of sperm flagella (Vogel et al., 2010). Significance of tubulin glutamylation contributed by TTLL6 group of proteins in regulating motility of cilia and length of cilia has been discussed in Chapters 3 and 4 respectively.

Tetrahymena thermophila **as a model**

Tetrahymena is a fresh water, free-living ciliate protozoa. Its scientific classification is as follows (Alfred Elliott, 1973, Biology of *Tetrahymena*):

Some of the aspects of *Tetrahymena* that make it a useful experimental model to work with are as follows:

1) *Tetrahymena*, inspite of being a single cell, shows 18 types of MTs, equivalent to the diverse MTs present in humans and other metazoans. Its cytoskeleton provides us with a wide array of functionally diverse MTs, including cilia, cortical MTs, and MTs required for cell and nuclear division during mitosis and meiosis. (Brown et al., 1999a; Gaertig, 2000; Gaertig and Cole, 2000).
2) *Tetrahymena* is easy to grow in the laboratory (Frankel, 2000).
3) *Tetrahymena* contains about 1000 motile cilia (conserved and important eukaryotic organelles), which beat in a coordinated manner to produce cell movement. Motile cilia of Tetrahymena have a conserved 9+2 axoneme structure with 9 outer doublet MTs and central pair, radial spokes, outer and inner dynein arms that regulate sliding of doublet MTs. Bending of cilia is generated by conserved dynein-based sliding of MTs. Defects in cilia can lead to several diseases such as polycystic kidney disease and primary ciliary dyskinesia. *Tetrahymena* is a good model system to study dynein-based motility as it has a potential to uncover many issues related to various ciliary diseases in humans.
4) *Tetrahymena* is suitable for reverse genetics. Genes in the *Tetrahymena thermophila* micronucleus or macronucleus can be mutated or deleted by process of homologous recombination (Cassidy-Hanley et al., 1997). The recent genome sequencing project has contributed enormously towards the identification of many genes that enable their targeting for overexpression, knockout and mutational studies (Eisen et al., 2006).

Tetrahymena is a good model to study PTMs, as other organisms, such as yeast, lack most of the PTMs (Alfa and Hyams, 1991). *Chlamydomonas* has a low level of homologous recombination, thus, it is difficult to introduce mutations or make gene knockouts in them. *Tetrahymena* is a great tool in genetics, as its genome has been sequenced, facilitating gene

identification followed by protein expression and gene knockouts. Exogeneous DNA can be integrated into its genome by homologous recombination enabling mutation of locus-specific genes (reviewed in (Turkewitz et al., 2002)).

There is a tremendous MT diversity in ciliates as *Tetrahymena* assembles up to 18 types of diverse and complex MTs such as cilia, cell cortex, cytoplasmic network, mitotic spindle, oral apparatus, MTs involved in developmental process like conjugation and nuclear MTs (Gaertig, 2000). *Tetrahymena* has a single α-tubulin gene *ATU1* (McGrath et al., 1994) that is essential and 2 redundant β- tubulin genes *BTU1* and *BTU2*, out of which atleast one is required for survival (Gaertig et al., 1993). These conventional tubulins are widely found in various microtubular structures and isoforms that are created by PTMs. In addition, *Tetrahymena* has 9 α-tubulin-like and 6 β-tubulin-like genes that show 45%-65% sequence identity to the conventional tubulins. *Tetrahymena* also has conserved alternative tubulins: γ, η, and ε tubulins.

Tetrahymena cells are polarized. The anterior side is marked by the oral apparatus (OA) and the posterior end has the contractile vacuole pore (CVP). The basal bodies (BB) most of which have cilia, are organized into longitudinal rows along the cell cortex. Longitudinal MT bundles (LMs), comprised of 7-9 MTs, run on the side of each ciliary row. The individual MTs that make up the LM bundle are relatively short and overlapping. The transverse MT bundle (TM) that originate from the anterior end of most BBs, lies perpendicularly to the LM and is composed of 8 MTs. From the posterior end of most BB, originates the post-ciliary MT (PC) and the non-microtubular kinetodesmal fiber run anterior to BBs. Cilia surrounding the oral cavity are linked into structures known as membranelles: undulating membrane (UM) and 3 associated membranelles: known as the adoral zone of membranelles (AZM. Thus, the oral apparatus has 4 hymens or membranes. The name *Tetrahymena* comes from these oral membranes : *Tetra*-four

and *hymena*-membrane. These cilia beat in a co-ordinated fashion to ingest and filtrate food particles (Chapman-Andresen and Nilsson, 1968).

Before the event of cell-division, a number of events, such as duplication and assembly of cellular structures, take place in a *Tetrahymena* cell. A new OA is synthesized in the mid-body region that lies below the future cleavage furrow area. New CVPs are assembled in the posterior part of the newly forming anterior daughter cell. Old CVPs and new OA are transferred to the old posterior daughter cell. LMs and BBs that lie in the cleavage furrow region develop breaks followed by constriction of the fission furrow. The cells then undergo rotokinesis, in which the anterior daughter cells rotate in a counter clock-wise manner that leads to breaking of the narrow ridge between the two cells. This leads to the formation of two new daughter cells (Brown et al., 1999a).

Basal bodies

Cilia and flagella arise from structures known as basal bodies (BBs). BBs are usually made up of 9 triplets of MTs that consist of a 13 protofilament A-tubule and 10-11 protofilament B and C-tubules. Exceptions include centrioles in *C.elegans* with 9 singlet MTs (O'Connell, 2000) and *Mastigamoebae* and *Drosophila* with 9 doublets (Callaini et al., 1997). Centrioles that form the centrosome are structurally similar to BBs. Moreover, there are some cell types that show an interconversion between the centrioles and the BB. During G1 in mammals, the mature mother centriole from the centrosomal location differentiates and migrates near the cell surface to form BBs for ciliogenesis. During mitosis, the assembled cilia resorb and the BB migrate back to the spindle pole to support mitosis (Dawe et al., 2007). In *Chlamydomonas*, prior to mitosis, BBs are severed from the axoneme (Rasi et al., 2009) and convert into centrioles to mediate the formation of mitotic spindle during mitosis (Beisson and Wright, 2003). However, in ciliates,

BBs are permanently positioned in the cortex of the cell and do not have direct functions in nuclear divisions. Thus, BBs in ciliates lack the ability to convert into centrioles.

Several proteins that are involved in ciliary diseases such as oriofacial digital syndrome protein, OFD1 (Ferrante et al., 2006), Meckel syndrome protein MKS1 (Kyttala et al., 2006), and Bardet-Biedl syndrome proteins (Ansley et al., 2003) are associated with various regions of the BB. Recently, 24 new BB proteins haven been identified in *Tetrahymena* (Kilburn et al., 2007). The BB proteome is comprised of 3 categories of proteins. First group of proteins are those that forms the core structural proteins that make up the BB such as tubulin and tektin (Hinchcliffe and Linck, 1998). The second group contains transiently associated proteins such as IFT and BBS proteins that do not co-purify with BBs on sucrose gradient and therefore may be loosely associated with BBs (Keller and Marshall, 2008). The third group of proteins are those that are permanently associated with fibers attached to the BB. For example, algal BBs contain fibrous protein known as rootletin (Geimer et al., 1998; Lechtreck and Melkonian, 1991; Lechtreck et al., 1999).

Tetrahymena cell has roughly 750 BBs. The older BBs act as site for the formation of a new BB. D.L. Nanney stained BBs in 1975 with protargol stain in dividing *Tetrahymena* and observed that proliferation of new BBs was more in the mid and posterior region of the cell as compared to the anterior region. Consequentially, the posterior daughter cell inherits more of new BBs as compared to the anterior offspring cell (Nanney, 1975). Thus, while the anterior and posterior offspring appear identical superficially, the anterior cell can be seen as a "mother" and the posterior cell could be a "daughter" if we consider the ratio of old to new structures in the cell cortex.

Cilia

Almost all eukaryotic organisms, except for higher plants, most fungi and slime molds, have highly conserved organelles known as cilia or flagella (Figure 2.5A). To avoid confusion with the completely structurally unrelated prokaryotic flagellum, I will refer to both eukaryotic cilia and flagella as "cilia". The axoneme is MT-based core of cilia and usually consists of 9 doublet MTs arranged into a circle and (in the case of most motile cilia) two central singlet MTs (Figure 2.5B). The diameter of the ciliary axoneme is about 0.25 μm but its length can vary ranging from a few μms (example, *Tetrahymena* cilia) to few millimeters (example *Drosophila* spermatozoid cilia). Cilia perform a number of crucial functions. In protists, cilia are used for collection of food and cell locomotion. In epithelial cells, that coat the human respiratory tract, cilia sweep the mucus along with dust and dead cells up towards the mouth mucociliary clearance). In ependymal cells, cilia sweep the cerebrospinal fluid in the spinal cord and brain ventricles. In the oviduct, cilia sweep ova into the uterine tract. Finally, cilia are essential for sperm motility. Some cells like sperm have a single motile cilium whereas respiratory epithelial cells and unicellular organisms like *Tetrahymena* have many cilia on their surface. Recently 360 conserved proteins have been identified in eukaryotic cilium proteome (Pazour et al., 2005). The actual number of ciliary proteins is likely much higher as additional proteins have been discovered in proteomes of other ciliate species through microarray studies. They consist of uncovered mRNAs upregulated during cilia formation (Pandey et al., 2008) and discovery of genes whose promoters have binding sites for transcription factors controlling ciliogenenesis (Ashique et al., 2009).

Each of the outer doublet MT is made up of complete A and incomplete B tubule with 13 and 11 protofilaments respectively while the central pair, C1 and C2 are made up of 13

protofilaments. C1 and C2 are enclosed in a central sheath of proteins that interact with radial spokes. The axoneme doublet MTs emerge from the A and B tubules of the BB while the C-tubule runs only up to the transition zone. The region that includes the distal end of the transition zone and beginning of the nine outer and central pair is called the proximal segment of the axoneme. After the proximal region is the middle and main region of the axoneme, containing 9+2 MTs. Towards the distal end, the diameter of the cilium is smaller as axoneme in this region contains only A-tubules with terminated B-tubules and the central pair (Snow et al., 2004).

The doublet MTs are held to each other via flexible nexin links that are considered to be part of the dynein regulatory complex (DRC) (Heuser et al., 2009). Radial spokes are projections that are positioned between doublet MTs and the CP. Near the radial spokes is a protein complex known as dynein regulatory complex. DRC along with CP and RS is required for co-ordinating dynein activity, as defects in DRC complex act can suppress paralysis induced by mutations in CP or RS (Huang et al., 1982; Luck et al., 1982). DRC and RS are known to contain the calcium binding proteins, centrin and calmodulin , respectively. Changes in calcium levels in cilia result in altered beating patterns (Dymek and Smith, 2007).

Like all other MTs, axonemal MTs have a distal plus growing end where incorporation of new subunits takes place (Johnson and Rosenbaum, 1992). The ends of axonemal MTs are connected to the ciliary plasma membrane via protein complexes called caps. The cap at the end of CP MTs is a ball-like structure that probably enables the attachment of MTs to the membrane. Doublets have caps that have a shape of filaments that are inserted into the end of the A-tubule of outer doublet and link them to ciliary membrane (Dentler, 1980).

In addition to motile functions, cilia also have sensory functions. In particular, mammalian cells often have solitary cilia called “primary cilia” that lack motility and function as

sensory organelles. Primary cilia lack CP and hence have 9+0 axonemal structure. Most primary cilia lack dynein arms (with exception of nodal cilia) that are imperative for the beating of cilia. Primary cilia are found in sensory cell types where they are critical for sensation, such as in the outer segments of rods and cones for light detection and in olfactory cells for chemoreception. In humans cilia are of crucial importance as their defects lead to a number of diseases such as diabetes, obesity, heart, sensory and skeletal defects, hypertension, neurological and developmental abnormalities (Hearn et al., 2005; Scholey, 2003). Polycystic kidney disease, infertility, laterality defects and hydrocephalus are also some important examples of diseases linked to defective cilia (Afzelius, 2004; Ibanez-Tallon et al., 2003). Unique 9+0 motile cilia, also known as nodal cilia, are found at the embryonic node during early development and contain dynein arms. Nodal cilia generate leftward movement of fluid that surrounds embryonic cells, and the flow generates the left-right symmetry of the embryo. A knockout of Kinesin-II protein in mice leads to block in the formation of nodal cilia, which consequently leads to *situs inversus,* a congenital condition in which position of organs is reversed (Nonaka et al., 1998).

Hedgehog signaling is required for proper development of an embryo. Components of the Hedgehog signaling pathway include various transmembrane proteins, transcription factors and associated proteins which localize to non-motile primary cilia. It is also important for normal development of vertebrates and invertebrates, as a defect in the hedgehog signaling pathway can lead to formation of tumors. Intraflagellar transport (IFT) is required for normal assembly and maintenance of cilia. Defects in IFT proteins leads to improper hedgehog signaling (Houde et al., 2006; Huangfu and Anderson, 2005; Huangfu et al., 2003). In kidneys, cilia may act as mechanosensors and flowmeters to monitor the composition and flow-rate of urine in nephrons. When urine flows, these cilia bend leading to increases of intracellular levels of calcium

(Praetorius and Spring, 2003). Polycystic kidney disease arises from defects in cilia of kidneys (Ong and Wheatley, 2003).

Intraflagellar transport (IFT)

The assembly and maintenance of most cilia takes places by a process known as the intraflagellar transport (IFT). IFT is a bi-directional transport of proteins in which the anterograde and retrograde motilities occur at different rates. IFT was discovered in paralyzed flagella of live *Chlamydomonas* cells (Kozminski et al., 1993b). As the axoneme grows, there is a delivery of tubulin and other ciliary proteins to the plus ends of axonemal MTs at the tips of cilia, where MT assembly takes place (Johnson Rosenbaum 1992). Different types of cargo such as radial spokes and tubulin are carried to the growing tip in association with protein complexes known as IFT particles (Qin et al., 2004). Motor proteins, including kinesin-2, carry the cargo forward to the tip where unloading of the required precursors takes place (Pan et al., 2006). Dynein1b, an isoform of cytoplasmic dynein, carries the recycled products (IFT particles and axoneme components that turnover as well as kinesin-2) back to the base of cilia (Pan and Snell, 2005; Pazour et al., 1999). The homodimeric kinesin is present in ciliated organisms like *Chlamydomonas* and *Tetrahymena* and is known as OSM-3. In *C.elegans* amphid channel cilia, OSM-3 along with kinesin-II, carry out the anterograde IFT along the middle segment of cilia that contains doublet MTs; OSM-3, alone, drives anterograde IFT in the distal region of cilia containing singlet MTs (Snow et al., 2004). The motor proteins work in association with multi-protein complex termed as IFT complex A and B and consist of roughly 20 subunits (Cole, 2003; Cole et al., 1998). The IFT complex A contains IFT144, IFT140, IFT139, IFT122A, IFT122B and IFT43 and is involved in retrograde transport. IFT complex B is made up of IFT172, IFT88, IFT81, IFT80, IFT74/72, IFT52, IFT57/55, IFT45, IFT27, IFT25 and IFT20 (Krock and Perkins, 2008; Scholey, 2003).

DIC microscopy of *Chlamydomonas* flagella showed the presence of IFT particles in a linear array lying between the flagellar membrane and the outer doublet (Kozminski et al., 1993b).

There are two types of kinesin-II motor complexes: heterotrimeric and homodimeric. In *Chlamydomonas*, the heterotrimeric kinesin-II consists of 90 and 85kDa subunits, encoded by FLA10 and FLA8 respectively, along with a 100kDa non-motor subunit termed kinesin-associated protein (KAP) that is encoded by FLA3 (Iomini et al., 2001; Kozminski et al., 1995; Pedersen et al., 2005). Kinesin-II is indispensable in *Chlamydomonas* as cells lacking functional FLA10 fail to assemble flagella (Adams et al., 1982). Studies on temperature sensitive FLA10 mutants revealed that kinesin-II is required for the maintenance of an already assembled flagellum (Kozminski et al., 1995). In mammals, the two subunits are called as KIF3A and KIF3B and their complete knockout in mouse embryos is lethal and embryos develop *situs inversus* (Marszalek et al., 1999; Nonaka et al., 1998; Takeda et al., 1999). . When 2 partially redundant kinesin-II genes were knocked out in *Tetrahymena*, it led to failure in assembly of cilia as well as lack of cytokinesis due to loss of cilia and the resulting ciliary paralysis (Brown et al., 1999b).

The cytoplasmic dynein 1b complex consists of 4 subunits: heavy chain (DYNC2H1), light intermediate chain (DYNC2LI1), intermediate chain (IC/WD) and light chain (LC). *Chlamydomonas* cells, with mutated homologue of DYNC2H1, developed short flagella with accumulated IFT particles, suggesting the importance of dynein in retrograde IFT (Pazour et al., 1999). A similar phenotype was observed in mice with a mutation in the heavy chain subunit and in *Chlamydomonas* with mutations in the light intermediate subunit (Hou et al., 2004). However, in *Tetrahymena*, elimination of dynein heavy chain 2 (DYH2) or dynein light chain (D2LIC) did not cause accumulation of particles or swelling of cilia. Cells lacking these dynein subspecies had

variable length cilia indicating importance of dynein-2 in length of cilia but not playing an essential function in the assembly or maintenance of cilia (Rajagopalan et al., 2009). A possible explanation is that cilia of *Tetrahymena* are shorter as compared to *Chlamydomonas* or *Trypanosoma*, and that the retrograde transport function can be served by a non-motor mechanism such as diffusion. As these non-motor mechanisms may be less efficient, this could lead to minor defects in cilia.

Interestingly, in *Drosophila*, sperm axonemes are assembled in the absence of IFT (Han et al., 2003), even though ciliary assembly in sensory neurons of this organism requires IFT (Sarpal et al., 2003). The sperm tail axonemes are exceptionally long and perhaps a non-IFT dependent mechanism is required for their assembly. It should be pointed out that the centrioles and basal bodies in principle self-assemble from soluble components. Thus, in some cases the entire axoneme could self-assemble without an ordered delivery of components. It should also be noted that once assembled, sperm has a limited life span, while cilia on other cell types survive for a longer period and require IFT for maintenance.

Ciliary motility

Typically, a motile cilium undergoes repeated cycles, consisting of a planar power stroke and a three dimensional recovery stroke. In the motile cilium, dynein motors that are permanently bound to A-tubule of doublets (with certain periodicity), in the presence of ATP, temporarily bind to B-tubule of an adjacent doublet. On hydrolysis of ATP, the globular heads of dynein arms move toward the minus end of the B-tubule, thereby producing a sliding force between the adjacent MT doublets in a cilium. (Brokaw and Kamiya, 1987; Gibbons and Gibbons, 1973, 1976). In an intact cilium, however, the doublet sliding is constrained due to the presence of nexin (dynein regulatory complex) links between doublet MTs and the attachment of the

axoneme to the basal body. These constraints are believed to convert the sliding force between doublet MTs into bending of the axoneme. Dynein on specific doublets (doublets 1 to 4) causes bending of flagellum on one side and dyneins on other doublets bend the cilia in the opposite direction when the cilium changes between the power and recovery stroke (doublets 6 to 9) (Satir, 1989; Satir and Matsuoka, 1989). This switching of dynein activity allows for changes between the power and recovery stroke, required for generation of productive motility.

Besides ATP-dependent dynein interactions, central pair and radial spokes have been shown to regulate ciliary motility, possibly by transmitting regulatory signals to the dynein arms (Kamiya, 2002; Porter and Sale, 2000; Smith and Yang, 2004). *Chlamydomonas* mutants lacking central pair or radial spokes (CP/RS) are paralyzed even though their axonemes undergo MT sliding on reactivation with ATP *in vitro* (Witman et al., 1978), indicating that the central pair and radial spokes are required for bending of cilia in a constrained state *in vivo*. In *Chlamydomonas* mutants lacking CP/RS become motile if combined with suppressor mutation in other loci (Huang et al., 1982). The suppressor were mapped to subunits of inner (Porter et al., 1992) and outer dynein arms(Porter et al., 1994). Some of these suppressor mutations lead to lack of regulatory subunits or certain axonemal dynein subtypes (Porter et al., 1994; Rupp et al., 1996). Also, in these suppressor mutants with defective CP/RS, the intermediate chain IC138 is hyperphosphorylated, which affects the rate of MT sliding (Hendrickson et al., 2004). All these studies described above indicate that CP/RS regulate dynein.

Dynein

Dynein motors convert the chemical energy contained in ATP into the mechanical energy of movement. Dynein transports various cellular cargoes by "walking" along cytoskeletal MTs towards the minus-end of the MT, which is usually oriented towards the cell center. Dyneins

comprise of heavy chains (HC), intermediate chains (IC) and light chains (LC). Dynein HC is the force producing subunit and is a large protein made up of approximately 4500 amino acids and its molecular weight is more than 540,000 Da. Dyneins are of two types: cytoplasmic dynein and axonemal dynein. Although, both the classes of dyneins have a similar structure, they vary in the number of HCs (and motor domains) as axonemal dyneins have either 2 or 3 heads while cytoplasmic dyneins consistently have 2 heads (Piperno, 1990). While cytoplasmic dyneins can processively move along MTs over long distances (Cho et al., 2008), axonemal dyneins are anchored at their non-motor end to the A-tubule in ATP-independent manner while their globular heads associate to the B-tubule transiently only in the presence of ATP (Johnson, 1983). The distinction between the two types of dyneins can get confusing as cilia also have the cytoplasmic dynein DH1B that drives retrograde IFT (Pazour et al., 1999).

Dynein heavy chains

In cilia, outer dynein arm complexes are made up of 2 or 3 heavy chains (HCs) joined together. In *Chlamydomonas*, the inner dynein arm contains 8 HCs, out of which 6 are monomeric and one is dimeric. Each HC contains N-terminal tail, ring formed by 6 AAA domains (in which hydrolysis of ATP takes place) and a coiled coil stalk that extends from the AAA ring (between 4^{th} and 5^{th} AAA). The part that connects AAA ring to the tail is called linker (Figure 2.6A). In ODA, the 3 HCs form globular heads designated as α, β and γ are 14 nm in diameter (Goodenough and Heuser, 1985). The globular head regions are connected to HC stem regions that also contain intermediate chains (ICs) and light chains (LCs). The stem region arises from basal region and is a complex of ICs and LCs. The basal region is required for cargo/adaptor binding in the case of cytoplasmic dynein or is bound to A-tubule in axonemal dyneins.

Cooperation between all the 3 HCs is required for efficient dynein activity, as in *Chlamydomonas,* ATPase activity of β-HC is decreased by association with α-HC and activity of αβ dimer HCs is affected by association with γ-HC. Studies on *Chlamydomonas* mutants lacking α, β or γ DHC, revealed that α HC is farthest from the A-tubule, γ closest to the IDAs and β is between the two (Liu et al., 2008; Sakakibara et al., 1991; Sakakibara et al., 1993) (Figure 2.6B).

Dynein HCs are members of the AAA (ATPases Associated with cellular Activities) family. The HC sequence is conserved and comprises of 2 domains: the (~one-third) N-terminal region that binds axonemal dyneins to A-tubule and (~two-thirds) of the C-terminal globular head region that contains 6 AAA nucleotide-binding domains forming a ring structure. The first four AAA domain consist of 4 nucleotide-triphosphate-binding motifs known as the P-loop (King 2000). The AAA ring of HCs is oriented parallel to the long axis of MT doublet (Ishikawa et al., 2007; Nicastro et al., 2005).Out of the 6 AAA domains, the first AAA domain I (from the N-terminal end) is the main nucleotide binding site (Imamula et al., 2007) and the third AAA domain is also involved in nucleotide binding (Cho et al., 2008; Kon et al., 2004). P-loop 1 is the main site for ATP hydrolysis in the β heavy chain and it is well conserved in all dynein HCs (Asai and Koonce, 2001; Gibbons et al., 1991). Mutational studies in *Drosophila* show that mutation of P-loop3 blocks ATP-mediated release of dynein from MTs, but does not affect ATP binding and hydrolysis at P-loop1(Silvanovich et al., 2003). Although the precise functions of rest of the P-loops is not yet known, it is speculated that they may serve as regulatory binding sites for ATP or related nucleotides such as UTP, TTP, ITP and GTP as seen in related cytoplasmic dyneins (Gibbons et al., 1994; Koonce et al., 1992). A stalk domain that protrudes from between the fourth and fifth AAA globular domain (Roberts et al., 2009) is required for the binding of dynein to the B-tubule track (Carter et al., 2008).

According to recent studies, 25 different DHCs have been reported in *Tetrahymena* (Wilkes et al., 2008). Two of them are cytoplasmic dynein HCs (DHC1 and DHC2) (Pfister et al., 2006), 18 HCs are predicted to be single headed inner dynein subunits (IDA) 2 HCs could be parts of a double headed IDA I1, 3 HCs are predicted to be in outer dynein arm (ODA) α, β and γ. Overall the dynein HCs of *Tetrahymena* can be categorized into 9 subclasses of ODAs/IDAs, out of which 6 classes are conserved across species and 3 of the classes are IDA HCs, that are divergent and may be ciliate-specific. The large number of IDA HCs may be required for producing a range of forces in different types of cilia (oral, posterior locomotory or anterior locomotory).

Intermediate chains

Intermediate chains (ICs) are required to keep the multi-subunit complex of dyneins, containing several HCs and LCs, together and are implicated in the attachment of the non-motor end to the A-tubule (or cargo in the case of cytoplasmic dynein). The IC/LC complex present at the base of ODA is crucial for the assembly, stability and attachment of ODA to outer doublets and may contribute towards regulating its motor activity (King et al., 1991; Mitchell and Kang, 1991). ODAs have 2 ICs, that contain 5 or 6 repeated regions at the C-terminal and belong to the WD-repeat family of proteins. WD repeats are speculated to be involved in protein-protein interactions and the assembly of multi-subunit complexes (Neer et al., 1994). These 2 proteins are known as IC1 and IC2 and are required for assembly of ODA as mutants lacking IC1 or IC2 fail to incorporate ODAs onto axonemal MTs (Mitchell and Kang, 1993). Both proteins directly interact with each other (King et al., 1995). IC1 (IC78/IC80) of *Chlamydomonas* directly interacts with tubulin (King et al., 1991) and may help in docking the ODA onto the A-tubule (King et al., 1995). IC1 seems to play a role in calcium regulation of ODA through calcium

dependent interaction with light chain, LC4 (Sakato et al., 2007).

