SIGNIFICANCE AND REGULATION OF MICROTUBULE-SEVERING *IN VIVO*

by

NEERAJ SHARMA

(Under the Direction of JACEK GAERTIG)

ABSTRACT

Katanin is a microtubule-severing protein complex that oligomerizes on the surface of microtubules and disassembles microtubules by inducing an ATP hydrolysis-dependent conformational change in the polymer lattice. Katanin is conserved from unicellular to multicellular eukaryotic organisms. It is a heterodimer consisting of p60, a severing subunit and p80, a non-catalytic subunit. Spastin is another related microtubule-severing protein involved in human diseases known as spastic paraplegia. It is unknown how katanin and spastin-mediated disassembly of microtubules is regulated *in vivo* to ensure that specific microtubules undergo severing at correct locations. In addition, the significance of microtubule severing is not well understood. To understand the functional significance of microtubule severing in the context of several types of microtubular organelles, the ciliate *Tetrahymena thermophila* was used in the present study. We engineered strains of *Tetrahymena* completely lacking katanin and spastin subunits. We show that katanin is essential for the cytokinesis and assembly of cilia. However, spastin is not essential for cell viability. Absence of katanin-mediated severing activity inhibited the assembly of the central pair and elongation of peripheral doublet microtubules in cilia. Loss of katanin also resulted in excessive polymerization and stability of cortical and cytoplasmic microtubules. We thus show that the function of katanin depends on the microtubule type and

subcellular environment. In the cell body, katanin reduces the mass and destabilizes the internal network of microtubules, whereas, it is required for the assembly of central pair as well as doublet microtubules inside the cilia. An overproduction of katanin disassembles ciliary and basal body microtubules in a non-random manner. Katanin preferentially severed polymodified (polyglutamylated and polyglycylated) microtubules in the axoneme, when overproduced. We also show that katanin regulates the levels of post-translation modifications on different types of microtubules. Previously, our laboratory showed that the polymodification sites present in β-tubulin are required for cytokinesis and assembly of cilia. We now show that the lack of katanin function gives the same phenotype as the one resulting from polymodification sites mutations. In the light of our results, we propose that post-translationally modifications of microtubules act as marks to guide the function of katanin to ensure spatial control of katanin activity.

INDEX WORDS: Katanin, Spastin, Ciliary assembly, Microtubules, Posttranslational tubulin modifications, Central pair

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CHAPTER 1

INTRODUCTION

Microtubules (MTs) are ubiquitous cytoskeletal elements that play essential roles in eukaryotic organisms. They are built by the self-association of α- and β-tubulin heterodimers in a head-to tail fashion leading to the formation of protofilaments of defined polarity. MTs are required for cell division, motility, cellular morphogenesis, secretion and cell surface specialization. To achieve diverse cellular functions, MTs are assembled into complex structures (microtubule-based organelles) such as cilia (flagella), basal bodies, centrioles, mitotic spindles, microtubule rootlets, axostyles, filose pseudopods, and other cytoskeletal structures. These organelles exist in a dynamic state of the polymer and maintenance of the dynamic properties appears to be critical for their functions.

One mechanism that can regulate the properties of microtubules and related organelles is the interaction with a variety of MT effectors. MT dynamics is reduced and MTs undergo stabilization and form bundles or even fused-wall multi-tubular MTs (doublets and triplets) due to the activity of a number of structural microtubule-associated proteins (MAPs). Besides MAPs, several small molecular compounds (such as paclitaxel) isolated from certain plants and marine organisms also promote the stability of MTs. Another class of MT effectors promotes the disassembly of MTs, and this class includes severing proteins, end-depolymerizing factors and tubulin-sequestering proteins. A number of natural compounds such as colchicine also cause disassembly of MTs. Such proteins and drugs, which affect the microtubule dynamics, will be described in detail in the literature review (chapter 2).

Katanin, the first MT-severing protein that was discovered, is an AAA ATPase protein that can sever microtubules *in vivo* and *in vitro*. Not much is known about the exact function and regulation of katanin. It is also unknown how microtubule severing is regulated *in vivo* from both spatial and temporal points of view. Few studies indicate that the carboxy terminal tail (CTT) domain of α- and β-tubulins is required for severing function of katanin. CTTs of α- and β-tubulin are highly acidic and flexible. There has been a longstanding interest in CTT owing to its presence on the outside of the microtubule and it's numerous interactions with a variety of microtubule effectors. The acidic nature of CTT is due to the presence of several evolutionary conserved glutamic acids residues. Mass spectroscopic analysis showed that these specific glutamic acids undergo unusual types of post-translational modifications (PTMs). PTMs are highly evolutionary conserved from protists to humans suggesting their yet unknown biochemical and physiological significance. Several of the PTMs such as acetylation, phosphorylation and palmitoylation are commonly present on different types of proteins. Other PTMs including polyglycylation, polyglutamylation and detyrosination exclusively occur on the CTTs of tubulins. Although, polyglycylation and polyglutamylation are two different types of modifications, they are collectively referred to *polymodifications* as they occur by polymerization of multiple glycine or glutamic acid residues to the glutamic acid residues present in the primary sequence of α- and β-tubulin CTT.

We have been developing *Tetrahymena thermophila* as a tool to identify and analyze the functions of tubulin polymodifications. *Tetrahymena* is a powerful model system to study the function of essential gene products. The ease of genomic DNA targeting which occurs exclusively by homologous recombination makes it an ideal organism for deletion of specific genes. Moreover, *Tetrahymena* assembles diverse and conserved types of microtubular

organelles including cilia, basal bodies and mitotic spindles, which are essential organelles in mammals as well.

Previous studies showed that the sites of polymodifications are essential for survival of *Tetrahymena thermophila*. Deficiencies in polymodification sites on tubulins strongly affected the organization of ciliary and cortical MTs. Based on the phenotypes of polymodification site mutants, we postulated that defective microtubule-severing activity is the downstream response to the lack of polymodification sites. Studies from other organisms have provided some indirect indications that katanin could be one of the interpreters of polymodification signals and may have a role in mediating the functions of PTMs.

In this present study, I have uncovered novel roles of katanin in the context of microtubule PTMs. This dissertation is organized into four chapters. Chapter 2 provides an overview of current knowledge about microtubules and the mechanisms that regulate their dynamics. Chapter 2 also describes contemporary mechanistic insights into the assembly of complex microtubular organelles such as cilia, which is highly relevant to my research. Chapter 3 includes multiple research projects based on the single theme of katanin and its potential relationship to PTMs. I focused on the novel findings that katanin serves as a microtubule assembly-promoting protein, and I have analyzed mutant phenotypes of katanin knockout cells and attempted to uncover the underlying mechanism of katanin regulation. In chapter 4, I discussed the significance of my research.

CHAPTER 2

REVIEW OF LITERATURE

Microtubules: Structure, Dynamics and Function

Microtubules (MTs) are noncovalent polymers of α- and β-tubulin heterodimers found in all eukaryotic cells. MTs have essential functions in various cellular processes such as cell division, cell morphogenesis, polarity, signal transduction, cell motility, and intracellular trafficking. Microtubules are hollow tubes made of a number of longitudinal protofilaments. Within a protofilament, α/β-tubulin dimers, are arranged head-to-tail, giving rise to polymer polarity. Tubulin dimers interact laterally with adjacent protofilaments forming a tubule most often with a lumen of 25 nm diameter. Microtubules, assemble *in vitro* from purified tubulin dimers and have a variable number of protofilaments, from 9 to at least 16 (Chretien and Wade, 1991), indicating a significant flexibility in the angle of lateral association between protofilaments within a MT. MTs nucleated *in vivo* have predominantly 13 protofilaments (Evans et al., 1985) although some specialized microtubules in selected cell types have a different number of protofilaments.

The first view of the 3D structure of αβ-tubulin heterodimer was obtained by electron crystallography (Nogales et al., 1998b). The 3D structure of a MT was resolved at the 20 Å resolution (Luduena et al., 1992; Nogales et al., 1999). These milestone structural studies have provided important insights into the structural and functional properties of MTs. Each monomer is very compact and formed by the tight interaction of three functionally distinct domains. The N-terminal domain (residues 1-206) is a nucleotide-binding domain (GTP) and is formed by six parallel β strands (S1-S6) alternating with helices (H1-H6). The nucleotide-binding pocket is

formed by a number of loops (loops T1-T6) at the N-terminal end of the core helix (H7). The core helix connects the nucleotide binding domain with the smaller, second domain, formed by three helices (H8-H10) and a mixed β sheet (S7-S10). The intermediate domain contains a loop (S9-S10) which is present at the inside surface of MTs. This loop in β-tubulin provides the binding site for the microtubule-stabilizing compound paclitaxel. The COOH-terminal domain is formed by two antiparallel helices (H11 and H12) that are located on the top of the previous two domains. GTP bound to the N-terminal domain of α-subunit is buried at the intradimer interface and is never hydrolyzed. However GTP bound to β-tubulin undergoes hydrolysis to GDP during MT assembly. It is relevant to my study that the structure of an important part of tubulin has not been resolved. Probably because of the high acidic nature of the carboxy-terminal tail domain (CTT), the last ten amino acid residues of α-tubulin and eighteen residues of β-tubulin were not visible in the crystallographic model of tubulin dimer (Sackett, 1995). I will discuss below the findings that CCTs are present on the surface of microtubules and undergo extensive post-translational modifications that play an important role in regulating the binding of effectors to microtubules.

MTs are intrinsically polar structures and this is a direct consequence of a head-to-tail association of the α/β- tubulin dimers within protofilaments. Thus, MTs have two structurally distinct ends: the plus end ends with a crown of β-tubulin and the minus end that ends with a crown of α-tubulin (Fan et al., 1996; Hirose et al., 1995; Mitchison, 1993; Nogales et al., 1999). This polarity is critical to the cellular functions of MTs and is reflected in the distinct properties of the both ends of the polymer. The minimal concentration of tubulin dimers required for polymerization (Cc) is lower for the plus end. Therefore, MTs polymerize primarily at the plus end. Furthermore, *in vivo*, the minus ends of MTs are usually anchored at a Microtubule-

Organizing Center (MTOC) such as a centrosome or a basal body (Brinkley, 1985; Dammermann et al., 2003; Tassin and Bornens, 1999) and usually the plus ends are available for polymerization/depolymerization (but not always). MTOCs are preferred locations for initiation of polymerization (nucleation) of MTs *in vivo* and *in vitro*. This is to a large extent the consequence of the presence of factors that stimulate the formation of new MTs (nucleation) such as γ-tubulin. Thus, in typical, well-studied mammalian cultured cells in interphase, MTs exhibit a radial pattern with the plus ends pointing toward the cell periphery and the minus ends pointing inward toward the centrosome, near the nucleus.

Each monomer of tubulin binds a guanine nucleotide, GTP. The tubulin dimer has two GTP binding sites; an exchangeable site (called E) and non-exchangeable site (called N). The N-site is located on α-tubulin, whereas the E-site is located on β-tubulin (Hesse et al., 1987). The exact role of the N site on α-tubulin is not clear but it is known that the binding of Mg^{2+} ion to the N-site is involved in stability of MT after its assembly (Menendez et al., 1998). Though, polymerization of MTs is closely coupled with hydrolysis of GTP at the E site on β-tubulin, GTP hydrolysis is not required for polymerization of MTs. After addition of a dimer to the growing polymer, GTP associated with E-site is rapidly hydrolyzed to GDP, which remains bound (Davis et al., 1994). The incoming tubulin dimer may act as a GTPase Activating Protein (GAP) (Nogales and Wang, 2006). Thus, tubulins represent an unusual case of G proteins that regulate their own GTP hydrolysis via self-polymerization. The state of the guanine nucleotide at the E site at the end of the polymer is of critical importance. Microtubule ends that have a GTP dimer have a straight conformation and can undergo further polymerization (Nogales et al., 1999). Recent cryo-EM reconstruction of unpolymerized GMPCPP-bound (GTP-like state) tubulin suggests that GTP-tubulin dimers have a straight conformation whereas GDP-tubulin dimers are

more curved (Wang and Nogales, 2005). The increase in the curvature is believed to cause depolymerization of the MT. At the concentration of dimers that is near Cc, the rate of addition of new dimers to the microtubule end may be too slow to balance the rate of GTP hydrolysis. Under these conditions, the MT can have a tubulin dimer with GDP at the E site exposed at the end. The terminal GDP dimer undergoes a conformational change that results in a tilt of α-tubulin relative to β-tubulin. This conformational change does not occur within the MT body as long as the plus end contains a collar of GTP dimers in a straight conformation (so called GTP cap) and is thought to act like a zipper. However, the energy released from GTP hydrolysis within the polymer is stored as a mechanical strain. This energy is released suddenly when the GTP cap is lost from the plus end. The loss of the GTP cap releases the mechanical strain within the lattice and this leads to rapid depolymerization of the plus end that involves peeling off of protofilaments (Nogales and Wang, 2006).

Importantly, at concentrations of tubulin slightly above Cc, the plus ends oscillate between phases of polymerization and depolymerization, which may reflect the stochastic changes in the local concentration of tubulin dimers near the ends. This behavior of microtubules is known as dynamic instability and occurs both *in vitro* and *in vivo*. Thus, the loss of a GTP cap from the plus end results in a "catastrophe" (Muller-Reichert et al., 1998), whereas the reacquisition of the cap by the depolymerizing end results in a "rescue" (Carlier and Pantaloni, 1981). The GTP bound to α-tubulin at N-site is non-hydrolyzable probably because it is trapped at the interface between the two subunits inside the dimer. However, the minus may be more stable because the terminal collar is made of α-tubulin subunits that have a GTP bound at the N site N-site.

Dynamic instability allows MTs to switch stochastically between growing and shrinking phases. This non-equilibrium behavior of MTs is crucial for several biological functions such as the formation of distinct MT arrays throughout the cell cycle, cell motility and cell morphogenesis (Kirschner and Mitchison, 1986; Valiron et al., 2001). Another type of behavior that MTs display is treadmilling (Margolis and Wilson, 1981; Rodionov and Borisy, 1997). Treadmilling is known to occur *in vivo* as well as *in vitro*. The main feature of this behavior is the addition of tubulin subunits at one end (plus) and removal of subunits from the other end (minus) leading to a flux of subunits along the polymer. The treadmilling behavior of MTs can be responsible for construction and function of bipolar mitotic spindles (Margolis et al., 1978), and plays an important role in the transport of chromosomes during mitosis (Garel et al., 1987; Margolis and Wilson, 1981). MT treadmilling mechanism can also have a role in generating the force necessary to produce tension in the mitotic spindle (Waters et al., 1996). The free tubulin subunit concentration plays a vital role in the assembly of MTs. For tubulin concentrations above a critical value Cc, tubulin dimers polymerize into MTs, while at the concentration below the Cc value, MTs depolymerize. *In vivo*, MTs with free minus ends can be generated by release from a MTOC (Abal et al., 2002; Keating et al., 1997), and breakage or severing of preexisting MTs. Severing functions of katanin and related spastin will be described later in detail.

The dynamic behaviors of MTs are tightly regulated at the cellular level (Kirschner and Mitchison, 1986). A variety of proteins collectively known as Microtubule-Associated Proteins (MAPs) and natural small molecule compounds are known to influence MT dynamics. Certain types of MTs are very stable in the cellular environment, and their stability is conveyed by structural MAPs. Structural MAPs are proteins that bind to the MT lattice in an ATP-independent manner. This class of MAPs includes MAP1, MAP2, and Tau in neurons and MAP4

in non-neuronal cells. Structural MAPs are known to bind, stabilize and promote the assembly of MTs. They bind along the entire MT length. Neuronal MAPs slightly increase the polymerization rate of tubulin dimers, but strongly suppress the catastrophe and promote rescues (Drechsel et al., 1992; Pryer et al., 1992; Trinczek et al., 1995). The net effect of binding of structural MAPs is a decrease in the turnover rate and an increase in the proportion of polymerized tubulin at steady state. In all these proteins, the MT binding domain is located near the COOH-terminus, whereas the N-terminal domain protrudes from the MT polymer surface and interacts with other proteins. The MT-binding site consists of highly conserved repeat motifs, which may be involved in cross-linking adjacent tubulin subunits (Lewis et al., 1988). In nerve cells, MAP2 is predominantly present in dendrites and the cell body while Tau is mostly present in the axon. This observation indicates the existence of additional mechanisms determining the localization of MAPs to a specific subset of MTs. Ultrastructural studies revealed that MAP2 and Tau bind along the sides of the MTs (Al-Bassam et al., 2002; Hirokawa et al., 1988) and increase MT rigidity and bundling. MAP2 and tau interact with the highly acidic CTTs of tubulins and these CTTs are known to have higher levels of polyglutamylation (Edde et al., 1990). The size of polyglutamylation side chains (see below for discussion of the role of microtubule modifications) could regulate the binding affinity of different neuronal MAPs (Bonnet et al., 2001). Overexpression of MAPs in cells can induce bundling of MTs, and the spacing of the MTs depends on the size of the projection domain (Chen et al., 1992). Overexpression of MAPs in fibroblast and neurons severely inhibits plus-end-directed MT transport (anterograde transport) mediated by kinesin (Ebneth et al., 1998; Stamer et al., 2002). For example when Tau was overexpressed, mitochondria accumulated in the cell body and failed to move into the axon. Interestingly, overexpression of microtubule-affinity regulating kinase

(MARK) rescues the anterograde transport defect by phosphorylating Tau and causing its dissociation from the MTs (Mandelkow et al., 2004). These observations suggest that saturation of MT surface by MAPs acts as a roadblock, which significantly impairs the microtubule-dependent transport. The inhibitory effects of phosphorylation on binding of MAPs were also observed in non-neuronal cells. For example in *Xenopus* oocytes, phosphorylation of MAP4 relieves its stabilization effect from MTs by lowering its affinity towards MTs. Dissociation of MAP4 probably enhances the MT dynamics that are essential for mitotic spindle formation and function (Faruki and Karsenti, 1994).

In contrast to the structural MAPs described above, another new class of MAPs strongly influences MT dynamics by interacting with the ends of MTs. These MAPs are called the +TIPs (plus-end-tracking proteins). +TIPs belong to several structurally unrelated families, which include MT plus- and minus-end-directed motors as well as non-motor MAPs. Generally speaking, +TIPs are responsible for regulating the properties of plus end such as the level of end dynamics and attachment to other objects in the cell. The first protein observed to dynamically track the plus end of MTs was CLIP-170 (Perez et al., 1999). Other plus-end tracking proteins include EB1, dynactin, and Lis1 (Lansbergen and Akhmanova, 2006). +TIPs are involved in mediating the anchorage of plus ends of MTs to kinetochores (during mitosis) and to the plasma membrane and are vital for chromosome segregation, vesicle transport, establishment and maintenance of cell polarity, and cell migration (Lansbergen and Akhmanova, 2006). +TIPs may be important regulators of dynamic instability of microtubule ends. For example, Stu2p, the yeast homolog of XMAP215, can rescue a depolymerizing end and promote a switch to polymerization (Tanaka 2005). A study in *Chlamydomonas* showed that EB1 localizes to the tip of flagella where it may have a role unloading the intraflagellar transport cargo (see below)

(Pedersen et al., 2003). The accumulation of +TIPs near the ends of microtubules could be regulated by two factors. First, some +TIPs could bind more strongly to tubulins that are not modified post-translationally and are enriched in the newly added portion of the polymer near the plus end (Honnappa et al., 2006). For example, the CAP-GLY domain MAPs bind more strongly to tubulins that still have a C-terminal tyrosine (did not undergo detyrosination) (Peris et al., 2006). Another mechanism for accumulation of +TIPs involves motor proteins. For example, the yeast homologs of +TIP CLIP170 are transported to the plus end by a kinesin motor Kip2/Tea2 (Bush et al 2004).