I1 IDA dynein in *Chlamydomonas* contains IC97, IC138, LC7b and flagellar associated protein-FAP120 (Bower et al., 2009; Ikeda et al., 2009; Wirschell et al., 2009). The C-terminal end of ICs is responsible for binding of HCs (Paschal et al., 1992) and the N-terminal region may confer functional specificity, such as directing ODA and IDA to their specific binding sites on A-tubule (Holzbaur and Vallee, 1994). ICs lie at the base of the dynein molecule and interact with each other as well as MTs.

Light chains

In terms of structure and functions, light chains (LCs) are quite diverse. LCs consist of 3 family of proteins: LC8, TcTex1/TcTex2 and LC7. LC8 is highly conserved across species and surprisingly is also present in rice and *C.elegans* that do not have cilia/flagella (King and Patel-King, 1995). Higher plants lack cilia and thus it was thought that they lack dynein, but lately it was found that rice (*Oryza sativa*) contains four dynein HCs and LC8 (King, 2002). LC8 has been found to be a component of flagellar radial spokes (Yang et al., 2001), myosin V (Espindola et al., 2000) as well as cytoplasmic dynein. LCs fall under two categories: those that bind to base of the ICs and to DHCs. The second category consists of 2 calcium binding proteins, centrins and LC4.

In cytosplasmic dyneins, LC8 and TcTex1 can bind at adjacent sites of ICs at the base of dynein motor complex. Using *in vivo* molecular trap method, Varma et al, showed that induced dimerization of LC8 and TcTex1 rapidly disrupted early endosomal and lysosomal organization as well as Golgi organization but no effect on mitotic progression. This indicates that LCs affect some, but not all, dynein-mediated processes (Varma et al., 2010). Also, in *Drosophila,* dynein LC1 is required for clearing out proteins by the process of autophagy that is required for

autophagic cell death and neurodegeneration (Batlevi et al., 2010). In *Aspergillus nidulans*, LIC is required for association of heavy and light chains as without the light chains, the assembly of dynein core complex is not possible (Zhang et al., 2009).

ODA HCs directly bind to a number of LCs that may play a regulatory role in axonemal beating (Wu and King, 2003). LC3 of *Chlamydomonas* binds to the N-terminal region of α, β or γ DHC; LC5 binds to N-terminal region of α and β DHC (Harrison et al., 2002; King et al., 1996) and LC4, a calcium binding protein, binds to the N terminal region of γ DHC (Sakato and King, 2003). Change in calcium levels in flagella from pCa6 to pCa4 causes a dramatic change in its waveform (Bessen et al., 1980). Interaction of LC4 and γ HC is dependent on calcium levels (Sakato et al., 2007), thus, LC4 acts as a calcium sensor. LC1 in *Chlamydomonas* outer arm is bound to γ HC of motor domain (Benashski et al., 1999).

Axonemal dynein

High salt extraction followed by sucrose density centrifugation of *Tetrahymena* ciliary extracts resulted in separation of 22S and 14S dyneins that correspond to outer and inner dynein arms, respectively (Toyoshima, 1987). Axonemal dyneins form a large protein complex (2 million daltons) containing 9–12 polypeptide chains. The outer and inner dynein arms are structurally different in the number of HCs, ICs and LCs. Also, ODAs basically regulate the sliding rate of MTs and beat frequency of a cilum while IDAs regulate the waveform of a cilium. I will now discuss the functions and properties of both the types of dynein molecules in detail.

Outer dynein arm

ODAs decorate the A-tubule at intervals of 24 nm. As I have mentioned above, ODA is a complex made up of 2 or 3 heavy (α, β or γ), 2-3 intermediate and 6-8 light chains. The structure

of ODA has been conserved during the course of evolution. On axoneme extraction, electron micrographs of ODA reveal 2-3 globular heads, each with projected stalks; their heads are connected together into a bouquet-like arrangement, by a flexible stem that arises from the base. In the actual ODA attached to axoneme, freeze-etch replicas show a single globular head with a single stalk projected and attached to B-tubule of the adjacent doublet and the base (proximal and distal) connected to the A-tubule.

ODAs are attached to the A-tubule via a docking complex (Casey et al., 2003b; Takada and Kamiya, 1994; Takada et al., 2002). The ODA docking complex (DC) is a MT-associated structure that facilitates anchoring of ODA onto flagellar axoneme. DC in *Chlamydomonas* consists of 3 subunits: 2 coiled-coil proteins DC1, DC2 and DC3, that is a calcium binding, calmodulin homologue. Normal assembly of ODA requires DC1 as well as DC2, but not DC3 as DC3 null mutants are able to assemble ODA partially (Casey et al., 2003a). DC1 and DC2 are directly associated with each other and do not require DC3 for assembly or localization of ODA. As DC3 binds to calcium, it seems to have additional regulatory functions such as forming disulphide bonds with other flagellar componenets (Casey et al., 2003b). How DC1, DC2 and DC3 specifically bind together to doublet MTs is still being investigated.

Inner dynein arm

In *Chlamydomonas*, IDAs are present at an interval of 96 nm on A-tubule (Figure 2.7). They are categorized into single-headed and double-headed IDAs. The arrangement of IDAs is quite complex and its composition across the length of flagellum is variable (Piperno et al., 1990). The double headed dynein, I1/f, contains 1α and 1β DHCs, 3 ICs and 5 LCs. The single headed chains, namely, a, b, c, d, e and g, are more complex in their composition and arrangement as they are localized in proximal as well as distal regions of flagella (Kagami et al.,

1990; Kato et al., 1993; Piperno, 1990). Several studies have shown that IDAs are responsible for generating and maintaining wave-form (Brokaw and Kamiya, 1987; Yagi et al., 2005).

Initially, 6 monomeric species were discovered using high salt extraction, however recently, 3 more minor species of this class have been identified (Yagi et al., 2009). They are located at the base of the flagellum and are much less abundant as compared to the other 6 species. All 6 species of dyneins have actin subunit that is required for assembly and docking of motor complex (Kato-Minoura et al., 1997). Species b, e and g contain centrin that is a calcium protein similar to calmodulin (Kagami and Kamiya 1992). Rest of the monomeric IDAs contain a LC known as p28 that binds to the stem region of HC and actin subunit (Hayashi et al., 2002). p28 is important for assembly and maintenance of IDA (LeDizet and Piperno, 1995).

I1, also known as IDA species f, is different from the rest of the single headed IDAs as it is the only species of IDA to exhibit the ability of translocating MTs *in vitro* in an assay where the motor tail is bound to glass (Smith and Sale, 1991). Also, dynein I1/f is known to play a crucial regulatory role in axonemes (Kotani et al., 2007; Wirschell et al., 2007) as it is a target for regulatory signals involved in flagellar motility (Porter and Sale, 2000; Wirschell et al., 2007). I1/f defective flagella show abnormal waveform and phototaxis (King and Dutcher, 1997; Okita et al., 2005). Dephosphorylation of ICs in I1 plays a role in ciliary reversal in *Tetrahymena thermophila* (Deckman and Pennock, 2004; Hennessey et al., 2002). Phosphorylation of IC138 is central to the regulatory role of I1 in MT sliding as phosphorylation results in inhibition of MT sliding causing cessation of flagellar motility (Pasquale and Goodenough, 1987). Failure in I1 assembly does not affect the assembly of other dynein isoforms (Kamiya et al., 1991; Mastronarde et al., 1992; Piperno, 1990). IDA-f has a very slow speed of translocation of MTs as compared to other IDA subspecies. Kotani and colleagues showed that isolated I1 slowed down

the rate of translocation of other IDAs by providing resistance (Kotani et al., 2007). Flagellar beat is regulated by mixture of IDAs and ODAs with different speeds. Faster moving ODAs and IDAs may be decelerated by action of slow moving dyneins such as I1 as well as by torque-generating actions of some IDAs.

ODA and IDA mutants

Two different methods have been employed to study the importance of various dyneins. First method is mutational studies in *Chlamydomonas* and second approach is using *in vitro* motility assays that contain various fractions of ODA and MTs. *Chlamydomonas* mutants missing the entire β HCs lack ODAs (Kamiya, 1988) indicating that β-HC is essential for ODA assembly. *Chlamydomonas* mutants lacking ODAs move 30-50% of WT speed but have a normal waveform, indicating that ODAs primarily regulate the beat frequency (Kamiya and Okamoto, 1985). Mutants lacking α-DHC, *oda-11*, swim slower than wild-type but faster than cells lacking entire ODA such as oda2 and oda4 (Liu et al., 2008).

The N-terminal region of β HC is important for the assembly of ODA complex as *oda4-s7* mutants that express only the N-terminal one-third maintain ODAs on their axonemes. However, the assembled ODA complex is not normal as one of the three globular heads is not present and the motility rate of these mutants is significantly lower than that in wild-type cells (Sakakibara et al., 1993). Out of the 3 DHCs, βDHC produces a bulk of the force generated during power stroke based on their *in vitro* ATPase activities (Pfister et al., 1982; Pfister and Witman, 1984).

Mutant flagella missing some of the IDAs beat at normal frequency, but with reduced shear amplitude and bend angle (Brokaw, 1994). Thus, IDAs primarily determine the waveform. *Tetrahymena* mutants with disrupted IDA HC6 exhibit slow swimming, defective waveform, inability to produce ciliary reversals in response to depolarizing stimuli, but a normal beat

frequency (Hennessey et al., 2002). Disruption of IDA HC7 in *Tetrahymena* resulted in altered waveform of cilia and reduced swimming velocity (Wood et al., 2007). Mutations in the IDA HC8, HC9 and HC12 of *Tetrahymena* resulted in slower swimming velocity and beat frequency as compared to wild-type cells (Liu et al., 2005).

Mutants lacking the entire outer dynein (*oda1*) or mutants lacking inner dynein subspecies f (ida1) or mutant lacking inner dynein subspecies a, c and d – all swim slowly (Kamiya et al., 1991). IDA mutants show reduction in shear amplitude and sliding velocity whereas ODA mutants mainly show reduction in beat frequency and sliding velocity, as the IDAs are required responsible for shear amplitude and ODAs are responsible for beat frequency (Brokaw and Kamiya, 1987). Combinations of double mutants of *oda1*, *ida1* are paralyzed (Kamimura and Kamiya, 1989; Kamiya et al., 1991). These results indicate that although functions of some DHCs are redundant, normal axonemal beating requires distinct activity contributed by each of the dynein arm species and subspecies. Some of the *Chlamydomonas* axonemal mutants are enlisted in Table 2.1.

One of the conditional mutants in *Tetrahymena* is *oad1* (outer arm deficient) mutant that grows and swims normally at 28C, but becomes non-motile at restrictive temperature of 38C, due to lack of ODA assembly. One of the possibilities for lack of ODA at higher temperature is that the *oad1* mutation may be affecting the binding sites of ODA (Attwell et al., 1992). I have used this mutant in my research (Chapter 3) to study how tubulin glutamylation affects IDAs in the absence of ODAs.

The mechanism of microtubule sliding in beating cilia

Until now it has been difficult to explain the exact correlation between structural and chemical events of the cross-bridge formation required for dynein force generation. For many

years, mechanisms for generating dynein force that would result in directional movement along MTs, as well the mechanisms involved in generation of spatial and temporal activation of dyneins to produce a ciliary bend, have been the subject of intense study.

The production of complex waveforms requires a sophisticated control of dynein activity as at any given moment only a subset of doublet MTs are active. One of the hypotheses to support this model is that there is a switch of active and inactive forms of dynein on particular subsets of doublet MTs to produce ciliary bends (Wargo et al., 2004). During the power stroke dynein molecules on particular set of doublets would be activated to bend the cilium in one direction. During the recovery stroke the next set of dynein molecules on rest of the doublets would be activated to bend the flagellum in the opposite direction (Lindemann and Lesich, 2010). Consequently a subset of dynein molecules are activated and inactivated in a coordinated fashion so that the cilia can move back and forth. What causes the regulation of dynein force production is not yet well understood.

The mechanochemical cycle of dynein

While performing its function, dynein alternates between the two mechanical states of pre-power and post-power stroke. It is the power stroke during which dynein produces force and movement. In axonemal dyneins, as seen in *Chlamydomonas*, during the power stroke, the AAA motor domain is observed to move by 8 nm towards the plus end of MT (Figure 2.8). The movement of motor domain is induced by changes in the linker (see below) that increases the distance between N-terminal tail that is bound to the A-tubule and the motor domain (so that the motor domain can reach farther). As the motor domain is connected to the B-tubule via microtubule-binding domain, the movement of motor domain drags the B-tubule towards the plus end by 8 nm via the stalk. All the while, the length of the stalk remains constant.Thus, dynein acts

as an ATP-dependent winch (Movassagh et al., 2010). The power stroke occurs after phosphate release and not after nucleotide hydrolysis or ADP release. After the power stroke the stalk (see below) merely acts as a grappling hook onto the B-tubule and is bound with high affinity during the power stroke. Following the power stroke there are structural changes in the motor domain to the pre-power stroke state. This causes the binding of stalk to change from high affinity to low affinity and subsequently dissociation of stalk from B-tubule. The linker also resumes back to its pre-power stroke state (now the distance between N-terminal tail and motor domain decreases). The swing of linker between pre-power stroke and post-power stroke produces the force for movement of dynein (Movassagh et al., 2010).

The microtubule binding stalk of dynein

For a moment lets divert to cytoplasmic dynein studies as the basic mechanism of all types of dynein is probably similar. The cytoplasmic dynein has a stalk that is composed of an elongated coiled-coil domain known as the shaft and a MT-binding domain (MTBD) at the tip (Gee et al., 1997). The stalk is 10-15 nm long and is located between the AAA4 and AAA5 modules of the globular head domain and consists of α and β helices. The stalk plays an important role in the function of dynein as it has an inherent ability 1) to change the affinity with which it can bind to MTs 2) to transfer the conformational changes from MTBD to catalytic sites in motor domain 3) to undergo rapid cyclical conformational changes.

Studies on the rat cytoplasmic dynein show that the stalk is α-helical and is shaped as an anti-parallel coiled coil (Hook et al., 2009). The movement of α and β helices, relative to each other, causes a shift in the confirmation of coiled-coil helices. This conformational change in the stalk allows for change in confirmation of MTBD for low binding and high binding affinity to MTs. These cyclical changes in the stalk region act as a communication between the catalytic

domains in AAA1 and AAA3 and tip of the stalk (Gibbons et al., 2005; Kon et al., 2009). One of the speculations is that dynein is attached to MTs such that the stalk is tilted, pointing towards the MT minus end.

Dynein linker

Linker is the region that connects AAA ring to the N-terminal tail of dynein (Burgess et al., 2003) (Figure 2.6A). Linker has a crucial role in motor activity as truncations in even small regions of N-terminal of linker region abolished motor activity (Reck-Peterson et al., 2006). Unlike in myosin and kinesin in which its lever arm movement produces motor motility, in dynein, conformational changes in N-terminal linker are proposed to drive the movement of dynein motor. To investigate this mechanism, Robert and colleagues, compared the position of linker domain in ATP bound dynein motor domain state (primed) and in no-nucleotide bound dynein motor domain state (unprimed). Studies involving recombinant cytoplasmic dynein from *Dictyostelium* revealed that, in the ADP-bound state, the linker runs across the head region from AAA1, ending near the base of stalk, near AAA4 and AAA5. In presence of ATP, during power stroke, there is a conformational change in the AAA+ ring which results in swinging of the linker by ~16-18 nm, close to AAA2 position (Burgess et al., 2003; Roberts et al., 2009). This kinetic energy of the linker is transferred to the stalk and moves to the tubulin subunit 8 nm ahead and pulls the cytoplasmic dynein forward (Carter et al., 2008; Ueno et al., 2008). Studies on axonemal dynein suggest that conformational changes in the linker induces shift of AAA+ ring by 8 nm towards the distal or plus end (Figure 2.8). This in turn could swing the N-terminal tail region (that is fixed on A-tubule) to propel the dynein towards minus end and drag the B-tubule towards plus end (Movassagh et al., 2010).

Although several independent studies confirm the sliding MT mechanism of ciliary motion, we yet exactly do not understand as to how the sliding of MTs is regulated by dynein activity.

Coordination of dynein arms during beating of cilia

Ciliary beat is comprised of several dynein cross-bridges that are initiated between adjacent outer doublets. As the flagella undergoes power-stroke and recovery stroke, each dynein molecule undergoes cycles of attachment to the MT, movement of MT and detachment of the dynein from the MT. Sugino and Naito suggested metachronal activation of dynein in which dynein beat sequentially rather than simultaneously to produce a ciliary beat (Sugino and Naitoh, 1982). There are ~200 dyneins on 1μm stretch of doublet MTs. Thus, there are 400 to 2000 dyneins on a flagella 2 μm to 10 μm long. ODAs slide at 200 μm /s while IDAs move at 5 μm/s (Seetharam and Satir, 2008). Kamiya suggested a model of cyclical bend propagation, in this model, dyneins in certain length of MTs associate with each other and cause sliding of adjacent doublets. When cilia bend, there is dissociation of dyneins at the base of the cilia and this dissociation spreads along the length of cilia. After complete dissociation, they re-associate from the base towards the tip (Aoyama and Kamiya, 2005). External stimulus can lead to forced bending, as seen in sea urchin sperm flagella, which in turn, leads to sliding events in doublet MTs (Hayashi and Shingyoji, 2008) as well as flagellar beating (Ishikawa et al., 2007). This suggests the importance of curvature of cilia in dynein engagement.

Recent studies by Movassagh show that, in nucleotide bound states, not all dyneins are seen in the same confirmation. They exist in clusters of two forms: apo state (absence of nucleotide) (80% found in this state) and ADP.Pi state (20% found in this state). Presence of the mixed clusters of dynein states on each MT doublet can provide a possible explanation for the

bending mechanism of cilia. Changes of all ODA from apo to nucleotide bound state would result in sliding of doublet MTs. If only some portion of ODA are in one state and the rest of the dyneins are in the other state, this would lead to formation of non-uniform forces, leading to bending of cilium (Movassagh et al., 2010).

Regulation of microtubule sliding by Inner dynein arms

Mutants lacking inner arm subspecies I1 and I2 cannot swim at all (Kamimura and Kamiya, 1989). Thus, IDAs seem to have a major contribution in the beating of flagella. Important information of properties of IDAs in regulating flagellar beat has been gained through *in vitro* motility assays. All IDA subspecies of *Chlamydomonas,* except subspecies I1/ f (0.1μm/s), show maximal MT translocation *in vitro* (2-11μm/s)and 5 out of 8 IDA subspecies cause MT rotation during translocation (Kagami et al., 1990). Significance of MT rotation by IDAs is not understood yet. However, it is speculated that torque generated by IDAs may modulate the position of adjacent outer doublets and thus, change the distance between ODAs and adjacent MTs (Kikushima and Kamiya, 2008). This may enable efficient force-production of IDAs during ciliary beat.

I1/f IDA is the only 2-headed IDA subspecies in *Chlamydomonas* and has a role distinct from the other subspecies. I1/f contains 3 ICs (IC97, IC138 and IC140) that are absent in other subspecies. IC138 undergoes phosphorylation by casein kinase (Yang and Sale, 2000). A model proposed by Wirschell suggests that signals emitted from CP are transmitted through RS. These signals affect the phosphorylation state of IC138 of particular outer doublets such as 5, 6 and 7 for example. This asymmetrical phosphorylation of outer doublets may cause localized inhibition of MT sliding (Dymek and Smith, 2007; Wargo et al., 2004; Wirschell et al., 2007). One of the

signals originating from CP could be calcium (Dymek et al., 2006; Wargo et al., 2005) or activation of axonemal kinases by RS (Smith and Yang, 2004).

Regulation of microtubule sliding by Central pair (CP) and Radial Spoke Complex (RS)

CP/RS are not absolutely required for axonemal beating (Huang et al., 1982), as eel sperm flagella and a male gamete of parasitic protozoan can beat and do not possess these structures (Gibbons et al., 1985; Prensier et al., 1980). However, several studies on *Chlamydomonas* flagella demonstrate importance of CP in regulation of flagellar beat. CP is located at 90 degrees angle to the major plane of beat (Afzelius, 1961; Gibbons, 1961). Early studies have shown that CP rotates as it extrudes from disintegrating axoneme (Kamiya, 1982). Also, its orientation is apparently correlated to the doublet that slides apart in disintegrating *Chlamydomonas* flagella (Wargo and Smith, 2003). Recent TEM studies on rapidly fixed *Chlamydomonas* flagella have confirmed these observations (Mitchell, 2003). There is another research that provides proof for helical structure of CP and suggests that its helical nature may cause it to rotate as the flagella beats (Mitchell and Nakatsugawa, 2004). Mutants lacking RS show severely reduced MT sliding rates, thus indicating regulation of dynein-driven activity by RS (Smith and Sale, 1992). In sea urchin sperm flagella, CP/RS is essential for calcium dependent regulation of dynein activity (Nakano et al., 2003). In *Chlamydomonas,* hydin is a CP-protein that is required for flagellar motility is possibly a part of CP/RS pathway for dynein activity regulation (Lechtreck and Witman, 2007). Intact RS complex is required for maintenance of rhythm of oscillatory beating and helical trajectory of flagella in *Chlamydomonas* (Wei et al., 2010).

Regulation of microtubule sliding by PTMs

We suspect the presence of other external factors that may regulate waveform and direction of a cilium. There may be several regulatory constraints along the length of a cilium

that impart a differential force and thus, a waveform to the cilium. Several studies have demonstrated the involvement of PTMs in regulating ciliary motility. Polyglycylation of tubulin is important in ciliary motility as anti-glycylation antibodies inhibited the reactivated motility in sea urchin spermatozoa (Bre et al., 1996). Similarly, anti-glutamylation antibodies in human epithelial cells caused a total cessation of ciliary beat (Million et al., 1999). Overexpression of polyglutamylase, TTLL6Ap in *Tetrahymena* resulted in paralysis of cell motility (Janke et al., 2005). We believe that differential distribution of polyglutamylation along the axonemal MTs would provide a simple means for regulating both the direction of the ciliary beat and the waveform of a cilium. In Chapter 3, I have discussed the significance of glutamylation in regulation of inner dynein arm-driven MT sliding.

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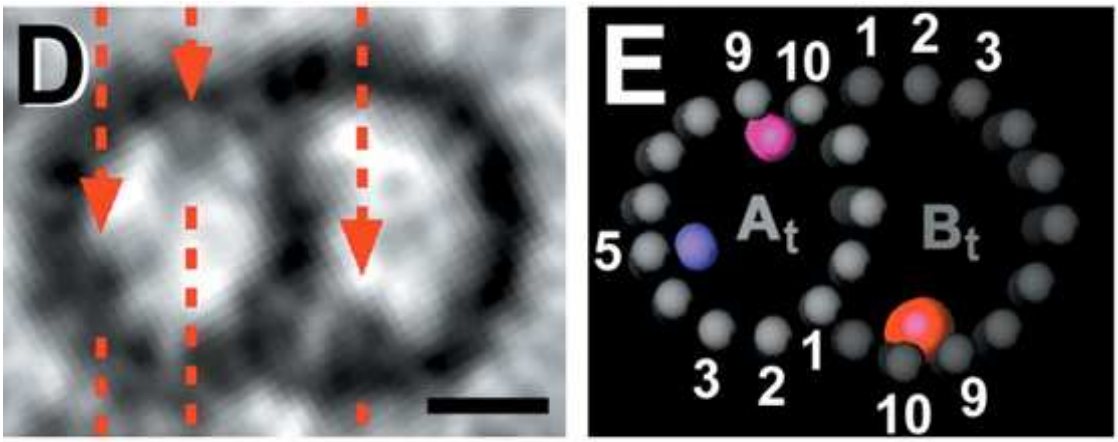

Adapted from "The molecular architecture of axonemes revealed by cryoelectron tomography" by Nicastro D, Schwartz C, Pierson J, Gaudette R, Porter ME, McIntosh JR, Science, 2006 Aug 18;313(5789):944-8.

Figure 2.1: Structure of microtubules

Microtubules are generally made up of 13 protofilaments. Cilia are made up of 9 outer doublets and central pair. Each of the outer doublets consists of A-tubule and B-tubule. The A-tubule of contains 13 protofilaments, while the B-tubule is incomplete tubules containing 11 protofilaments. The arrangement of the tubules is such that the B-tubule is fused to the side of A-tubule.

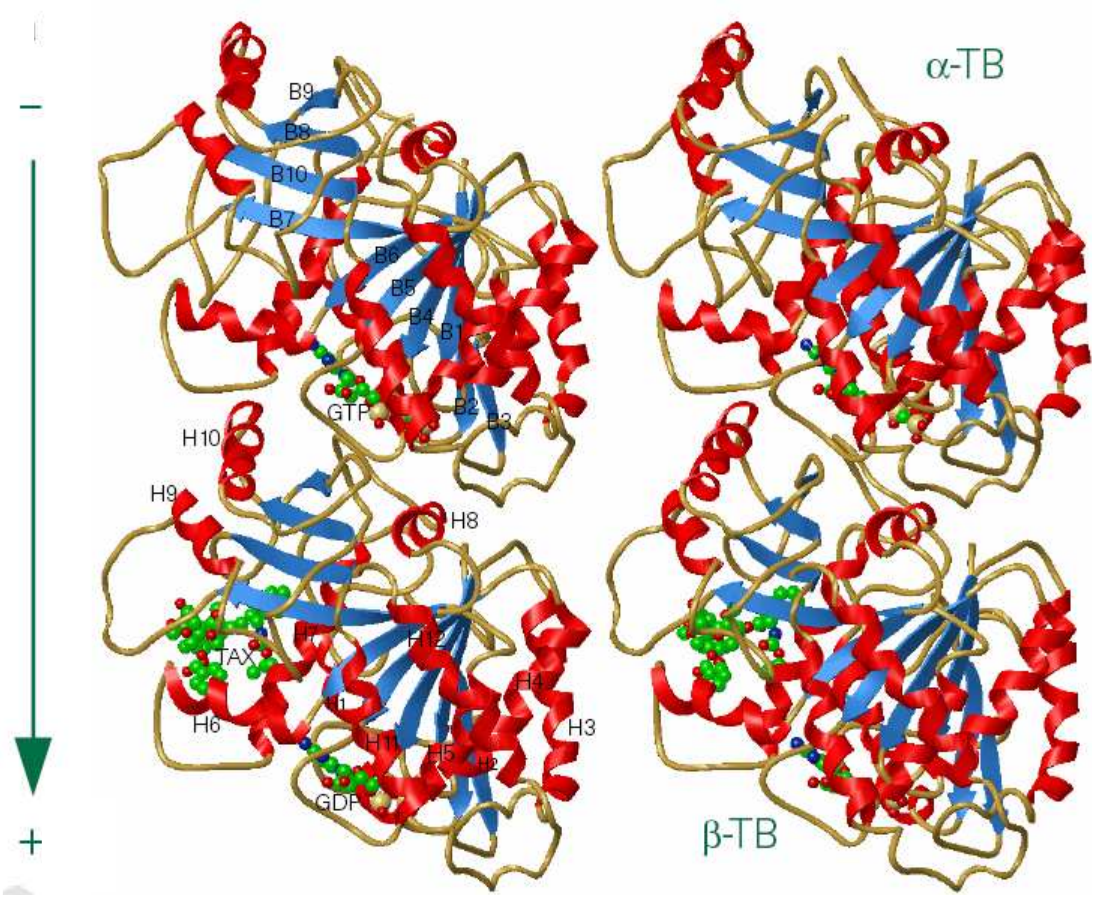

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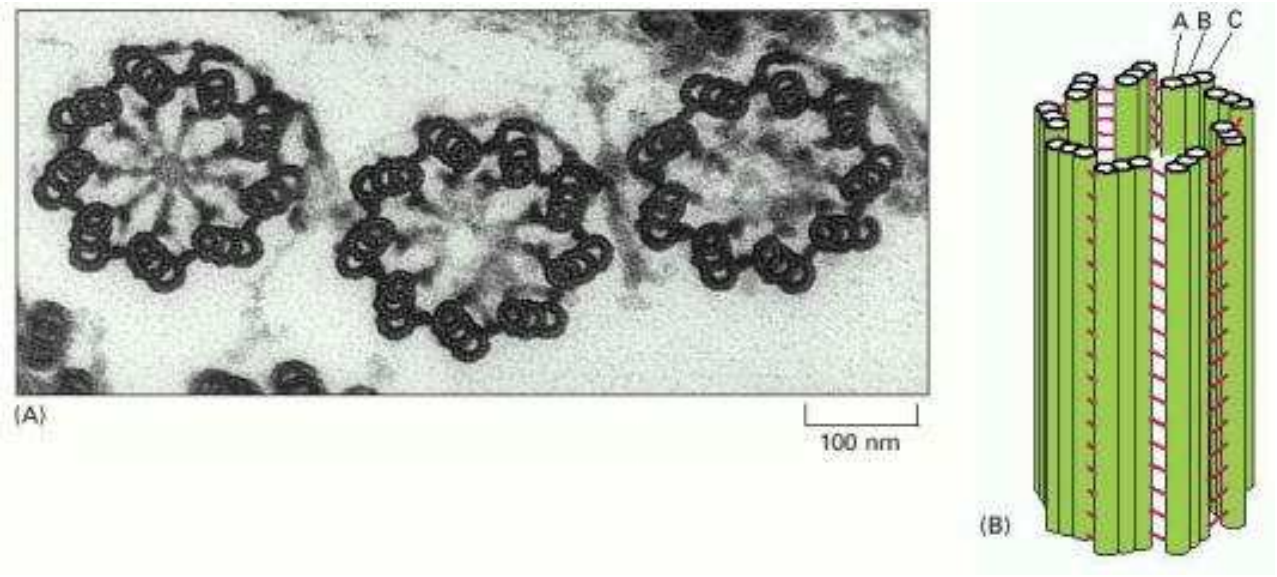
Figure 2.2: Ribbon diagram of tubulin dimer showing α-tubulin with bound GTP



Adapted from Molecular Biology of Cell by Alberts, 2002

Figure 2.3: The cartwheel structure of triple microtubules

Structure of basal bodies is same as that in centrioles. They contain nine groups of fused triplet microtubules arranged in a cartwheel.

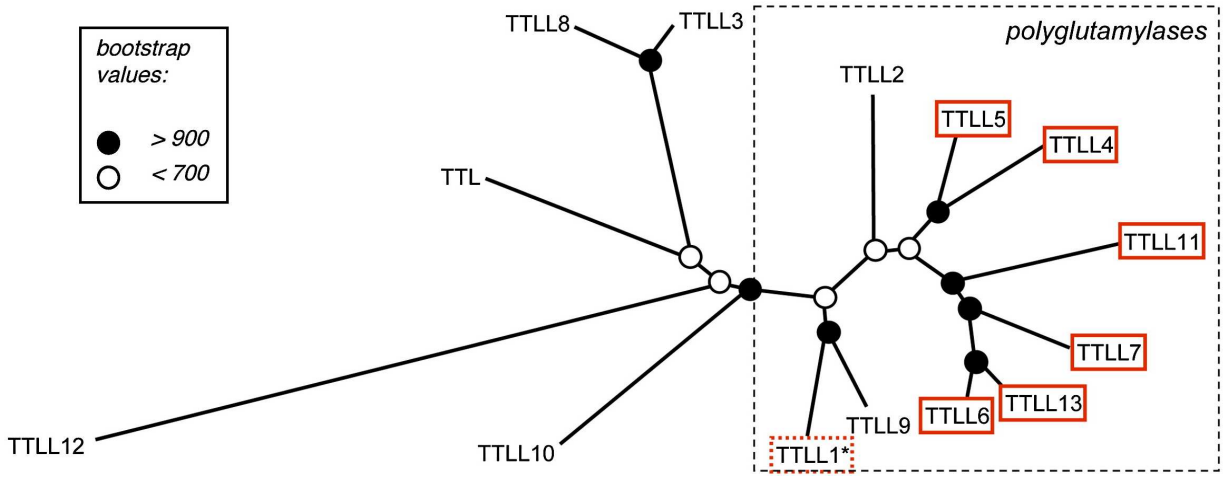


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Figure 2.4: Phylogenetic tree of murine TTLL group of proteins

There are 13 TTLL proteins named TTLL1 through TTLL13. TTLL1, 4, 5, 6, 7, 9, 11 and 13 are E-ligases. TTLL3, 8 and 10 are glycylases. Function of TTLL12 is unknown.

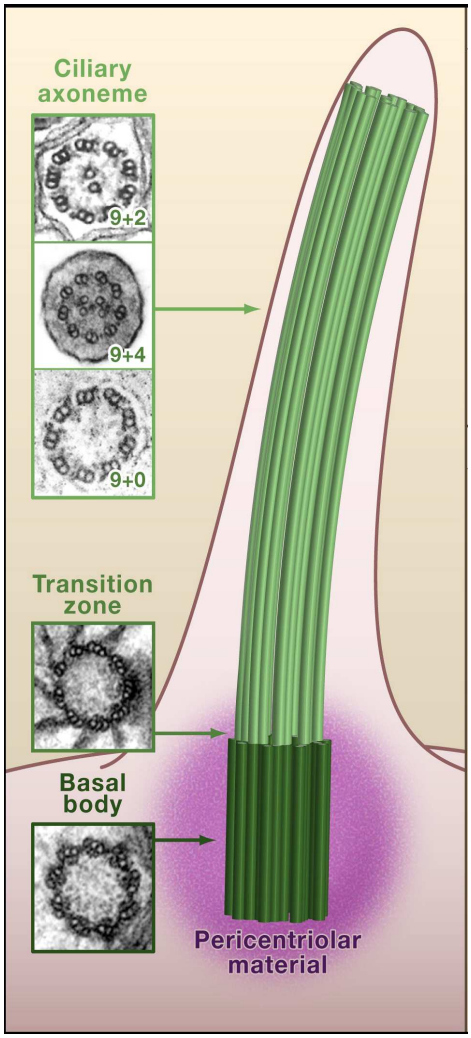

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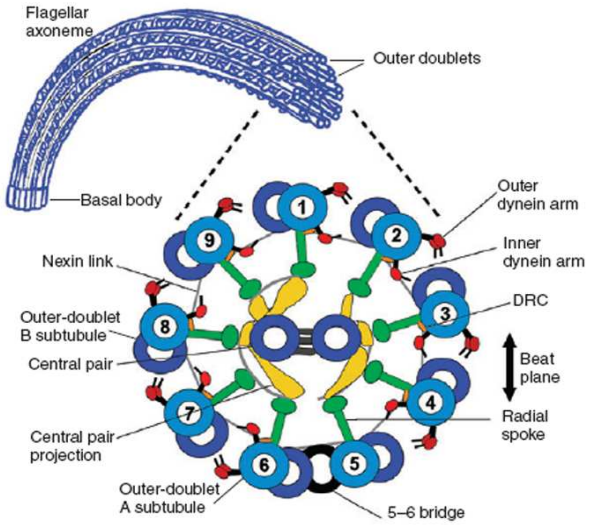
Figure 2.5: A) Longitudinal and cross section of a cilium

Cilium emerges from basal body that is surrounded by pericentriolar material. Basal body is made up of nine triplets (A+B+C) of MTs, which continues upto the transition zone, above which, the C tubule discontinues. Rest of the ciliary axoneme consists of doublet MTs (A+B).

Depending on type of cilium, the axoneme consists of no central pair (9+0) as seen in sensory cilia or with central pair (9+2) as seen in motile cilia or 2 pair of central pairs (9+4) as seen in notochordal plate of rabbit embryo.

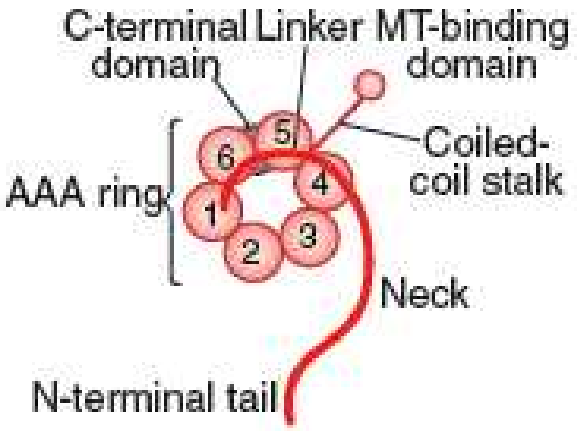
B)



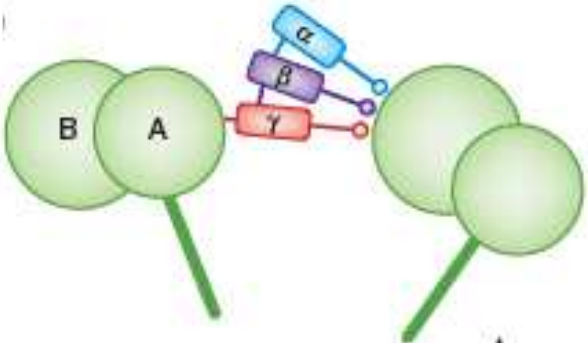
Adapted from "Flagellar and ciliary beating: the proven and the possible" by Lindemann CB and Lesich KA, 2010, JCS, Feb 15;123(Pt 4):519-28.

Figure 2.5B: Axoneme consists of 9 outer doublets that are numbered in a particular order and central pair of doublets that are surrounded by central pair projections. Doublets 5 and 6 are permanently linked to each other. Nexin links connect the outer doublets. A-tubules contain outer and inner dynein arms along with dynein regulatory complex near the inner dynein arms.

A)



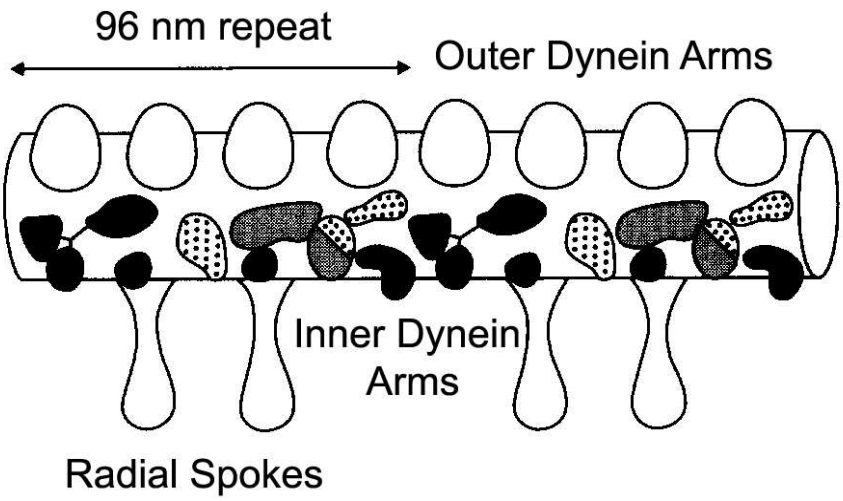
B)



Adapted from "Axonemal dyneins winch the cilium"by Stephen King, Nature Structural and Molecular Biology, Vol 17, June 2010

Figure 2.6: Organization of dynein heavy chain subdomains

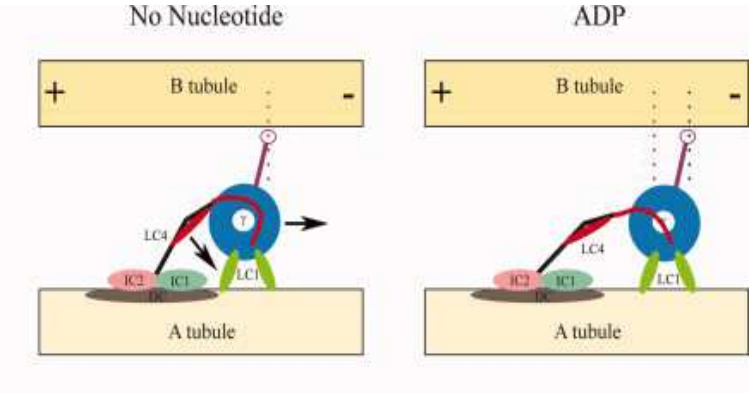
A) Six AAA domains make up a ring-like structure. Coiled-coil stalk is arises between AAA4 and AAA5 and is connected to microtubule-binding domain. Linker arises from AAA1 and is connected to N-terminal tail region via neck. B) Diagram of cross-section through axonemal doublets that shows the orientation of three heavy chains, a, b and g within *Chlamydomonas* outer dynein arm.



Adapted from "Axonemal dyneins: assembly, organization, and regulation" by Porter M.E. Curr Opin Cell Biol. 1996 Feb;8(1):10-7.

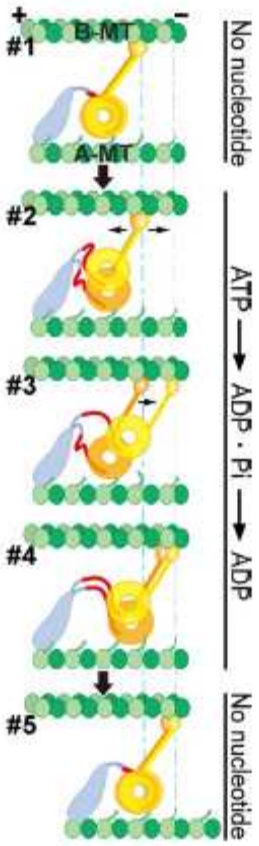
Figure 2.7: Diagrammatic representation of the arrangement of outer and inner dynein arms on axonemal doublets

Outer dynein arms are placed at regular intervals of 96 nm, while the inner dynein arms are found at intervals of 24 nm on the A-tubule. The inner dynein arms consist of single-headed and double-headed dyneins (I1).



Both copies of LC1 are bound simultaneously to the A-tubule and act as a loose tether. Thus, during a cycle of ATP binding and hydrolysis, the Y HC motor unit moves with respect to the A-tubule. The N-terminal domain alters its orientation during this process.

Adapted from "Axonemal dyneins winch the cilium"by Stephen King, Nature Structural and Molecular Biology, Vol 17, June 2010



Adapted from "Dynein pulls microtubules without rotating its stalk" by Ueno H, Yasunaga T, Shingyoji C, Hirose K, 2008 Proc Natl Acad Sci U S A. 2008 December 16; 105(50): 19702–19707

Figure 2.8: Models explaining movement of dynein on MTs

Table 2.1: *Chlamydomonas* mutants of axonemal components

Mutant strains	**Missing structures**	**Proteins encoded by WT gene**
oda1	Outer dynein arm, ODA-DC	DC2
oda2/pf28	Outer dynein arm	γ HC
oda3	Outer dynein arm, ODA-DC	DC1
oda4/s7	β HC motor domain	β HC
oda5	Outer dynein arm	Oda5
oda6	Outer dynein arm	IC2
oda6-r88	LC2, LC6, LC9	IC2
oda7	Outer dynein arm,	ODA7
oda8	Outer dynein arm,	?
oda9	Outer dynein arm,	IC1
oda10	Outer dynein arm,	?
oda12	Outer dynein arm,	LC2+LC10
oda14	Outer dynein arm (partial)	DC3
oda15	Outer dynein arm (partial)	LC7
oda16	Outer dynein arm (partial)	ODA16
Ida1/pf9	Inner arm I1	α HC
ida4	Inner arms a,c,d	pf28
pf14	Radial spokes	RSP3
pf18	Central pair	?

Source: Kamiya R, 2007, International Review of Cytology, Vol. 219

CHAPTER 3

TUBULIN GLUTAMYLATION REGULATES CILIARY MOTILITY BY ALTERING INNER DYENIN ACTIVITY

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[1]Suryavanshi S, Eddé B, Fox LA, Guerrero S, Hard R, Hennessey T, Kabi A, Malison D, Pennock D, Sale WS, Wloga D, Gaertig J. This work has been published in Curr Biol. 2010 Mar 9;20(5):435-40.

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Abstract

How microtubule-associated motor proteins are regulated is not well understood. A potential mechanism for spatial regulation of motor proteins is provided by posttranslational modifications of tubulin subunits that form patterns on microtubules. Glutamylation is a conserved tubulin modification (Edde, Rossier et al. 1990) that is enriched in axonemes. The enzymes responsible for this posttranslational modification, glutamic acid ligases (E-ligases), belong to a family of proteins with a tubulin tyrosine ligase (TTL) homology domain (TTL-like or TTLL proteins) (Janke, Rogowski et al. 2005). We show that in cilia of *Tetrahymena*, TTLL6 E-ligases generate glutamylation mainly on the B-tubule of outer doublet microtubules, the site of force production by ciliary dynein. Deletion of two TTLL6 paralogs caused severe deficiency in ciliary motility associated with abnormal waveform and reduced beat frequency. In isolated axonemes with a normal dynein arm composition, TTLL6 deficiency did not affect the rate of ATP-induced doublet microtubule sliding. Unexpectedly, the same TTLL6 deficiency increased the velocity of microtubule sliding in axonemes that also lack outer dynein arms, in which forces are generated by inner dynein arms. We conclude that tubulin glutamylation on the B-tubule inhibits the net force imposed on sliding doublet microtubules by inner dynein arms.

Materials and Methods

Germ Line Targeting

To prepare targeting plasmids, we amplified fragments of macronuclear DNA of *Tetrahymena thermophila* for each locus and subcloned them on either side of a selectable drug resistance cassette (Table 3.1). The gene targeting plasmids were made based on the macronuclear genome sequence of *Tetrahymena thermophila*, available at the *Tetrahymena*

Genome Database (Eisen, Coyne et al. 2006). Two fragments of macronuclear DNA were amplified for each locus. The fragments were designed to flank the genomic sequence that encodes the catalytic domain of TTLL6 (Table S1). Fragments were subcloned on either side of a selectable drug resistance cassette (*neo3* (Shang, Song et al. 2002) or *mtt1-rpl29* (Bowen and Gorovsky, personal communication). The cassette was embedded in a reverse transcriptional orientation. The targeting fragments were separated from the rest of the plasmid using restriction enzymes and transformed biolistically into mating CU428 and B2086 strains (Cassidy-Hanley, Bowen et al. 1997) at 3, 3.5 and 4 hr after mixing. After shooting, cells were incubated in SPP and 2.5 μg/ml $CdCl_2$ for 3 hr, and transformants were selected with appropriate drugs (*neo3*, paramomycin-100 μg/ml with $CdCl_2$ 2.5 μg/ml; *mtt1-rpl29*, cycloheximide 15 μg/ml with $CdCl_2$ 2.5 μg/ml). Putative germline transformants were identified by co-resistance to 15 μg/ml 6-methylpurine. Knockout heterokaryons were generated by allowing the disrupted alleles to assort from the macronucleus via phenotypic assortment and by making the micronucleus fully homozygous using a star cross (Hai, Gaertig et al. 2000). Double knockout strains were made by multiple rounds of standard crosses. Total homozygotes were created by crossing appropriate heterokaryons and isolating progeny. The absence of the targeted genomic regions in the homozygote was confirmed by PCR of genomic DNA with primers designed to amplify junctions between selectable cassettes and the nontargeted gene specific regions (Table 3.2). For rescue, vegetatively growing 6AF-KO cells were biolistically transformed with a fragment encoding MTT1-GFP-TTLL6A targeted to BTU1 (Shang, Song et al. 2002). A strain lacking *TTLL6A* and *TTLL6F* and homozygous for the *oad1-1* mutation (Attwell, Bricker et al. 1992) was constructed by standard crosses.

Immunofluorescence and electron microscopy

The cells were stained by immunofluorescence as described in (Wloga, Camba et al. 2006) with the following primary antibodies: 12G10 (1:50), SG polyclonal anti-total *Tetrahymena* tubulin (1:600), and polyE (1:100). *Chlamydomonas reinhardtii* cells (strain 137c) were fixed with cold methanol, and labeled with a mixture of 12G10 (1:10) (Janke, Rogowski et al. 2005), and polyE antibodies (1:100) at 4^{o}C overnight, followed by goat-anti-mouse-FITC and goat-anti-rabbit-Cy3 antibodies (Zymed, 1:200). Cells were viewed on a Leica TCS SP confocal microscope.

For standard transmission electron microscopy (TEM), cells were fixed as described (Sharma, Bryant et al. 2007).For immunogold TEM, cells carrying an MTTT1-GFP-TTLL6A transgene were grown in 5 ml of SPP medium (2 x 10^5 cells/ml) and induced with 2.5 μg/ml $CdCl_2$ for 3 hrs, spun down briefly and fixed in an Eppendorf tube and stained as described above for immunofluorescence with anti-GFP antibodies (Abcam, 1:10,000) overnight followed by anti-rabbit IgG-10 nm gold (1:60) (Amersham Pharmacia) for 2 hr at room temperature. Cells were concentrated by centrifugation to 100 μl and post-fixed with 1 ml of 2% glutaraldehyde (in 0.1M sodium cacodylate buffer, pH7.2) on ice for 1h. Cells were washed 5 times in cold sodium cacodylate buffer (10 min each on ice) and fixed with 1 ml of 1% osmium tetroxide for 1 hour on ice. The pellet was washed 5-18 times in water, followed by dehydration in ethanol concentration/water series and embedding in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and analyzed on JEOL 1200 EX transmission electron microscope. For immunogold whole mount TEM, cilia were purified from 50 ml of cells (3 X 10^5 cells/ml) and cilia were demembranated in 500 μl of 1% NP40 in axoneme buffer. After 5 min on ice, axonemes were collected at 23,000 g (15 min, 4^{o}C), washed with 1 ml of axonemal buffer and,

centrifuged and suspended in the axoneme buffer at 10 mg tight pellet/ml of. To initiate sliding of axonemal microtubules, 0.5 µl of 2 mM ATP was added to 50 µl of axonemal suspension (final concentration 40 µM). After incubation for 3 min at room temperature, 10 µl of suspension was placed onto a formvar-coated grid for 1 min and the excess was absorbed by filter paper.

Whole mount immunogold TEM

For immunogold whole mount TEM, axonemes were purified and reactivated with ATP as described in the main text, and labeled with antibodies according to (Wloga, Rogowski et al. 2008) with minor modifications. Ten µl of the ATP-reactivated axonemes were placed on top of a formvar-coated copper EM grid, allowed to settle for 1 min, the excess of liquid was drained with a filter paper and the material was immediately fixed by floating the grid on top of a 50 µl drop of 2% paraformaldehyde in PHEM buffer. The grids were washed by dipping in 10 mM HEPES (pH 7.4) about 15 times and blocked by covering with 10 µl of 3% BSA, 0.01% Tween20 in PBS for 15 min. The grids were incubated in 50 µl drops of primary antibodies (1:100 polyE in the blocking buffer). The grids were washed by dipping in PBS about 15 times, incubated in 50 µl drops of the secondary antibody, anti-rabbit IgG-10 nm gold (GE Healthcare) (1:60 in the blocking buffer), washed 15 times in PBS, and negatively stained with 2% uranyl acetate.

Phenotypic Studies

The multiplication, cell motility, and phagocytosis rates were measured as described (Wloga, Rogowski et al. 2008). To assay swimming behavior, we added 2 ml of cells (2.3×10^5 cells/ml) to 100 ml of wash buffer (10 mM Tris, 50 µM $CaCl_2$, MOPS pH 7.2), centrifuged them at 1000 g for 2 min, and suspended them in 2 ml. After 30 min of adaptation, cells were assayed in 70 ml drop on a two-ring slide with or without SDBS (20 µg/ml), and video recordings were

done under a dissecting microscope with a Moticam 480 digital camera. To measure the beat frequency, we recorded cells (2 X 10^5 cells/ml) at 500 frames/s by a Photronics 1280 PCI FastCam on a Nikon Eclipse E600 microscope.

Biochemical Studies

Partial purifications of Ttll6Ap and Ttll1p from overproducing *Tetrahymena* strains and *in vitro* glutamylation assays with taxotere-stabilized microtubules made of the murine brain and HeLa tubulin were performed as described (Wloga, Rogowski et al. 2008). To purify cilia, we grew *Tetrahymena* cells to a density of 3 X 10^5 cells/ml in 500 ml of super proteose-peptone, washed them with 10 mM Tris (pH 7.5), and suspended them in 40 ml of 10 mM Tris, 50 mM sucrose, and 10 mM $CaCl_2$ with protease inhibitors (Complete, Roche). Deciliation was initiated by adding 600 µl of 0.5 M acetic acid, followed after 2 min by 550 µl of 0.6 M KOH. Cell bodies were removed (1,860 X g; 5 min), and cilia were collected (23,300 X g; 15 min; 4^{o}C) and suspended in 500 µl of the axoneme buffer (20 mM potassium acetate, 5 mM $MgSO_4$, 0.5 mM EDTA, 20 mM HEPES, pH 7.6).