In addition to the MAPs that stabilize MTs, there are natural compounds that are known to promote stabilization of MTs. While their function in nature is unclear, these compounds are important as research tools as well as medicinal drugs, in particular to treat cancer. Paclitaxel (commonly known as taxol) is an antimitotic agent and has clinical significance for the treatment of many cancers (Rowinsky et al., 1990). Paclitaxel inhibits mitosis (Jordan et al., 1993), induces MT bundles in interphase cells (Fuchs and Johnson, 1978) by stabilizing MTs (Schiff and Horwitz, 1980). In agreement with the finding that the paclitaxel binding site is present in β-tubulin (Nogales et al., 1998b), paclitaxel interferes with the dynamic instability of MTs. Paclitaxel probably stabilizes MTs by inhibiting the protofilament curling at the ends of MTs during depolymerization (Arnal and Wade, 1995).

It needs to be mentioned that compounds with a MT stabilizing activity are rare, while numerous compounds were identified (both natural and synthetic) that depolymerize MTs. For example, the anti-mitotic compounds, colchicine and vinblastin modulate MT dynamics by inhibiting MT polymerization (Wilson et al., 1999). The specific site of colchicine binding is not known but appears to be located at the interface between α- and β-subunits in the tubulin dimer

(Nogales et al., 1999). The vinblastin binding site is distinct from the colchicine site and it appears to be located at the β-tubulin exposed at the plus end of MTs (Nogales et al., 1999). Nocodazole, one of the synthetic anti-tubulin drugs, rapidly depolymerizes MTs *in vivo* and *in vitro* (Vasquez et al., 1997). Nocodazole binds to tubulin dimers and MT polymer (Lee et al., 1980; Vasquez et al., 1997) and increases the GTPase activity of pure unpolymerized tubulin dimers (Lin and Hamel, 1981). Even a small increase in the soluble pool of GDP-tubulin dimers significantly reduces the rate of MT elongation and increases the catastrophe frequency probably because incorporation of GDP dimers rapidly destabilizes the ends of MTs (Caplow and Shanks, 1995; Vandecandelaere et al., 1995; Vasquez et al., 1997). Other studies showed that both plus and minus ends of MTs experience concentration-dependent shortening, increase in catastrophe frequency and decrease in rescue frequency in the presence of nocodazole (Vasquez et al., 1997). The self-assembly of tubulin either bound or unbound to associated proteins, can be blocked by nocodazole in a reversible manner (Samson et al., 1979). Furthermore, treatment with nocodazole disrupts fast axoplasmic transport and results in a decreased density of axonal MTs while increasing the density of neurofilaments (Samson et al., 1979). Another class of MT-depolymerizers are dinitroanilines, which bind to tubulin and disrupt MTs (Hugdahl and Morejohn, 1993; Morrissette et al., 2004; Traub-Cseko et al., 2001). Based on studies done in *Toxoplasma gondii*, *Plasmodium falciparum* and *Leishmania major,* the binding site for these compounds is located below the H1-S2 loop and includes residues on helix H7 and on the T7 loop in α-tubulin and this may lead to disruption of lateral association of protofilaments and subsequent microtubule disassembly (Mitra and Sept, 2006). Interestingly, These compounds are effective against plant and protozoan tubulin but do not affect mammalian and fungal tubulin (Chan and Fong, 1990; Chan et al., 1991; Hugdahl and Morejohn, 1993). For instance, one of the

dinitroanilines, oryzalin, disrupts MTs from *Leishmania* whereas rat brain tubulin polymerizes normally. The tubulin of *Tetrahymena* is highly sensitive to oryzalin (Stargell et al., 1992).

I will now discuss properties of proteins that promote depolymerization of MTs *in vivo*, and thus act in the manner similar to depolymerizing drugs. Recent studies have uncovered a group of kinesin motor related proteins known as kinesin-13 that are probably not true motors but ATPases that destabilize the plus end of MTs and promote their disassembly. Kinesin-related proteins can be subdivided into three main subfamilies (kinesin N, kinesin I and kinesin C) based on the location of the motor domain (N-terminal, Internal or C-terminal). Kinesins N and C can move along MTs in the plus end (N) or minus end (C) direction, respectively (Vale and Fletterick, 1997). Thus, in kinesins, the location of the motor domain is predictive of the motor directionality. Interestingly, kinesin-13 proteins are Kin I type and are not known to have an ability to move along MTs. Instead, kinesin-13 proteins bind to the plus ends and depolymerize MTs, and have a significant impact on the MT dynamics *in vivo* (Hertzer et al., 2003; Ovechkina and Wordeman, 2003). The depletion of XKCM1, the first identified kinesin-13, in *Xenopus* egg extracts resulted in long MTs incapable of forming mitotic spindles (Walczak et al., 1996). The discovery of kinesin-13 as MT-depolymerizing agents led to the identification of other members of this family that influence MT dynamics *in vivo* and *in vitro* (Homma et al., 2003; Maney et al., 1998; Tournebize et al., 2000). XKCM1 and its mammalian homologue MCAK functions specifically at kinetochores during mitosis, where they are required for proper chromosome segregation by inducing depolymerization of plus ends of spindle microtubules and allowing chromosomes to move toward the spindle poles while being attached to a depolymerizing MT end (Walczak et al., 2002; Wordeman and Mitchison, 1995). The catalytic core of kinesin-13 is sufficient for promotion of catastrophes but the neck region is required for the depolymerizing

activity (Desai et al., 1999; Moores et al., 2002; Okada and Hirokawa, 2000; Ovechkina et al., 2002). At the MT end, kinesin-13 binds to the protofilament and promotes the curved conformation of the protofilaments (while hydrolyzing ATP) causing a catastrophe event. Interestingly, the ATP-independent diffusion along MT surface can direct kinesin-13 either to the plus or minus end (Helenius et al., 2006). Importantly, the CTT of β-tubulin is essential for depolymerizing activity of Kin I kinesins (Moores et al., 2002), which also is a general feature of motoric kinesins, that require CTTs of tubulins for their movement along the MTs (Thorn et al., 2000; Wang and Sheetz, 2000). Other kinesin superfamily members, including kinesin-8 proteins (Kip3) and kinesin-14 (Kar3p) also have a MT end-destabilizing activity (Cottingham and Hoyt, 1997; DeZwaan et al., 1997; Meluh and Rose, 1990; Miller et al., 1998). Kinesin-8 (Kip3p) destabilizes only the plus end of MTs (Varga et al., 2006). However, kinesin-14, Kip3 can also use ATP hydrolysis to move towards the plus ends of MTs. Thus, kinesin-14 proteins have a dual activity: they are capable of moving along MTs and destabilizing the plus end (Ems-McClung et al., 2007). As a result, kinesin-14, Kar3p also has a preference for destabilization of the plus end (Sproul et al., 2005).

HIV protein Rev has also been implicated in disassembly of MTs *in vitro*. *In vitro* HIV-1 Rev interacts at the surface of MTs within its N-terminal domain and disassembles MTs to form stable bilayer rings (Watts et al., 2000). The N-terminal domain of Rev protein has sequence similarity to the tubulin-binding region of the motor domain of kinesin-13. A small acidic polypeptide, MINUS, purified from cultured neural cells and bovine brain also inhibits MT formation (Fanara et al., 1999).

Another MT-destabilizing factor, Op18/Stathmin was purified from *Xenopus* egg extracts and initially was proposed to have a function in promoting MT depolymerization by increasing

the rate of catastrophes (Belmont and Mitchison, 1996). However, stathmin possesses a strong tubulin dimer sequestering activity, which prevents the MT growth by reducing the intracellular pool of unpolymerized tubulin available for polymerization (Jourdain et al., 1997). Thus, stathmin appears to be a bifunctional protein and its N-terminal regulatory domain possesses catastrophe-promoting activity while the C-terminal domain contains tubulin-sequestering activity (Howell et al., 1999). The catastrophe-promoting activity occurs at the plus end of polymerized MTs and could be based on stimulation of hydrolysis of GTP with subsequent loss of the GTP cap and depolymerization (Howell et al., 1999). Structural studies with stathmin and tubulin suggest that stathmin can bind unpolymerized as well as polymerized tubulin and either prevents polymerization of αβ-tubulin heterodimers or induces depolymerization of MTs (Gigant et al., 2000; Larsson et al., 1999; Wallon et al., 2000). Importantly, stathmin can bind to free tubulin dimers when they have GDP bound to the E site and are in a curved conformation. The interaction with GDP tubulin dimers is the basis for sequestering activity of stathmin. The MT-destabilizing and sequestering activity of stathmin is switched off by phosphorylation when cells enter into mitosis, allowing MTs to polymerize and assemble mitotic spindles (Marklund et al., 1996). Interestingly, overexpression of active stathmin depolymerizes interphase MTs, but does not interfere with the formation of mitotic spindles. On the other hand, overexpression of mutant stathmin, which cannot be inactivated by phosphorylation, results in severe defects in the assembly of mitotic spindles as well as arrest in cell cycle at G2/M phase (Marklund et al., 1996; Rubin and Atweh, 2004). Inhibition of stathmin expression causes accumulation of cells in G2/M phases and difficulty in the exit from mitosis (Mistry and Atweh, 2001). MT severing represents another unique mechanism that offers a new means of destabilization and disassembly of MT

cytoskeleton. In the following section the known structure and functions of MT-severing proteins; katanin and spastin are discussed.

Katanin

The first clue that a microtubule severing factor exists emerged from observations of mitotic extracts of *Xenopus laevis* which could sever stable MTs along their length (Vale, 1991). This activity was relatively weak in interphase cytoplasmic extracts. An increase in the MT severing activity could play a role in the reorganization of MTs during the transition from interphase to mitosis.

A microtubule-severing protein, katanin was purified and characterized from mitotic sea urchin eggs, as a complex of 60 kDa (p60) and 80 kDa (p80) subunits, exhibiting severing activity on taxol stabilized MTs *in vitro* (McNally and Vale, 1993). Importantly, katanin can not sever MTs assembled from subtilisin digested tubulin heterodimers that lack the CTTs (McNally and Vale, 1993). The p60 subunit is a member of the AAA ATPase superfamily (Confalonieri and Duguet, 1995) and its MT-stimulated ATPase activity is responsible for the disassembly of MTs (Hartman et al., 1998; Hartman and Vale, 1999). The p60 subunit displays an ATPase activity and can sever microtubules in the absence of p80 (McNally et al., 2000). The highly conserved AAA domain sequence of p60 contains the “Walker A” (P loop) and “Walker B” signature motifs found in many ATPases (Walker et al., 1982). Coexpression of both subunits in baculovirus-transfected insect cells led to the formation of active p60/p80 complexes (Hartman et al., 1998) that severed MTs *in vitro* assays. Rotary-shadowing electron microscopic imaging revealed that purified p60 alone and the p60-p80 complex form 14-16 nm and 20 nm hexameric ring structures, respectively (Hartman et al., 1998). Fluorescence Resonance Energy Transfer and hydrodynamic studies of the p60 subunit indicated that p60 forms a hexameric ring in the

presence of ATP (Hartman and Vale, 1999). Such oligomerization of p60 katanin requires the presence of a MT. Based on their observations Hartman and Vale, 1999 proposed a model for the mechanism of katanin-mediated disruption of tubulin contacts within a MT wall. According to this model, an ATP bound katanin p60 (but not katanin-ADP); has an enhanced ability for oligomerization only in the presence of MTs. The p60 ring binds to MTs with high affinity, and then its ATPase activity is stimulated. ATP hydrolysis and subsequent phosphate release change the conformation of the bound katanin ring, which produces a mechanical strain that is conveyed onto bound tubulins and destabilizes tubulin-tubulin contacts within the MT lattice. Similar conformational changes (hexameric ring formation) were observed for other AAA proteins not involved with MT severing (Hanson et al., 1997).

The role of the p80 subunit is less clear but it appears that its function may be primarily in regulating the p60 activity and in targeting the complex to specific subcellular sites. For example, katanin is often found enriched at the centrosomes (near the minus ends of many microtubules) and this led to a suggestion that katanin could be involved in stimulation of microtubule flux by severing near the minus end and removing the γ-tubulin cap (Waters et al., 1996). In fact, the WD40 domain of p80 is required for targeting of katanin complex to the centrosome (Hartman et al., 1998; McNally et al., 2000). Human p80 concentrates at the interphase centrosome even after complete depolymerization of MTs with nocodazole, suggesting that the WD40 domain of p80 interacts with a resident centrosomal protein without the need for MTs (Hartman et al., 1998). WD40 repeats have been implicated in protein-protein interaction for other proteins (Komachi et al., 1994; Wall et al., 1995). Furthermore the carboxy terminal domain of p80 binds to microtubules and enhances the action of the amino terminal MT

binding domain of p60 (McNally et al., 2000) (Hartman and Vale, 1999). Thus, p80 may play a role in localization as well as activity of the katanin complex.

In *Xenopus laevis*, two ATP-independent MT-severing proteins, p56 (Shiina et al., 1992) and EF1α (Shiina et al., 1994) also contribute to an increase in MT severing activity at the interphase/prophase transition. However, the majority of the M-phase severing activity in *Xenopus* extract is caused by katanin, suggesting that cyclin B/cdc2 (directly or indirectly) activates katanin (McNally and Thomas, 1998). The p60-p80 katanin complex localizes to spindle poles in human and *Xenopus* cells and its centrosome localization appears to be cell type specific (McNally et al., 1996; McNally and Thomas, 1998). Interestingly, the bulk of katanin is cytosolic which indicates that katanin might be stored in an inactive state. Furthermore, higher concentrations of MTs inhibit the oligomerization of katanin heterodimers by sequestering katanin heterodimers to individual MT polymer (Hartman et al., 1998; McNally and Thomas, 1998). However, the WD40 domain of p80 may reverse this inhibition by interacting with specific centrosomal proteins, thereby increasing the local concentration of katanin heterodimers and further promoting the multimeric ring formation near the centrosome. Also, when cyclin B/cdc2 activity increases during early M-phase, this could promote the recruitment of katanin to the spindle poles to release free MT minus ends from their attachment sites in the γ-tubulin ring complexes in the pericentriolar material (Moritz et al., 1995; Zheng et al., 1995). This release could promote depolymerization of MTs minus ends during the poleward flux, an event required for the maintenance of the mitotic spindle structure (Waters et al., 1996) as well as during the separation of chromosomes at anaphase (Desai et al., 1998; McNally et al., 2000). In agreement with this model, it is known that the minus ends of MTs that are nucleated from γ-tubulin ring complexes (γ-TuRc) are physically capped (Dammermann et al., 2003; Wiese and Zheng, 2000)

and that γ-TuRc caps stabilize the minus ends by preventing both minus-end depolymerization and polymerization (Keating and Borisy, 2000). On the other hand, a minus end depolymerization has been directly observed on single MTs which lack a centrosome association in fish melanophores *in vivo* (Rodionov and Borisy, 1997) and it is well established that mitotic spindle microtubules undergo poleward flux of tubulin polymer (Mitchison, 1989). Inhibition of katanin by overexpression of a dominant-negative form with a mutation in the ATPase domain did not affect the assembly of mitotic spindle, anaphase or cytokinesis (Buster et al., 2002). This was surprising, but it is not clear that the method used resulted in significant decrease in katanin activity. Another possibility is that mammalian cells can use the related protein, spastin, for some activities that also involve katanin. However, in the same study, the spindle MTs disassembled at a lower rate in nocodazole when katanin was inhibited or mislocalized (Buster et al., 2002) suggesting that katanin-mediated severing of MTs in the spindle results in an increased number of uncapped MT minus ends that are more sensitive to nocodazole. It thus appears that the role of katanin in mitosis is subtle but a definitive answer will be obtained in experiments on mammalian mitotic cells lacking katanin (and spastin) subunits that could be created either by gene targeting or by RNAi.

Based on the currently available data, katanin appears to be more important during meiosis. In *Caenorhabditis elegans* mei-1/mei-2 katanin (p60/p80) severing activity is strongly involved during meiosis in oocytes, but not during mitosis in embryos (Srayko et al., 2000; Yang et al., 2003). A loss of function mutation of the MEI-1 gene results in a highly disorganized meiotic spindle but mitosis in these mutants is normal (Clandinin and Mains, 1993; Mains et al., 1990). In a normal worm embryo, mei-1 protein is present during meiosis but undergoes degradation after fertilization before the embryo enters mitosis. A gain of function mutation in

MEI-1 causes abnormal persistence of mei-1 protein into mitosis. In these mutant worms, meiosis is normal but mitotic spindles are disintegrated (Clark-Maguire and Mains, 1994; Pintard et al., 2003). Normally, in *C. elegans*, the meiotic spindles are much shorter than the embryonic mitotic spindles. Thus the mei-1 mutant phenotypes suggest that function of mei-1 p60 is to keep the spindle MTs shorter during meiosis (McNally et al., 2006; Srayko et al., 2000). The *C.elegans* homologue of p80 subunit, mei-2, was originally identified as a suppressor of a mei-1 mutant (Mains et al., 1990). Subsequent genetic studies showed that mei-2 functions as an activator of mei-1 (Clandinin and Mains, 1993; Clark-Maguire and Mains, 1994). Coexpression of mei-1 and mei-2 disassembled MTs in HeLa cells (Srayko et al., 2000). Furthermore, the loss of function of MEI-2 phenocopies the loss of function of MEI-1, indicating that both subunits function as a severing complex during meiosis and that each subunit is essential for katanin activity *in vivo* (Lu et al., 2004; Mains et al., 1990). Recent studies show that katanin-mediated severing produces many short MTs around the meiotic chromatin, which increases the MT polymer mass of the *C. elegans* meiotic spindle (Srayko et al., 2006). Wild-type meiotic spindles consist of a large number of short, overlapping MTs lying along the spindle axis whereas mei-1 null spindles contain fewer and longer MTs (Srayko et al., 2006). Srayko et al, 2006 and McNally et al 2006 suggest that during oocyte meiosis, MTs fragments generated by katanin do not depolymerize immediately but act as a template for elongation resulting in an increased mass of the total polymer. Thus, unexpectedly, a "destructive" activity of katanin emerges as an important factor that stimulates microtubule polymer assembly and therefore ultimately acts as a positive regulator of microtubule mass.

It is not known how katanin selects MTs for severing and what cellular cues regulate the function of katanin. There is also uncertainty regarding the spatial control of katanin activity. In

M-phase cells katanin is concentrated at the spindle poles. This led to a suggestion that katanin primarily acts near the minus ends of microtubules. However, recent high resolution TEM study in meiotic embryos of *C. elegans* identified widespread lattice defects or holes in the wild-type spindle MTs but not in the mei-1 mutant embryo (Srayko et al., 2006). This would suggest that despite its enrichment at the spindle poles, katanin is active throughout the entire spindle where its role could be in generating short microtubules from pre-existing microtubules. However, we should mention that meiotic oocytes and embryos are unusual due to the absence of centrosomes (that are later introduced by sperm) and in these cells, the spindle assembly occurs primarily at the surface of chromosomes that carry factors that stimulate microtubule polymerization and organization into spindle halves. Thus, katanin may be less restricted during meiosis in oocytes. Alternatively, katanin pool at the centrosome may represent a stored form that is released and activated by an unknown mechanism and katanin could be acting in locations that are far from the centrosome. It also remains unclear, whether the site of severing activity is regulated primarily by availability of active katanin near in the MT vicinity or by structural cues that are already present on the MT. Biophysical studies based on single MT severing and mathematical modeling suggest that katanin severing is non-random and could occur preferentially at pre-existing defects in the microtubule lattice (Davis et al., 2002). However, these studies involved MTs that were polymerized in vitro and it is not clear whether natural MTs have pre-existing lattice defects different from those caused by katanin itself. Additional clues regarding spatial regulation of katanin activity came from studies on nerve cells (see below), which suggest that competing MAPs that stabilize MTs and protect against katanin restrict katanin activity.