Microtubule Sliding in Isolated Axonemes

Cilia were suspended at 0.1 mg protein/ml in the axoneme buffer (without protease inhibitors). For experiments on axonemes purified from strains carrying the *oad1-1* mutation, all strains were grown for 12 hr at 38^{o}C with inocula adjusted according to individual growth rates to collect cells at 3 X 10^5 cells/ml. Cilia were suspended at 0.1 mg protein/ml in 500 µl of the motility buffer (1 mM DTT, 50 mM potassium acetate, 5 mM $MgSO_4$, 1 mM EGTA, 30 mM HEPES, PEG 1%, pH 7.6). To demembranate, we added 10 µl of 1% NP-40 in motility buffer to 50 µl of diluted cilia. The axoneme suspension was pipetted into a perfusion chamber

constructed with a glass slide and cover-slip separated by double-stick tape. The perfusion chamber was washed with 50 μl of axoneme buffer followed by perfusion with 50 μl of 1 mM of ATP in the motility buffer. The sliding of microtubules was recorded on a Zeiss Axiovert 35 microscope equipped with dark field optics (403 PlanApo) on a silicon-intensified camera (VE-1000, Dage-MTI). The video images were converted to a digital format with Labview 7.1 software (National Instruments). The sliding velocity was determined manually by measuring microtubule end displacement as a function of time on tracings calibrated with a micrometer (Okagaki and Kamiya 1986).

Results and Discussion

TTLL6 Enzymes Generate Tubulin Polyglutamylation in Cilia

The genome of *Tetrahymena* contains six genes encoding TTLL6 paralogs, namely Ttll6Ap through Ttll6Fp (Figure 3.1A). Ttll6Ap is targeted to cilia (Janke, Rogowski et al. 2005; Wloga, Dave et al. 2010). To characterize the enzymatic properties of Ttll6Ap, we overexpressed GFP-Ttll6Ap in *Tetrahymena* and partially purified and assayed the enzyme for glutamylation of microtubules *in vitro*. Glutamylation involves two distinct steps: initiation and elongation. To distinguish between the two reactions, we used microtubules with varying levels of preexisting glutamylation: high (adult murine brain tubulin), intermediate (young murine brain tubulin), and low (HeLa tubulin) (Regnard, Fesquet et al. 2003). Enriched GFP-Ttll6Ap strongly modified microtubules made of adult brain tubulin, less efficiently modified microtubules made of young brain tubulin, and failed to detectably modify microtubules made of HeLa tubulin (Figure 3.1B). The activity was primarily on β-tubulin, as seen earlier (Janke, Rogowski et al. 2005). Moreover, enriched GFP-Ttll6Ap did not modify un-polymerized adult brain tubulin (Figure 3.1B). As a

control, we used another partially purified E-ligase, Ttll1p (Wloga, Rogowski et al. 2008), with the same microtubule substrates, and detected a distinct enzymatic profile (Figure 3.1B). These and earlier studies (van Dijk, Rogowski et al. 2007; Wloga, Dave et al. 2010) are consistent with Ttll6Ap acting primarily as a glutamyl side chain elongase for β-tubulin in microtubules.

In *Tetrahymena*, glutamylation occurs on most types of microtubules, but the length of the glutamyl side chain is spatially regulated (Wloga, Rogowski et al. 2008), presumably by localized activities of elongases such as Ttll6Ap. Although the modification is detectable on most, if not all, microtubules, only microtubules of cilia and basal bodies are labeled by the polyE antibodies that recognize elongated (poly) glutamyl side chains ((Wloga, Rogowski et al. 2008); Figure 3.1Dc). Knocking out the *TTLL6A* gene by DNA homologous recombination neither changed the levels of tubulin glutamylation (Figure 3.1C) nor affected the gross phenotype. *TTLL6F* encodes a close paralog (Figure 3.1A). Cells with a deletion of *TTLL6F* showed no reduction in the levels of tubulin glutamylation (Figure 3.1C). However, a double-knockout strain, 6AF-KO, had strongly reduced levels of elongated side chains recognized by polyE antibodies (Figure 3.1C), indicating that Ttll6Ap and TtllFp act synergistically. Consistent with this result, 2D gel electrophoresis of axonemal proteins showed a prominent reduction in the abundance of protein isoforms migrating as a smear on the more acidic side of the major β-tubulin spots in 6AF-KO cilia (Figure 3.5B). Immunofluorescence with polyE antibodies showed a decrease in tubulin polyglutamylation signal in cilia and basal bodies of 6AF-KO cells imaged side by side with wild-type cells (Figure 3.1Da–1Dc). The levels of tubulin glutamylation recognized by the GT335 antibody that detects an epitope at the base of the glutamyl side chain and probably recognizes side chains of any length (Wolff, de Nechaud et al. 1992) appeared unchanged in 6AF-KO cilia based on immunofluorescence (Figures 3.1Ea–

1Ec; Figure 3.5A) and western blotting (Figure 3.1C). These data indicate that the absence of Ttll6Ap and Ttll6Fp leads to shortening but not complete loss of glutamyl side chains, which agrees with the enzymatic profile of Ttll6Ap obtained *in vitro*. In cilia of 6AF-KO cells, the levels of tubulin acetylation and glycylation appeared nearly normal (Figure 3.1C). Thus, Ttll6Ap and Ttll6Fp together contribute to β-tubulin glutamylation in cilia and are responsible primarily, if not exclusively, for the chain elongation *in vivo*.

Ttll6Ap and Ttll6Fp Affect Ciliary Motility

The 6AF-KO cells have a normal density of cilia that appear only slightly shorter than wild-type cilia (wild-type, 5.6 + /- 0.8 μm, n = 150; 6AF-KO, 5.0 +/- 0.5 μm, n = 150). However, the 6AF-KO cells moved at only one-fifth of the wild-type rate (Figure 3.2A). In ciliates, phagocytosis requires the motility of ciliary membranelles that sweep food particles into the oral cavity. Although the 6AF-KO cells assemble oral membranelles (Figures 3.1Da–1Ec, arrowheads), they exhibited a greatly reduced rate of formation of food vacuoles (Figures 3.2C and 3.2D), consistent with malfunction of oral cilia. 6AF-KO cells also showed a reduced rate of multiplication (Figure 3.2B). *Tetrahymena* cells require motile cilia for conjugation (unpublished data). When starved 6AF-KO cells (grown earlier for over 100 generations to reach sexual maturity) were mixed with wild-type cells, few pairs formed, and these pairs dissociated quickly (Figure 3.2E). Thus, all functions dependent on normal ciliary motility appear to be severely affected in 6AF-KO cells.

Biolistic bombardment of 6AF-KO cells with a GFP-Ttll6Ap transgene (targeted to an unrelated locus) resulted in the appearance of cells with vigorous motility (at the frequency of 0.014%), and no such cells were found in the mock transformed population ($n = 10^7$). The rescued cells had a GFP signal in cilia and basal bodies (data not shown) and recovered a nearly

normal rate of motility, multiplication (Figures 3.2A and 3.2B), and phagocytosis (Table 3.3). Thus, the dramatic loss of ciliary functions seen in 6AF-KO cells is caused by the loss of TTLL6 protein activity.

High-speed video microscopy showed that in wild-type cells, locomotory cilia had an asymmetric waveform, and rows of cilia were engaged in metachronal waves. In contrast, in 6AF-KO cells many cilia appeared straight, and some were seen rotating around a central pivot point, often colliding with each other in an uncoordinated motion. Furthermore, immunofluorescence images indicated that 6AF-KO cilia are more straight than wild-type (Figures 3.1Da–1Ec). In some 6AF-KO cultures grown for over 480 generations, the wave-form was partly restored to normal. In these ‘‘adapted’’ 6AF-KO cells (6AF-KO^{A}), the beat frequency could be measured and was found to be 60% of wild-type (Figure 3.2F). Exposure of wild-type *Tetrahymena* cells to 20 μg/ml of sodium dodecyl benzene sulfonate (SDBS) causes rapid avoidance reaction associated with backward motility, likely by depolarizing the ciliary plasma membrane (T.H., unpublished data). Although wild-type cells showed rapid SDBS-induced avoidance responses (based on deviations from the linearity of swimming paths), the 6AF-KO cells failed to swim backward and instead slightly increased the rate of forward motility (Figures 3.2Ga–3.2Hb). The responses of 6AF-KO cells to other plasma membrane-depolarizing treatments (1 mM Ba^{2+}, 20 mM Ca^{2+}) were similar to SDBS (data not shown). At the time of addition of SDBS, some 6AF-KO cells showed a slight turn (Figure 3.6), indicating that the signal detection pathways that regulate motility are at least partly functional. These data suggest that the response to signals, which requires proper modulation of activity of dynein arms, is affected. However, ultrastructural studies revealed that 6AF-KO axoneme cross-sections have a normal morphology (Figures 3.3A and 3.3B, n = 209). No difference was found in the frequency

of outer (ODA) and inner (IDA) dynein arms on wild-type and 6AF-KO axoneme cross-sections (Figure 3.3C). Thus, we thought that TTLL6 enzymes, via tubulin glutamylation, could be affecting the activity ciliary dyneins.

Ttll6Ap and Ttll6Fp Primarily Modify the B-Tubule of Outer Microtubules

Immunogold transmission electron microscopy (TEM) studies showed that overexpressed GFP-Ttll6Ap localized to the outer doublets and not to central microtubules in all cross-sections examined (Figures 3.3D and 3.3E, n = 38). We evaluated the distribution of polyE epitopes on isolated doublet microtubules that were extruded from the axoneme with 40 mM ATP (see below). In negatively stained doublet microtubules viewed on edge, the A-tubule can be identified based on its increased width (as compared to the B-tubule) and based on dynein arms projecting from its surface (Figures 3.3G and 3.3H, arrowhead). In wild-type doublets, the majority of gold particles were detected in proximity to the B-tubule (Figures 3.3F and 3.3G; Figure 3.7), and the signal was dramatically reduced in 6AF-KO axonemes (Figures 3.3F and 3.3H; Figure 3.7). Thus, Ttll6Ap and TtllFp primarily generate glutamylation on the B-tubule. Our observations are consistent with earlier microscopic and biochemical studies showing enrichment of tubulin glutamylation on the B-tubule (Kann, Prigent et al. 1995; Multigner, Pignot-Paintrand et al. 1996; Lechtreck and Geimer 2000). Because the B-tubule serves as a track for ciliary dynein, the primary role of tubulin glutamylation in cilia could be to regulate the motor activity of ciliary dynein.

Tubulin Glutamylation Strongly Affects the Inner Dynein Arm-Driven Microtubule Sliding in Isolated Axonemes *In Vitro*

When isolated *Tetrahymena* axonemes are exposed to ATP, outer doublet microtubules undergo unconstrained dynein-driven microtubule sliding (rather than reactivated bending), and

the velocity of ATP-induced axonemal microtubule sliding can be used to assay the activity of ciliary dynein *in situ* (Summers and Gibbons 1971; Sale and Satir 1977; Okagaki and Kamiya 1986). With 1 mM ATP, microtubules underwent sliding at similar rates in wild-type and 6AF-KO axonemes (Figure 3.4B). In wild-type axonemes, the microtubule sliding velocity is believed to be primarily determined by the activity of ODAs (reviewed in (Kamiya 2002)). To test whether the TTLL6-mediated tubulin glutamylation affects the microtubule sliding generated specifically by IDAs, we constructed a strain that lacks *TTLL6A* and *TTLL6F* and is homozygous for the temperature-sensitive *oad1-1* allele. When *oad1-1* mutants are grown at restrictive temperature (38ºC), ODAs fail to assemble, and cells are nearly paralyzed (Attwell, Bricker et al. 1992; Ludmann, Schwandt et al. 1993). We assessed microtubule sliding in axonemes isolated from wild-type, *oad1-1*, 6AF-KO, and 6AF-KO;*oad1-1* triple mutant cells, all grown for 12 hr at 38ºC. We confirmed that 6AF-KO;*oad1-1* cells had fewer ODAs as compared to 6AF-KO cells grown at the same temperature (Figure 3.4A). As reported (Seetharam and Satir 2005), *oad1-1* axonemes showed a decreased rate of microtubule sliding as compared to wild-type (Figure 3.4C), whereas wild-type and 6AF-KO axonemes showed nearly the same microtubule sliding velocity, as seen earlier for axonemes from cells grown at the standard temperature (compare Figures 3.4B and 3.4C). Unexpectedly, the 6AF-KO;*oad1-1* axonemes showed a nearly 2-fold increase in the rate of microtubule sliding as compared to oad1-1 axonemes (Figure 3.4C). These data indicate that tubulin glutamylation generated on the B-tubule by Ttll6Ap and Ttll6Fp has a restraining effect on the microtubule sliding velocity generated by the net activity of IDAs.

To conclude, we report that tubulin polyglutamylation generated by TTLL6 enzymes plays a major role in ciliary motility and that the mechanism appears to involve regulation of inner dynein arm activity. Earlier studies have implicated tubulin glutamylation in axoneme assembly (Campbell, Waymire et al. 2002; Pathak, Obara et al. 2007). Deletion of additional TTLL6 paralogs in *Tetrahymena* led to major shortening of the axoneme (unpublished data). Thus, tubulin glutamylation affects both axoneme assembly and motility, and the latter function may require a higher dose of TTLL6 activity. A recent study of a *Chlamydomonas* mutant defective in TTLL9, an α-tubulin-preferring E-ligase, also revealed that tubulin polyglutamylation controls ciliary motility by affecting inner dynein arm activity (Kubo, Yanagisawa et al. 2010). Thus, both studies link tubulin glutamylation, mediated by two conserved E-ligases on either α- or β-tubulin, to regulation of IDA activity. The abnormal waveform and lack of ciliary reversals that we observed in the 6AF-KO cells are also consistent with malfunctioning IDAs in *Tetrahymena* (Hennessey, Kim et al. 2002; Wood, Hard et al. 2007). The mechanochemical properties of ODAs and IDAs are distinct, but the underlying structural basis is not well understood (reviewed in (Kamiya 2002)). For example, in *Tetrahymena*, the 22S dynein fraction from the salt extract of axonemes (mainly ODAs) produces a linear gliding of microtubules at the rate of 8 μm/s, whereas the 14S dynein fraction (presumably IDAs) produces a motility at the rate of 4 μm/s associated with microtubule rotation (Vale and Toyoshima 1988). Importantly, two IDA subtypes that were studied in *Chlamydomonas* (dyneins c and f/I1) display processive movements along microtubules (Sakakibara, Kojima et al. 1999; Kotani, Sakakibara et al. 2007). Kotani and colleagues proposed that in the bending cilium, processive IDAs, acting simultaneously with faster ODAs, impose a drag on sliding microtubules and could increase the axoneme curvature (Kotani, Sakakibara et al.

2007). We speculate that tubulin glutamylation is important for the processive motility of IDAs. One unusual feature of dynein motor domain is that it contacts the microtubule track by a conserved stalk domain (Gee, Heuser et al. 1997). Recent studies indicate that the stalk acts as a tether that allows for pulling of parts of the dynein molecule toward the microtubule during the power stroke (Carter, Garbarino et al. 2008; Ueno, Yasunaga et al. 2008). Thus, tubulin glutamylation could regulate the affinity of the stalk in inner dynein arms to the B-tubule. The patterns of glutamylation vary between axonemes of different species (Hoyle, Turner et al. 2008). Thus, specific beating patterns could be dependent on diverse patterns of tubulin glutamylation.

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Authors Contribution

1) Jacek Gaertig and Dorota Wloga: Fig. 3.1A (Phylogenetic tree of TTLL6)
2) Bernard Eddé: Fig. 3.1B (Invitro glutamylase reaction)
3) Stella Guerrero: Fig. 3.2 E (Pair formation during conjugation)
4) Amrita Kabi and David Pennock: Fig. 3.2F (Beat Frequency)
5) Todd Hennessey and Robert Hard: Fig. 3.2 G, H, Fig. 3.6 (Avoidance Reaction)
6) Laura Fox and Winfield Sale: Fig. 3.4 B, C (Microtubule sliding rate)
7) David Malison: Fig. 3.5B (2D-gel electrophoresis)

All the remaining panels were exclusively done by Swati Suryavanshi

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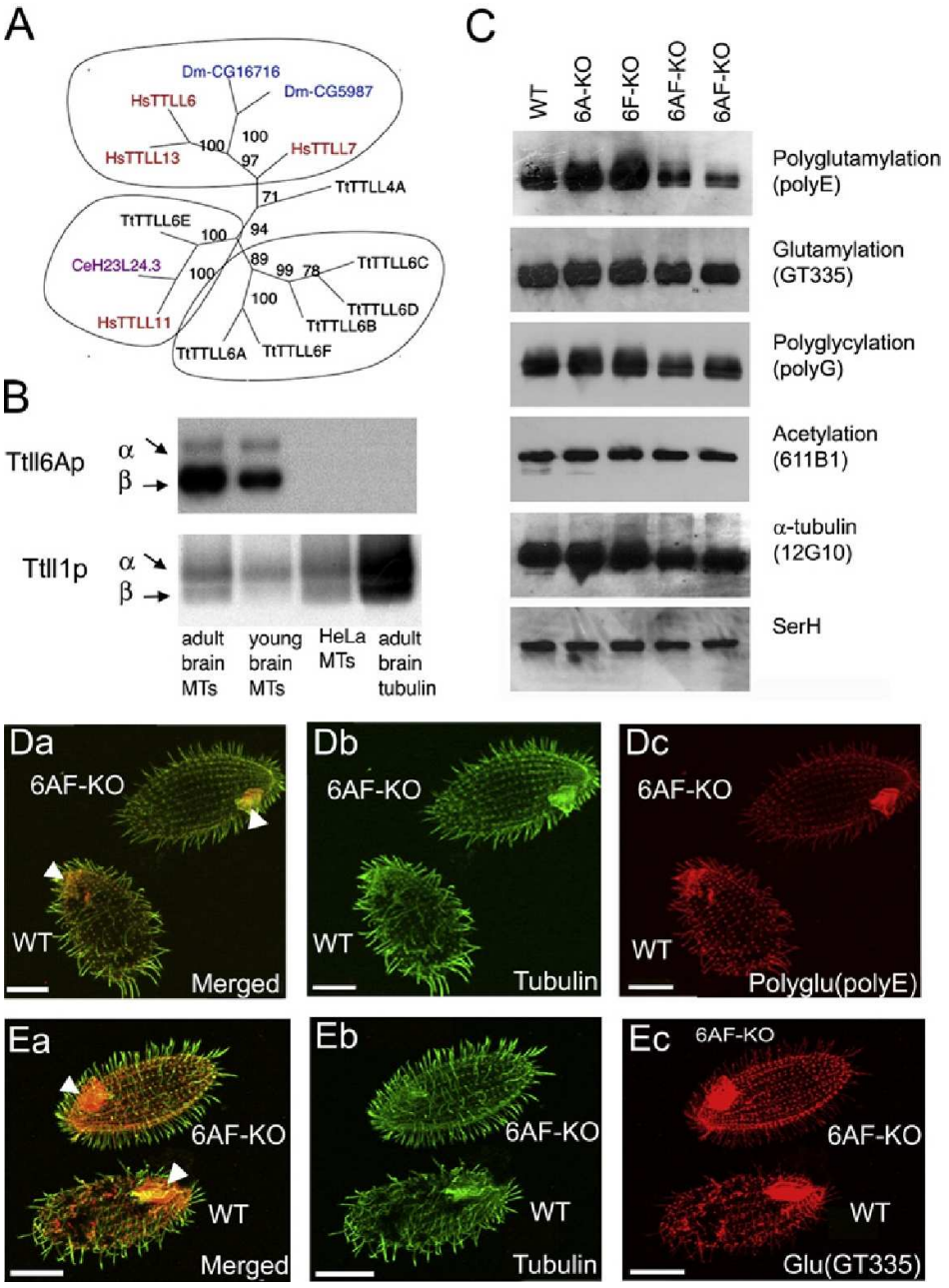

Figure 3.1: Deletion of Ttll6Ap and Ttll6Fp Leads to a Loss of Tubulin Glutamylation in Cilia. (A) A neighbor-joining phylogenetic tree based on the catalytic domain of TTLL6 E-ligases (Wloga, Rogowski et al. 2008). Tt-Ttll4Ap was used as an outgroup. The following abbreviations of species are used: Hs, *Homo sapiens*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Tt, *Tetrahymena thermophila*. (B) A fluorogram of mammalian

microtubule proteins (20 mg) separated by SDS polyacrylamide gel electrophoresis after in vitro glutamylation with partially purified GFP-Ttll6Ap, GFP-Ttll1p, ATP, and ^{3}H-glutamate, (C) A western blot of cilia proteins. The anti-SerH antigen antibodies were used as a loading control. (Da–Ec) Immunofluorescence images of pairs of wild-type and 6AF-KO cells imaged side by side. Wild-type cells were prefed with India ink to reveal dark food vacuoles. Cells were labeled with 12G10 anti-α-tubulin mAb and polyE anti-polyglutamylation antibodies (Da–Dc) or with SG anti-total tubulin antibodies and GT335 anti-glutamylated tubulin mAb (Ea–Ec). Arrowheads mark oral membranelles. Scale bar represents 10 mm. Quantitative data are shown in Figure 3.5A.

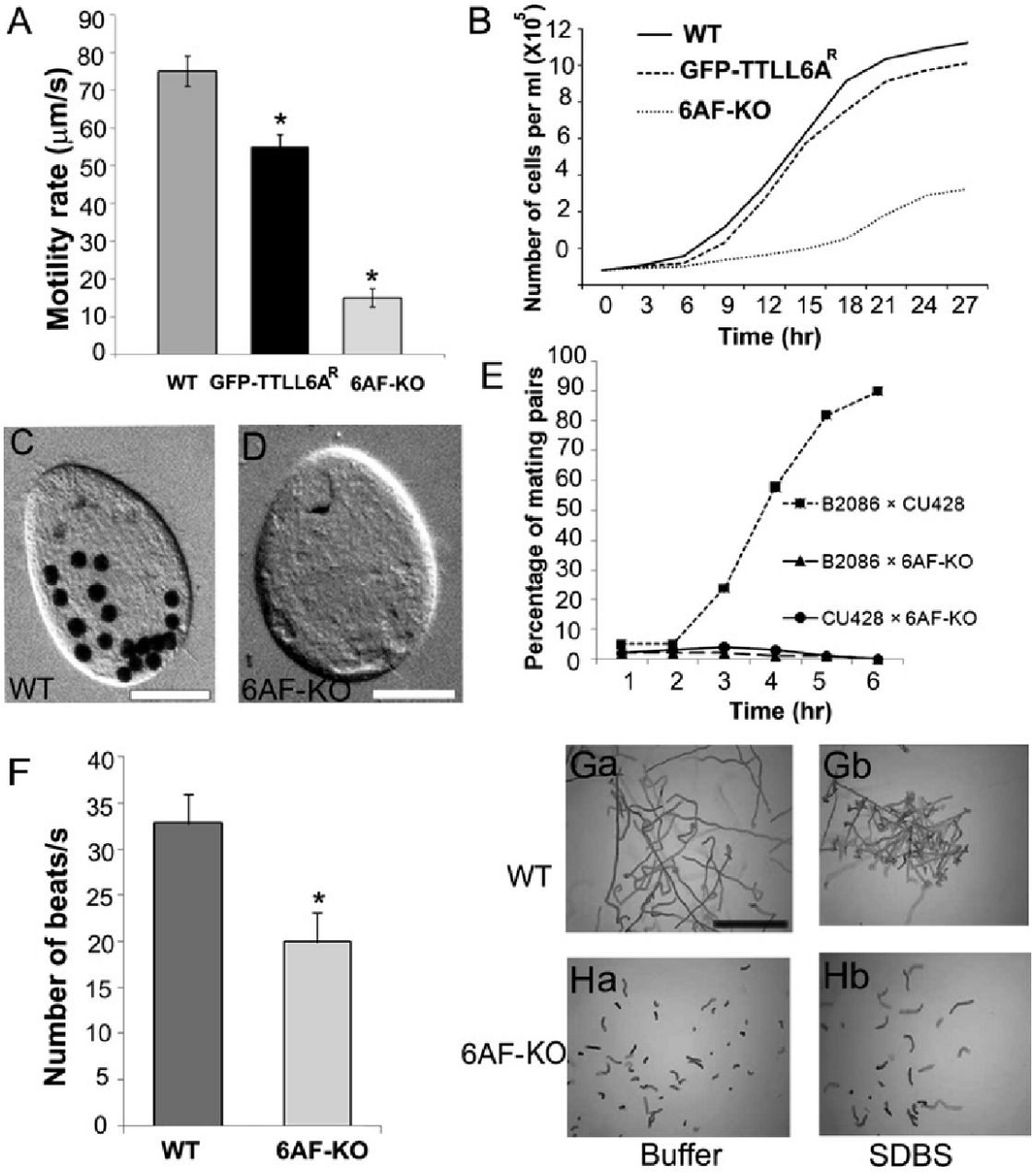

Figure 3.2: Cells Lacking Ttll6Ap and Ttll6Fp Display a Loss of Cilia-Dependent Functions

(A) A histogram shows the average linear cell motility rate during 5s for wild-type, 6AF-KO, and 6AF-KO cells rescued with a GFP-Ttll6Ap transgene (6AF-KO^R) (n = 40 for each strain).

Bars represent standard deviations. *$p < 0.001$. (B) Culture growth curves. (C and D) Images of a wild-type (C) and 6AF-KO (D) cell exposed to India ink for 30 min. Scale bar represents 20 mm. (E) The graph shows the percentage of paired cells following mixing of either two starved wild-type strains (CU428 and B2086) or 6AF-KO cells with either of the two wild-type strains. (F) The average ciliary beat frequency for wild-type ($n = 27$) and 6AF-KO ($n = 27$, $p < 0.0001$) cells. Error bars represent standard deviations. (Ga–Hb) Swimming responses to sodium dodecyl benzene sulfonate (SDBS). Wild-type (Ga and Gb) or 6AF-KO (Ha and Hb) cells were exposed to either a buffer alone or SDBS (20 mg/ml), and the paths of live cells were recorded for 1s. Scale bar represents 1 mm.

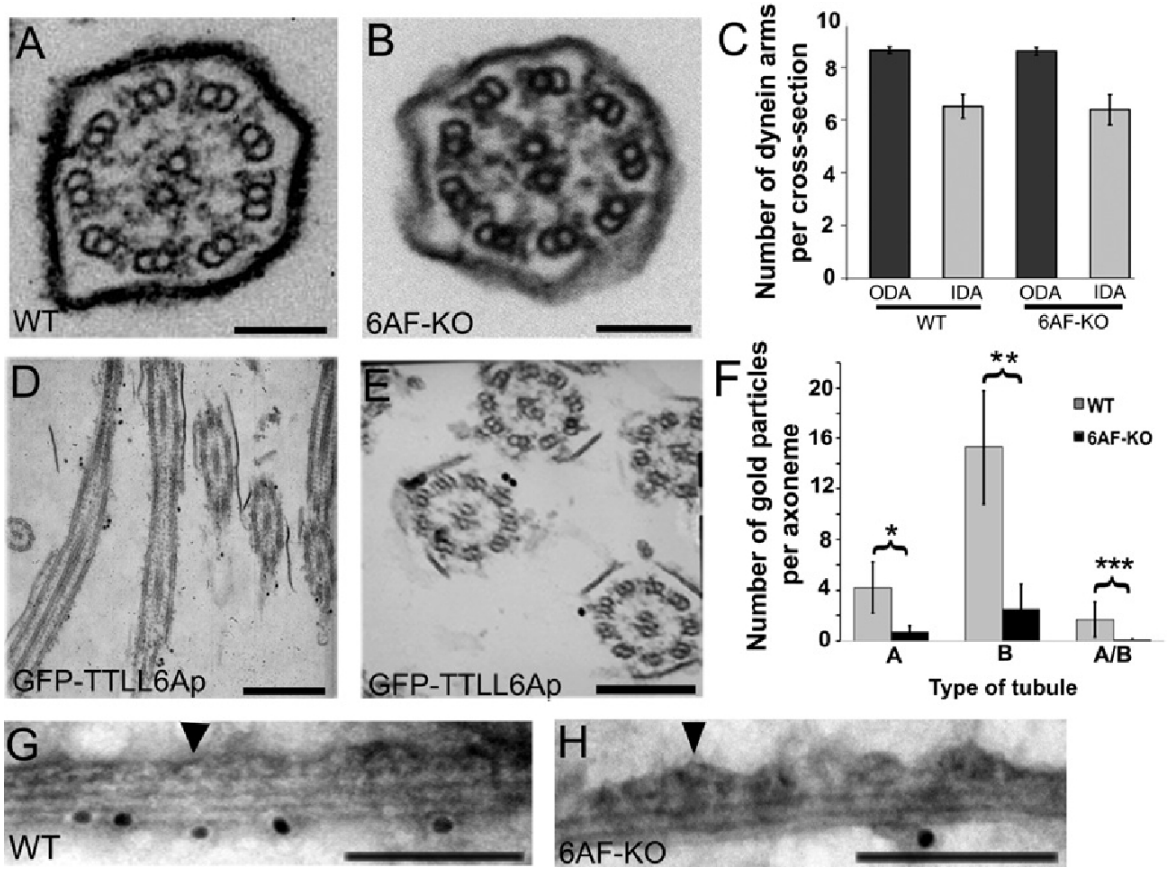

Figure 3.3: Ttll6Ap and Ttl6Fp Generate Polyglutamylation Primarily on B-Tubule of Outer Microtubules

(A and B) Cross-sections of wild-type (A) WT and 6AF-KO (B) cilia of cells grown at 30³ C. Scale bar represents 100 nm. (C) A graph that documents the average number of inner dynein arms (IDAs) and outer dynein arms (ODAs) per axoneme cross-section (wild-type, n = 27; 6AF-KO, n = 27). Error bars represent standard errors. (D and E) Sections of cells expressing GFP-Ttll6Ap that were labeled with anti-GFP anti-bodies with immunogold transmission electron microscopy (TEM). Scale bar in (D) represents 750 nm. Scale bar in (E) represents 250 nm. (F) A graph that quantifies the localization of pol-yglutamylated tubulin epitopes in doublet micro-

tubules labeled by whole-mount immunogold microscopy (shown in Figures 3G and 3H and Figure 3.7) with polyE antibodies and anti-rabbit IgG 10 nm gold conjugates. Each gold particle was scored as associated more closely with either the A- or B-tubule or the intertubule junc-tion (A/B). Error bars represent standard devia-tions. *p = 0.0001, **p < 0.0001, ***p = 0.0085. Twelve wild-type and 12 6AF-KO axonemes were scored.

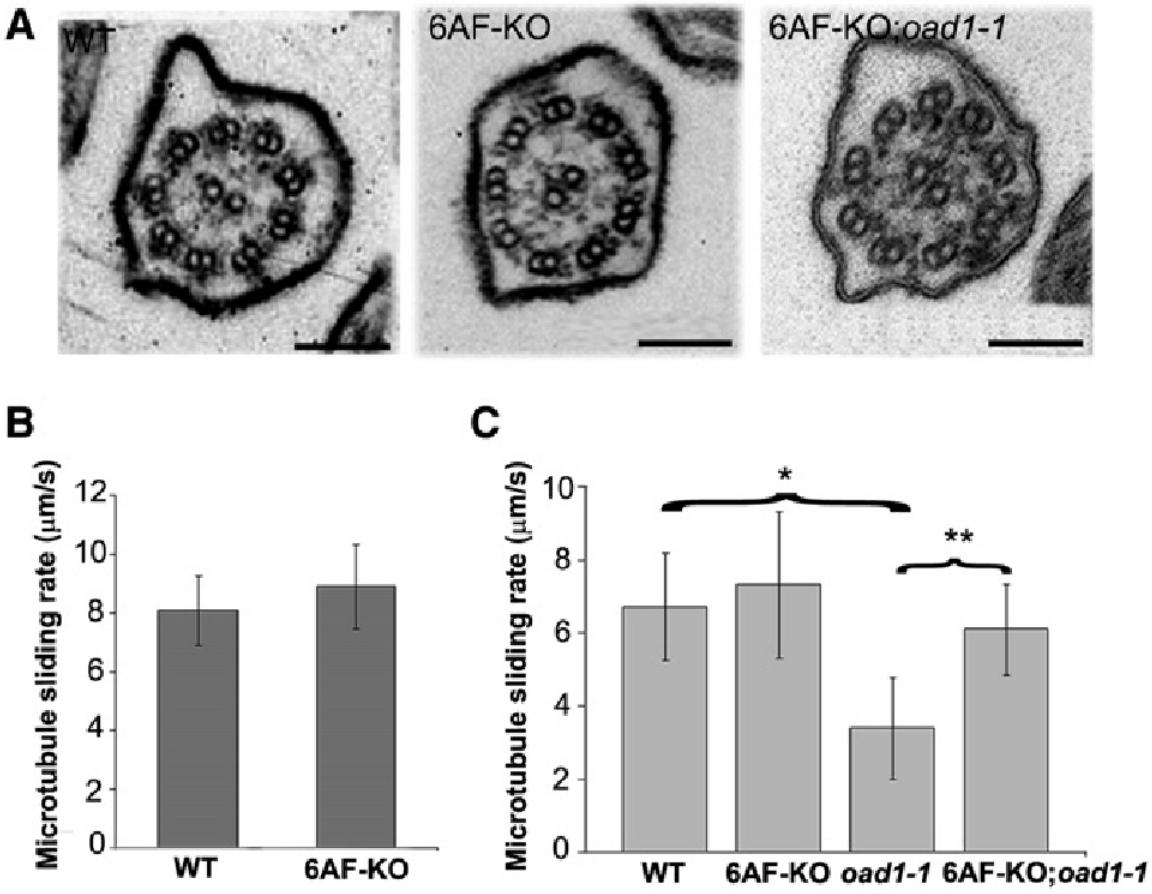

Figure 3.4: Tubulin Glutamylation Regulates the Velocity of Inner Dynein Arm-Driven Microtubule Sliding in Axonemes *In Vitro*

(A) TEM cross-sections of wild-type, 6AF-KO, and 6AF-KO;*oad1* axonemes grown for 12 hr at 38^{o}C. Scale bar represents 125 nm. The average numbers of dynein arms on scored axoneme cross-sections were as follows: 6AF-KO: 8.6 6 0.2 ODA, 6.4 6 0.6 IDA per section, n = 15; 6AF-KO;*oad1-1*: 2.8 6 0.5 ODA, 6.5 6 0.2 IDA per section, n = 15. (B) Graph shows the average sliding velocity of wild-type (n = 40) and 6AF-KO (n = 40) axonemes obtained from cells grown at the standard temperature (30^{o} C). Error bars represent standard deviations. Data were collected in three independent experiments. (C)A graph that documents the average sliding velocity of

wild-type (n = 74), oad1-1 (n = 41), 6AF-KO (n = 73), and 6AF-KO;*oad1-1* (n = 46) axonemes obtained from cells grown at 38^{o}C to induce the loss of ODAs in cells that are homozygous for the oad1-1 allele. Error bars represent standard deviations. *$p < 0.0001$ for 6AF-KO;*oad1-1* versus wild-type; **$p < 0.0001$ for *oad1-1* versus wild-type. Data were collected in three independent experiments. Slide and coverslip separated by double-stick tape. The perfusion chamber was washed with 50 ml of axoneme buffer followed by perfusion with 50 ml of 1 mM of ATP in the motility buffer. The sliding of microtubules was recorded on a Zeiss Axiovert 35 microscope equipped with dark field optics (403 PlanApo) on a silicon-intensified camera (VE-1000, Dage-MTI). The video images were converted to a digital format with Labview 7.1 software (National Instruments). The sliding velocity was determined manually by measuring microtubule end displacement as a function of time on tracings calibrated with a micrometer (Sale and Satir 1977).

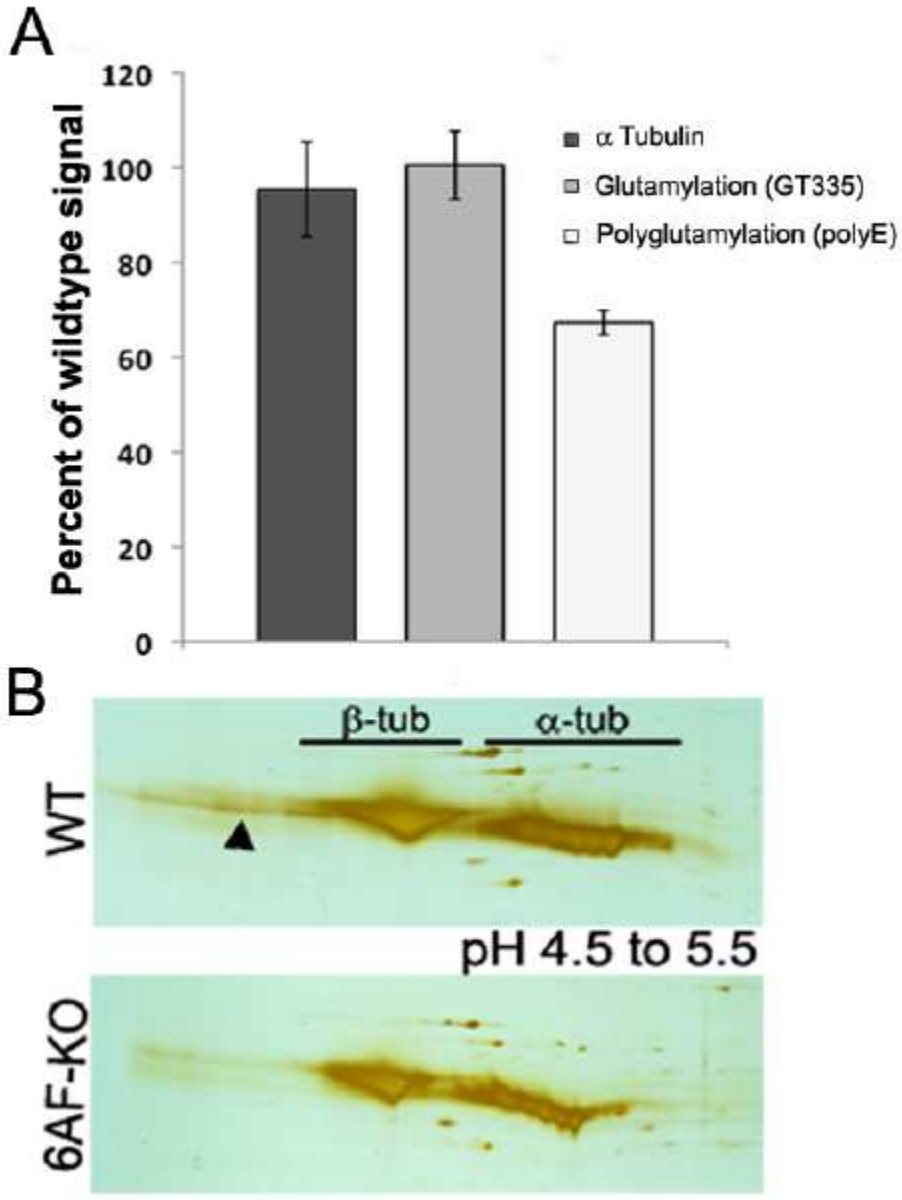

Figure 3.5: Loss of Ttll6Ap and Ttll6Fp Leads to a Loss of Elongated Glutamyl Side Chains in Axonemes

(A) A histogram that documents the average intensity of fluorescence signals detected for antibodies against tubulin or glutamylated tubulin using quantitative immunofluorescence. Wild type (labeled with India ink) and 6AF-KO cells were mixed and processed for double

immunofluorescence for total tubulin and glutamylated tubulin (using either GT335 or polyE antibodies). Confocal images were obtained of pairs of wild type and mutant cells located near each other. Signal intensities were measured for individual axonemes using ImageJ. A total of 4 images (8 cells) were analyzed, Between 73 and 196 axonemes were measured for antibody. For any given pair of cells, the mutant signal average was calculated as a percentage of the adjacent wild type cell signal. The histogram contains an average of each image value. Error bars represent standard errors. (B) Images of portions of tubulin regains of 2D gels of axonemal proteins. The arrowhead marks a region that contains a string of highly acidic protein forms migrating near the main spots of tubulin in the wild type that are missing in the 6AF-KO cilia. Note that on SDS-PAGE, ciliate tubulin migrates in an inverted order as compared to most other species (β-tubulin migrates more slowly).

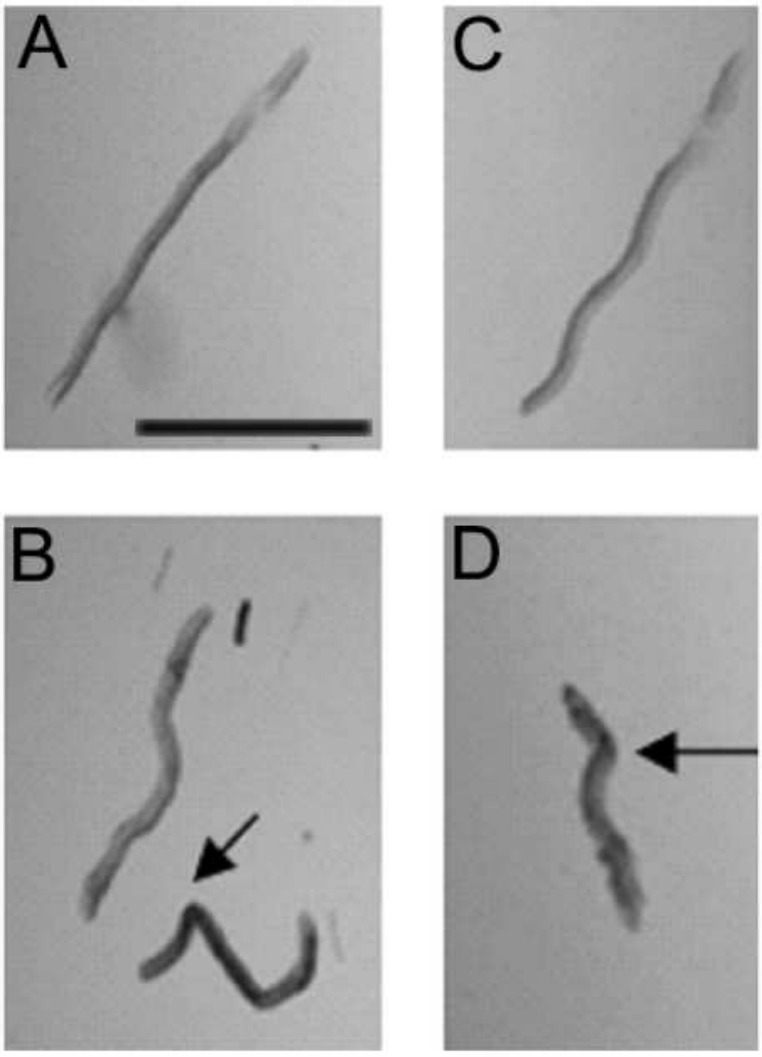

Figure 3.6: AF-KO Mutants Respond to Membrane-Depolarizing Treatments by Turning Paths of live 6AF-KO cells in the control solution (A, B) and upon exposure to 20 μg/ml SDBS. The slight turn in the path that occurs immediately after addition of SDBS is marked with an arrow. Bar - 200 μm.

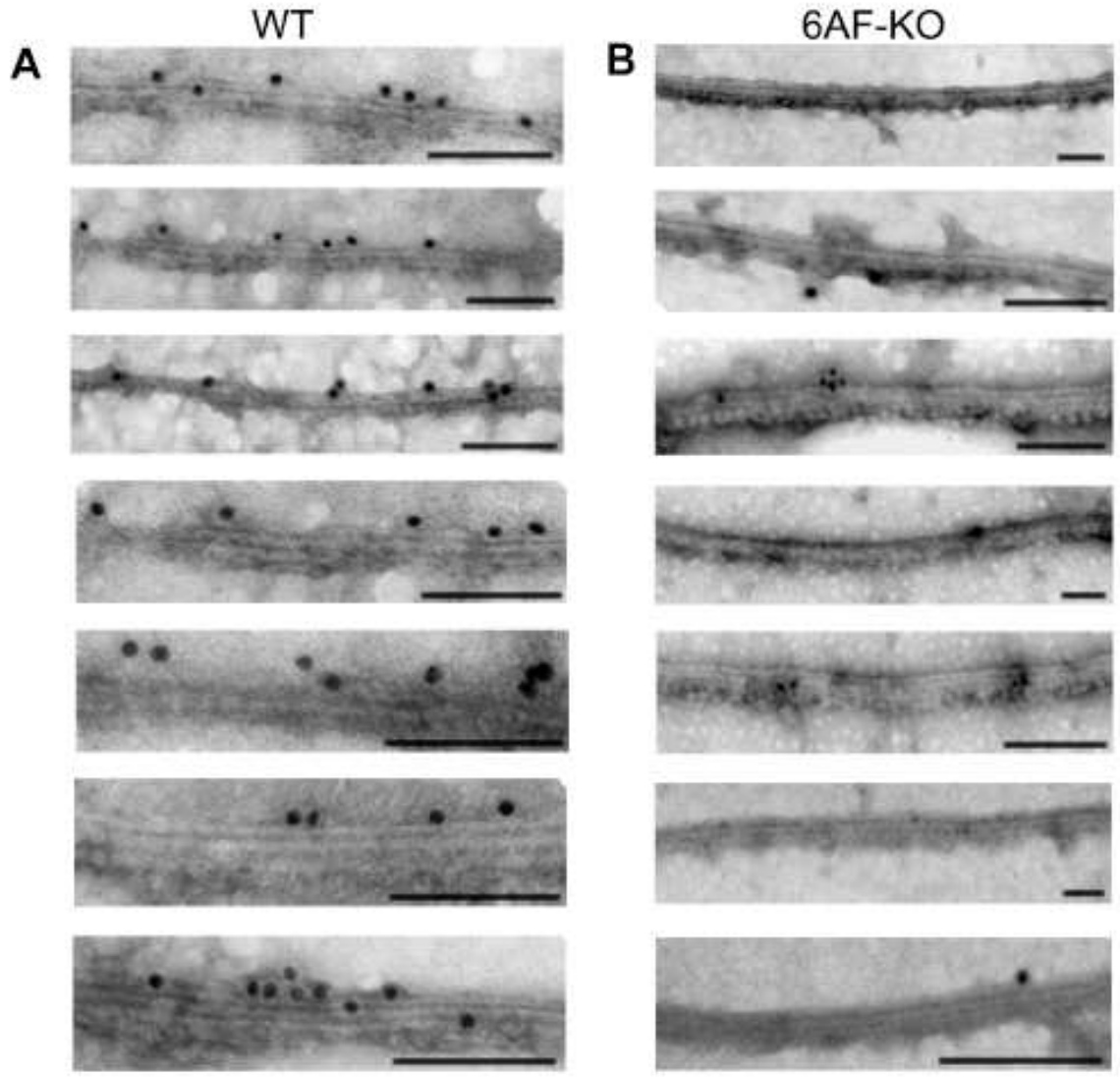

Figure 3.7: Ttll6Ap and Fp Primarily Affect the Polyglutamylation Levels on the B-Tubule or Outer Doublets

Images of negatively stained immunogold-labeled (using polyE antibodies) wild-type and 6AF-KO axonemes. Bar = 100 nm.

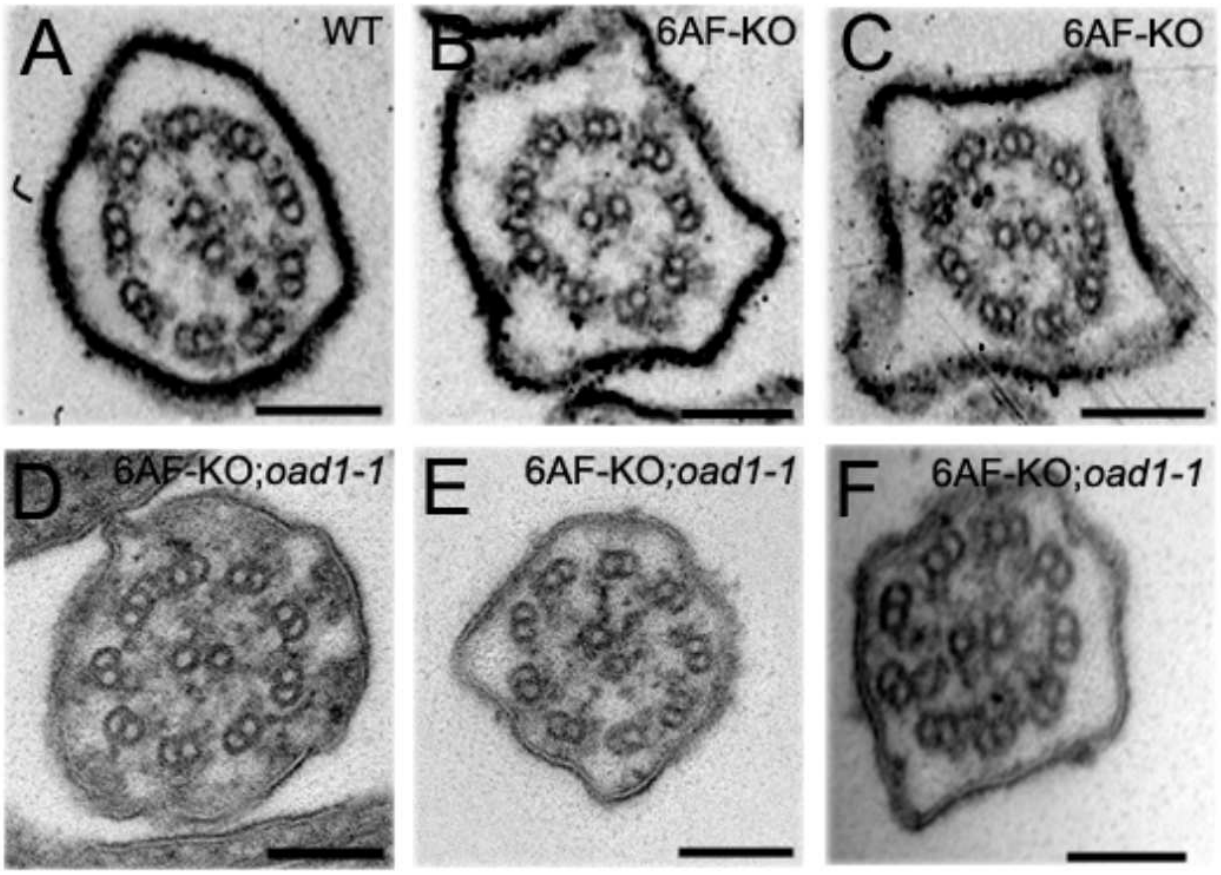

Figure 3.8: *Oad1-1* Allele Decreases the Frequency of Outer Dynein Arms in 6AF-KO Cells

TEM crossections of wild type (A), 6AF-KO (B, C) and 6AF-KO;*oad1-1* cilia (38^{o}C) (D-F).

Table 3.1 Primers Used for Construction of Targeting Fragments.

Gene	Primer sequence (5'-3')	Restriction sites used to release the targeted fragment	Size of targeting fragement	Selectable cassette	TTLL catalytic domain region replaced
TTLL6A	Forward:ATATTGGGCCCGAGGAAGATGAT GATGAGA Reverse:ATAAACCCGGGGCTAAAGAAAAC ATACCAG Forward:AATTTACTAGTAGCCATGGGTTT TAGAAGT Reverse:ATTATGAGCTCCTTTTGGAAGTA ATGTCAG	ApaI / SacI	1.5 Kb 1.45 Kb	Neo3	926 bp
TTLL6F	Forward:ATATTGGGCCCGAGCTAATCAAA CATACGA Reverse:ATTATATCGATTTCCTAGCTATTC TGGTTA Forward:ATATTCCCGGGAAAAAGCCTGAT GTTGAAG Reverse:ATATTCCGCGGGGCTACAAATAA AGTCCAT	ApaI / SacII	1.47 Kb 1.44 Kb	mtt1-rpl29	1.6 Kb

Table 3.2 Diagnostic Primers Used for Verification of Gene Disruptions (Amplify Deleted Regions)

Type of Gene	
TTLL6A	Forward 5'-TATCTTTTGGACTGATAATGCT-3' Reverse 5'-CTCTTAATATCTTTCCACAG-3'
TTLL6F	Forward 5'-AGATCTCTAAAGGAAAATGC-3' Reverse 5'-TTCATGTAGTTATCTGGTTG-3'

Table 3.3: Phagocytic Activity Is Restored by Reintroduction of GFP-Ttll6Ap Transgene into 6AF-KO Cells

	Wild Type	**6AF-KO**	**GFP-TTLL6A^{R}**
Average number of labeled food vacuoles/cell	42.8	0	36.3
Standard deviation	3.7	-	3.5
N =	25	25	25

Food vacuoles were labeled with India ink for 30 min, cells were fixed and the average number of food vacuoles per cell was determined.

CHAPTER 4

TUBULIN GLUTAMYLATION REGULATES THE LENGTH OF CILIA

Suryavanshi S, Wloga D, Gaertig J. To be submitted to Journal of Cell Biology

Abstract

Post translational modifications have been implicated in regulation of diverse functions of microtubules. Glutamylation is one of the most conserved and ubiquitously present post-translational modifications of microtubules that affects both α- and β- tubulin of various microtubular structures such as centrioles, basal bodies, cilia, mitotic spindle and neurites. Recently, catalytic subunits of tubulin glutamic acid ligases (E-ligases) have been identified as conserved enzymes related to tubulin tyrosine ligase and are known as tubulin tyrosine ligase like proteins (TTLL). In *Tetrahymena,* the TTLL6 subtype of E-ligases consists of 6 proteins. Deleting several of these genes resulted in progressive loss of ciliary motility, slow multiplication rate and defective phagocytosis. In a previous study, we showed that deletion of two TTLL6 paralogs inhibits ciliary motility by altering the function of dynein arms, with apparently no effect on axoneme assembly. In this study we show that deletion of additional 3 TTLL6 paralogs in one strain leads to axoneme shortening. Thus, TTLL6 mediated tubulin glutamylation has at least two functions in cilia: motility and axoneme elongation. These two functions are dependent on the dose of TTLL6 activity, with axoneme elongation requiring a lower dose of such activity.