Katanin has been implicated in the formation of axonal projections in the neuron. It should be mentioned that axons and dendrites are filled with MTs that are not associated with a

centrosome. These MTs play a crucial role in the growth of neural extensions and support high rate of organellar traffic in differentiated neurons. Katanin is concentrated at the neuronal centrosome and is also widely distributed throughout the neuron but its levels change depending on the developmental stage. For example, elevated levels of katanin are detected during the growth phase of axon and the levels drop down when axon reaches the target cells (Karabay et al., 2004). Inhibition of katanin using function-blocking antibodies delivered by microinjection resulted in accumulation of MTs at the centrosome and also prevented the growth of neuronal processes (Ahmad et al., 1999; Karabay et al., 2004). Furthermore, expression of a dominant-negative (ATPase deficient) form of p60 inhibited axonal growth (Karabay 2004). These observation led Bass and collaborators to propose the "cut and run" hypothesis to explain the organization of MTs in the axons (Baas et al., 2005). They propose that katanin generates short MTs by severing microtubules nucleated at the centrosomes in the cell body. The severed MTs could be transported into the axons by motor proteins using long microtubules as a track. Although katanin would primarily work inside the cell body and around the centrosome, its activity inside axons could play a role in reorganizing bundles of MTs during axonal branching (Ahmad et al., 1999; Baas et al., 2005). Interestingly, the studies done in neurons reveal potential complexity in the interactions between p60 and p80 and suggest additional roles for p80. The ratio of p60 and p80 subunits varies significantly at different phases of neuron development, in different regions of neuron as well as in different tissues and organs (Yu et al., 2005). In contrast to uneven and variable p60 localization, p80 subunit localizes evenly to all compartments of the neurons, suggesting that p80 may have a function other than targeting katanin to centrosome and negatively regulating p60 severing function (McNally et al., 2000; Yu et al., 2005). Overexpression of katanin p60 destroyed MTs and also inhibited axonal outgrowth (Karabay et

al., 2004). Interestingly, in neurons overproducing p60, the axonal MTs were more resistant to severing compared to MTs of the cell body and dendrites (Qiang 2005). This could be a result of protection of axonal microtubules by Tau, a structural MAP that is highly enriched in axons. Indeed when Tau was depleted in neurons using RNAi, axonal MTs showed increased sensitivity to overproduced katanin (Qiang et al., 2006). These observations suggest that katanin severs MTs in a non-random fashion and is strongly influences by pre-existing MAPs that could sterically inhibit katanin binding to MTs.

Recent studies uncovered a possible role of katanin in the assembly of MTs in cilia, another type of cytoplasmic MTs-rich projections. The structure of cilia will be discussed in detail below, but it should be mentioned that cilia resemble axons in that they contain microtubules with plus ends oriented toward the distal end of projections. In *Chlamydomonas*, the *PF15* gene encodes p80. Strikingly, an insertional mutation of *PF15* resulted in paralyzed cilia that lacked the central pair MTs but otherwise appeared normal (Dymek et al., 2004). This result suggested that katanin is required for assembly and/or stability of central pair MTs. Interestingly antibodies against p80 detected this protein primarily inside cilia but in the fraction containing outer doublets (Dymek et al., 2004). Little if any p80 was found associated with the central pair even though these MTs fail to assemble in the absence of functional p80. The presence of p80 inside cilia suggested a direct role in the assembly of ciliary MTs, but this role is difficult to conceive because cilia unlike axons do not undergo branching and internal breakage of MTs. Another puzzling aspect is that the expression pattern of p80 in *Chlamydomonas* is atypical for a flagella (or cilia)-related gene. Most if not all genuine ciliary proteins are upregulated when *Chlamydomonas* cells regenerate flagella after experimental deciliation primarily due to increase in the levels of transcription. However, the mRNA levels of PF15 (p80)

decreased after deciliation, which is not a typical property of a ciliary gene involved with assembly of these organelles (Dymek et al., 2004).

Another important question that emerged from this *Chlamydomonas* study was whether the PF15 mutant phenotype represents a deficiency in katanin function. The regions close to the COOH-terminus of *Chlamydomonas* and animal p80s are only weakly similar. Given the fact that the p60 binding domain of p80 is present near the carboxyl terminus of p80 (McNally et al., 2000), it was possible that Pf15p has alternative binding partners and that its role in ciliary assembly is not related to katanin p60 function and severing of MTs. I have addressed this question by deleting the p60 gene in *Tetrahymena* (see Chapter 3).

Interestingly, earlier studies led to the suggestion that katanin plays a role in the process of shedding of cilia known as deciliation (or deflagellation). Ciliated protist cells shed the entire cilia or flagella in response to chemical stress and this process resembles the loss of the flagellum when mammalian sperm enters the oocyte during fertilization (Quarmby, 2004). Deciliation involves a breakage of the outer doublet MTs at a specific site in the distal region of the transitional zone (a connection area between the ciliary axoneme and the basal body (Blum, 1971). Earlier studies suggested the involvement of centrin, a calcium-binding basal body/centrosome protein that is associated with stellate fibers of the transition zone in *Chlamydomonas* (Sanders and Salisbury, 1989). A mutation in the centrin gene in *Chlamydomonas* led to inefficient deflagellation and indicated that calcium induced contraction of stellate fibers provides torsional and shear stress on the outer doublet MTs that causes them to break. Since deletion of centrin is lethal (Susan Dutcher, personal communication; Lynne Quarmby, personal communication), the role of centrin in deflagellation was not conclusively established. However, Quarmby and colleagues claimed that deciliation in the centrin mutant is

normal and postulated that katanin might be responsible for deflagellation in *Chlamydomonas* (Lohret et al., 1998; Lohret et al., 1999). Three pieces of evidence indicated a possible role of katanin in deflagellation: 1) katanin purified from sea urchin severed MTs of the axoneme *in vitro,* 2) immunogold electron microscopy detected (based on anti-human p60 antibodies) detected p60 katanin at the transitional zone (the site of severing during deciliation), and 3) antibodies against the human p60 katanin inhibit calcium-induced axonemal severing in purified basal body-axoneme complexes in vitro. RNAi studies of p60 katanin were repeatedly unsuccessful in *Chlamydomonas*, indicating an essential role of katanin in *Chlamydomonas* (M. Q. Rasi, 2006). Interestingly, a successful RNAi-based knockdown of p60 katanin could be achieved in a flagella-less IFT88 mutant, suggesting that cells lacking flagella have a permissive state for the knockdown of katanin (M. Q. Rasi, 2006). These results indicate that katanin is essential for survival in *Chlamydomonas* only when flagella are present. In *Chlamydomonas*, cilia are completely resorbed prior to mitosis and basal bodies convert into centrioles and function in mitosis as spindle poles. It is conceivable that in *Chlamydomonas*, katanin is required for complete disassembly of axonemal MTs to enable release of basal bodies so that they can be converted into mitotic centrioles. To conclude, there is a considerable level of confusion regarding the role of katanin in cilia. Some data suggest that katanin plays a role in assembly of cilia, while other data indicate additional roles in deciliation and resorption of cilia prior to mitosis. One of the goals of the research in this thesis was to re-evaluate the role of katanin in *Tetrahymena*, a ciliate cell type in which gene knockouts are easily obtained and methods are at hand to establish the function of katanin even if it is essential using an experimental heterokaryon approach that allows for studies of inducible lethal phenotypes (Cassidy-Hanley et al., 1997).

Spastin

Hereditary spastic paraplegia (HSP) is a group of clinically and genetically heterogenous diseases characterized by neuronal degeneration at the distal ends of the longest axons of the central nervous system resulting in spasticity and weakness of the lower limbs (Salinas et al., 2007). The most common causes of autosomal dominant HSP (AD-HSP) are mutations in the gene encoding spastin, *SPG4* (Hazan et al., 1999). Spastin is an AAA ATPase that has significant sequence similarity with katanin p60 (Frickey and Lupas, 2004; Lupas and Martin, 2002). The AAA domain contains Walker A and B motifs as katanin does. The N-terminal half of human spastin contains predicted nuclear localization signals and a so-called microtubule interaction and trafficking (MIT) domain (Beetz et al., 2004; Ciccarelli et al., 2003). Numerous mutations, including missense, nonsense, and splice site point mutations, deletions and insertions, have been reported in the AAA domain of SPG4 in AD-HSP patients (Fonknechten et al., 2000). The homology with katanin suggested that spastin is also a MT-severing protein and that a deficiency in severing exists in AD-HSP.

In proliferating mammalian cells, spastin localizes to the centrosome in different cell types during all stages of the cell cycle and its localization does not require MT integrity (Svenson et al., 2005). Initial evidence that spastin regulates MT integrity came from the observation that overexpressed spastin disrupted the MT network (Errico et al., 2002). Overexpressed spastin localized to spindles poles, the midbody and the distal portions of the motor neurons during cell division. Spastin plays an important role in neuronal differentiation. Inhibition of spastin causes axonal undergrowth, reduces the synaptic area and increases the density of MTs at the synapse whereas spastin overexpression reduces the synaptic strength and amount of synaptic MTs. Interestingly, the phenotype of overexpression of spastin was partially

rescued by treatment with a MT-stabilizing drug (paclitaxel), while the phenotype of spastin deficiency was reversed by a treatment with a MT destabilizing drug (nocodazole) (Trotta et al., 2004). This study suggested that spastin inhibits the accumulation of MTs in the synaptic region of the axon, and that spastin destabilizes MTs. Furthermore, MTs that accumulated near the synapse had excessive levels of MTs post-translational modifications such as acetylation on Lys-40 (Trotta et al., 2004). It is widely accepted that this modification accumulates on MTs as a function of their longevity (Takemura et al., 1992). Thus, the results indicated that spastin negatively regulates accumulation of modifications on MTs by making the polymer more dynamic. In the absence of spastin, MTs in certain locations could have increased half-life and accumulate modifications. In contrast, another study in *Drosophila* showed that spastin-null larvae contain of fewer MTs at the neuromuscular junction (Sherwood et al., 2004). It is not clear why the two papers investigating spastin in *Drosophila* gave conflicting results for the same location (neuromuscular junction). The Trotta paper is strengthened by the fact that a deficiency in spastin in the mouse led to a similar phenotype. Mice lacking functional spastin (equivalent mutation of spastin as observed in AD-HSP patients) led to axonal swelling that progressively caused axonopathy of the central nervous system (Tarrade et al., 2006). Consistent with the view that spastin is a MT-severing protein, lack of spastin resulted in accumulation of stable modified detyrosinated MTs. Neurite swellings were more prominent in those regions which frequently show MT transition between stable (detyrosinated tubulin) and dynamic (tyrosinated tubulin) isoforms, indicating that spastin affected MT dynamics in mutant neurons. These swellings were caused by impaired retrograde transport, most likely associated with abnormal association of MTs with minus end directed dynein motor.

The ATPase activity of spastin is required for MT-severing *in vivo* and *in vitro*. Many HSP mutations produce ATPase-deficient forms of spastin that do not sever MTs (Evans et al., 2005). In addition to its MT-severing, spastin promotes the bundling of MTs *in vitro* and *ex vivo*, independently of its ATPase activity (Salinas et al., 2005). This novel observation suggests that spastin may have two functions; ATP independent MT-bundling activity and ATP dependent MT-severing activity. A recent study showed that spastin assembles into a hexamer in the presence of ATP and recognizes the CTT domains of tubulins for an effective MT-severing (White et al., 2007). Thus, both spastin and katanin strongly interact with the CTTs of tubulins. This result is important because the interaction of MT severing factors with CTTs brings a possibility that both of these severing proteins are affected by post-translational modifications (PTMs) of microtubules.

Posttranslation tubulin modifications (PTMs)

Most known PTM types affect the CTTs of tubulins on assembled microtubules. These PTMs are highly conserved and interestingly, exist in organisms that have spastin and katanin but are absent in organisms that lack severing factors such as fungi. The cellular role of PTMs is poorly understood. Earlier research from our laboratory established that mutations at sites of PTMs known as polymodifications on β-tubulin led to hyperstable, abnormally long cortical microtubules in *Tetrahymena thermophila* (Thazhath et al., 2004; Thazhath et al., 2002). This observation suggested that polymodifications on β-tubulin could regulate a MT depolymerizing or specifically severing activity. Thus, an important goal of this thesis was to evaluate whether katanin, spastin and PTMs act in the same pathway. The simplest idea that we considered was that PTMs mark MTs to create preferred sites for katanin or spastin severing activity and

therefore provide a spatial control of microtubule severing. The most likely PTMs to influence katanin and spastin are those that are present on CTTs (polyglycylation, polyglutamylation and detyrosination). What is known about these PTMs is provided below. Some information regarding acetylation of Lys-40 is also provided. While this modification occurs in the lumen of a microtubule (Odde, 1998), it is commonly used as a marker of the dynamic state of microtubules (Takemura et al., 1992; Westermann and Weber, 2003) and this PTM was assayed in this thesis work.

Both α- and β-tubulin are affected by various types of modifications including phosphorylation, palmitoylation, acetylation, detyrosination, polyglutamylation and polyglycylation (Westermann and Weber, 2003). These PTMs are highly conserved from protist to humans, but their significance is not well understood. Very few functional studies have been done to understand their importance. The majority of PTMs occur on the CTTs of α- and β-tubulin so they are predicted to mark the outside surface of MT polymer (Nogales et al., 1999; Nogales et al., 1998b). One exception is α-tubulin acetylation that occurs at the N-terminal Lys-40 residue, which appears to be located inside the lumen of MTs (Odde, 1998). Another exception is phosphorylation of Ser-172 on β-tubulin because this PTM occurs on unpolymerized tubulin and inhibits the ability of dimers to polymerize (Fourest-Lieuvin et al., 2006). In the next sections specific focus is on PTMs that are likely to occur on the tail domains of tubulins, the sites of interactions with spastin and katanin.

Polyglycylation

Tubulin polyglycylation is the addition of multiple units of glycine to the conserved glutamic acid residues present in the CTT of α- and β-tubulin polypeptides. Polyglycylation

appears to be restricted to ciliated or flagellated cells (Iftode et al., 2000). Several potential glycylation sites on CTT of α- and β-tubulin were mapped in *Paramecium* using mass spectrometry (Vinh et al., 1999). Later similar sets of sites were confirmed in *Tetrahymena* by *in vivo* mutagenesis (Xia et al., 2000). Xia et al 2000 revealed that sites of polyglycylation on α-tubulin are dispensable whereas the sites on β-tubulin are essential for cell survival. Later, Rupal Thazhath performed mutational analysis of polyglycylation sites present in the CTT of β-tubulin by using germline heterokaryons methodology (Cassidy-Hanley et al., 1997). Knockout heterokaryons strains were constructed that introduce a triple site mutation in CTT of β-tubulin ($\beta DDDE_{440}$) into the transcriptionally silent micronucleus. Upon completion of conjugation, the mutated gene is expressed in subsequent progenies. $\beta DDDE_{440}$ mutants failed to complete cytokinesis and assemble immotile cilia (Thazhath et al., 2002). The immotile ciliary phenotype was due to inability of mutants to assemble the central pair (9+0 axoneme). In addition, several other structural abnormalities were detected in the axoneme of $\beta DDDE_{440}$ mutants such as incomplete peripheral doublets, displacement from peripheral position to the center, and large gaps in the transition zone. The basal bodies, however, which are rich in polyglutamylated tubulin, appeared to be unaffected in these mutants. In contrast to the lack of assembly in axoneme, a hypertrophy of MTs was detected in the cortex of these mutants, which potentially caused an arrest in cytokinesis. These mutant cells failed to complete cytokinesis and this resulted in formation of cell chains. The hyperassembly severely affected a specialized type of MTs in the cortex called longitudinal microtubules (LMs) bundles. LMs are bundles of non-overlapping MTs (5-8 MTs), which run along the ciliary rows from and undergo breakage during cytokinesis in WT *Tetrahymena* cells. In $\beta DDDE_{440}$ mutants, LMs bundles consist of 11-15 MTs indicating their impaired depolymerization that in turn could have caused cytokinesis arrest

(Thazhath et al., 2004). Such differential effects caused by deficiency of polyglycylation sites could be attributed to different MAPs. One possible explanation is that some axoneme specific MAPs may require polyglycylation sites for the stability of axoneme and its subcomponents. In the cell cortex, sites of polyglycylation may regulate MT-severing factors such as katanin or spastin to maintain the proper length of LM segment MTs and as a consequence to control the thickness of these bundles. A recent study from *Chlamydomonas* provided new clues to the mechanism of central pair loss in $\beta DDDE_{440}$ mutants. An insertional mutagenesis of non-catalytic subunit of katanin, p80 led to assembly of normal length 9+0 paralyzed flagella (Dymek et al., 2004). A MT-severing activity may therefore be a major effector of polyglycylation sites in both the axoneme assembly as well as severing of LM in the cell cortex. If this is the case, lack of MT-severing activity appears to be straightforward for cortical MTs whereas the exact mechanism involving severing factor(s) in the assembly of cilia remains unclear. Enzymes responsible for the glycylation of α- and β-tubulins have been recently identified (K. Rogowski and D. Wloga personal communications) and their functions are under investigation.

Polyglutamylation

In this type of PTM, a polyglutamate side chain of variable length is attached through an isopeptide bond to the γ-carboxyl group of a glutamic acid in the CTT domain of α- and β-tubulin (Westermann and Weber, 2003). This unusual PTM was first identified in mammalian brain α-tubulin (Edde et al., 1990) and the biochemical nature of these side chains was characterized (Redeker et al., 1991). This PTM is highly conserved from protists to mammals. Glutamylation appears to be correlated with the presence of cilia and centrioles. In protists, polyglutamylation is abundant in stable microtubular structures such as ciliary axoneme and

basal bodies (Bre et al., 1994; Lechtreck and Geimer, 2000). In mammals, this modification is present on non-ciliary MTs as well. The majority of tubulin in the adult mammalian brain is polyglutamylated suggesting that this PTM plays a vital role in neural development (Wolff et al., 1992). In non-neural vertebrate cells, glutamylated tubulins are concentrated in centrioles, mitotic spindle, midbody, and primary cilium (Bobinnec et al., 1998b). In addition to α- and β-tubulin, polyglutamylation also occurs on the nucleosomal assembly proteins, NAP1 and NAP2, suggesting a function of this PTM in regulating chromatin structural proteins (Regnard et al., 2000). Injection of monoclonal antibodies specific to glutamylated tubulin caused disassembly of centrioles in HeLa cells (Bobinnec et al., 1998a). Similar antibodies blocked the motility of reactivated sea urchin sperm flagella suggesting that glutamylated tubulin plays a role in dynein-based motility (Gagnon et al., 1996). Mice lacking a subunit of an α-tubulin-specific glutamylase showed degradation of sperm axonemes and decreased male-male aggression, suggesting a major role in the assembly of axonemes and a subtle role for neuronal MTs in the brain (Campbell et al., 2002). A recent study showed that in these mice, KIF1a kinesin localization, which is required for normal synaptic transmission in neurons is perturbed (Ikegami et al., 2007). Tubulin polyglutamylation also regulates the binding affinity of MAPs to MTs (Bonnet et al., 2001; Boucher et al., 1994). Polyglutamylated α-tubulin is present at similar levels in the brain during development from neonate to adult. On the other hand, levels of polyglutamylated β-tubulin are higher in adult brain suggesting that β-tubulin polyglutamylation is required for the maturation of the nervous system (Audebert et al., 1994). The catalytic subunits of tubulin polyglutamylase enzymes contain a conserved TTL domain (Janke et al., 2005) that was originally identified in the enzyme responsible for addition to tyrosine residue to the α-tubulin (Barra et al., 1988; Ersfeld et al., 1993). Recent studies identified enzymes responsible for glutamylation of α- and

β-tubulin in mammals and *Tetrahymena* (Ikegami et al., 2006; Janke et al., 2005). The murine TTLL7, a β-tubulin polyglutamylase, accumulates in MAP2-enriched neurons and is required for growth of neurite in PC12 cells (Ikegami et al., 2006). Excessive polyglutamylation caused by overexpression of TTLL6Ap inhibited the cell proliferation and affected dynein-based ciliary motility in *Tetrahymena* (Janke et al., 2005). Elimination of specific α-tubulin glutamylase genes in *Tetrahymena* affected cell growth, cilia-dependent phagocytosis and development of certain microtubular structures (D. Wloga unpublished results). Remarkably, deletion of some β-tubulin specific glutamylase genes blocked assembly of B-tubules of ciliary doublets (S. Suryavanshi, unpublished data).

Detyrosination

Most α-tubulin contains a tyrosine residue at the extreme COOH-terminus. The removal of tyrosine residues by a yet unidentified tubulin tyrosine carboxypeptidase (TTCP) is called detyrosination (Argarana et al., 1978). The tyrosine residue is incorporated into the α-tubulin polypeptide chain in a transfer-RNA independent manner, earlier demonstrated in brain homogenates (Arce et al., 1975; Barra et al., 1973). MT polymerization kinetics is independent of the presence or absence of tyrosinated-tubulin at the COOH-terminus. Like acetylation, detyrosination preferentially accumulates on stable MTs but does not cause stability of MTs (Khawaja et al., 1988). In mammalian cells, the detyrosinated MTs preferentially associate with the vimentin intermediate filaments (VIFs) and the distribution of VIFs is dependent on kinesin motors, which may interact with detyrosinated α-tubulin in a selective manner (Kreitzer et al., 1999; Liao and Gundersen, 1998). Inhibiting the detyrosination in myoblasts with 3-nitrotyrosine blocked morphological differentiation and led to accumulation of muscle-specific factors (Chang et al., 2002). The reverse reaction, addition of tyrosine to the penultimate glutamic acid residue

at the extreme COOH-terminus is an ATP dependent reaction catalyzed by tubulin tyrosine ligase (TTL) (Ersfeld et al., 1993). A knockout of the TTL gene in mice led to lethality shortly after birth, most likely due to defective neuronal organization during prenatal development (Erck et al., 2005). Thus, the addition of tyrosine residue appears to be important, but the *in vivo* significance of detyrosination in mammals is not known. α-tubulin also incorporate 3'-nitrotyrosine, a modified amino acid that is generated by the reaction of nitric oxide species with tyrosine. This ligation reaction is also catalyzed by TTL enzyme *in vivo* and *in vitro* (Kalisz et al., 2000) in an irreversible manner. Such abnormal accumulation of modified tyrosine led to MT dysfunction and cellular injury in epithelial lung cancer cells (Eiserich et al., 1999). In *Tetrahymena*, the α-tubulin COOH-terminus ends in the sequence E-G-Y, unlike in animals where the COOH-terminus sequence is E-Y. *Tetrahymena* do not possess an enzyme, which exhibits classical TTL activity. Surprisingly, recent mass spectrometry (MS) analysis showed extensive detyrosination of nearly all α-tubulin in cilia in *Tetrahymena* (Redeker et al., 2005) indicating the presence of tubulin carboxypeptidase activity in this organism. Interestingly, in *Tetrahymena*, blocking the removal of terminal tyrosine residue by addition of a tag of 6 Histidine residues did not produce a change in the phenotype (J. Gaertig personal communication).

Acetylation

Acetylation is the only PTM that does not affect the CTT domain of either α-or β-tubulin. It occurs on the epsilon-amino group of a conserved lysine 40 residue in one of the loops of the N-terminal domain of α-tubulin. Electron microscopic crystallographic studies predicted that modified lysine residue points towards the lumen of the MT (Nogales et al., 1999). The PTM was first discovered in the flagellar MTs of *Chlamydomonas* (L'Hernault and Rosenbaum,

1985) and later found in most eukaryotic organisms (Piperno and Fuller, 1985; Piperno et al., 1987). Acetylation occurs on stable microtubular structures such as axonemes, but is probably not directly involved in stability of these structures. The enzymes responsible for α-tubulin acetylation are unknown but a partial tubulin acetyltransferase activity was purified from *Chlamydomonas* flagella (Maruta et al., 1986). HDAC6, , is mammalian tubulin deacetylase that is related to histone deacetylases (Hubbert et al., 2002). It deacetylates tubulin and MTs *in vitro* and *in vivo* (Matsuyama et al., 2002). Overproduction of HDAC6 significantly decreases the levels of tubulin acetylation, stimulate the chemotactic movement of fibroblasts (Hubbert et al., 2002). Consistently, an inhibitor of HDAC6, tubacin decreased cell motility (Haggarty et al., 2003). However, expression of non-acetylatable α-tubulin in *Chlamydomonas* (Kozminski et al., 1993a) and replacement of the single conventional α-tubulin gene with a nonmodifiable variant in *Tetrahymena* (Gaertig et al., 1995) did not affect the assembly of MTs and related organelles. Also, a disruption of HDAC6 deacetylase activity in embryonic stem cells increased the level of tubulin acetylation, but did not significantly affect cell proliferation or differentiation (Zhang et al., 2003). One study showed that a certain subpopulation of MTs exhibit increased stability after HDAC6 inhibition, suggesting that acetylation influences the stability of MTs (Matsuyama et al., 2002). In contrast, Palazzo et al 2003 showed that tubulin acetylation does not increase MT stability but it may accumulate on MTs stabilized by some other mechanism (Palazzo et al., 2003). These results are consistent with earlier observations that tubulin acetylation does not affect the assembly of MTs and acetylated tubulin can be detected in long-lived MTs (Webster and Borisy, 1989). Palazzo et al 2003 also suggested that cell motility is not affected by MT stability but is influenced by the level of acetylation on MTs. Another tubulin deacetylase, SIRT2, has been identified in humans as an ortholog of Sir2p (silent information regulator 2

protein). Human SIRT2 shows a striking preference for acetylated-tubulin peptide in comparison to acetylated histone H3 peptide, which suggest that it may partly involved in tubulin deacetylation along with HDAC6 (North et al., 2003). Interestingly, recently study showed that MT acetylation stimulates the recruitment of kinesin-1 motor to neurite MTs (Reed et al., 2006). Thus, acetylation could function as a mark that directs motors to the axon and not to dendrites in nerve cells.

Cilia

Cilia (note the term cilia is used for eukaryotic flagella here; in principle cilia and flagella are the same organelle and differ only in length) are highly conserved organelles that emanate from the surface of eukaryotic cells (Davenport and Yoder, 2005; Gibbons, 1981). Cilia are present in all eukaryotes except most higher plants, some apicomplexans such as *Cryptosporidium*, fungi and slime molds. It is assumed that organisms such as *Arabidopsis thaliana*, *Saccharomyces cerevisiae*, and *Dictyostelium discoideum* have lost cilia (as well as standard centrioles/basal bodies) during evolution as a specialization to their specific life styles (Stechmann and Cavalier-Smith, 2002). The cilium consists of a MT-rich axoneme covered by a specialized plasma membrane that is a continuation of the plasma membrane. Cilia can be classiffied into three categories. Motile cilia usually occur in groups on cells and have an axoneme composed of nine pairs of outer doublet MTs surrounding a single pair of central MTs (9+2 arrangement). Examples of motile cilia are those present on the epithelial lining of lungs, on ependymal cells lining brain ventricles, on the surface of motile protists *Tetrahymena*, *Paramecium, Chlamydomonas*, and on mammalian sperm. Nodal cilia and primary cilia, both have an axoneme with a 9+0 arrangement of MTs as these cilia lack the central pair of MTs.

Nodal 9+0 cilia occur as solitary cilia on cells of the embryonic node in vertebrates, but have dynein arms and exhibit a rotational movement that establishes the left-right asymmetry in the developing mammalian embryo (Yost, 2003). Immotile 9+0 primary cilia occur on most epithelia and stromal cells in mammals. Primary cilia perform a variety of sensory functions including chemosensation, photoreception and olfaction and are assumed to be a result of evolutionary loss of structures required for motility (such as dynein arms, radial spokes, and central pair) and acquisition of signaling pathways required for sensation (Perkins et al., 1986).

The 9+2 structure of motile cilia was first revealed by transmission electron microscopy (Fawcett, 1954; Nicastro et al., 2006). The structure and molecular composition of axoneme are well conserved among eukaryotic motile cilia from protozoans to human. Ciliary axoneme represents highly complex microtubular structure consisting of about 400 distinct proteins (Gherman et al., 2006; Inglis et al., 2006; Pazour et al., 2005). Cilia are nucleated by basal bodies (BB). The BB is composed of nine triplets MT and is in principle a modified centriole that serves as a template for assembly of the cilium. In mammals, the BB is formed by differentiation and migration of a centriole from the centrosomal location to the plasma membrane (Dawe et al., 2007). In *Chlamydomonas*, the BB converts back into a centriole and serves during mitosis. However, in ciliates, BBs have lost their mitotic function,are permanently located in the cell cortex and specialized in ciliary biogenesis. The ciliary outer doublet MTs are extensions of the A- and B-tubule of the basal body (BB) MT triplets. Major structures in motile 9+2 cilium, which interact with MTs, are central pair-associated projections, outer dynein arms (ODA), inner dynein arms (IDA) and radial spokes (Nicastro et al., 2006). Unlike peripheral doublet MTs, the central pair singlet MTs are not a continuation of the BB triplet MTs and are more labile than doublet MTs (Dutcher, 1995). The central pair microtubules nucleate from an electron-dense

structure located on top of the basal body. Importantly, the mechanism, of nucleation of the central pair is distinct from the one responsible for assembly of ciliary doublets. Specifically, the assembly of the central pair is sensitive to a knockdown of γ-tubulin, while doublets assemble under the same conditions. This could be a simple consequence of the fact that the doublets polymerize as extensions of triplets of the BB while the central pair assembles *de novo* and requires γ-tubulin as the nucleating complex at the central structure (McKean et al., 2003). The two MTs of the central pair are composed of 13 protofilaments, but they are not identical and can be distinguished (as C1 and C2) based on the length of associated projections and stability (Linck et al., 1981). In addition to αβ-tubulin, the central pair complex consists of at least 23 polypeptides (most of which are probably MAPs bound directly to MTs), which are highly conserved across the organisms possessing motile cilia or flagella (Adams et al., 1981; Dutcher et al., 1984). In the part of my doctoral research that is not described in the present dissertation, I produced gene deletions in *Tetrahymena* orthologs of two central pair components, PF20 and PF16, and obtained cells with nearly paralyzed cilia (unpublished results). Mutations in other central pair proteins invariably have led to ciliary paralysis (Mitchell, 2004). In some cell types such as in the ciliate *Paramecium* and *Chlamydomonas*, the central pair undergoes rotation as the cilium bends (Omoto and Kung, 1980; Omoto and Witman, 1981). This led to a hypothesis that the rotating central MTs regulate the activity of ciliary dynein that is associated with outer doublet MTs (Mitchell and Nakatsugawa, 2004). The rotating central pair could be transmitting signals to doublets via radial spokes, possibly using a system of kinases/phosphatases that are associated with the radial spokes (Porter and Sale, 2000; Yang et al., 2006). A kinesin related protein, Klp1, could play a role in the rotational motility of the central pair as this kinesin is located along one of the two central pair MTs and its knockdown lead to defective ciliary

motility (Yokoyama et al., 2004). While the central pair is important for ciliary motility, the rotation-based model is uncertain and may not be general. This is because some cell types have motile 9+2 cilia but the central pair is in a fixed position (Gadelha et al., 2006). Furthermore, suppression mutations were identified that restore motility in 9+0 flagella of *Chlamydomonas* (Huang et al., 1982).

Doublet MTs can be distinguished from central pair MTs based on their physical and structural properties. Importantly, the mechanism of assembly of doublet MTs must be different from the one responsible for assembly of the more common singlet MTs. A similar mechanism may be responsible for the formation of triplet MTs in centrioles/basal bodies. Delta-tubulin is a tubulin-related protein that is required for the formation of triplet MTs (specifically the C-tubule) in BBs and centrioles (Dutcher 1998, Garreau et al 2001) but its role in the formation of doublet MTs. It is possible that delta-tubulin is a nucleating factor for the C-tubule of BBs/centrioles. The loss of another tubulin protein epsilon-tubulin, in *Paramecium* inhibited the formation of both the C and B-tubules (Depuis-Wiliams 2002). Thus, it is possible that epsilon tubulin provides a nucleation function for the B-tubule. The formation of doublets of the cilium would require a simple extension of the A and B-tubule while the C-tubule could be capped on the top of the basal body. However, assembly of doublet MTs is not a simple matter of extension of the A and B tubules of the BB. *In vitro*, when tubulin dimers are added to axonemal seeds, only singlet MTs form. One possible reason for that is that doublet MTs are made not just of tubulins but also of a number of additional proteins that may stabilize the doublet structure. Recent cryoelectron tomography studies of axonemes provided a detailed insight about the structure of doublet MTs (Nicastro et al., 2006; Sui and Downing, 2006). The peripheral doublet consists of complete A-tubule and an incomplete B-tubule, which comprise of 13 protofilaments (numbered

PF A1-A13) and 10 protofilaments (numbered PF B1-B10), respectively. In their highest resolution model, Sui and Downing illustrated the presence of increased density of non-tubulin proteins between the partition zone of A and B tubule. Previous and current studies established that stable ribbons formed by three or four adjoining protofilaments arise from the partition region (Nojima et al., 1995; Stephens et al., 1989; Sui and Downing, 2006). Besides α- and β-tubulin, the major components of ribbons are tektin (tektin A, B, and C) and Sp77, Sp83 and Rib72 proteins (Linck and Norrander, 2003). CryoEM analysis of doublet MTs confirmed earlier immuno electron microscopic observations that Sp77, Sp83 and Rib72 are the components of the linker structure (a part of the partition region), which connect A and B tubule at every 16 nm periodicity. Rib72, a *Chlamydomonas* homologue of Sp77, may be involved in Ca^{2+} dependent regulation of ciliary and flagellar beating.

Intraflagellar Transport

The assembly and maintenance of cilia and flagella is dependent on a highly conserved type of organellar motility known as intraflagellar transport (IFT). Since cilia are devoid of ribosomes, IFT transports all structural components of the axoneme and cilia signaling components from the cell body to the tip of the axoneme and retrieves recycled components back to cell body (Kozminski et al., 1993b; Scholey, 2003). Multimeric protein complexes called IFT particles are transported bidirectionally from the BB region to the tips of flagella and back, and can be seen *en route* between the ciliary membrane and the outer doublet MTs of cilia. IFT particles, purified from *Chlamydomonas* cells, are composed of at least 16 polypeptides. IFT particles exist in two complexes: complex A (4 proteins) and complex B (12 proteins) (Cole et al., 1998). IFT is dependent on coordinated functions of two IFT motor proteins (Cole et al., 1998; Ou et al., 2005). IFT particles are moved from the basal body to the tip of cilium (anterograde movement)

by one or two plus end directed kinesin-2 motors and move in the reverse direction (retrograde transport) using a minus end directed cytoplasmic dynein type motor called IFT-dynein (cytoplasmic dynein heavy chain 1b/light-intermediate chain complex, cDhc1b/LIC complex). IFT motors, IFT particles and their associated cargo accumulate around the BB prior to entering the axoneme (Deane et al., 2001; Iomini et al., 2001). In *Chlamydomonas*, IFT complexes initially associate with the transitional fibers that extend from the distal portion of the basal body to the plasma membrane and could function as a loading dock for IFT (Deane et al., 2001). Following assembly of the transport machinery, the anterograde kinesin-2 motors moves the IFT particles and associated cargo of ciliary precursors along to the tip of the axoneme. Earlier studies showed that assembly of ciliary components occurs at the distal end of cilia that contain plus ends of axonemal microtubules (Johnson and Rosenbaum, 1992). TEM analysis of the *Chlamydomonas* flagellum revealed that IFT particles move along the B subfiber of outer doublet MTs (Kozminski et al., 1993b). The main function of the anterograde movement is to transport the building blocks required for the elongation of cilia from the BB to the growing tip of the cilium. The clue that kinesin-II functions as an anterograde IFT- motor came from studies in *Chlamydomonas*. The FLA10 gene encodes a kinesin-2 motor subunit and a member of the kinesin-2 family of heterotrimeric, microtubule-plus-end-directed kinesins (Scholey, 1996). A temperature-sensitive mutant of flagellar *fla10*, cannot assemble flagella at nonpermissive temperature and existing flagella undergo shortening and complete disappearance at nonpermissive temperature in these mutants. Under restrictive conditions in *fla10* mutants, IFT particles can not be transported flagella cannot be assembled or maintained (Adams et al., 1982; Kozminski et al., 1995; Rosenbaum and Witman, 2002). When temperature-sensitive *fla10* cells maintaining full-length flagella at the permissive temperature were shifted to restrictive

temperature and deflagellated, they cannot regenerate their flagella. Injection of antibodies against a motor subunit of kinesin-2 into sea urchin blastulae led to the formation of short paralyzed cilia due to lack of central pair of MTs (Morris and Scholey, 1997). A knockout of two partially redundant kinesin-2 genes led to the lack of cilia in *Tetrahymena thermophila* (Brown et al., 1999b). Furthermore, kinesin-2 deficient *Tetrahymena* often failed to complete cytokinesis. This is due to the fact that in *Tetrahymena*, ciliary motility is required for the abscission of the cytokinetic connection at the end of cell division (Brown et al., 1999a). In mouse embryos, the nodal cilia did not assemble when either KIF3A or KIF3B subunits of kinesin-II were knocked out (Marszalek et al., 1999; Nonaka et al., 1998).

Two related kinesin motors; kinesin-2 and OSM-3 are required for the anterograde transport to build sensory cilia of neurons in *C. elegans* (Signor et al., 1999b). Kinesin-2, which is a typical heterotrimeric IFT motor, is expressed in all ciliated neurons whereas homodimeric OSM-3 expression is restricted to chemosensory neuronal cilia (Tabish et al., 1995). It is necessary to mention here that in these nematode dendritic cilia compartmentalization is different from other typical cilia and flagella. The chemosensory cilia of dendrites can be divided in three different sections; 1 μm long proximal segment that is the functional counterpart of a transition zone, 4 μm long middle section contains doublet MTs and 2.5 μm distal segment of singlet microtubules. Both motors (Kinesin-2 and OSM-3) coordinately move anterograde IFT particles at a rate of 0.7 μm/sec in the middle segments, whereas OSM-3 alone moves the particles with a speed of 1.3 μm /sec along the singlet microtubules of the distal segment. These observations indicate that Kinesin-2 is sufficient for the formation of doublet MTs, additional OSM-3 motor is required for the further elongation of single MTs in the distal region. A dual motor system may

be needed for IFT if there are structural differences between the distal and proximal axonemal tracks (doublet MTs vs. single MT).