Introduction

Microtubules constitute an important part of the cytoskeleton and are polymers made up of α and β-dimers. Microtubules perform diverse functions and one of the major factors for microtubule diversity are conserved post-translational modifications (PTMs) of tubulin. PTMs occur mainly after the microtubule assembly and mediate the functions of microtubules (Verhey and Gaertig, 2007). However, both the functions of many PTMs and the precise mechanisms of how PTMs affect microtubules are not well understood. Most of these PTMs affect the carboxy-

terminal tail (CTT) domain of tubulin on the outside surface of the microtubule (Nogales et al., 1999). Glutamylation adds multiple glutamates as a polypeptide side chain to tubulin, NAP1 and a few other proteins (Edde et al., 1990; Regnard et al., 2000; van Dijk et al., 2007). Glutamylation of tubulin is conserved from protists to mammals and occurs on diverse microtubules including axonemes (Bre et al., 1994; Wolff et al., 1992). Glutamylation is enriched on microtubules of cilia, centrioles/basal bodies, the mitotic spindle and neurites. Discovery of glutamic acid ligases (E-ligases) has helped accelerate functional studies of tubulin glutamylation. All known E-ligases belong to a family of proteins that have a tubulin tyrosine ligase (TTL) homology domain, known as TTL-like (TTLL) proteins (Janke et al., 2005; van Dijk et al., 2007; Wloga et al., 2008). A knockdown of TTLL6 mRNA in zebrafish led to loss of olfactory cilia (Pathak et al., 2007). Mice lacking a non -catalytic subunit of TTLL1 complex (Regnard et al., 2003) have disorganized sperm axonemes (Campbell et al., 2002). Thus, tubulin glutamylation appears to be important in the axoneme assembly or stability but its molecular function is unknown. By deleting several paralogous TTLL6 E-ligases in *Tetrahymena* in this study, we show that tubulin glutamylation has two functions that are glutamylation dose-dependent in cilia: axoneme motility and elongation.

Materials and Methods

Phylogenetic studies

The phylogenetic analyses of sequences of TTLL6 paralogs were done as described in (Wloga et al., 2008).

Gene disruptions in *Tetrahymena*

Two fragments of macronuclear DNA, flanking the genomic sequence encoding the catalytic domain of TTLL6 genes (based on the *Tetrahymena* Genome Database), were amplified

from genomic DNA using primers listed in Table 1 (Eisen et al., 2006). These amplified fragments were then cloned on the either side of a drug resistance cassette (*neo3* (Shang et al., 2002) , *mtt1-rpl29* (Bowen and Gorovsky, personal communication) or *bsr* (Brown et al., 1999)), digested and transformed biolistically into mating CU428 and B2086 strains (Cassidy-Hanley et al., 1997). The transformed cells were then incubated in SPP and 2.5 μg/ml CdCl2 for 3 hr and selected with appropriate drugs (*neo3*, paramomycin-100 μg/ml with CdCl2 2.5 μg/ml; *bsr*, blasticidine 120 μg/ml; *mtt1-rpl29*, cycloheximide 15 μg/ml with CdCl2 2.5 μg/ml). The resulting putative germline transformants were selected by adding 15 μg/ml of 6-methylpurine. The drug-resistant transformants were allowed to assort phenotypically to allow assortion of disrupted alleles from macronucleus and by making the micronucleus fully homozygous using a star cross (Hai et al., 2000). Double, quadruple and quintuple knockout strains were made by multiple rounds of standard crosses. Since *TTLL6A*, *TTLL6D* and *TTLL6E* were disrupted using the same selectable marker, *neo3*, the progeny clones containing both disrupted alleles were identified by extracting total genomic DNA and PCR amplification with primers that were designed to replicate junctions between *neo3* and gene specific regions (Table 4.2). Total homozygotes were created by crossing appropriate heterokaryons and isolating progeny.

Phenotypic studies

Multiplication rate of mutants and wild-type cells was found out by growing their cultures in 25 ml of SPP in 250 ml Erlenmeyer flasks at 30°C with shaking (150 rpm). For determining the motility rate, a drop of culture (2 x10^5 cells/ml) was observed under a microscope by placing the drop on a slide without a coverslip. Paths of moving cells were recorded and measured as described (Wloga et al., 2008). Phagocytosis rate was measured by

adding 3 µl of India ink to 1 ml of growing cells and counting the number of cells with food vacuoles containing ink for 2 hrs at the interval of 30 minutes.

Biochemical studies

Cilia purification was done by growing cultures of *Tetrahymena* to a density of 3 x 10^5 cells/ml in 500 ml of SPP, followed by a wash with 10 mM Tris pH 7.5. The cell pellet was then suspended in 40 ml of 10 mM Tris, 50 mM sucrose, 10 mM CaCl2 with protease inhibitors (Complete, Roche). To deciliate cilia 600 µl of 0.5 M acetic acid was added to this suspension of cells, followed by 550 µl of 0.6M KOH. The deciliated cells were then centrifuged at 1860 x g for 5 min. The resulting cell pellet containing cell bodies was discarded while the supernatant containing cilia were collected and re-centrifuged at 23,300 x g for15 min at 4°C. The resulting pellet containing cilia was resuspended in 500 µl of the axoneme buffer (20 mM potassium acetate, 5 mM MgSO4, 0.5 mM EDTA, 20 mM HEPES, pH 7.6). Western blots were done as described (Janke 2005), with the primary antibodies at the following dilutions: GT335 anti-glutamylated tubulin (1:1,000) (Wolff et al., 1992), ID5 anti-polyglutamylated tubulin (1:50) (Rudiger et al., 1999; Wloga et al., 2008), 12G10 anti-α-tubulin (1:10,000) (Developmental Studies Hybridoma Bank), polyE anti-polyglutamic acid (1:1,000) (Shang et al., 2002), polyG anti-polyglycine (1:5,000) (Duan and Gorovsky) and 6-11-B1 anti-acetyl-K40 on α-tubulin (1:10,000) (Sigma Aldrich) .

Immunofluorescence and electron microscopy

Immunofluorescence studies were done as described in (Wloga et al., 2006) with the following primary antibodies: GT335 (1:10,000), polyE (1:100), 12G10 (1:50), SG polyclonal anti-total *Tetrahymena* tubulin (1:600). A Leica TCS SP2 confocal microscope was used for

viewing labeled cells. For standard transmission electron microscopy (TEM), cells were fixed as described by (Sharma et al., 2007).

***In vitro* microtubule sliding**

Tetrahymena cells were grown in 50 ml of culture and cilia were purified as described above. Cilia were membranated by adding 500 µl of 0.1% NP-40 in the axoneme buffer to the pellet of cilia and centrifuged at 23,300 g for 5 min. The resulting pellet containing axonemes was washed with 500 µl of the axoneme buffer and suspended at 2 mg/ml concentration. 1mM ATP was added to slide doublet microtubules out of the axonemes and the sliding velocity was measured according to (Okagaki and Kamiya, 1986)**.** Sliding was recorded using a Zeiss Axiovert 35 microscope equipped with dark field optics, 40 X PlanApo lens and a silicon intensified camera (VE-1000, Dage-MTI, Michigan City, IN). The video images were converted to a digital format using Labview 7.1 software (National Instruments, Austin, TX).

RESULTS

TTLL6 family of proteins in *Tetrahymena* consists of 6 paralogs and they contribute to tubulin glutamylation in cilia

In *Tetrahymena ,*TTLL6 proteins constitute one of the several conserved subtypes of E-ligases, and modify ciliary tubulin (Janke et al., 2005; van Dijk et al., 2007; Wloga et al., 2008). Phylogenetic analysis reveals the presence of 6 paralogous genes encoding TTLL6, namely, *TTLL6A*, through *TTLL6F*, in *Tetrahymena*, (Fig. 4.1A). Ttll6Ap of *Tetrahymena* is a β-tubulin-preferring E-ligase that exhibits a strong side-chain elongating activity. Ttll6Ap is targeted to cilia and has been studied extensively. *TTLL6F* gene is a closely related paralog of *TTLL6A* (Suryavanshi et al., 2010; Wloga et al., 2010). *TTLL6B, TTLL6C* and *TTLL6D* are also paralogs

and are closely related to each other. *TTLL6E* is closely related to the human TTLL6 type proteins. We suspected a significant level of functional redundancy among various TTLL6 paralogs in *Tetrahymena*. Murine TTLL6 type proteins are highly expressed in tissues with ciliated cells (van Dijk et al., 2007); the full length Ttll6Ap localizes to cilia of *Tetrahymena* (Suryavanshi et al., 2010; Wloga et al., 2010). Our earlier study reveals that glutamylation contributed by Ttll6Ap and Ttll6Fp is required for normal ciliary motility (Suryavanshi et al., 2010). However, tubulin glutamylation is also present in non-motile primary cilia (Wolff et al., 1992) and organisms that have exclusively non-motile sensory cilia, such as *C. elegans*, have TTLL6 orthologs. In zebrafish depletion of a TTLL6 ortholog by morpholinos caused shortening and loss of a subset of cilia. Thus, it appears that TTLL6-mediated tubulin glutamylation has another function in cilia, distinct from its role in cell motility. Furthermore, it is possible that the second ciliary function is covered by the 4 TTLL6 paralogs that were not disrupted in our original study. We test this idea by knocking out the additional TTLL6-type genes in cells already lacking *TTLL6A* and *TTLL6F* genes.

We created strains with single knockouts of *TTLL6B*, *TTLL6D*, and *TTLL6E,* using DNA homologous recombinations. Eliminating these genes individually neither changed the levels of tubulin glutamylation levels nor affected the gross phenotype (data not shown). Since the proteins encoded by these genes are closely related, our conjecture was that they may act synergistically among themselves or with earlier studied Ttll6Ap and Ttll6Fp. Thus, we created a double knockout strain for *TTLL6B* and *TTLL6D* (6BD-KO) and combined these mutations with the already available genetic background to create a quadruple knockout strain lacking *TTLL6A*, *TTLL6B*, *TTLL6D*, and *TTLL6F* (6ABDF-KO) and a quintuple knockout strain also lacking *TTLL6E* (6ABDEF-KO). Except for 6BD-KO, cells appeared to have a wild type gross

phenotype (data not shown). As expected, based on the phenotype of 6AF-KO cells the 6ABDF-KO and 6ABDEF-KO cells showed a dramatic phenotype of extremely slow cell motility and growth. To determine whether deletion of paralogs other than TTLL6A and F has additional effects we performed western blot assay on ciliary tubulin of wild-type and mutant cells (Figure 4.1B). The polyE antibodies probably recognize glutamyl side chains that are =>3 per side chain (Wloga et al., 2008). A western blot with polyE showed that, the reduction in polyglutamylation is proportional to the number of TTLL6 proteins eliminated; quintuple knockouts show greater loss in polyglutamylated tubulin as compared to quadruple and double knockouts. The GT335 monoclonal antibody mAb is proposed to recognize glutamyl side chains of any length (Wolff et al., 1992). A small reduction in the GT335 signal is apparent. However, it appears that the elimination of 5 TTLL6 paralogs caused a strong reduction in the signal of long side chains and only small reduction in the signal of total side chains. With the limitation that some side chains may not be detectable by antibodies due to steric effects between adjacent sites, it appears that all theTTLL6 paralogs studied here, contribute mainly to the side chain elongation and not initiation. Thus, we propose that all these TTLL6 paralogs, like earlier studies with Ttll6Ap, are side chain elongases. The small loss of GT335 signal could reflect a low level of side chain initiation activity of one or more of the studied enzymes or their association with another E-ligase (e.g. TTLL1) that has side chain-initiating activity. Alternatively, a decrease of the side chain length due to lack of elongation activity could increase the activity of a deglutamylase enzyme. Furthermore, a decrease in side chain length could have an effect on the axoneme dynamics or other properties that indirectly inhibit side chain initiating E-ligase of a subtype distinct from TTLL6 (such as TTLL1).

These above biochemical results were confirmed by immunofluorescence in which mutant cells were mixed with wild-type cells that were earlier fed with Indian ink (Figure 4.1C-D) and imaged side-by-side. This mixture of cells was labeled with anti-α tubulin (12G10), anti-polyglutamylated tubulin (polyE) and anti-glutamylated tubulin (GT335) antibodies. Double (top panel), quadruple (middle panel) and quintuple (lower panel) mutants showed a dramatic decrease in levels of polyE in cilia and basal bodies, indicating their synergistic mechanism (Figure 4.1D). GT335 signals were only slightly lower in quadruple and quintuple mutants as compared to that of wild-type (Figure 4.1C).

Loss of TTLL6 proteins affects cilia-dependent functions in a gene dose-dependent manner.

With elimination of each of the additional paralog, the motility defect worsens and the multiplication rate decreases (Fig. 4.2A-B). Quadruple and quintuple knockout mutants multiply every 8 hrs as compared to wild-type cells that double in 3 hours (Figure 4.2A). 6AF- KO mutants move at one-fourth speed of wild-type while 6ABDF- KO and 6ABDEF- KO mutants move about one-eighth speed of wild-type (Figure 4.2B). In ciliates, phagocytosis occurs inside the oral apparatus and requires the motility of ciliary membranelles that sweep food particles. To analyze their phagocytosis ability, we employed an assay by adding Indian ink to the cultures of wild type and the mutant cells. With time, wild-type cells show an increasing number of food vacuoles filled with ink particles. On the other hand, knockout cells showed none to very few ink-filled food vacuoles; quadruple and quintuple knockouts being more severely affected as compared to double knockouts, consistent with the loss of function of oral cilia (Figure 4.2C-G). All these data confirm that most of the cilia based functions are affected in strains depleted of TTLL6 E-ligases.

TTLL6 proteins in *Tetrahymena* are required for formation of normal length cilia

To determine the cause of progressive loss of cilia-dependent functions in the series of TTLL6 mutants, we analyzed the organization of cilia by immunofluorescence. The number of cilia in all the mutants seemed unaffected in all mutants studied (Figure 4.3F). On measuring the lengths of various axonemes of all the mutants using immunofluorescence images, it is apparent that axonemes become progressively shorter with elimination of increasing number of TTLL6 genes. Thus, in 6AF-KO cells, the average cilia length is ~10% shorter that wild-type, 6ABDF-KO cilia are ~13% shorter than wild type, while 6ABDEF-KO cilia are ~28% shorter than wild-type cilia. Particularly, among all types of cilia, the reduction in lengths of the posterior end cilia of all the mutant strains was more pronounced (Figure 4.3A-E). The posterior cilia of 6ABDEF-KO cells are 32% shorter than the posterior cilia of wild-type cells (Figure 4.3E). Next, we investigated whether the short axonemes in mutant strains have defects in the axoneme structure. Transmission Electron Microscopy analysis revealed that 6ABDF-KO axonemes appear normal (Figure 4.3G, H). Thus, at least these 4 paralogs contribute to axoneme elongation but their activities are not required for assembly of the 9+2 organization.

Thus, we reveal that glutamylation contributed by TTLLL6 proteins is required for regulating lengths of axoneme. This function is fulfilled redundantly by 5 TTLL6 paralogs but requires a lower dose of glutamylation as compared to higher dose of TTLL6-type E-ligases necessary for ciliary motility (Suryavanshi et al., 2010).

TTLL6 deficient cells display slow cilia regeneration rate

Intraflagellar transport (IFT) is a bidirectional trafficking of protein complexes between the basal body and the tip of cilia that occurs along the axoneme and required for assembly in growing cilia as well as for maintenance of cilia (Marshall et al., 2005; Scholey, 2003). Since

cilia in TTLL6 mutant series are progressively shorter, this opens up a possibility that TTLL6-mediated glutamylation of tubulin regulates the kinetics of IFT. Alternatively, the rate of cilia assembly could be normal, but cilia may not reach the normal length due to a defect in length regulation that could be independent of IFT. To determine whether the rate of cilia elongation is affected in TTLL6 series of mutants, we deciliated starved wild-type and mutant cells by a pH shock method, and allowed cells to regenerate cilia while periodically analyzing these cells by immunofluorescence using anti-tubulin and anti-glutamylation antibodies at fixed intervals of time (Figure 4.4A to E). It is apparent that in the quadruple and quintuple mutants detectable ciliary stubs appeared with a great delay as compared to wild-type or 6AF-KO mutants. For example, wild-type and double mutants started regenerating short stubs of cilia after 30 minutes whereas the quadruple and quintuple mutants started regenerating only after 75 minutes. The slopes of curves that document the length of cilia have a lower angle indicating that the actual rate of cilia elongation is reduced in TTLL6 mutants in a gene dose dependent manner. These data indicate that the rate of axoneme assembly most likely the rate of IFT as the major underlying mechanism is reduced in the TTLL6 mutants.

Tubulin glutamylation does not affect the ODA-driven sliding of axonemal microtubules

We wanted to test if a dramatic loss in β-tubulin glutamylation by deleting several TTLL6-type E-ligases has any effect on outer dynein activity. For this, we isolated axonemes from 6AF-KO, 6ABDF-KO, 6ABDEF-KO and wild type cells, induced dynein-dependent sliding of outer microtubules *in vitro* with 1mM ATP (Summers and Gibbons, 1971) and determined the velocity of sliding of microtubule ends (Figure 4.5A). Wild-type axonemes underwent disintegrations by sliding of doublet microtubules at the average rate of 8.4 +/- 1.0 μm/sec. As for the mutants, fewer axonemes underwent sliding, and among those that did reactivate the average

sliding velocity rate was similar to that of wild-type: 9.38 +/- 1.4 μm/sec for 6AF-KO, 9.52 +/- 1.23 μm/sec for 6ABDF-KO and 9.83 +/- 1.29 μm/sec for 6ABDEF-KO. MT sliding velocity is believed to be primarily determined by outer dynein arms. The lack of difference in sliding velocities of wild-type and mutants suggests that β-tubulin glutamylation does not regulate ODAs but IDAs, as determined earlier for 6AF-KO mutants.

DISCUSSION

Glutamylation occurs in two steps- chain initiation and elongation. During initiation, the first glutamate is linked by an amide bond between its α-amino group and the γ-carboxyl group of the glutamate residue in the primary sequence. During the elongation step, additional glutamates are linked by isopeptidic bonds (Redeker et al., 1991; Wolff et al., 1994). Previous studies showed that specific E-ligases contribute to either a chain initiation or chain elongation activity (Janke et al., 2005; van Dijk et al., 2007; Wloga et al., 2008), except for murine TTLL7, which can catalyze initiation as well as elongation reaction in vitro (Mukai et al., 2009).

In this work we have characterized the function of tubulin glutamylation generated by TTLL6 type E-ligases in *Tetrahymena*. Strong elongating enzymes often have associated weak initiation activity and strong initiating enzymes have associated weak elongating activity. But previous studies showed that, i*n vitro*, Ttll6Ap shows an exclusive elongase activity when expressed in *Tetrahymena* (Suryavanshi 2010) and in *E. coli* (van Dijk et al., 2007). We also showed that elimination of Ttll6Ap and its closest homolog Ttll6Fp, leads to apparent shortening of the glutamyl side chains *in vivo* but not their complete loss. Here we have extended these observations by eliminating additional TTLL6 paralogs. Interestingly, deletion of 5 TTLL6 paralogs also resulted in small loss of number of glutamyl side chains, in addition to a major loss in long glutamyl chains. It is possible that *in vivo* some or all of these proteins acquire initiation

activity in presence of an adapter unit. Alternatively, these enzymes may require a rate-limiting post-translational modification to enable an intrinsic side chain initiation activity *in vivo*. Another possibility is that the loss of elongase activity alone causes reduction in the total number of side chains in a less direct manner, for example by activating a deglutamylase enzyme or by changing the microtubule polymer dynamics. Thus, it appears that TTLL6 proteins are involved primarily, if not exclusively, in side chain elongation in *Tetrahymena*. This is by no means an expected result, since there are other TTLL6 homologs, such as murine TTLL7, that not only catalyze elongation reaction but also initiates glutamyl side chains on tubulin. Thus, even relatively small changes in the primary sequence of tubulin could determine the nature of catalytic activity (initiation or elongation) of these enzymes. In order to confidently predict the type of activity of E-ligases it is necessary to investigate this matter further by doing additional experiments.

Earlier, we show that the combined activity of Ttll6Ap and Fp is required for normal ciliary motility, by regulating both, the waveform and beat frequency (Suryavanshi et al., 2010). These effects are likely mediated by modulation of the activity of inner dynein arms. Moreover, we showed that glutamylation is enriched on outer doublets, and more precisely on the B-tubule that serves as a track for dynein arms during force production. A similar conclusion was reached in an independent study in *Chlamydomonas* for TTLL9 E-ligase (Kubo et al., 2010). Here we show, that at a lower dose, tubulin glutamylation mediated by TTLL6 proteins is also required for elongation of the axoneme. Thus, our observations agree with the data reported earlier by Pathak and colleagues, who showed that a morpholino knockdown of TTLL6 in zebrafish led to shortening of olfactory cilia (Pathak et al., 2007). Mutations in the CTTs of β-tubulin, that are used for both glutamylation and glycylation also lead to shorter cilia, albeit these cilia are also disorganized and most notably lack the central pair (Thazhath et al., 2004; Thazhath et al., 2002).

Maintenance and growth of cilia requires IFT. IFT is required for delivering cargo in the form of tubulin and other components to the tip of the cilia via two types of motor proteins: kinesin-2 and cytoplasmic dynein 2b (Scholey, 2008). Kinesin-2 carries the cargo in an anterograde direction, from basal body towards the tip of cilium, while dynein is responsible for the retrograde transport. Both of these transports occur along the sides of doublet microtubules (reviewed in (Scholey, 2003)). Defects in motor proteins, IFT proteins, or a reduction in frequency or rate of IFT leads to shorter lengths of cilia (Kozminski, 1995; Pedersen et al., 2005; Piperno et al., 1998; Porter et al., 1999). IFT occurs on the outer doublets of ciliary microtubules (Kozminski et al., 1995). Polyglutamylation of tubulin is also enriched particularly on outer doublets (Suryavanshi et al., 2010). One of the reasons for shorter ciliary lengths and slow growth of cilia in TTLL6 deficient cells could be that loss of tubulin glutamylation deregulates IFT by affecting IFT motors. Glutamylation on ciliary microtubules has been implicated in regulating IFT as IFT particles accumulate near outer doublets in hypomorphic mutants lacking sites for polymodifications in the C-terminus of tubulin (Redeker et al., 2005). In the absence of tubulin glutamylation, the frequency or speed of IFT components may slow down the transport of cargo required for axonemal assembly.

There is already some evidence that motor proteins can be affected by tubulin glutamylation. Kinesin motor moves processively on ciliary microtubules before detaching itself from the microtubules. Kinesin is bound to microtubules in such a way that it does not restrict its diffusion along microtubules and yet keeps it processive by its attachment to the glutamyl rich CTT of tubulin (Okada and Hirokawa, 2000). Using single-motor motility assays, it was shown that lack of CTT of tubulin does not affect the binding of kinesin motors to the tubulin but reduce the processivity of the kinesin motors. CTTs have been shown to interact with and enhance not

only the processivity of kinesin but also that of cytoplasmic dynein (Thorn et al., 2000; Wang and Sheetz, 2000). Polyglutamylation of tubulin has been shown to be differentially sensitive to different types of motors. Ikegami and colleagues showed that ROSA mutant mice, lacking PGs1, a tubulin glutamylase, show reduced trafficking of kinesin-3 motor cargo in neurons (Ikegami et al., 2007). Mutations of the polymodification sites on CTT of tubulin affected binding of kinesin-1 to MTs (Reed et al., 2006). Axonemal inner dynein arm is subjected to regulation by tubulin glutamylation (Kubo et al., 2010; Suryavanshi et al., 2010). Thus, eliminating tubulin glutamylation in cilia, normally executed by TTLL6 proteins, could adversely affect the interaction of motor proteins due to reduction in negatively charged CTTs.

While it is certainly plausible that tubulin glutamylation mediated by TTLL6 affects IFT motors, we need to be open to alternative explanations. One observations that is inconsistent with the IFT model is that *Plasmodium* species contain TTLL6 sequences and assemble cilia without IFT (Chen et al., 2006). An alternative explanation of the axoneme shortening in mutants deficient in TTLL6 proteins is that tubulin glutamylation changes the rate of turnover of tubulin subunits across the axoneme. Axonemes slowly turnover tubulin and this activity could play a role in size regulation (reviewed in (Gaertig, 2008)). Thus, a shortened axoneme could result from an improper level of subunits turnover within the axoneme. The results of experiments on potential ciliary factors that promote MT turnover support this general idea.

The post-translationally modified CTTs of tubulin are known to strongly interact with microtubule-severing factors such as katanin and spastin, (McNally and Vale, 1993; Roll-Mecak and Vale, 2008). Importantly, mutations in katanin subunits led to shortening of the axoneme in *Tetrahymena* and lack of assembly of central microtubules in *Tetrahymena* (Sharma et al., 2007) and *Chlamydomonas* (Dymek et al., 2004). In both species, katanin subunits were localized to

the outer doublet microtubules (Dymek et al., 2004; Sharma et al., 2007). These observations indicate a counter-intuitive mechanism, where a certain level of tubulin turnover stimulated by katanin inside cilia is required for their assembly and elongation. Without either katanin or tubulin glutamylation, axonemes could have a lower turnover rate which could lead to decreased length and under more severe deficiency affect the microtubule structure. Wild-type glutamylation levels in basal bodies and axonemes are required for slow turn-over and stability of these microtubules (Thazhath et al., 2004). When Ttll6Ap was overexpressed in *Tetrahymena*, it resulted in hyper-glutamylated axonemes that were short and defective with 9+0 structure. When these cells were treated with paclitaxel, a significant increase in the length of cilia was observed. Also, when Ttll6Ap-overproducing cells were treated with paclitaxel, increase in ciliary glutamylation levels was seen. This suggests that excessively long glutamyl chains may destabilize microtubules and increased glutamylation increases the turn-over rate of tubulin cilia. (Wloga et al., 2010). Thus, excessive or reduced polyglutamylation levels on microtubules could decrease the tubulin turn-over rate, leading to shorter and unstable microtubules. On the other hand, over-expression of inactive G-ligase, Ttll3p in *Tetrahymena*, resulted in shorter cilia with defective structure. These mutants not only have reduced glycylated ciliary microtubules but also hyperglutamylated axonemes. It is known that glycylation and glutamylation negatively regulate each other. Increase in inactive G-ligase appeared to induce hyperglutamylation which may have caused slower tubulin turnover or lattice destabilization leading to shorter axonemes (Wloga et al., 2009). It is likely that both excessive and insufficient tubulin turnover affect the axoneme length. Glutamylation may be regulating the tubulin turnover in these structures. Thus, in all cilia, motile and non-motile, tubulin glutamylation could regulate the turnover of tubulin, that is, the rates at which new subunits are incorporated and removed from the lattice. Perhaps, pulse chase

labeling studies in hypoglutamylated / hyperglutamylated mutant cells would shed light on the precise mechanism by which tubulin glutamylation regulates tubulin turnover rate (Song and Dentler, 2001; Stephens, 1999).