The retrograde motor for IFT, IFT-dynein was discovered in sea urchin embryos, as a protein whose mRNA is upregulated following deciliation, suggesting its role in ciliary assembly (Gibbons et al., 1994). Mutations in genes encoding IFT-dynein heavy chains, light intermediate chains in *Chlamydomonas* or *C. elegans* lead to assembly of short cilia. (Pazour et al., 1998; Porter et al., 1999; Signor et al., 1999a). These short cilia have bulged tips filled with IFT particles. In retrograde motor IFT-dynein (cytoplasmic dynein 1b motor) mutants, IFT particles can move into the flagellum by the action of kinesin-2, but cannot be returned to the base of the flagellum owing to the absence of the retrograde motor (Pazour et al., 1998; Signor et al., 1999a). The movement of IFT particles is continuous in both directions with transport at 2.5 μm/sec and 4.0 μm/sec in the anterograde and retrograde directions, respectively. Kinesin-2 is carried as an IFT cargo from the ciliary tip to the base by IFT-dynein. Conversely, IFT dynein initially travels as a cargo using kinesin-2 dependent transport. However, it is unknown how IFT-particles assemble and disassemble at the base and tip of the cilium and what is the mechanism that governs the loading and unloading of molecular motors at designated locations. At the tip, the size of IFT particles is reduced possibly due to unloading of axonemal precursors (Kozminski et al., 1993b; Piperno et al., 1998), the kinesin-2 motor becomes cargo and cytoplasmic dynein 1b takes over to transport the particles back to the BB region. IFT particles remodeling occurs at the tip and base of the cilium, suggestive from slower movement of anterograde IFT and more numerous retrograde particles (Iomini et al., 2001). Because the IFT particles move unidirectionally without pausing, the regulator proteins that turn kinesin-2 on and IFT-dynein off must be concentrated at the base of the flagellum. Studies on various flagellar tip mutants

suggest that complexes A and B dissociate from each other when they are released into the tip compartment for the subsequent reorganization of IFT particles and for association of complex A with active IFT-dynein prior to retrograde transport (Iomini et al., 2001; Pedersen et al., 2006; Pedersen et al., 2005). The exact mechanism of loading and unloading of IFT particles and their association with specific motor proteins is unknown. Ciliary MTs possess polarity with their plus ends toward the tip of the cilium and minus end directed to the base of the cilium. EB1 protein was initially identified as MT-plus end tracking protein, which binds to the plus ends of cytoplasmic, spindle and astral MTs (Berrueta et al., 1998; Mimori-Kiyosue et al., 2000; Morrison et al., 1998). Later EB1 was identified as a molecular component of axonemes in connecting cilium of rod photoreceptor cells which encouraged IFT researchers to analyze the role of EB1 in trafficking of IFT at distal tip of the cilia (Schmitt and Wolfrum, 2001). Pederson and colleagues identified an orthologue of EB1 in *Chlamydomonas*, which localizes to the tip of flagella and the proximal region of the basal bodies (Pedersen et al., 2003; Sloboda and Howard, 2007). Complete depletion of EB1 from the flagellar tip, led to accumulation of IFT complex A polypeptides near the tip, a region where remodeling of IFT particles is believed to occur (Pedersen et al., 2003). The exact cause of such abnormal accumulation of IFT particles in mutants lacking EB1 at the tip is not clear. However, in the light of the function of other homologs of EB1 (Carvalho et al., 2003), it is possible that *Chlamydomonas* EB1 promote dynamic instability of axonemal MTs at the tip of the flagellum, thereby regulating the flagellar assembly and rates of turnover axonemal proteins (Qin et al., 2004).

Knowledge about the nature of cargo that is bound to IFT particles is still incomplete. It is assumed that the cargo represents most if not all-axonemal components. Presumably, the cargo is required for the assembly, motility, and signaling properties of cilia and flagella, and thus IFT

particles are likely to transport structural and force-generating components of the axoneme, as well as sensory ciliary membrane receptors and associated signaling molecules. It is known that IFT machinery is required for delivery of inner dynein arms (Piperno et al., 1996). Recently a direct connection was established between one of the IFT proteins and outer dynein arms. Witman and others revisited the role of IFT 46 protein previously identified as a component of complex B (Cole et al., 1998). *Chlamydomonas* insertional mutants of IFT46 show reduced levels of complex B particles and also assembled truncated paralyzed flagella defective in the assembly of dynein arms and central pair MTs (Hou et al., 2007). Analysis of IFT46 suppressor mutation revealed that IFT46 protein is specifically involved in targeting of outer dynein arms into the flagella via IFT pathway.

Mutations in IFT particles also affect the transport of tubulin (Marshall and Rosenbaum, 2001), and aurora-like kinase (Pan and Snell, 2003). In vertebrate retina, IFT delivers signaling molecules involved in photoreception to rod outer segments, which is a modified cilium (Marszalek et al., 2000; Pazour et al., 2002). Importantly, many ciliary proteins have been found preassembled in cytoplasm before delivering them to cilia. For example, radial spokes, complexes of 17 polypeptides and outer dynein arms, which consist of at least 15 polypeptides, preassemble in the cytoplasm before their hauling to axoneme by IFT particles (Diener, 1996; Fowkes and Mitchell, 1998). The most plausible explanation for such process is that preassembly is not possible within the cilia due to spatial constraint.

It is a long lasting enigma, whether tubulin is transported to cilia as dimeric subunit, short pieces of MTs or as polymer. The form of tubulin transported to cilia is not precisly understood. Studies done in axons of nerve cells have provided some clue because like cilia, axons also lack the protein synthesis machinery and both structures require continuous flow of tubulin for proper

maintenance of their structures. Earlier studies suggested that tubulin is transported to the axon as soluble subunits (Hirokawa et al., 1997; Miller and Joshi, 1996) or as stable polymers (Baas and Brown, 1997). Recently *ex vivo* studies were performed on fluorescent-labeled tubulin in squid giant axons to investigate the molecular mechanism of transport and the form in which tubulin is transported (Terada et al., 2000). Based on diffusion time difference between different states of tubulin (soluble vs. polymerized), it is quite convincing that tubulin is transported as an oligomer but not as dimer or polymer to the end of the axons. Their studies also revealed that tubulin transport inside axons is dependent on the kinesin motor because inhibition of kinesin function by injection of anti-kinesin antibody blocked tubulin transport significantly.

In addition to assembly and maintenance of the cilium, IFT also plays an important role in signaling and development. In *Chlamydomonas*, IFT participates in activation of cGMP dependent protein kinase which is further required for gamete activation and cell-cell fusion (Wang et al., 2006). Mutation in IFT particles causes defects in chemotaxis and osmotic avoidance behavior as well as results in extended life span in *C. elegans* (Schafer et al., 2006; Scholey and Anderson, 2006). Growing evidences indicate that functional IFT components are essential for normal development of kidney, cellular metabolic pathways and left-right patterning (Davenport and Yoder, 2005; Pazour and Rosenbaum, 2002).

Cilia and Intraflagellar transport associated diseases

Consequences of structural or functional aberration in cilia or flagella depend on the cell type. Mice lacking a central pair protein, SPAG6, murine orthologue of *Chlamydomonas* PF16, show severe symptoms of hydrocephalus (due to immotile ependymal cilia), and infertility (caused by immotile sperm flagella) (Sapiro et al., 2002). Epithelial motile cilia present in the

human respiratory tract and female oviduct contribute to the propagation of mucus and suspended particles across the tissue surface (mucociliary clearance). Genetic defects in motile cilia that affect the beating cause Primary Cilia Dyskinesia (PCD), which is associated with various clinical manifestations including respiratory diseases, infertility and *situs invertus* (Chilvers et al., 2003). Reduced mucociliary clearance is the primary cause of recurrent infection of lower and upper respiratory tracts. About 50% of PCD patients are diagnosed with *situs invertus* (Kartagener syndrome), which is a complete left right reversal of internal organs. It has been well established that defects in ciliary motility is the primary cause of *situs inversus* (Yost, 2003). Asymmetric fluid flow in mammalian node initiates the switching of bilateral symmetry to left-right asymmetry (Hirokawa et al., 2006). According to the mechanosensory model of development of left-right asymmetry, embryonic node is comprised of motile (9+0 monocilium) as well as non-motile cilia (9+0 primary cilium) (McGrath et al., 2003). Motile cilium, which contains the motor protein left-right dynein, is centrally located at the node. Non-motile cilia, lacking the dynein motor, are distributed around the periphery of the node. The motile cilium in the center generates nodal flow from right to left and peripheral primary cilia detect leftward nodal flow through mechanosensor, pkd-2 (product of polycystic kidney disease type 2 gene) present in the ciliary membrane. However, a recent model suggests that left-right asymmetry is generated by leftward movement of membrane-sheathed particles by nodal flow (Tanaka et al., 2005). This flow may specifically increase Ca^{2+} concentration on the left side that can further activate hedgehog-signaling pathway for the establishment of left-right asymmetry.

One of the cyst diseases is polycystic kidney disease, inherited as a recessive (ARPKD) or dominant (ADPKD) trait that results from mutations in pkhd1 and pkd-1 or pkd-2, respectively (Pazour, 2004; Yoder et al., 2002a). In the PKD condition, defective cilia-mediated

sensing of urine flow in the collecting tubule results in overproliferation of cells, which led to formation of cysts. The ciliary membrane is rich in receptors, ion channels and signaling molecules, which could be activated by chemical, mechanical or environmental cues. In the kidney, primary cilia are present on most cells of the nephron (except intercalated cells) where they act as mechanosensors, responding to fluid-flow via membrane proteins, such as polycystins (polycystin-1 and polycystin-2) (Nauli et al., 2003; Nauli and Zhou, 2004). A unifying theme that has emerged from studies of PKD is that affected proteins localize to the cilium or basal body (Calvet, 2003a; Ong and Wheatley, 2003). The identification of the *Chlamydomonas* Complex B subunit IFT88, as the homologue of the murine Tg737 gene was a striking discovery, which linked structural abnormality of primary cilia to the development of PKD (Pazour et al., 2000; Yoder et al., 1995; Yoder et al., 2002b). IFT88 also known as Tg737, Polaris or OSM-5, is required for cilia function, is a component of the IFT complex B. Mice with insertional mutation in $Tg737^{orpk}$ develop PKD and have defects in left-right patterning and retinal degeneration. Further, disruption of KIF3a (IFT anterograde kinesin) confirmed that proper cilia formation is required for normal renal function (Lin et al., 2003). It is quite clear that increased cell proliferation is a key feature of PKD disease, but the mechanism of uncontrolled cell division mediated by defective cilia or its function is still not clear.

The primary cilium occurs on the surface of differential cells, anchored to the BB beneath the plasma membrane (Wheatley et al., 1996). The BB develops from the mother centriole of the centrosome in a manner that is coordinately regulated with the cell cycle. As cells reenter the cell cycle, the cilium and BB are disassembled, enabling centrioles to function as nucleating center for the mitotic spindles. Once a cell completes the cell division and becomes quiescent, the mother centriole transitions to the BB and assembles the cilium (Quarmby and Parker, 2005).

Further evidence that cilium, BB and centrosome are associated structurally and functionally comes from the recent observations that certain proteins linked with ciliary diseases localize to all three structures (Badano et al., 2005). The recent renaissance of primary cilium has uncovered it from being a vestigial organelle to one of a central importance in a number of development processes and diseases (Calvet, 2003b; Pazour and Rosenbaum, 2002).

Tetrahymena thermophila as a model organism

Tetrahymena thermophila is well-suited organism to study the functions of katanin and spastin in the context of various types of microtubular organelles (such as cilia) and associated tubulin posttranslational modifications. Yeast lacks katanin, spastin and tubulin polymodifications and *Chlamydomonas* does not have a high level of homologous recombination. *Tetrahymena* is a free-living, ciliated protist that exhibits typical eukaryotic features and some unusual adaptations. Recent completion of *Tetrahymena* 100 MB genome sequence (Eisen et al., 2006) and development of novel molecular genetic methodologies make it a very useful eukaryotic model organism. It provides a very strong system for reverse genetic analysis by utilizing several methods such as germline knockout or gene replacement, overexpression of proteins, somatic knockout, and antisense inhibition (Chilcoat et al., 2001; Clark et al., 2001; Hai et al., 2000). The availability of a genomic database provides an additional tool for identification of genes and proteins and their subsequent functional studies (Stover et al., 2006).

Tetrahymena consists of two non-equivalent nuclei: micronucleus (MIC) and macronucleus (MAC). The diploid MIC is transcriptionally inactive during vegetative growth and is germline. The MAC is a polyploid nucleus and controls the phenotype of *Tetrahymena* by

constant expression of genes during its vegetative stage of the life cycle. The new MAC develops from the MIC during conjugation and parental MAC is resorbed. During cell division MIC divides mitotically whereas MAC divides amitotically with random segregation of its chromosomes. This segregation of chromosomes in MAC lead to phenotypic assortment where heterozygous MAC eventually becomes homozygous (Doerder et al., 1992).

Genetic information of *Tetrahymena* is stored in the MIC and transmitted to the sexual progeny during conjugation. The conjugation is a developmental process in ciliates, where nutritionally starved cells of different mating types undergo a phase of sexual reproduction (Frankel, 2000). After fusion of two cells, MICs undergo meiosis and produce four haploid nuclei. Three postmeiotic nuclei undergo degeneration and only one of the nuclei undergo an additional mitotic division. The mitotic division haploid nuclei produce two genetically equivalent pronuclei; migratory and stationary pronuclei. Each member of the conjugating pair exchange migratory pronuclei through the conjugal junction to fuse with the resident stationary pronuclei. The resulting diploid zygotic micronucleus undergoes two subsequent mitotic division to produce four nuclei which are further positioned as pairs at anterior and posterior poles. Two postzygotic nueleic located at the anterior pole differentiate into two new MACs while the two posterior nuclei become MICs. Following the separation of exconjugants (carrying two new MICs, two new MACs and one parental MAC), parental MAC and one new MIC undergo degradation by apoptosis. During the first cell division after separation, the remaining MIC divides mitotically while the MACs are segregated to subsequent daughter cells without division. Thus the conjugation process leads to degradation of old MAC and development of new MAC, which is a product of zygotic MICs. As a result of conjugation, genes that were once

transcriptionally silent in the MIC are brought to transcription in progenies. This process has provided an intelligent way of producing knockouts of genes essential for cell viability.

In *Tetrahymena*, germline (MIC) and somatic (MAC) gene disruption can be routinely achieved by biolistic bombardment of conjugating cells undergoing meiosis using linearized targeting vector DNA. Knockout heterokaryons can be created by disrupting gene to be studied in the MIC while retaining corresponding WT alleles in the MAC (Hai et al., 2000). Heterokaryon strains can be maintained indefinitely in the vegetative life cycle because mutated gene is silent in MIC. Two such homozygous germline-heterokaryons can be mated to one another, and phenotype of the resulting progeny can be studied. This approach is very useful even when the gene is essential and the introduced mutation or gene disruption lead to lethal phenotype. Upon completion of conjugation, the lethal mutations are expressed and WT phenotype is replaced by mutant phenotype in the progeny due to gradual replacement of WT gene products with mutant ones. Thus, knockout heterokaryons approach is very useful to achieve: 1) single step inducible germline gene knockout; 2) provide a window of opportunity to analyze the progression from the WT to terminal phenotype; 3) phenotypic rescue by introducing WT or mutant versions of the gene into the conjugating heterokaryons, without any flanking selectable marker. This method has been used with considerable success, in cases, where terminal phenotype is lethal. Using this approach, others and we analyzed the effects of eliminating and mutating several genes in *Tetrahymena* (Brown et al., 1999b; Xia et al., 2000) (Hai and Gorovsky, 1997; Thazhath et al., 2002). I used the germline gene knockout heterokaryon method to analyze the functions of microtubule-severing proteins, katanin and spastin.

Another genetic methodology is the use of an inducible gene expression system. A cadmium chloride-inducible, *MTT1* promoter is available for *Tetrahymena*, which is based on the non-essential metallothionein (*MTT1*) gene (Shang et al., 2002b). *MTT1* gene product neutralize and accumulate cadmium and is induced by cadmium. Shang et al prepared a construct where coding region of gene of interest can be flanked by 5' and 3'UTRs of *MTT1* gene, under control of the *MTT1* promoter. The *MTT1* promoter can be used to construct conditional lethal mutants as well as an expression system and it provides fine control of gene expression (Thazhath et al., 2004). As mentioned earlier, the levels of katanin in neurons vary significantly depending on the developmental stage of the axon. Furthermore, growth of axons is highly sensitive to the level of katanin (Karabay et al., 2004). The inducible system provides an ideal tool to study katanin expression in *Tetrahymena*, in a non-lethal manner. The *MTT1* based expression system was used to generate lethal phenotype when p60 is overexpressed. This approach is also used to study the localization of katanin when it is expressed at lower levels (results described in chapter 3).

Ciliates have a wide array of complex microtubular organelles in a single cell. *Tetrahymena* assembles conserved types of structures such as cilia, basal bodies, mitotic spindles, as well as specialized types of MT-based structures associated with cell cortex, nucleus, development, cytoplasm and oral apparatus (Gaertig, 2000). Previous studies and the recently sequenced genome revealed the presence of a single essential alpha-tubulin (*ATU1*), two redundant β-tubulin (*BTU1* and *BTU2*) and a single essential gamma-tubulin (GTU1) in *Tetrahymena* (Gaertig et al., 1993; Shang et al., 2002a). In addition, *Tetrahymena* also contains nine noncanonical α-like (three) and β-tubulin like (six) genes, which may have redundant roles in assembly or function of highly specialized MTs. The gene product of *BLT1*, a β-tubulin like gene is localizes to a small subset of MIC specific MTs but is not a part of axonemal MTs (K.

Clarke and M. Gorovsky personal communication). The deletion of *BLT1* and one α-tubulin like gene did not affect the phenotype of *Tetrahymena* (R. Xie and M. Gorovsky, Personal communication). Interestingly, all of the noncanonical tubulins lack the characteristic tail domain for posttranslation modifications, polyglycylation and polyglutamylation. The conventional tubulins of *Tetrahymena*, α-tubulin (Atu1p) and β-tubulin (Btu1p and Btu2p) are incorporated in most of the major microtubular structure and they occur in a large number of isoforms, created by various types of PTMs.

In the following section, the localization of three types of PTMs: acetylation, glutamylation and glycylation are discussed. The specific monoclonal or polyclonal antibodies are used to probe these modifications. The α-tubulin acetylation occurs on stable MTs. In *Tetrahymena,* this PTM is detected by the monoclonal 6-11 B-1 antibody and known to accumulate on ciliary, cortical and MIC-specific MTs. This modification is not detectable on highly dynamic cytoplasmic MTs (Gaertig et al., 1995). The side chain length of tubulin glycylation can be distinguished by specific antibodies against single or >1 glycine residues. Tubulin monoglycylation (single glycine residue as a side chain) can be probed with monoclonal TAP952 antibodies whereas tubulin polyglycylation (more than one glycine residues) can be detected with monoclonal AXO49 antibodies. Monoglycylation occurs on most type of MTs. Monoglycylation accumulates at the tip of the cilia, which is devoid of polyglycylation. Polyglycylation covers most of the ciliary shaft but is also present on cortical MTs and BB.

Immunogold electron microscopy has been used to dissect the patterns of glycylation and glutamylation within the axoneme of *Paramecium tetraurelia*. (Bre et al., 1994; Bre et al., 1998; Gaertig et al., 1995; Libusova and Draber, 2006). Polyglycylated tubulin is more concentrated above the transition zone andits concentration decreases toward the distal end of the

cilia. A similar polyglycylation gradient was observed for sea urchin spermatozoa flagella when labeled with AXO49 antibodies. However, polyglutamylation is more concentrated at the distal part of the axoneme and is nearly absent from transition zone. The basal body also strongly labels for polyglutamylated tubulin. In mammalian spermatozoa, peripheral doublets can be distinguished based on the accumulation of specific type of PTMs. Outer doublets 1-5-6 predominantly contain polyglutamylated tubulin epitope whereas doublets 3-8 are more concentrated with monoglycylated tubulin epitope (Fouquet et al., 1997; Kann et al., 1998). Furthermore, B tubules in sea urchin spermatozoa have more glutamylated tubulin compared to A tubule (Plessmann and Weber, 1997). *Tetrahymena* proved to be very useful for the research described here in this dissertation; notably, creating knockouts of various genes (including katanin and spastin), evaluating the effect of loss of katanin function upon different microtubular organelles by immunofluorescence and electron microscopic analysis, and overexpression and localization studies by epitope tagging.

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CHAPTER 3

KATANIN REGULATES DYNAMICS OF MICROTUBULES AND BIOGENESIS OF MOTILE CILIA

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Abstract

The *in vivo* significance of microtubule severing and mechanisms that govern its spatial regulation are not well understood. We engineered null mutations of subunits of microtubule severing proteins, katanin and spastin in *Tetrahymena*, a cell-type with elaborate microtubule arrays. We show that spastin is dispensable while katanin is essential. The net effect of katanin on the polymer mass depends on the microtubule type and location. While katanin reduces the mass and destabilizes the internal network of microtubules, its activity increases the mass of ciliary microtubules. We also show that katanin reduces the level of several types of post-translational modifications on microtubules. Furthermore, katanin deficiencies phenocopied a mutation of β-tubulin that prevents deposition of polymodifications (glutamylation and glycylation) on microtubules. We propose that katanin preferentially severs older, post-translationally modified segments of microtubules.

Introduction

Microtubules are ubiquitous cytoskeletal polymers made of α/β-tubulin heterodimers that are required for cell motility, intracellular transport and mitosis. Little is known about how organelle-specific properties of microtubules are generated. The length of microtubules determines the size of microtubule-based organelles, and affects the distribution of forces generated by microtubule-dependent motors. The length of microtubules can be regulated either by addition/loss of subunits at the polymer ends, or by internal breakage. Katanin, a dimer of p60 and p80 proteins (McNally and Vale, 1993), has a strong microtubule-severing activity. p60 is a catalytic subunit that oligomerizes and breaks microtubules while hydrolyzing ATP (Hartmann and Vale, 1999). Katanin has been implicated in the organization of microtubule arrays *in vivo*.

Katanin is required for assembly of the meiotic spindle in *Caenorhabditis elegans* (McNally et al., 2006; Srayko et al., 2000). Surprisingly, katanin deficiency led to a decrease in the microtubule polymer mass in the meiotic embryo of *C. elegans*, probably because katanin generates microtubule fragments that can serve as seeds for nucleation of new microtubules (Srayko et al., 2006). Katanin was implicated in generation of short microtubules destined for transport to neural extensions (Baas et al., 2005). In plants, katanin is required for normal assembly of cortical bundles (Bichet et al., 2001; Burk et al., 2001). Furthermore, a mutation in p80, the non-catalytic subunit of katanin, inhibited assembly of central pair microtubules in cilia of *Chlamydomonas reinhardtii* (Dymek et al., 2004). Thus, katanin has emerged as a positive regulator of the microtubule polymer mass that is critical for assembly of microtubule arrays.

On the other side, katanin activity increases during the mitotic prophase, suggesting that katanin plays a role in the disassembly of interphase microtubules to allow for reassembly of tubulin into the mitotic spindle (McNally and Thomas, 1998). Thus, katanin could also function as negative regulator of the microtubule mass. The net effect of katanin on the polymer could depend on the size of microtubule products that are released by severing. Specifically, katanin could negatively regulate the polymer mass, if the product of severing is a tubulin dimer or a short microtubule that cannot seed assembly.

Here we show that katanin plays a negative regulatory role in the management of non-ciliary microtubules in *Tetrahymena*. We show that katanin reduces the polymer mass as well as increases the level of its dynamics in the cell body. However, in the same cell, katanin promotes assembly of ciliary microtubules, and therefore its effects are organelle-type specific. We also show that katanin-mediated severing is non-random *in vivo*. Furthermore, katanin mutations phenocopy a mutation of the domain of β-tubulin involved in polymeric post-translational

modifications (PTMs), glutamylation (Eddé et al., 1990) and glycylation (Redeker et al., 1994) (*polymodifications*). We propose that katanin regulates the longevity of non-ciliary microtubules by preferentially depolymerizing post-translationally modified segments of the polymer.

Results

Katanin is required for disassembly of cell body microtubules and assembly of motile cilia. To identify microtubule-severing factors, we searched the genome of *Tetrahymena thermophila* for AAA type ATPase sequences. We identified two sequences encoding p60 katanin, representing the predicted genes *KAT1* and *KAT2*, and a spastin encoding sequence, *SPA1*. Both predicted Kat1p and Kat2p proteins have a central AAA domain, but Kat2p also has an N-terminal LisH domain (Fig. 3.1A). Kat1p and Kat2p belong to conserved clades and Kat1p is more closely related to the well-studied vertebrate p60s (Fig. 3.2). The *Tetrahymena* genome also contains a sequence encoding p80, the putative non-catalytic subunit of katanin, *KAT3*.

To evaluate the role of microtubule severing *in vivo*, we constructed strains lacking either *KAT1*, *KAT2,* or *SPA1*. *Tetrahymena* has two nuclei, the germline micronucleus, and the somatic macronucleus, and the macronuclear genome determines the phenotype. Heterokaryon strains were made that were homozygous for gene disruption in the micronucleus and had wild type alleles in the macronucleus. Heterokaryons are normal during vegetative propagation, but when two such strains conjugate, they produce cells with a new macronucleus that express a gene disruption phenotype. Heterokaryon progeny lacking either *KAT2* or *SPA1* appeared normal (Fig 3.4). However, progeny of *KAT1* knockout heterokaryons underwent only 2-3 normal divisions and formed multinucleated "cell-chains" by repeated failure to complete cytokinesis (Fig. 3.5A). During cytokinesis of normal *Tetrahymena* cells, cortical longitudinal microtubule (LM) bundles

(Fig, 3.1B) undergo partial depolymerization as the contractile ring ingresses (Thazhath et al., 2002). Cell-chains lacking zygotic Kat1p had abnormally thick LMs, many of which span adjacent subcells, and failed to undergo depolymerization during cytokinesis (Fig. 3.6C). It thus appears that the hyperstable LMs block the ingression of the cleavage furrow. Furthermore, based on immunofluorescence, we observed an about 2-fold increase in the mass of insoluble tubulin in the cell body, which indicates that the mass of internal cytoplasmic microtubules increases in the absence of zygotic Kat1p (Fig. 3.7).

While normal *Tetrahymena* swim rapidly due to the beating of cilia, the *KAT1* knockout cell-chains were nearly paralyzed. Immunofluorescence showed that the cell-chains had two types of cilia: a few normal length cilia that were more near the ends of cell-chains, and a majority of excessively short cilia (Fig. 3.6B,C & 3.11E,F). In the ciliary rows that cover the cell surface of *Tetrahymena*, new cilia-templating basal bodies form near old basal bodies, and old units are not resorbed (Allen, 1969). Most likely, the normal length cilia are older parental units, while the short cilia were assembled in the absence of zygotic Kat1p. This hypothesis is consistent with the known preference for insertion of new basal bodies within the mid-posterior region (Frankel et al., 1981; Thazhath et al., 2004). TEM showed that the majority of ciliary cross-sections (80%, n=205) had 9 doublet microtubules, but lacked the central pair (Fig. 3.8A-C). The 20% of 9+2 cilia likely represent pre-existing cilia.

In other organisms, katanin functions as a complex of p60 and p80, a noncatalytic subunit (McNally and Vale, 1993; Srayko et al., 2000). The progeny of heterokaryons lacking the *KAT3* (p80) gene developed into paralyzed cell-chains that were remarkably similar to those produced by the lack of *KAT1* (Fig. 3.5B). The simplest explanation is that Kat1p and Kat3p work in the same pathway, and both are essential for katanin activity.

Importantly, a strikingly similar paralyzed cell-chain phenotype was earlier observed for the bDDDE$_{440}$ mutation of β-tubulin in which 3 polymodifiable glutamic acids in the tail domain were replaced by aspartic acids (Fig. 3.5C and reference (Thazhath et al., 2002)). The only difference is that the bDDDE$_{440}$ mutation also inhibits the assembly of a subset of B-tubules of peripheral doublets (Thazhath et al., 2002). Thus, katanin and polymodifications on β-tubulin could function in the same pathway.

Kat1p (p60) has selective microtubule-severing activity *in vivo*.

To test whether Kat1p has microtubule severing activity, we overproduced GFP-Kat1p using an inducible promoter at a high copy number. After induction, GFP-Kat1p accumulated in cells (Fig. 3.9A) and cell multiplication was inhibited (Fig. 3.9D). After 4-6 hrs, overproducing cells had fewer microtubules. Nearly all microtubules disappeared after 12 hrs (Fig. 3.9B). We were intrigued by the rapid loss of ciliary axonemes (Fig. 3.9C) because katanin was earlier implicated in deciliation (a rapid shedding of cilia caused by low pH, based on severing of outer doublets at the transitional zone, the region between the basal body and the ciliary axoneme)(Lohret et al., 1998; Quarmby, 2004). TEM study showed that many cilia of overproducing cells had severed axonemes, but the breakage points were present outside of the transitional zone (Fig. 3.8D-G). Cross-sections of cilia frequently showed partial or complete loss of outer doublet microtubules, suggesting that GFP-Kat1p nicks axonemes. We frequently observed a loss of only the B tubule (Fig. 3.8N-O). Strikingly, on most cross-sections the central pair microtubules were intact (Fig. 3.8H-O). Thus, the A-tubule of the doublet and the central microtubules are more resistant to katanin-mediated severing. We also observed numerous cilia with two axonemes profiles adjacent to each other (Fig. 3.8H-M). These images point at the likely mechanism of loss of cilia.

Initially, an outer doublet could undergo nicking, introducing a point of structural weakness, and the beating of cilia could break the axoneme (Fig. 3.8D-G). Next, the distal portion of the axoneme could slide past the proximal portion. The residual axoneme and the broken fragment could undergo further fragmentation (Fig. 3.8F, G, J-M).

Overexpressed GFP-Kat1p also affected the basal body microtubules. By inspecting the undulating membrane (UM) of the oral apparatus, we found that there is selectivity in the Kat1p activity. In the UM, the basal bodies are arranged into two rows with the same orientation, with only the outer row being ciliated. Strikingly, basal bodies of the outer row showed defects in a subset of triplets that were located at the same circumferential positions (Fig. 3.8P). Thus, Kat1p is a genuine severing protein, but the enzyme has preferred sites of activity on microtubules *in vivo*.

GFP-Kat1p localizes to basal bodies, LMs, and axonemal microtubules. Attempts at generating polyclonal antibodies against Kat1p were unsuccessful. We therefore tagged Kat1p with GFP by rescuing progeny of *KAT1* heterokaryons, with a GFP-KAT1 gene targeted to the native locus. However, the rescue strains did not have detectable GFP expression. Thus, the native promoter-driven Kat1p could be below the detection limit. Next, we rescued *KAT1* heterokaryons with a fragment encoding GFP-Kat1p under the control of a cadmium-dependent promoter maintained at a low copy number. The GFP-Kat1p rescue strains grew more slowly in the absence of exogenous cadmium, but had a normal rate of growth with cadmium (Fig. 3.10A). In immunofluorescence with anti-GFP antibodies, GFP-Kat1p was detected near the basal bodies (Fig. 3.10B). In fixed cells that were not processed for immunofluorescence, we detected GFP-Kat1p fluorescence as lines on a side of ciliary rows (Fig 3.10C), indicating that GFP-Kat1p

preferentially associates with LMs (see Fig, 3.1B). Using light microscopy, we could not detect GFP-Kat1p in cilia. However, using post-embedding immunoelectron microscopy we detected GFP-Kat1p epitopes in two locations: near the basal bodies (Fig. 3.10D a, b) and within cilia. Strikingly, GFP-Kat1p was seen almost exclusively near the outer doublets (Fig 3.10D c-e).

Lack of katanin stimulates microtubule PTMs.

The fact that katanin null mutations phenocopied a β-tubulin polymodification domain mutation indicated that katanin interacts with post-translationally modified microtubules. To explore further the relationships between microtubule PTMs and katanin, we examined the levels of PTMs in Kat1p-null cells. In normal cells, polymodifications (glutamylation and glycylation) are present on most microtubules. However, on the relatively dynamic internal microtubules, the side chains are limited to a single G (Xia et al., 2000) or E (unpublished data). We used antibodies which recognize side chains with 3 or more units (ID5 for polyglutamylation (Rudiger et al., 1999) and AXO49 for polyglycylation (Levilliers et al., 1995)). Unlike normal cells, most Kat1p-null cell-chains had polyglycylated and polyglutamylated internal cytoplasmic microtubules (56 and 75%, respectively) (Fig. 3.11B,C). In the cell cortex of normal cells, polyglutamylation is detectable only in basal bodies and cilia (Fig. 3.14), while cortical bundles are only monoglutamylated. However, in Kat1p-null cell-chains, LMs were labeled with an anti-polyglutamylation antibody (Fig. 3.14).

In normal cells, a short cilium in the process of assembly labels strongly with an anti-polyglutamylation antibody ID5 and the labeling decreases as the cilium elongates and matures. The anti-polyglycylation antibody AXO49 gives a complementary pattern with a high signal in mature cilia (Fig. 3.11D). In the Kat1p knockout cell-chains, the short mutant cilia had high level

of detectable polyglutamylation and low level of polyglycylation as compared to pre-existing cilia present mostly at cell extremities (Fig. 3.11E,F).

In *Tetrahymena* cells, the internal cytoplasmic microtubules are dynamic, judged by their sensitivity to microtubule-depolymerizing compounds (Stargell et al., 1992) and lack of lys-40 α-tubulin acetylation (a marker of stable microtubules) (Gaertig et al., 1995). Strikingly, the Kat1p-null cell chains accumulated strong acetylation on the internal cytoplasmic microtubules (Fig. 3.11A). Ninety eight percent (n=76) of Kat1-null cell-chains had acetylated cytoplasmic microtubules and a several fold increase in the signal of acetylated tubulin was detected in the cell body (Fig. 3.7C). Western analysis of total cell-chains (Fig. 3.11G) showed levels of tubulin acetylation similar to normal cells, but cell-chains have excessively short cilia and therefore lack a major source of acetylated tubulin found in normal cells (Gaertig et al., 1995). These data are consistent with an increase in acetylation on cell body microtubules in mutants. The accumulation of hyperacetylated microtubules is not an indirect consequence of absence of cytokinesis, because cells blocked in cytokinesis by a distinct mechanism did not accumulate hyperacetylated cytoplasmic microtubules (Fig. 3.13). Thus, the absence of katanin strongly affects the levels of PTMs on microtubules. For all locations and PTMs types (with exception of glycylation on ciliary axonemes) we observed an increase in the levels of PTMs. Thus, katanin functions as a negative regulator of microtubule PTMs.

Katanin increases microtubule dynamics *in vivo*. Internal cytoplasmic microtubules of *Tetrahymena* depolymerize in the presence of nocodazole while cortical and ciliary microtubules do not(Stargell et al., 1992). The accumulation of acetylation and polymodifications on internal microtubules in the Kat1p-null cell-chains indicated that these microtubules were hyperstable.

Indeed, after 1 hr treatment with 40 mM nocodazole, all wildtype cells lacked detectable cell body microtubules (Fig. 3.12A,C), while in 84% (n=75) of Kat1-null cell-chain cytoplasmic microtubules were abundant (Fig. 3.12B,D). Thus, the phenotype of *KAT1* deficiency could be caused to some extent by hyperstability of internal MTs. To test this, we incubated a population of cell-chains with either 2 mM oryzalin or 10 mM paclitaxel to either destabilize or hyperstabilize MTs, respectively. Remarkably, oryzalin increased the number of complete divisions undergone by cells lacking zygotic Kat1p (Fig. 3.12F). The treatment with paclitaxel blocked multiplication of Kat1p-null cells (Fig. 3.12F). At these concentrations, neither of the drugs affected the growth of wildtype cells (Fig. 3.12E). This pharmacological profile indicates that katanin-deficient cells have hyperstable microtubules and that katanin increases dynamics of microtubules *in vivo*.

Katanin is not required for deciliation in *Tetrahymena*. Deciliation is a rapid shedding of cilia in response to chemical stresses, based on breakage of the axoneme within the transitional zone. Katanin was implicated in severing of axonemes in *Chlamydomonas* (Lohret et al., 1998). Surprisingly, the Kat1p-null cell-chains deciliated normally in low pH. Nearly all cilia were lost, indicating that the short cilia that assemble under katanin deficiency also shed (Fig. 3.15 A-C). The deciliated Kat1p-null cells regenerated uniformly short cilia within 2 hrs and were paralyzed, indicating that the regenerated cilia were 9+0. Double knockout cell-chains lacking *KAT1* and *KAT2* also deciliated (Fig. 3.15 D,E) and regenerated cilia (unpublished data), indicating that in *Tetrahymena* this process is not dependent on katanin. The ability of katanin-deficient cells to regenerate cilia indicates that the absence of katanin does not lead to a general deficiency in the pool of unpolymerized tubulin. This was confirmed by a western blot that showed that a

population of cell-chains had a normal level of soluble (Triton- X100 extractable) tubulin to wild type (Fig.3.11G).

Discussion

Katanin can act as a negative regulator of microtubule mass and dynamics

To our knowledge, our study is the first functional evaluation of all known microtubule-severing factors in a single organism. The use of *Tetrahymena* enabled us to establish the significance of microtubule severing in a cell type with diverse microtubular systems, including cytoplasmic networks, cortical arrays and axonemes. We show that katanin affects the microtubule polymer mass differentially depending on intracellular location. Specifically, we show that katanin decreases the polymer mass of non-ciliary microtubules (internal and cortical), and increases the mass of ciliary microtubules.