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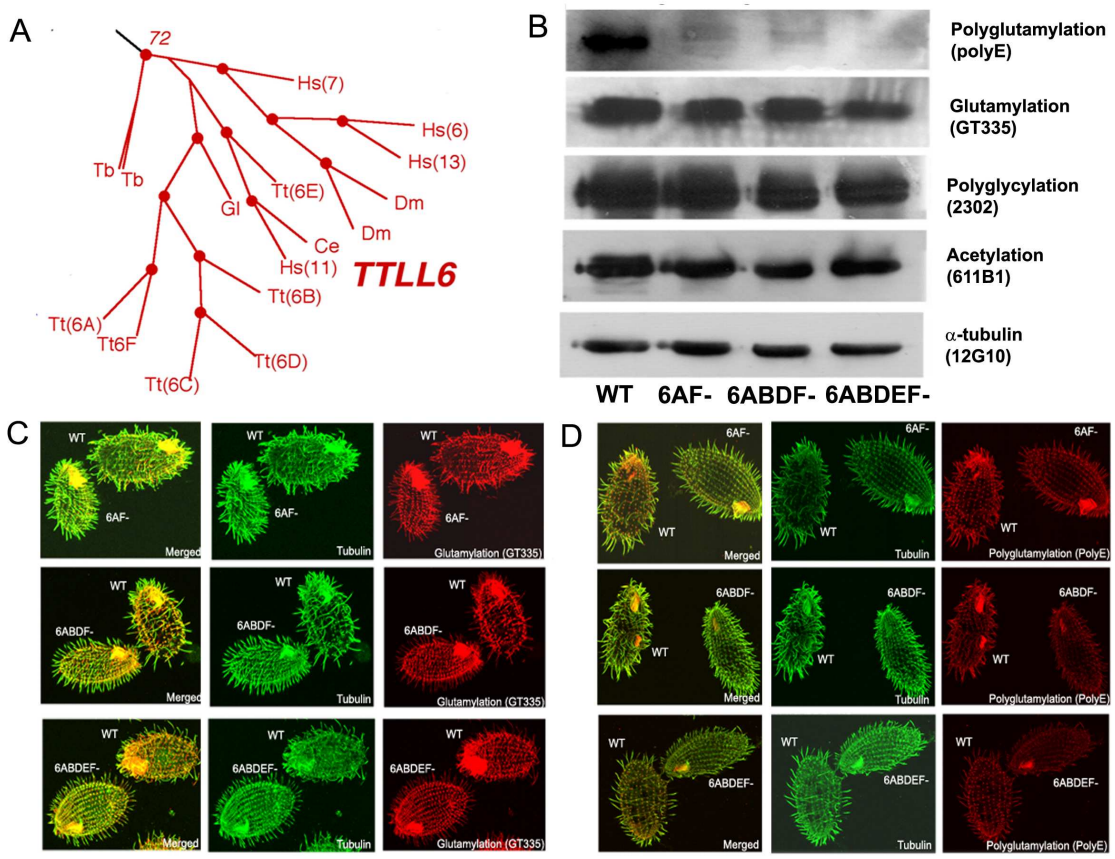

Figure 4.1: TTLL6 group consists of 6 proteins and their deletion leads to a major loss of tubulin glutamylation in cilia and basal bodies

(A) A phylogenetic tree based on the conserved catalytic domain of TTLL6 E-ligases from several model organisms using sequences listed in (Wloga, Rogowski et al. 2008). The TTLL6 subfamily in *Tetrahymena* has 6 paralogs. The following abbreviations of species are used: Hs, Homo sapiens; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; GI, *Giardia*; Tb, *Trypanosoma brucei* ; Tt, *Tetrahymena thermophila* (B) A western blot of ciliary proteins obtained from wild-type and knockout cells. Note that there is a strong reduction in the polyE signals in all the knockouts whereas the levels of GT335 are slightly lower in 6AF-KO and

6ABDF- KO cells and significantly lower in 6ABDEF-KO cells as compared to wild-type. (C-D) Confocal immunofluoroscence images of wild-type and 6AF-KO (top panel), wild-type and 6ABDF-KO (middle panel), wild-type and 6ABDEF-KO (lower panel) side by side. To distinguish wild-type cells from mutants, the WT cells were prefed with India ink to reveal dark food vacuoles. Cells were fed with (C) 12G10 anti-α-tubulin mAb and GT335 anti-glutamylated tubulin mAb or (D) with SG anti-total tubulin antibodies and polyE anti-polyglutamylation antibodies Note: There appears to be only a slight decrease in the GT335 signal in the knockout cells. A drastic reduction in the levels of glutamylated tubulin in the knockouts is seen; severe reduction in signal in quadruple and pentanal knockouts seen as compared to double knockouts. Tubulin is the loading control. No obvious changes seen in the acetylation and polyglycylation signals.

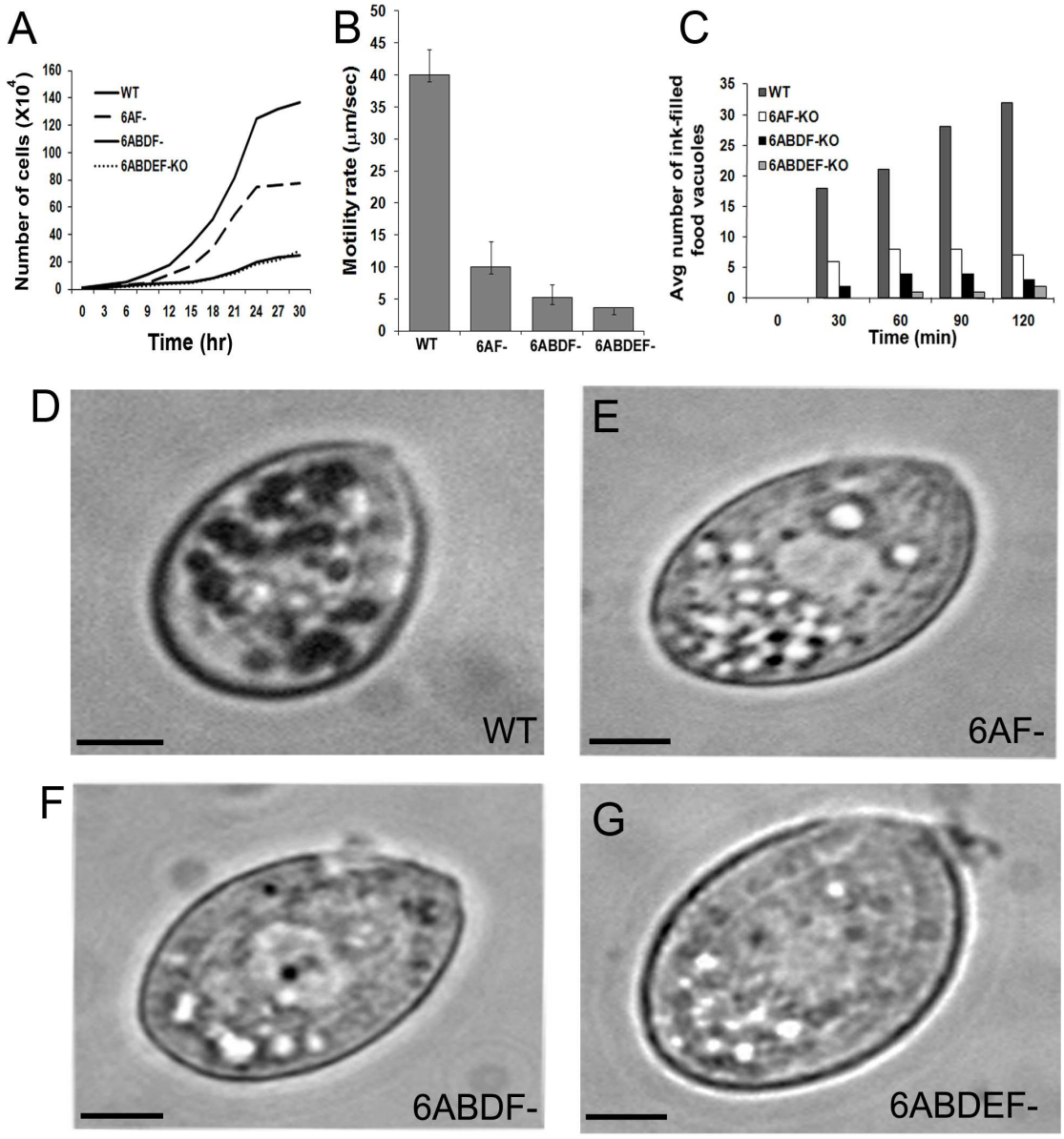

Figure 4.2: TTLL6 deficient cells display a loss of ciliary-dependent functions

(A) Culture growth curves for wild-type, double, quadruple and pentanal mutants. Quadruple and pentanal knockouts grow at similar rate that is much slower than that of wild-type cells (B) A histogram that shows the average linear cell motility rate for wild-type, 6AF-, 6ABDF- and 6ABDEF- cells (n = 50 for each strain). Paths of moving cells were measured for 5 sec. (C)

Histogram showing the rate of phagocytosis in WT and mutants. (D-G) Images of wild type (C), 6AF-KO (D), 6ABDF-KO (E) and 6ABDEF-KO (F) cells that were exposed to India ink in the culture medium for 60 min. TTLL6 mutants show few or no food vacuoles with ink particles as compared to several dark food vacuoles in wild-type cells. Scale bar corresponds to 20 μm.

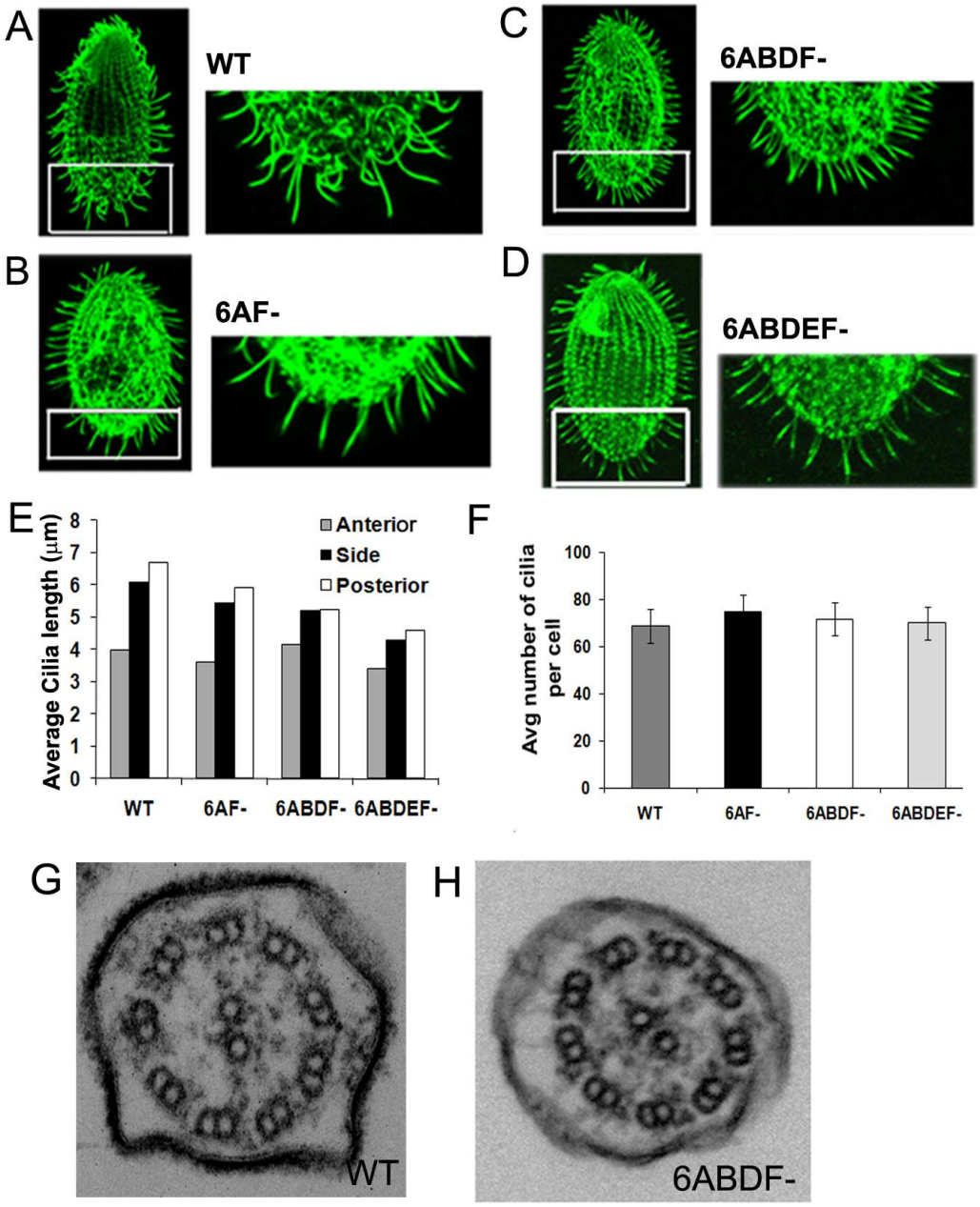

Figure 4.3: TTLL6 proteins affect axoneme length in a gene dose dependent manner.

(A-D) Immunofluoroscence images showing wild-type (A), and knockout cells labeled with a mixture of anti-tubulin antibodies (12G10 anti-α-tubulin and SG, anti-total tubulin). Insets mark regions of the posterior cortex that are shown at higher magnification. Note that most cilia in the

6AF-KO (B), 6ABDF-KO (C) and 6ABDEF-KO (D) cells are straight consistent with a defect in the waveform. In addition, the 6ABDF-KO and 6ABDEF-KO cells have significantly shorter cilia, especially in the posterior end. (E) A graph that compares the lengths of (side, anterior and posterior) cilia in wild type and knockout cells. Wild type side (n=120), anterior (n=80), and posterior cilia (n=60), 6AF-KO side (n=132), anterior (n=100), and posterior cilia (n=82), 6ABDF-KO side (n=110), anterior (n=65), and posterior cilia (n=62), 6ABDEF-KO side (n=100), anterior (n=60), and posterior cilia (n=50) were scored. (F) Histogram showing the average number of cilia in wild-type and mutant cells (n = 32 for each). (G)Cross sections of wild-type and (H) 6ABDF-KO axonemes show a normal 9+2 structure (60,000 X).

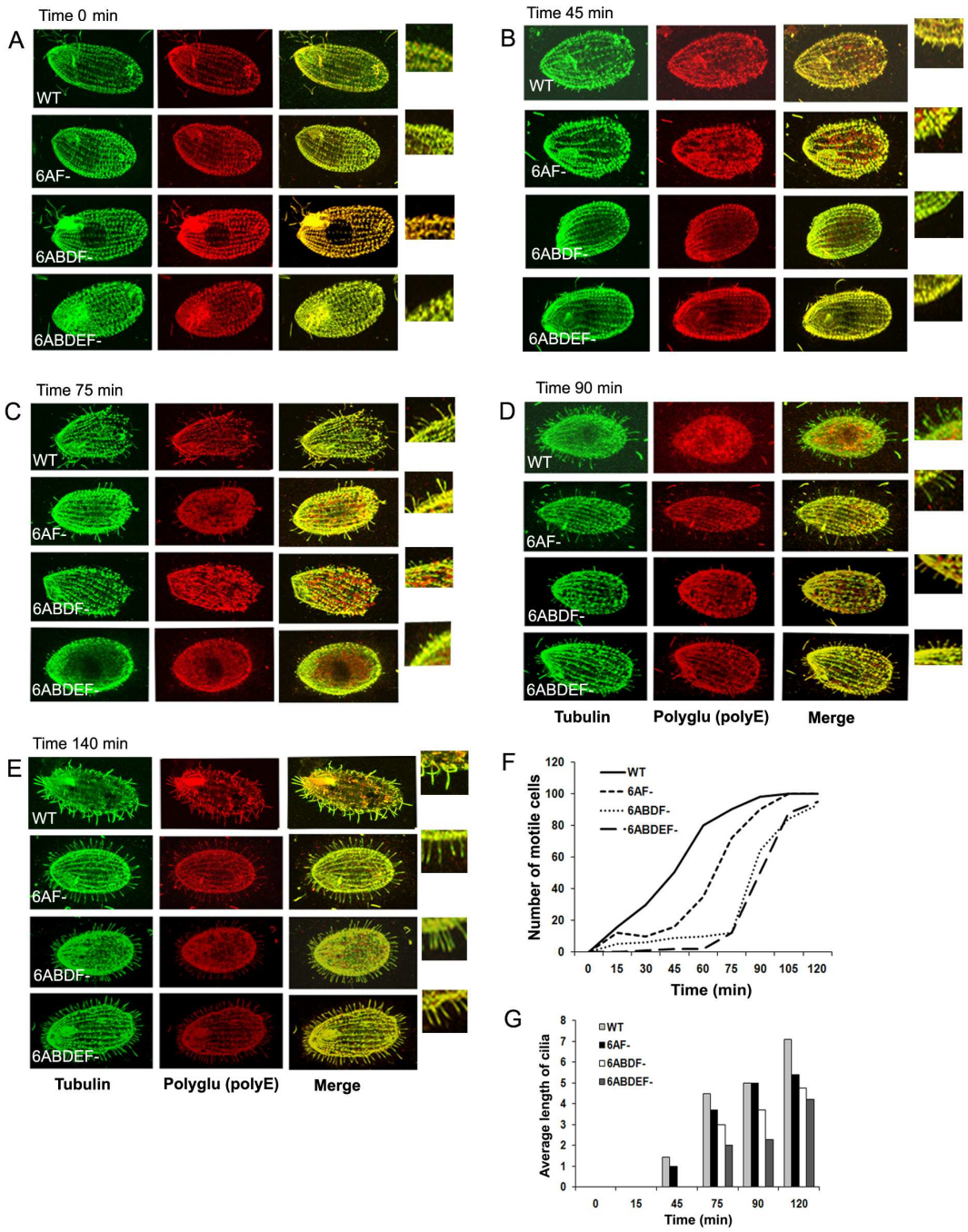

Figure 4.4: TTLL6 protein activity is required for normal cilia regeneration.

(A-E) Confocal images of wild-type and knockout cells labeled with mixture of anti-tubulin antibody (12G10) and anti-polyglutamylation antibody (polyE) immediately after deciliation, and at time points 15, 45, 75, 90 and 120 min, following deciliation. Insets mark regions with

regenerating cilia at higher magnification. All cells lack cilia at time 0 (A) and 15 min (not shown) after deciliation. Only wild-type and double mutants regenerate short cilia by 45 min after deciliation (B) whereas quadruple and mutant cells start regenerating short cilia only after 75 min (C-D). By 140 min, all strains regenerate cilia to the maximum lengths characteristic to each strain (E). (F) Graph exhibiting the time course of moving cells undergoing ciliary regeneration in different strains. (G) Histogram showing the average lengths of regenerated cilia in each strain at all time points (n=20 for all strains).

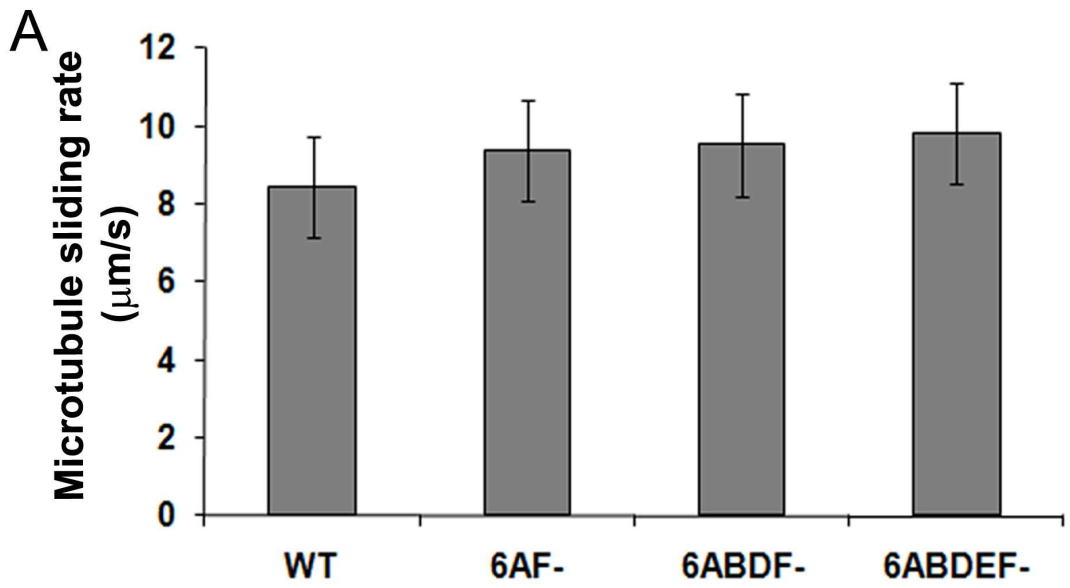

Figure 4.5: Average sliding velocity of axonemal microtubules in wild-type and TTLL6 deficient cells.

Purified axonemes were reactivated with 1mM ATP and their sliding velocity determined using dark-field video microscopy. (A) A graph that documents the average sliding velocity of wild type (n=38), 6AF-KO (n=37), 6ABDF-KO (n=37) and 6ABDEF-KO (n=37) axonemes. Error bars represent standard deviations. Data are composed of three independent experiments.

Table 4.1: Primers Used for Construction of Targeting Fragments.

Type of Gene	Primer sequence (5'-3')	Restriction sites	Restriction sites to release cassette and sites	Size of fragment	Type of cassette	TTL coding region replaced
TTLL6A	5'UTR-Forward ATATTGGGCCCGAGGAAGAT GATGATGAGA	ApaI SmaI	ApaI / SacI	1.5 Kb	pNeo3	926 bp
	5'UTR-Reverse ATAAACCCGGGGCTAAAGAA AACATACCAG			1.45 Kb		
	3'UTR-Forward AATTTACTAGTAGCCATGGGT TTTAGAAGT	SpeI				
	3'UTR-Reverse ATTATGAGCTCCTTTTGGAAG TAATGTCAG	SacI				
TTLL6B	5'UTR-Forward ATATTCCGCGGATTTATGAGT TGGCTGCTA	SacII	SacII / ApaI	1.54 Kb	p4B2-1	804 bp
	5'UTR-Reverse ATATTCCCGGGGCGCAAATC ATCTTCGTTA	SmaI		1.56		

	3’UTR-Forward TTATT ATC GAT ATT CCA TTG TGA TTC ACC AT	ClaI		Kb		
	3’UTR-Reverse ATTATGGGCCCATTGAGGAA GATTGAGGTC	ApaI				
TTLL6D	5’UTR-Forward ATTAAGGGCCCGATTCAAAG CTAGACTTCCAA	ApaI	ApaI / SacI	1.5Kb 1.9Kb	pNeo3	876 bp
	5’UTR-Reverse ATATTCCCGGGTAAGGCCATC TATGAGAAATG	SmaI				
	3’UTR-Forward ATTTAGTAGACCATTCCATTG TGATTCACCAT	AccI				
	3’UTR-Reverse TAAATGAGCTCGGTTAATGC AACTCTTACCTA	SacI				
TTLL6F	5’UTR-Forward ATATTGGGCCCGAGCTAATC AAACATACGA	ApaI	ApaI / SacII	1.47 Kb	pM-rpl-B	1.6 Kb
	5’UTR-Reverse ATTATATCGATTTCCTAGCTA TTCTGGTTA	ClaI				

	3'UTR-Forward ATTAT ATC GAT CAA GGT ATC AAT TCA TCA AT	ClaI		1.44 Kb		
	3'UTR-Reverse ATATTCCGCGGGGCTACAAA TAAAGTCCAT	SacII				
TTLL6E	5'UTR-Forward ATTAT GGG CCC TGA ATT AGA AGC GTT GGT TTC	ApaI	ApaI/SacI		pNeo3	120 bp
	5'UTR-Reverse ATATA CCC GGG C AAT AAG AGG ATC AAG AGA AC	SmaI				
	3'UTR-Forward ATTAA GTA GAC C AAG GTA TCA ATT CAT CAA TCA	AccI				
	3'UTR-Reverse AAAAT GAG CTC T GTT GCA CAT AAA ACT AAC TTA	SacI				

Table 4.2: Diagnostic Primers Used for Verification of Gene Disruptions (Amplify Deleted Regions)

Type of Gene	**Primers used to confirm presence of knockout**
TTLL6A	Forward primer 5'-TATCTTTTGGACTGATAATGCT-3' Reverse primer 5'-CTCTTAATATCTTTCCACAG-3'
TTLL6B	Forward primer 5'-ATTAATCATTTCCCAGGTTCT-3' Reverse primer 5'-ACGTTATCTTAGTTGGCATTT-3'
TTLL6D	Forward primer 5'- ATATTTATGTATCCTGAAGG-3' Reverse primer 5'-CTAAAACTTCAAAACACATT-3'
TTLL6F	Forward primer 5'-AGATCTCTAAAGGAAAATGC-3' Reverse primer 5'-TTCATGTAGTTATCTGGTTG-3'
TTLL6E	Forward primer 5' ATATTTATGTATCCTGAAGG-3' Reverse primer 5' ACGTTATCTTAGTTGGCATTT-3'

CHAPTER 5

CONCLUSIONS

The focus of my research was to identify the function of TTLL6-type tubulin E-ligases using *Tetrahymena thermophila* as a model. The two novel findings that I have made include 1) polyglutamylation by TTLL6 E-ligases regulates axonemal dynein and 2) polyglutamylation by TTLL6 E-ligases regulates the length of ciliary axonemes. Glutamylation is a post-translational modification on the CTT of tubulin that involves initiation and elongation of glutamate side-chains. During initiation, the first glutamate is linked by an amide bond between it's α amino group and the γ carboxyl group of the modified glutamate residue in the primary peptide backbone of the protein. The elongation step involves addition of glutamate units that are linked by isopeptidic bonds, thus leading to the formation of a linear side chain of glutamate residues (Alexander et al., 1991; Redeker et al., 1991; Wolff et al., 1994).