In *Caenorhabditis elegans* meiotic embryos, deficiency in katanin decreased the polymer mass, likely because in this cell type, katanin produces shorter microtubules that provide new free microtubule ends for polymerization (McNally et al., 2006; Srayko et al., 2006). The different outcome observed for the cell body compartment can be reconciled with the *C. elegans* studies, if, in *Tetrahymena*, katanin releases tubulin dimers or short microtubules that cannot prime assembly. Thus, the consequences of katanin activity could depend on both the nature of the katanin activity (and the resulting size of severing products) as well as on the cellular context that determines the fate of severed microtubules. The consequences of the loss of spastin were similar to what we observed here for katanin. A mutation of murine spastin induces swellings in axons that were enriched in detyrosinated, stable microtubules (Tarrade et al., 2006). A knockdown of spastin mRNA in *Drosophila* caused stabilization of microtubules (Orso et al.,

2005; Trotta et al., 2004) although another study reported a reduced the number of microtubules in spastin-deficient *Drosophila* (Sherwood et al., 2004). Thus, most of the studies on spastin, taken together with our data indicate that katanin and spastin increase the level of dynamics and reduce the levels of post-translational modifications on non-ciliary microtubules.

Katanin severs preferred microtubule sites in vivo

We show that katanin displays a high level of selectivity *in vivo*. GFP-Kat1p associated with only a subset of microtubule locations. We detected tagged Kat1p around basal bodies, near LMs and doublet microtubules in cilia. The basal body-associated katanin may be involved in severing of minus ends of internal cytoplasmic microtubules (Torres et al., 1991). Cortical LMs are bundles of partly overlapping microtubules with a uniform polarity (Pitelka, 1961). Katanin may be involved in the turnover of the LM segment microtubules and its deficiency could lead to abnormal persistence of LM microtubules and block cytokinesis.

While it is unclear whether katanin under physiological conditions severs stable microtubules of cilia and basal bodies, these structures were severed by overproduced katanin. Remarkably, katanin preferentially severed specific microtubule triplets within the basal bodies, confirming that centrioles have radial asymmetry (Beisson and Jerka-Dziadosz, 1999). Inside cilia, katanin severed doublet microtubules, while the central pair microtubules were unaffected. Within the doublets, the B-tubule was more prone to severing compared to the A-tubule.

Two mechanisms could restrict katanin activity: 1) differential binding of MAPs that sterically block katanin, or 2) differential marking of microtubules with post-translational modifications. It is already known that Tau MAP protects axonal microtubules against katanin severing (Qiang et al., 2006). However, MAPs are strongly affected by PTMs (Bonnet et al.,

2000; Peris et al., 2006; Reed et al., 2006). Thus, katanin could be directly or indirectly regulated by PTMs of microtubules.

Katanin and microtubule PTMs could regulate each other

Previously, we described the consequences of mutations that affect the sites of polymodifications on β-tubulin (Thazhath et al., 2004; Thazhath et al., 2002). We now show that nearly all these defects are also present in the katanin null mutants. A simple explanation is that katanin requires polymodified microtubules for its transport to proper sites of activity. Recently, acetylation of α-tubulin at Lys-40 was implicated in transport mediated by kinesin-1 (Reed et al., 2006). Alternatively, polymodifications could mark preferred sites for katanin-mediated severing. Importantly, polymodifications occur on the C-terminal tail domains of tubulins and both katanin and spastin require tubulin tails for severing activity (McNally and Vale, 1993; Roll-Mecak and Vale, 2005; White et al., 2007). Furthermore, in *C.elegans*, a substitution of a potentially polymodifiable glutamic acid in the tail domain of β-tubulin rescued lethality associated with overproduction of katanin (Lu et al., 2004), supporting the idea that a polymodified tail of β-tubulin increases katanin activity. Importantly, the known pattern of glutamylation *in vivo* correlates with the preferred sites of katanin activity revealed by our study. For example, the B-tubules have higher levels of glutamylation, as compared to the A-tubules and the central pair (Fouquet et al., 1996; Lechtreck and Geimer, 2000; Multigner et al., 1996). The transitional zone lacks glutamylation (Kann et al., 2003; Lechtreck and Geimer, 2000) and was resistant to katanin in our study. Furthermore, microtubule types that become hypertrophic in the absence of zygotic katanin in *Tetrahymena* (internal and LMs) are at least monoglutamylated in normal cells. The

recent identification of tubulin glutamylases (Janke et al., 2005) should allow for a direct test of the role of polymodifications in katanin activity.

Importantly, we show here that katanin is a negative regulator of PTMs, including polymodifications and acetylation. Katanin could regulate PTM levels directly by recognizing polymodified segments of microtubules. Alternatively, katanin could regulate PTMs indirectly, by increasing the polymer turnover and reducing the time of exposure to modifying enzymes. Thus, katanin and PTMs could regulate each other. While polymodifications could regulate katanin, it is clear that the resulting severing activity regulates the levels of PTMs on microtubules.

Katanin plays a role in cilia biogenesis

We show that both subunits of katanin are required for assembly of motile cilia. In *Tetrahymena*, disruption of p60 blocked the assembly of the central pair and inhibited elongation of outer doublets. A mutation of p80 also inhibited the central pair formation in *Chlamydomonas* (Dymek et al., 2004). As shown here, Kat1p p60 has a strong microtubule severing activity *in vivo*. It is therefore reasonable to propose that microtubule severing is required for assembly of cilia. Two (non mutually exclusive) models can be proposed. First, the role of katanin in cilia biogenesis could be indirect and based on the dependence of this process on proper dynamics of microtubules in extra-ciliary locations. Katanin-mediated severing could generate a pool of tubulin dimers or oligomers in the cell body, which could then be used as precursors for ciliary assembly. While Kat1p-deficient cell-chains had normal levels of soluble tubulin and could regenerate 9+0 cilia after deciliation, katanin may generate a subpool of precursor tubulin required for the central pair formation. Alternatively, katanin could function inside cilia. Ciliary

protein precursors are delivered by the intraflagellar transport (IFT) pathway (Scholey, 2003). It is possible that precursor tubulin transported to ciliary tips by IFT has a form of oligomers or very short microtubules, as has been proposed for tubulin transported inside neural extensions (Baas et al., 2005; Terada et al., 2000). Katanin may be needed inside cilia for fragmentation of an oligomeric tubulin cargo prior to its assembly. It is intriguing, however, that overexpressed GFP-Kat1p preferentially bound and severed the outer doublets. This activity appears unrelated to deciliation response as severing occurred outside of the transition zone. Furthermore, cells lacking zygotic p60 underwent deciliation, indicating deciliation does not involve katanin. The association of katanin with doublets could be dismissed as an artifact of an abnormally high level of GFP-Kat1p and the potential high affinity of katanin for polymodified tubulin where it could serve a function unrelated to severing. In *Chlamydomonas*, however, antibodies detected p80 specifically in the outer doublet compartment (Dymek et al., 2004). Thus, the ciliary function of katanin could require its association with the outer doublets, possibly based on a novel form of severing of these microtubules that could provide supply of tubulin required for the central pair assembly. Interestingly, we recently found that several Nima A type kinases, NRKs, promote depolymerization of axonemal microtubules when overproduced, but accumulate in assembling cilia when expressed at a normal level (Wloga et al., 2006). Thus, an increased turnover of axonemal microtubules could play a role in the assembly of cilia.

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Materials and Methods

Strains and cell culture conditions

Tetrahymena thermophila strains B2086.1 and CU428.1 (from Peter Bruns, Cornell University) were grown in either SPP(Gorovsky et al., 1975), or MEPP medium (Rasmussen and Orias, 1975). The CU522 strain (from Donna Cassidy-Hanley, Cornell University), was used for introduction of MTT1-GFP-KAT1 transgene at a high copy number using the negative selection method with paclitaxel (Gaertig et al., 1999).

Germline knockouts

Targeting plasmids for disruption of *KAT1, KAT2*, *KAT3* and *SPA1* were prepared as described in the Supplemental Material and Methods. Fragments disrupted by either *neo2* or *neo3* selectable cassettes were introduced into the micronucleus of mating cells using biolistic gun and heterozygous transformants were obtained (Cassidy-Hanley et al., 1997). Heterokaryons were

generated by bringing the micronucleus to homozygosity using a star cross while allowing the disrupted alleles to assort from the macronucleus (Cassidy-Hanley et al., 1997).

Expression of GFP-tagged Kat1p

To overexpress Kat1p at a high copy number, the coding region of *KAT1* was amplified with primers carrying MluI (5'-TATATACGCGTCATGTCAAATTCAGATAAACAATTA-3') and BamHI (5'-TAATTGGATCCCTATCAAACAGAACCAAATTCT-3') sites and cloned into pMTT1-GFP to create pMTT1-GFP-KAT1. Starved CU522 cells were bombarded with a linearized BTU1-MTT1-GFP-KAT1-BTU1 fragment obtained by SacI and XhoI digestion of pMTT1-GFP-KAT1, and transformants were selected on SPP with 20 mM paclitaxel (Gaertig et al., 1999). Using this approach, the transgene integrates by homologous recombination into the non-essential *BTU1* gene that carries a mutation conferring sensitivity to paclitaxel. The copy number of the transgene was increased by allowing cells to assort the mutant *BTU1* allele during vegetative propagation in the presence of paclitaxel.

To express GFP-Kat1p as a sole Kat1p at a low copy number, we rescued mating *KAT1* heterokaryon progeny from death, by introducing a *KAT1* transgene but without applying any selection directly to increase the transgene copy number (Hai et al., 1999). The *KAT1* knockout heterokaryons strains were allowed to complete conjugation during 24 hrs, transformed biolistically with a BTU1-MTT1-GFP-KAT1-BTU1 fragment. Transformants that integrated the transgene into the *BTU1* locus were selected with paromomycin (120 mg/ml) and cadmium chloride (2.5 mg/ml) (based on cadmium-dependent resistance to paromomycin conferred by the *neo3* gene inserted into the native *KAT1* locus).

Microscopy

We modified the immunofluorescence protocol described in (Thazhath et al., 2002) for analysis of Kat1p null cell-chains. The immunofluorescence localization of GFP-Kat1p in rescue cells, was done as described in (Wloga et al., 2006). The following primary antibodies were used: 12G10 anti-α-tubulin (mouse monoclonal 1:10 dilution), 20H5 anti-centrin (mouse monoclonal 1:100), anti-total *Tetrahymena* tubulins SG (rabbit polyclonal 1:600), AXO49 anti-polyglycylated tubulin (Levilliers et al., 1995) (mouse monoclonal 1:100), ID5 anti-polyglutamylated tubulin (mouse monoclonal 1:10) (Rudiger et al., 1999), anti-GFP (rabbit polyclonal 1:500) (Abcam), 6-11B1 anti-acetylated α-tubulin (mouse monoclonal 1:20) (Sigma) (Ledizet and Piperno, 1991). FITC or Cy-3 conjugated secondary antibodies (Zymed) were used at 1:100. Nuclei were stained with DAPI (sigma). Cells were viewed with a Leica TCS SP2 spectral confocal microscope (using 63X water immersion with 1.2 N.A). Images were assembled in Adobe Photoshop 8.0.

For quantitative immunofluorescence, we manually picked mutant cell-chains, mixed them with normal cells and processed for immunofluorescence. We used ImageJ to determine the pixel intensity value for areas of cell bodies in mutant and wildtype cells positioned side-by-side on the same confocal section images. One hundred randomly selected rectangular areas of 30 x 30 pixels were used to obtain an average pixel intensity, for a total of 10 mutant and 10 adjacent wildtype cells. The average background value was also determined and subtracted from the corresponding intensity values obtained for cell bodies.

For transmission electron microscopy of *KAT1* knockouts, ~5000 mutant cell-chains were isolated and washed 2 times with 10 mM Tris HCl buffer at pH 7.5. The cells were fixed in 2% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.2 for 1 h at 4^{o}C. Fresh tannic acid was

added to 0.01% for 1 h at 4°C. Cells were washed 5 times with 10 mM Tris and postfixed in 1% OsO_4 for 1 h at 4°C, washed 5 times in 4°C with water before dehydration through a graded ethanol series. Cells were transitioned to Epon 812 resin with acetone at 33%, 66% and 100% intervals. Cells were infiltrated with 100% Epon for 8 h at 25°C. Fresh 100% Epon was added and allowed to polymerize at 60°C. Ultrathin sections of 50-60 nm were collected and post-stained with uranyl acetate and lead citrate. Sections were visualized on JEOL 100CXII, JOEL 1200 EX, or FEI Technai 20 transmission electron microscopes.

Immunogold labeling was performed on cells expressing GFP-Kat1p at a low copy number as a result of rescue of mating *KAT1* knockout heterokaryons. A post-embedding procedure was performed as described (Ueno et al., 2003). Ultrathin sections were immunolabeled with the polyclonal anti-GFP antibody (1:500) (Abcam ab6556-25), followed by 10 nm colloidal gold-conjugated goat anti-rabbit IgG (Amersham Pharmacia Biotech UK Ltd). Immunolabeled sections were stained with 2% aqueous uranyl acetate for 30 min and washed several times with distilled water until no signal was detectable in the control sections made for cells not expressing GFP.

Phenotypic tests

To measure the multiplication rate, cells were diluted to 10^4 cells/ml from a feeder culture of 2 x 10^5 cells/ml and grown without shaking in 10 ml of SPP. The cell density were measured every 2 hrs. In some experiments, to induce MTT1-driven GFP-Kat1p expression, $CdCl_2$ was added at 2.5 mg/ml.

The number of cilia was determined in cells that overproduced GFP-Kat1p. WT cells and uninduced cells were used as controls. Cells were labeled with the anti-tubulin SG antibody

(1:500). Cilia number was determined using single confocal section that traversed the widest diameter of the macronucleus. For each time point, 10 different cells were analyzed.

To test the effects of oryzalin and paclitaxel upon the growth of Kat1-null cell-chains, the *KAT1* null phenotype was induced *en mass* as follows. *KAT1* knockout heterokaryons were grown to the density of 2 x 10^5 cells/ml. Cells were starved in 10 mM Tris-HCl for 24 hrs and 5 ml of each of the two heterokaryon strains were allowed to mate in 50 ml conical flask for 24 hrs. Cells were spun down and suspended in 10 ml SPP to a final concentration of 10^4 cells/ml. Paromomycin (120 mg/ml) and cadmium chloride (2.5 mg/ml) were added to inhibit the growth of non-mating cells (note that between 5-10% of heterokaryon cells do not mate and therefore retain a wildtype phenotype; these cells were prevented from overgrowing the population by addition of paromomycin). The resulting suspension highly enriched in exconjugant cells lacking zygotic Kat1p, was incubated in SPP with or without 10 mM paclitaxel, 2 mM oryzalin or 1% DMSO as a control for 80 hrs. Ten ml of cell suspension was scored in the DIC microscope and the total number of cells was determined (note that we scored cell chains as 1 cell regardless of the number of subcells) at 24 hr, 36 hr, 50 hr, 65 hr, and 80 hrs.

Bioinformatics

Sequences of AAA domain proteins were obtained from NCBI databases. Gene accession numbers of sequences used for phylogenetic analyses are listed in the legend of Fig S2. The AAA domain sequences were aligned using Clustal X 1.82 and corrected manually in Seaview (Galtier et al., 1996). A tree was calculated using the Phylip package (Felsenstein, 1997). One thousand replicates of the sequence set were created using SEQBOOT. The distances were calculated in PROTDIST, trees were reconstructed using NEIGHBOR. The Jones-Taylor-

Thorton (JTT) substitution model was used. A consensus tree was obtained using CONSENSE and the tree was plotted using DRAWGRAM.

Gene Disruption

To prepare a targeting fragment for disruption of *KAT1*, a 4.3 kb macronuclear genomic DNA fragment of *KAT1,* was amplified with addition of SacI and ApaI sites and cloned into pBluescript (SK+). The primers used were:
5'- TTATAGAGCTCCTATGTATTTTGAGCAGGTC-3' and 5'-TATAAGGGCCCGGCTTTTAATGTTCTCTTGA-3'. The pMNBL plasmid (Shang et al., 2002) carrying the *neo3* gene was modified to reduce the size of the *MTT1* promoter to 0.9 kb. The shortened *neo3* cassette of pMNBL starting at the AccI in *MTT1* promoter was subcloned giving pTvec-neo3. pTvec-neo3 was digested with SpeI to release *neo3* sequence. The cloned *KAT1* genomic fragment was digested with SpeI and *neo3* was inserted resulting in pTvec-neo3R-KAT1. The targeting plasmid was digested with SacI and ApaI and used to transform mating CU428.1 and B2086 strains by biolistic bombardment as described (Cassidy-Hanley et al., 1997).

To disrupt *KAT3*, a 1.3 kb fragment of 5' UTR was amplified with addition of SmaI and ApaI restriction sites (primers: 5'-ATAATGGGCCCTACTTAAAATCTTCTTCTTCTA-3', 5'-AATATCCCGGGTGTTTCTATTTAATGGTTTGTC-3') and cloned into pTvec-neo3. The resulting plasmid was digested with ClaI and SacI and used to insert an amplified 1.5 kb of the 3' UTR of *KAT3* (primers 5'-TAATAATCGATGTTTAACGTTGATGGAGAT-3' and 5'-AATAAGAGCTCGCATCCATAACATAACAAGG -3') to create pKAT3-neo3R. The plasmid was digested with ApaI and SacI and used for biolistic bombardment and creation of *KAT3* knockout heterokaryons as described above for *KAT1*.

To prepare a plasmid for disruption of *KAT2*, 1.4 kb of 5'UTR was amplified with addition of SacI and BamHI sites (primers: 5'-AATTTGAGCTCTGCAAAGCTACTACCAAGAT -3' and 5'-ATATTGGATCCTTCATACGAGATTCACCTTC -3') and cloned into p4T2ΔHindIII, a *neo2* cassette plasmid, using SacI and BamHI sites. The resulting plasmid was digested with XhoI and ApaI and used to insert a 1.8 kb of 3' UTR of *KAT2* (primers: 5'-AATAACTCGAGGTAGACCAAAATAACACACT -3' and 5'-TATATGGGCCCCCTTTGTTTCTTTGGATTTG -3'), to create pNeo2R-KAT2. pNeo2R-KAT2 was digested with SacI and ApaI prior to biolistic transformation using methods described above.

To disrupt *SPA1*, its 5' UTR was amplified with addition of SmaI and ApaI sites (primers 5'-TATATGGGCCCAAAGTAGTAAACAAACCTCAAT -3' and 5'-AATTACCCGGGATTACTTTTTACACTATTCAGC -3'), and cloned into pTvec-neo3. The resulting plasmid was digested with ClaI and SacI and used to clone a 3' UTR of *SPA1* flanked with ClaI and SacI (primers 5'-TATATATCGATCCATAAGAAATAGACTCAGC- 3' and 5'-ATAAA*GAGCTC*CTAAATAATTGATGTGAACTGA-3').

Expression of GFP-Kat1p in the native locus

To place the GFP-KAT1 coding region in the native locus, the 5'-BTU1-MTT1 fragment of pMTT1-GFP-KAT1 was replaced with 5' UTR of *KAT1* and the 3'BTU1 sequence was replaced by 3'UTR of *KAT1* to create pKAT1-GFP-KAT1. Primers used for amplification of 5'UTR of KAT1 were 5'- TTATTGAGCTCTTGGTTCATAATTACTGTTCAT -3' and 5'-ATTATAAGCTTTGAAAGTGAGTGTGTTTGAG -3' flanked by SacI and Hind III sites

respectively. Primers used for amplification of 3'UTR were 5'-ATTATGGATCCGCGATTTCAATAAGTTTTAC -3' and 5'-TATAAGCGCCCGGCTTTTAATGTTCTCTTGA -3' flanked by BamHI and XhoI sites respectively. The sizes of respective 5' and 3' UTR were 1.6 kb and 695 bp. The resulting KAT1-GFP-KAT1 fragment was used to rescue mating *KAT1* mating heterokaryons as described above and transformants that integrated the transgene into the native *KAT1* locus by homologous recombination where selected with paromomycin.