Until 2005, due to difficulties in purification, the enzymes responsible for tubulin PTMs remained unidentified, which made it difficult to study the significance and mechanism of PTMs. Just before I started working on my project, tubulin E-ligases were discovered (Janke et al., 2005). Bernard Eddé and colleages were able to purify tubulin E-ligase from mouse brain extract using monoclonal antibodies generated against the partially purified enzyme that blocked its activity. They found this enzyme to be a complex of five subunits (p24/PGs5, p32/PGs1, p33/PGs2, p49/PGs3 and p79/PGs4), out of which PGs3 was found to be an orthologue of human protein TTLL1 (Janke et al., 2005). The peptide sequence of TTLL1 shares 17% identity

with that of TTL, an enzyme that catalyzes ligation of tyrosine to C-terminal glutamate of deytrosinated α-tubulin (Ersfeld et al., 1993). Just like tyrosination involves addition of tyrosine residue to tubulin primary sequence, glutamylation involves addition of another residue, glutamic acid. Thus, there was a strong suspicion that TTLL1 is the enzyme responsible for glutamylation. However, in spite of several efforts, a recombinant murine TTLL1 did not produce any tubulin glutamylase activity when expressed in prokaryotic and eukaryotic systems such as *E.coli*, insect and mammalian cell lines (Janke et al., 2005). All this work was being done in collaboration with our lab. Subsequent genomic analyses in *Tetrahymena* revealed that out of the five subunits of the murine brain E-ligase complex, only one, TTLL1, has homologs in *Tetrahymena*. When an N-terminal GFP or C-terminal HA fusion of Ttll1p (*Tetrahymena* TTLL1) was over-expressed in *Tetrahymena,* it localized to basal bodies, contractile vacuole pores and oral deep fibers (all microtubule structures), but its overexpression did not increase the levels of tubulin glutamylation and the phenotype remained normal (Janke et al., 2005). Even *in vitro* glutamylation activity in cell extracts of over-expressed cells did not show any increase (Janke et al., 2005). The failure to increase tubulin glutamylation levels in *Tetrahymena* could be because of Ttll1p, like murine TTLL1 orthologue, could be a part of a multi-subunit complex, with other subunits required for catalytic activity. However, eliminating *TTLL1* gene resulted in the reduction of tubulin glutamylation in basal bodies (Janke et al., 2005; Wloga et al., 2008). Using anti-GFP antibodies, in immunoprecipitation assay of cell extracts from over-expressed cells, showed α-tubulin-preferring primarily chain-initiating E-ligase activity (Wloga et al., 2008).

Later, using human TTLL1 protein sequence as query, phylogenetic analyses showed the presence of additional TTLL genes in *Tetrahymena* and other organisms. The analyses revealed presence of 50 *Tetrahymena* TTLL proteins including Ttll1p. The presence of so many TTLL proteins maybe required for regulating the assembly and functions of diverse MT structures in *Tetrahymena*. Thus, it was reasonable to speculate that *Tetrahymena* could have several more tubulin E-ligases, including α-tubulin and β-tubulin E-ligases, belonging to the TTLL family. Among the TTLL family of proteins, *Tetrahymena* has 6 paralogous genes belonging to TTLL6 group of proteins, namely, Ttll6Ap, through Ttll6Fp. Most organisms have TTLL6 proteins including most, if not all, ciliated species (Janke et al., 2005). When Ttll6Ap-GFP was overexpressed in *Tetrahymena,* it localized mainly to cilia and little extent to basal bodies and cell body MTs (Janke et al., 2005). There was a striking increase in levels of polyglutamylation of β-tubulin *in vivo* as well as *in vitro*. The phenotype was dramatic as the Ttll6Ap-overexpressed cells displayed loss of movement as well as cessation of growth (Janke et al., 2005). We wanted to know if the increase in tubulin glutamylation levels was a direct result of the catalytic activity of Ttll6Ap or an indirect effect of Ttll6Ap as an adapter for some other protein. This is where my work began, when I created a mutant version of Ttll6Ap carrying a substitution of highly conserved E residue to G in the TTL-like catalytic domain. On over-expressing the inactive version of Ttll6Ap, we observed no difference in the glutamylation levels of tubulin or in the growth and motility patterns, indicating that it was indeed Ttll6Ap that was capable of catalyzing polyglutamylase activity of tubulin autonomously.

We were excited to observe that hyperglutamylation of ciliary MTs in Ttll6Ap over-expressed *Tetrahymena* cells resulted in inhibition of ciliary beating. This study for the first time reflected the significance of tubulin glutamylation in regulating MT functions in cilia. We had a

strong suspicion that glutamylation could regulate dynein-based ciliary motility due to the ciliary paralysis observed in Ttll6Ap over-expressed cells. These studies encouraged me to investigate the role of Ttll6Ap in depth (Chapter 3).

Previous studies showed that a number of E-ligases display primarily either a chain initiation or chain elongation activity when expressed in a eukaryotic host such as mammalian cells or *Tetrahymena* (van Dijk et al., 2007). Ttll6Ap is unique in that in *in vitro* assays, this enzyme showed an exclusive elongase activity not only when expressed and partially purified from *Tetrahymena* (this study) but also when produced in *E.coli* (van Dijk et al., 2007). Biochemical characterization of Ttll6Ap showed us that Ttll6Ap can only catalyze elongation activity of glutamyl side chains of polymerized MTs which have pre-existing glutamylation.

To evaluate the significance of TTLL6 type enzymes on tubulin, I decided to create a strain that lacked *TTLL6A* gene. To our dismay, *TTLL6A-KO* cells appeared to have no obvious mutant phenotype. *TTLL6F* is a closely related paralog of *TTLL6A*. So I decided to make a single knockout of *TTLL6F* and again the *TTLL6F-KO* cells seemed to have a normal phenotype. As Ttll6Ap and Ttll6Fp are closely related, there was a strong possibility of them being functionally redundant. So I made a strain that lacked *TTLL6A* as well as *TTLL6F* genes. We were very excited to see that the double knockout cells moved and multiplied extremely slowly as compared to wild-type cells. These mutants also lack ciliary reversals, have a defective ciliary waveform and reduced beat frequency. Biochemical analyses showed drastic reduction in levels of elongated side chains of cilia and basal bodies, indicating that Ttll6Ap and Ttll6Fp act synergistically and that in vivo their activity is primarily in chain elongation (consistent with *in vitro* assays). Loss in ciliary motility was to some extent unexpected because glutamylated tubulin is present in both motile (Bre et al., 1994) and non-motile axonemes (Bobinnec et al.,

2000). We know that tubulin glutamylation competes with tubulin glycylation, as these two PTMs utilize the same or adjacent sites on CTTs (Wloga et al., 2010; Wloga et al., 2009). However, the 6AF-KO cilia have normal levels of tubulin glycylation in ciliary MTs. The simplest explanation is that the loss of Ttll6Ap and Ttll6Fp has shortened the side chains due to lack of elongation but that the initiation step is still in place, and the single glutamic acid in the side chain is sufficient to inhibit competing glycylation, This is important, as it is unlikely that the deficiency in ciliary motility observed in the 6AF-KO cells is caused by excessive tubulin glycylation.

Since the normal cilia function was disturbed, it was possible that the reason for defective function was an abnormal axonemal structure. However to our surprise, the TEM analysis of mutant cilia revealed an apparently normal 9+2 structure. Other studies have shown that cilia with defective functions could have a normal 9+2 structure. *Chlamydomonas* mutants lacking ortholog of human TTLL9 E-ligase, show defective flagellar motility but also have normal axonemal structure (Kubo et al., 2010). Also, in *TTLL1*-null mice, the respiratory tract cilia do not function normally, but seem to have a normal axonemal organization despite of a very strong loss of tubulin glutamylation (Ikegami et al., 2010). The only exception is sperm axoneme, that is severely disorganized in *TTLL1* mutants in mice (Vogel et al., 2010). Thus, we can conclude that so far the axoneme organization in most contexts is not affected by severe loss of tubulin glutamylation, but ciliary motility and length are.

The fact that overproduction (Janke et al., 2005) as well as absence of Ttll6Ap (Suryavanshi et al., 2010) leads to abnormal ciliary motility, made us speculate that tubulin glutamylation could regulate ciliary dynein. The reduction in the beat frequency is consistent with an ODA deficiency (Kamiya, 2002) but some IDA mutants also have slightly reduced beat

frequency (Brokaw and Kamiya, 1987). On the other hand, the altered waveform and lack of ciliary reversal are consistent with IDA defects (Ashizawa et al., 1994; Hennessey et al., 2002; Wood et al., 2007). The double 6AF-KO mutants displayed reduced beat frequency, lack of ciliary reversal as well as altered waveform opening up the possibility that lack of glutamylation could affect ODAs or IDAs or both. To investigate this matter further, we conducted MT sliding assays on wild-type and mutant axonemes. When isolated axonemes are exposed to ATP, activation of ciliary dynein motors causes an unrestricted sliding of outer doublet MTs (rather than reactivated bending), that leads to axoneme disintegration (Satir, 1985; Summers and Gibbons, 1971). Thus, the ATP-induced sliding of axonemal MTs can be used to assay the activity of ciliary dynein *in situ*. With 1 mM ATP, MTs underwent sliding at similar rate in wild-type and 6AF-KO axonemes. To test how the TTLL6-mediated tubulin glutamylation affects the MT sliding generated specifically by IDAs, we assayed the MT sliding in axonemes from wild-type, conditional mutants *oda1-1* that lack outer dynein arms at restrictive temperature, 6AF-KO and 6AF-KO;*oda1-1* cells. Unexpectedly and to our surprise, the 6AF-KO;*oda1-1* axonemes showed a nearly two-fold increase in the rate of MT sliding as compared to *oda1-1* axonemes.

The simplest explanation of our observations is that tubulin glutamylation affects IDA (but not ODA) activity by modifying its track on the B-tubule. Additional observations that we have made here reveal a strong link between IDAs and tubulin glutamylation. Firstly, the 6AF-KO cells do not produce ciliary reversals, a phenotype that was earlier reported for an IDA mutation (Hennessey et al., 2002). Secondly, axoneme reactivation experiments indicate IDAs being affected. We show that in an axoneme with a normal dynein arm composition, TTLL6 enzymes have little effect on the rate of MT sliding *in vitro*. Under these conditions, the rate of

sliding is primarily determined by ODAs. Strikingly, in axonemes depleted in ODAs (*oda1-1*), deletion of TTLL6 enzymes led to an increase in the rate of MT sliding.

How is it that doublet MTs of mutants missing ODAs and reduced glutamylated tubulin slide faster than MTs of mutants missing ODAs with normal level of tubulin glutamylation? Sliding of MTs measures ability of dynein molecules to move on MTs. In intact axonemes, under restriction of nexin-links and basal bodies, this linear sliding of MTs is converted to ciliary bend. ODAs and IDAs have distinct functional properties (reviewed in (Kamiya, 2002)). For example, in *Tetrahymena,* the 22S fraction of dynein salt extract (ODAs) produces a linear gliding of MTs at the rate of 8 μm/s, while the 14S fraction (IDAs) produces a motility at the rate of 4 μm/s associated with MT rotation (Vale and Toyoshima, 1988). The duty ratio is defined as the ratio of time spent by motor attached to its MT track and total ATPase cycle time. Thus, processive motors reflect higher duty ratio. Two IDA subtypes that were studied in *Chlamydomonas* (dynein c and f/I1) are high duty ratio motors that display processive movements along MTs (Kotani et al., 2007; Sakakibara et al., 1999), a quite unexpected property for a filament sliding motor (e.g. in the contracting muscle, myosin motors are non processive (Taft et al., 2008)). Kotani and colleagues proposed that in the bending cilium, IDAs impose a drag on MTs that are pushed strongly by ODAs and increase the axoneme curvature (Kotani et al., 2007). We can speculate that tubulin glutamylation is required for the high duty ratio motility generated by IDAs. Bending of cilia in one direction may require the action of some faster moving dyneins (such as ODA) and some 'restraining' dyneins to keep in control the excessive force of fast dyneins. Thus, restraining dyneins could be a major factor in causing increased curvature of cilia. Glutamylation on α- or β-tubulin of B-tubule could serve as 'glue' on the B-tubule to increase the duty ratio and hence the proccessivity of these restraining IDAs. It is known that multiple non-processive motors slide

filaments faster as compared to processive motors (Higuchi and Endow, 2002). A group of non-processive motors acting on a single filament can be compared to a group of humans pulling a rope in a tug-of-war game. Thus, in the absence of glutamylation, IDAs may become less processive and as a result produce a more robust net force on doublet MTs. Regardless of the mechanism, we show that tubulin glutamylation has a strong restraining effect on the MT sliding velocity generated specifically generated by IDAs. Further insights into the mechanism of regulation of dynein by tubulin glutamylation can be obtained in the future in single molecule assays. These assays would require highly purified dynein arms and MTs with different levels of tubulin glutamylation. Such polymodified MTs could perhaps be prepared by *in vitro* glutamylation by combining purified TTLL6 enzyme and hypoglutamylated TTLL6-KO axonemes (Gennerich et al., 2007).

Although, we have shown that tubulin glutamylation affects ciliary dynein, we cannot exclude a possibility that this PTM affects non-motor components that interact with the B-tubule. Nexin bridges connect the A- and B-tubule and are believed to act as elastic links that promote axoneme bending (Goodenough, 1989; Nicastro et al., 2006; Witman et al., 1978). Also, electron-dense structures are present inside the lumen of the B-tubule (Nicastro et al., 2006). While the TTLL6-deficient axonemes appear normal in standard TEM, a subtle structural defect of either the surface or luminal feature could be revealed in the future by electron tomography (Nicastro et al., 2006; Sui and Downing, 2006). It is also possible that polyglutamylation may affect the assembly of one of the classes of inner dynein arms, which would be challenging to discern at the level of TEM resolution, owing to high density of inner dynein arms on A-tubule. However, studies by Kubo and colleagues have shown that *Chlamydomonas* mutants lacking TTLL9-dependent glutamylation on α-tubulin were strongly defective in flagellar motility but ion exchange

chromatography analysis revealed normal composition of inner dynein arm subspecies (Kubo et al., 2010). Also our own 2-D gel studies reflect that any protein differences, if any, are minor (D. Malison, S. Suryavanshi, J. Gaertig unpublished data). Thus, structural changes in the TTLL6AF-KO mutants are unlikely.

Dynein is unusual among motor proteins because its motor domain contacts the MT track via a stalk domain (Gee et al., 1997). Recent studies indicate that the stalk acts as a tether that allows for pulling of parts of the dynein molecule toward the MT during the power stroke (Carter et al., 2008; Ueno et al., 2008). It is tempting to speculate that tubulin glutamylation regulates the affinity of the dynein stalk to the B-tubule.

Since we have demonstrated that tubulin polyglutamylation regulates activity of inner dynein arm, the future goal would be to explore the precise mechanism of regulation as well as find out the precise inner dynein arm subspecies in *Tetrahymena* that is regulated by glutamylation. *Tetrahymena* has a complex composition of IDA species with at least 8 subtypes, containing processive double headed and non-processive single headed dyneins (Angus et al., 2001; Xu et al., 1999). It is possible that, due to distinct intrinsic properties of dynein molecules, various subspecies of IDAs may respond differently to polyglutamylation. Differentiating between subspecies of IDAs that are modulated by this PTM would mainly involve two methods of exploration. We may be able to answer this question by making double mutants that lack elongating E-ligase as well as double-headed or single-headed IDA subspecies. If mutants lacking TTLL6A and double-headed IDA show higher MT sliding rate as compared to mutants lacking only 2-headed IDA, then it would mean that glutamylation usually restricts activity of 1-headed IDA sub-species. This approach may be more difficult and time consuming as *Tetrahymena* has several double- headed and single-headed dyneins. Constructing knockouts for

each of them and crossing them with glutamylation lacking strains to make double knockouts could be arduous. Alternatively, we may be able to obtain important information by screening the functional properties for each of the IDA subspecies in an *in vitro* motility assay (Kotani et al., 2007). Performing this assay would need purified IDA subspecies from *Tetrahymena* and hypo-glutamylated MTs purified from mutant TTLL6A-KO. *In vitro* assays would be less time-consuming and more precise in answering our questions.

As mentioned previously, strains lacking two TTLL6 paralogs, TTLL6A and TTLL6F, (Chapter 3) and strains lacking five TTLL6 proteins TTLL6A/B/D/E and F (Chapter 4) in *Tetrahymena* resulted in inflexible cilia that lacked curvature, altered waveform and reduced beat frequency. Ciliary beat is asymmetric and consists of power stroke and recovery stroke. In the absence of asymmetric ciliary beat, the cilia cannot function normally. In mice lacking Ttll1p, the tracheal cilia of mice lose axonemal curvature (become straight), as a result of which, there is accumulation of mucus in nasal cavity due to loss of ciliary function (Ikegami et al., 2010; Vogel et al., 2010). We have also observed that *Tetrahymena* TTLL6 double and multiple KOs have excessively straight axonemes (Chapter 3). Ikegami and colleagues suggest that tubulin glutamylation is required for the structural curvature of ciliary axonemes that generates asymmetrical ciliary beat (Ikegami et al., 2010). Although not spelled out directly, Ikegami model appears to suggest a role for tubulin glutamylation in MT curvature that does not require dynein activity. The speculation that tubulin glutamylation in cilia may majorly contribute to the microtubule curvature is strengthened by the study, in which immotile human airway epithelial cilia that lacked outer dynein arm had normal structural curvature (Fliegauf et al., 2007). In another study, mice that were deficient in ODAs and some IDAs had immotile cilia but normal axonemal curvature (Omran et al., 2008). In the future it should be possible to determine whether

the level of glutamylation affects the curvuture of microtubules assembled *in vitro* from pure tubulin. It is possible that tubulin polyglutamylation affects axoneme curvature directly and via regulation of IDAs. Tubulin glutamylation could curve axonemes in a relaxed state (without dynein bound to MTs) which could indeed give bias to the direction of power stroke (Ikegami et al., 2010). However, having polyglutamylation could enhance the curvature of axoneme produced by the 'pushing' of restraining IDA on MTs. Thus, both the models: restraining IDA causes bending of cilia, and tubulin glutamylation is an axoneme curvature-determinant, could be correct but the mechanisms may be different at both stages. The reason for glutamylation regulating asymmetric cilia beat could be the asymmetric distribution of this modification with axonemes. In mice sperm flagella, glutamylated tubulin is mostly found on outer doublets 1(positioned on the side of recovery stroke), 5 and 6 (positioned on the side of power stroke) (Fouquet et al., 1994). Also, labeling of axonemes with anti-polyglutamylated tubulin antibodies in *Chlamydomonas* and *Tetrahymena* cells reveals an asymmetric pattern. The gradient of polyglutamylation is proximo-distal with maximum labeling at the base and tips of cilia (Suryavanshi, unpublished data).

In the second part of my thesis, I have explored the significance of other TTLL6 paralogs in *Tetrahymena* (Chapter 4). As mentioned before, TTLL6 family of proteins consist of 6 proteins, namely *TTLL6A* through *F*. We knew that Ttll6Ap localizes to cilia (Janke 2005) and catalyzes polyglutamylation of β-tubulin of ciliary MTs along with its close paralog Ttll6F (Chapter 3). Earlier studies have suggested a role of polyglutamylation in assembly and maintenance of axonemal MTs (Ikegami et al., 2007; Pathak et al., 2007; Wloga et al., 2010). There is a functional redundancy between Ttll6A and 6F proteins. We had a strong suspicion that their other related paralogs, namely *TTLL6B, TTLL6C, TTLL6D* and *TTLL6E* may be functionally redundant. To test this hypothesis, I first made single knockouts of each of these by

eliminating additional TTLL6 paralogs such as *TTLL6B, TTLL6C, TTLL6D* and *TTLL6E*. Knockouts of these genes were made by replacing approximately 800 bp of the conserved TTL region by drug resistance cassettes (*bsr* cassette for 6B, *neo3* for 6C, 6D and 6E). Disappointingly, none of the single knockouts gave us any obvious mutant phenotype. So, using standard crosses, I created strains lacking 4 or 5 of the TTLL6 genes, namely, TTLL6ABDF-KO and TTLL6ABDEF-KO. Owing to some technical difficulties, we were not successful in making a strain that lacked all of the 6 paralogs. Expectedly, the quadruple and quintuple knockouts exhibited a severe mutant phenotype in which these cells grow and multiply very slowly. Western blot and immunofluoroscence assays of both mutants revealed even more drastic reduction in levels of elongated side chains than that in double mutants. Moreover, ciliary lengths (especially posterior cilia) of quadruple and quintuple mutants were short by 22% and 32%. This was a significant reduction in cilia lengths induced by lack of glutamylases.

Next we wanted to determine if the mutant cilia were assembled short or become short due to lack of stable axonemal MTs. In other words, we wanted to know if polyglutamylation of tubulin affects the assembly of axonemes, its rate of elongation or maintenance of stable MTs. For this purpose, I deciliated the mutant cells by pH shock method and allowed them to regenerate. We were excited to see that quadruple and quintuple mutants showed delayed growth of ciliary stubs as compared to wild-type cells. These data indicate that the rate of axoneme assembly, most likely the rate of IFT as the major underling mechanism, is reduced in the TTLL6 mutants.

Thus, we show that in a dose dependent manner, tubulin glutamylation mediated by TTLL6 is required for normal length of the axoneme. Recently (Pathak et al., 2007) showed that a morpholino knockdown of TTLL6 in zebrafish led to shortening of olfactory cilia. *TTLL1*-null

mice have short and defective sperm axonemes (Ikegami et al., 2010; Vogel et al., 2010). I would like to propose two models to explain the possible reasons for short and slow growing cilia. According to the first model, tubulin polyglutamylation may regulate intra flagellar transport (IFT). Defects in motor proteins, IFT proteins or reduction in frequency or rate of IFT leads to shorter lengths of cilia (Kozminski, 1995; Pedersen et al., 2005; Piperno et al., 1998; Porter et al., 1999). IFT seems to occur on the B-tubule of outer doublet (Kozminski et al., 1993; Pigino et al., 2009; Redeker et al., 2005). β-tubulin glutamylation on B-tubule, contributed by TTLL6 proteins, may serve as a track for the movement of anterograde IFT motor kinesin-2. Already there is evidence that motor proteins can be affected by tubulin glutamylation. Kinesin-2 is the anterograde motor that carries cargo, in the form of tubulin subunits and other precursors required for ciliary assembly, from basal body towards the distal tip of cilia (Scholey, 2008). Any defect in the anterograde transport would lead to shorter cilia (Brown et al., 1999). Kinesins contain conserved positively charged basic residues and they interact with negatively charged carboxyl terminus of tubulin that is enriched with PTMs. Site directed mutagenesis studies of basic residues of kinesin have demonstrated that processivity of kinesins is influenced by the electrostatic association between kinesin neck region and CTT (Westermann and Weber, 2003). *In vitro* assays have shown that kinesin shows strongest affinity for MTs that contain polyglutamate chain of 3 residues in length (Larcher et al., 1996). In the absence of B-tubule polyglutamylation contributed by TTLL6 proteins, kinesin may fail to be processive due to loss of interaction with highly negatively charged CTTs, leading to slower IFT. Polyglutamylation of α- tubulin contributed by PGs1, non-catalytic subunit of *TTLL1*, is required for regulating entry of KIF1 kinesin-3 into neurites as the long positively charged K loop of kinesin (Okada and Hirokawa, 1999) can strongly interact with highly negatively charged CTT containing

polyglutamylation (Okada and Hirokawa, 2000).

According to the second model, polyglutamylated tubulin could regulate tubulin turnover subunits in growing axoneme. Turnover of axonemes due to polymerization and depolymerization events of ciliary MTs could play a role in regulation of axonemal lengths (reviewed in (Gaertig, 2008)). Thus, a shortened axoneme could result from slow turnover of tubulin subunits within the axoneme. Tubulin CTTs that are enriched with PTMs are known to strongly interact with MT-severing factors such as katanin and spastin, (McNally and Vale, 1993; Roll-Mecak and Vale, 2008). Importantly, mutations in katanin subunits led to short axonemes with 9+0 structure in *Tetrahymena* (Sharma et al., 2007) and *Chlamydomonas* (Dymek et al., 2004). In both species, katanin subunits were localized to the outer doublet MTs (Dymek et al., 2004; Sharma et al., 2007). These observations indicate that activity of katanin is required for certain level of tubulin turnover inside cilia to facilitate the assembly and elongation of axonemes. Without either katanin or tubulin polyglutamylation, axonemes could have a lower turnover rate, which could lead to decreased length and under more severe deficiency affect the MT structure.

To have a better insight into the possible mechanism of glutamylation modulated tubulin turn-over, pulse chase labeling studies in hypoglutamylated / hyperglutamylated mutant cells would shed light on the precise mechanism by which tubulin glutamylation regulates tubulin turnover rate (Song and Dentler, 2001; Stephens, 1999). Also, studying the localization patterns of each of these paralogs, (except Ttll6Ap whose localization (ciliary) has already been studied) will give us additional information about their sites of action and mechanism.

Recently, reversible enzyme involved in glutamylation, deglutamylase has been discovered in *C.elegans* and mice (Kimura et al., 2010). According to the authors, over-

expression of cytosolic carboxypeptidase CCPP-6 in *C.elegans* and *CCP-5*, a functional homologue of nematode *CCPP-6* in mice, results in decrease in tubulin glutamylation levels of sensory cilia tubulin and cortical neurons, respectively. Perhaps, deleting one of the *CCPP-6* homologue in *Tetrahymena* could rescue TTLL6-knockout phenotypes. It is possible that in absence of deglutamylases, loss of tubulin glutamylation contributed by TTLL6 proteins in TTLL6-knockouts could be replaced, to some extent, by other unidentified β-tubulin E-ligases. This substituted glutamylation could be removed by deglutamylases leading to severe mutant phenotype. In *Tetrahymena,* if we delete a major deglutamylase in the background of our mutants, then the glutamylation by other TTLLs maybe able to rescue the mutant phenotype created by TTLL6-knockouts.

Thus in conclusion, glutamylation of tubulin seems to be critical to the assembly, maintenance of structure and functions of cilia.

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