Immunofluorescence

For analyses of *KAT1* knockout progeny, 100-200 cells were isolated in 20 ml of 10 mM Tris, pH 7.5, in an Eppendorf tube. A mixture of 2 % paraformaldehyde in PHEM buffer at pH 6.9 (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM magnesium chloride, 1 mM paclitaxel, 0.5 mg/ml leupeptin, 10 mg/ml E-64, 10 mg/ml chymostatin, 12.5 mg/ml antipain) and 0.5% Triton X-100 in PHEM buffer was prepared shortly before use in a ratio of 10:9. Cells were fixed and permeabilized simultaneously by adding 20 ml of the above mixture. The entire content of the tube was spread on a coverslip and air dried at 30^{o}C. Coverslips with cells were processed for immunofluorescence as described (Gaertig et al., 1995). For immunofluorescence of GFP-Kat1p in rescued cells, about 100 cells were isolated into a drop on a slide of 10 mM Tris pH 7.5 and fixed with 15 ml of 2 % paraformaldehyde in PHEM buffer for 10 sec followed by permeabilization with 10 ml of 0.5 % Triton X-100. For immunolocalization of GFP-Kat1p in overproducing cells (in the CU522 strain background), cells were grown to a density of 10^5 cells/ml without paclitaxel and induced with 2.5 mg/ml of cadmium chloride for 3 h, fixed and processed for immunofluorescence as described above.

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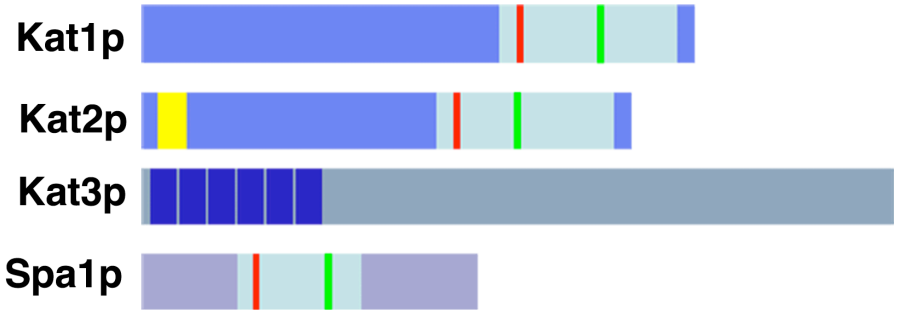
Figure Legends

Figure 3.1. ***Tetrahymena* cytoskeletal organelles and domain organization of katanin and spastin.** (A) Domain organization of predicted katanin and spastin subunits of *Tetrahymena*. (B) A scheme showing the main cytoskeletal organelles of *Tetrahymena*. Abbreviations: oa, oral apparatus; bb, basal body; lm, longitudinal microtubule bundle; mic, micronucleus; ma, macronucleus; tm, transverse microtubule bundle; pc, postciliary microtubule; kd, kinetodesma (non-microtubular); cvps, contractile vacuole pore.

Figure 3.1

A


Kat1p
Kat2p
Kat3p
Spa1p
Walker A
Walker B
AAA domain
WD 40 domain
LiSH domain

B

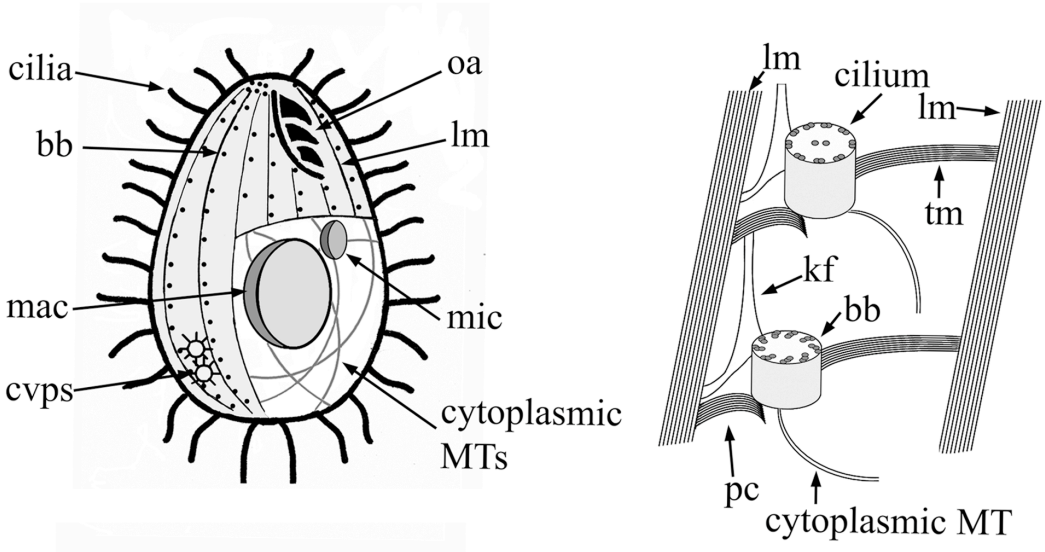

cilia
oa
bb
lm
mac
mic
cvps
cytoplasmic MTs
lm
cilium
lm
tm
kf
bb
pc
cytoplasmic MT

Figure 3.2**. Phylogeny of AAA domain proteins based on the sequence alignment shown in Fig. 3.3**. Numbers above branches represent bootstrap values above 50%. Abbreviations: At, *Arabidopsis thaliana*; Ce, *Caenorhabditis elegans*; Cr, *Chlamydomonas reinhardtii*; Dm, *Drosophila melanogaster*; Dr, *Danio rerio*; Gg, *Gallus gallus,* Hs, *Homo sapiens*; Lm, *Leishmania major;* Mm, *Mus musculus*;; Os, *Oryza sativa*; Sc, *Saccharomyces cerevisiae*; *Tb, Trypanosoma brucei*; Tc, *Trypanosoma cruzi*; Tt, *Tetrahymena thermophila*; Xl, *Xenopus laevis*.

Figure 3.2

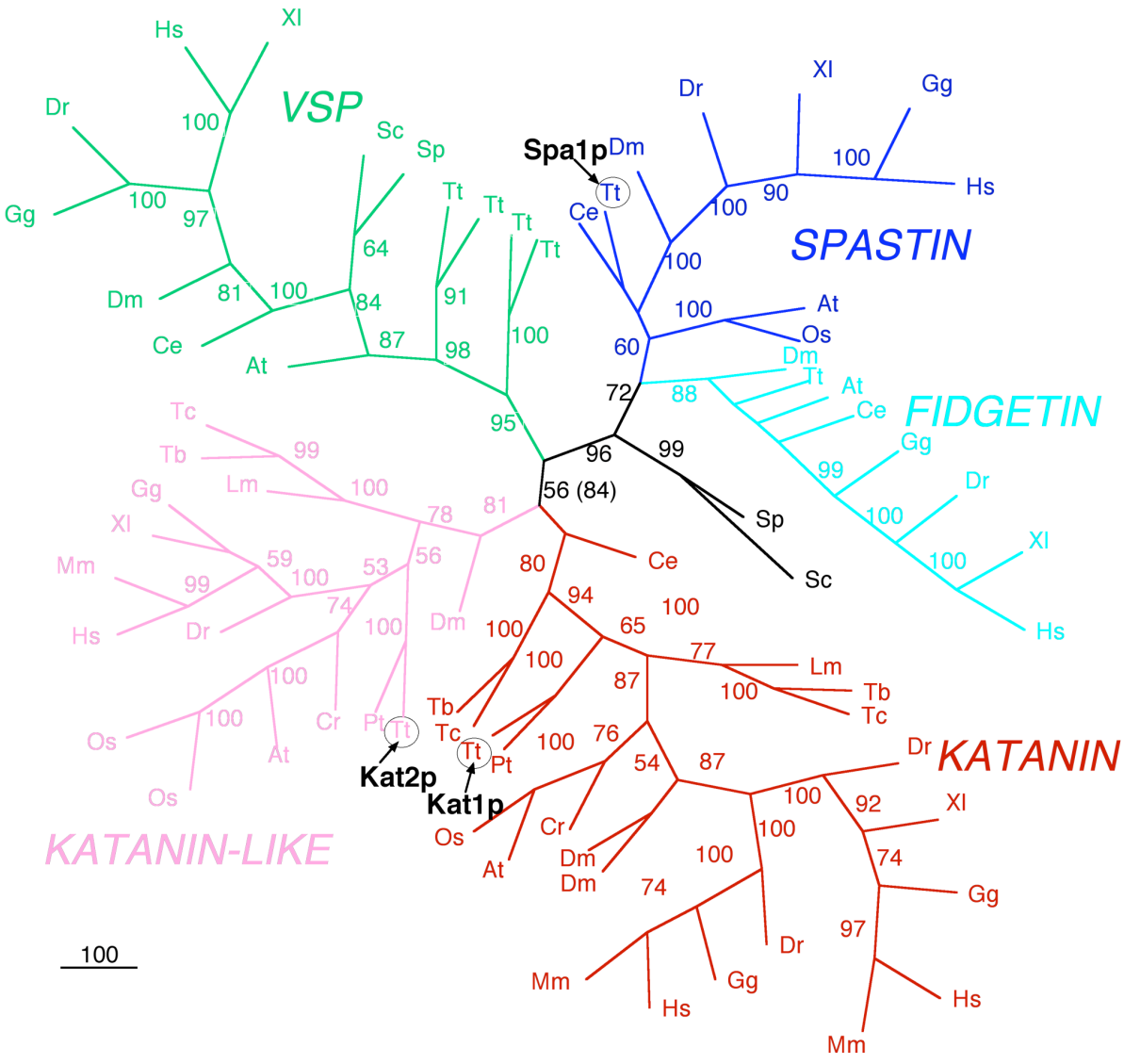

VSP
SPASTIN
FIDGETIN
KATANIN
KATANIN-LIKE
Spa1p
Kat2p
Kat1p
Hs
Xl
Dr
Gg
Dm
Ce
At
Sc
Sp
Tt
Os
Cr
Pt
Tc
Tb
Lm
Mm
100

Fig 3.3 **A multiple sequence alignment of AAA domain proteins from the genome of *Tetrahymena thermophila* and other organisms.** The accession number of sequences used are as follows: spastins: Hs (NP_955468.1), Gg (NP_001026232.1), Xl (AAH77358.1), Dr (AAQ74774.1), Dm (AAL39667.1), Ce (NP_741586.1), Tt (XP_001030702), Os (BAD44799.1), At (NP_182074.3). Fidgetins: Hs (AAH27856.1), Gg (XP_426585.1), Xl (AAH77410.1), Dr (XP_698026.1), Ce (NP_504197.1), Tt (XP_001012750), At (BAB01094.1). AAA proteins in fungi: Sp (CAA17029.1), Sc, (NP_015251.1). p60s katanin subunits: Os (BAB86043.1, NP_916186.1, BAD73365.1), At (BAB87822.1, NP_565791.1), Cr (AAF12877.1, e_gwh.4.60.1) , Tt (XP_001012977, XP_001007193), Pt (CAK68014.1, CAK76484.1|), Tb (XP_828909.1, XP_822379.1, XP_828466.1), Tc (XP_810191.1, XP_814174.1, XP_815288.1), Lm (CAJ04919.1, CAJ02878.1), Hs (NP_008975.1, AAH00612.1, AAH34999.2), Mm (AAH09136.1, AAH30434.1, BAB30604.1), Gg (ABF21049.1, XP_417114.1, XP_414699.1), Xl (AAD53310.1, AAI06553.1), Dr (XP_683438.1, AAH85416.1, AAI08057.1), Dm (AAF52059.2, CG1193, CG10793), Ce (NP_492257.1). Vps4: Hs (AAH47932.1), Gg (CAG31054.1), Xl (AAH81138.1), Dr (NP_957200.1), Dm (NP_573258.1), Ce (NP_490816.4), Sc (NP_015499.1), Sp (CAA91171.1), Tt (XP_977097, XP_001022453, XP_001026351, XP_001026651), At (At2g27600.1, At1g80350.1).

```
69   300
TtSpastin   LQT-DPLVQQ INLT-MLEKK NTIKFEDIAG LKEVKEALYE SIIYPNLRPD IFQGIRAPPR
TtFign      LKGMDQKLID LIENEIVENA ANVKWEDIAG LSSAKESVKE TIVWPMLNPQ IFTGIRAPPK
HsFign      LKNLEPKMIE LIMNEIMDHG PPVNWEDIAG VEFAKATIKE IVVWPMLRPD IFTGLRGPPK
XlFign      LKNIEPKMIE LIMSEIMDHG PPLNWDDIAG LEFAKTTIKE IVVWPMLRPD IFTGLRGPPK
DrFign      LKNFEPKIIE LIMSEIMDHG PPVAWDDIAG LEFAKATIKE IVVWPMLRPD IFTGLRGPPK
GgFign      LKNTDTHLID LVTNEIINQG PPVDWNDIAG LDLVKAVIKE EVLWPVLRSD AFNGLTALPR
AtFind      LRNLEPRLIE HVSNEIMDRD PNVRWDDIAG LEHAKKCVTE MVIWPLLRPD IFKGCRSPGK
DmFingCG33  LAHLDSKMVD HILGESMHDF KPVAWEDIAG LESAKSTFLE AIIMPLRRPD LFTGVRCPPR
CeFign      LKHFDENIIS LIESEIMSVN NEIGWADVAG LEGAKKALRE IVVLPFKRPD VFTGIRAPPK
HsSpastin   FRNVDSNLAN LIMNEIVDNG TAVKFDDIAG QDLAKQALQE IVILPSLRPE LFTGLRAPAR
GgSpastin   FRNVDSNLAN LILNEIVDSG PAVKFDDIAG QELAKQALQE IVILPSLRPE LFTGLRAPAR
XlSpastin   LRNVDSNLAN LILNEIVDSG PSVKFADIAG QDLAKQALQE IVILPSIRPE LFTGLRAPAR
DrSpastin   FKNVDSKLAS LILNEIVDSG SVVRFDDIAG QDLAKQALQE IVILPALRPE LFTGLRAPAR
DmSpastin   VKGVEQKLVQ LILDEIVEGG AKVEWTDIAG QDVAKQALQE MVILPSVRPE LFTGLRAPAK
OsSpastin   GANYDDKLVE MINTTIVDRS PAVKWEDVAG LDKAKQALME MVILPTKRRD LFTGLRRPAR
AtSpastin   GNVYDDKLVE MINTTIVDRS PSVKWDDVAG LNGAKQALLE MVILPAKRRD LFTGLRRPAR
CeSpastin   LNGVDKVIGE RLLDEVLDNT G-VRMDDVAG CHSAKAALEE AVILPALNPN LFKGLRQPVK
Sc          VQGVDRNACE QILNEILVTD EKVYWEDIAG LRNAKNSLKE AVVYPFLRPD LFKGLREPVR
Sp          QTTPSSDFEY AIMNEIISNH EPVYWSDIAG LDDAKNSLKE AVIYPFLRPE LFQGLREPVQ
HsVPS       DNPEKKKLQE QLMGAVVMEK PNIRWNDVAG LEGAKEALKE AVILPIKFPH LFTGKRTPWR
XlVPS       ENPEKKKLQE QLMGAIVMEK PNVRWNDVAG LEGAKEALKE AVILPIKFPH LFTGKRTPWR
GgVPS       EDPEKKKLQN QLQGAIVMER PNVKWSDVAG LEGAKEALKE AVILPIKFPH LFTGKRTPWR
DrVPS       EDPEKKKFQN QLSGAIVMEK PNIKWNDVAG LEGAKEALKE AVILPIKFPR LFTGKRTPWR
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CeVPS       EDEDKKKFQD KLSGAIVMEK PNVKWTDIAG LEGAKEALKE AVILPIKFPQ LFTGNRKPWQ
ScVPS       GGEDNKKLRG ALSSAILSEK PNVKWEDVAG LEGAKEALKE AVILPVKFPH LFKGNRKPTS
SpVSP       LDSDAKKLRS ALTSAILVEK PNVRWDDIAG LENAKEALKE TVLLPIKLPQ LFSHGRKPWS
AtVSP       EDPEQSKLRA GLNSAIVREK PNIKWSDVAG LESAKQALQE AVILPVKFPQ FFTGKRRPWR
TtVSP1      NKDENTKFKE ALQETIVPEK PNIKWDDIAG LVKAKESLKE AVILPIRFPE IFKGARKPWK
TtVSP2      GDKDNAKFEQ ALGEAIVTEK PNVHWSDVAG LENAKKALNE AVILPIRFPH IFQGMIKPWR
TtVSP3      TLKYIQDLKR SLKNCIVKKK PNVTFNDIAG NEYAKKQIQQ SFKLPLLYPN LFSGQPSPWR
TtVSP4      QQKYLKDLKS QLKSCILTKK PPIKFKDIAG NDYAKKCIEE NFILPSVLPN LFQGKPKPFN
AtKat1      YEGPDEDLAA MLERDVLDST PGVRWDDVAG LSEAKRLLEE AVVLPLWMPE YFQGIRRPWK
OsKat1      YEGPDMDLAA MLERDVLDST PGVRWDDVAG LSEAKRLLEE AVVLPLWMPE YFQGIRRPWK
HsKat1      STGYDKDLVE ALERDIISQN PNVRWDDIAD LVEAKKLLKE AVVLPMWMPE FFKGIRRPWK
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DrKat1      RGGEDKDLID ALERDIISQN PNVTWDDIAD LEEAKKLLKE AVVLPMWMPE FFKGIRRPWK
HsKat1L     GAGYDKDLVE ALERDIVSRN PSIHWDDIAD LEEAKKLLRE AVVLPMWMPD FFKGIRRPWK
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DmKat1LCG1  NNHIEAELVD ILERDILQKD PKVRWSDIAD LHDAKRLLEE AVVLPMLMPD YFKGIRRPWK
CrKat1      YSGPDQELAA MLERDIVDQG TSVKWDDIAG LEEAKRVLNE ALVLPMIMPD FFTGIRRPVK
TbKat1      PRSGEEELVA LIEADMHVGS LSVGWEDVAG LQDAKGLLEE AVVYPVLMPE YYQGIRRPWK
TcKat1      ARAGEEELVS LIESDMNAGP MKVSWDDIAG LEEAKGLLEE AVVYPVLMPE YFQGIRRPWK
LmKat1      GRAGEEELVQ LIEADMHVGK LPVTWDDIAG LEEAKRLLEE AVVYPVLMPD YYQGIRRPWK
DmKat1CG11  PLGYEVHLVD TLEKDILQRH PCIKWTDVAG LNEAKTILQE AVVLPVIMPE FFKGIRRPWR
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TcKat3      PSGINAEFAD RIESEIIERS PNVQWEDIAG IPDAKRLLKE AVILPLLVPE LFTGVVQPWK
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AtKat2      -SAETRTLAE SLSRDIIRGN PNIKWESIKG LENAKKLLKE AVVMPIKYPT YFNGLLTPWK
HsKat2      MNSEMRELAA VVSRDIYLHN PNIKWNDIIG LDAAKQLVKE AVVYPIRYPQ LFTGILSPWK
MmKat2      MNSEMRELAA VVSRDIYLHN PNIKWNDIIG LDAAKQLVKE AVVYPIRYPQ LFTGILSPWK
GgKat2      MNGEMRELAT VVSKDIYLHN PNVKWDDIIG LDAAKRLVKE AVVYPIRYPQ LFTGILSPWK
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CrKat2      LQGELRELGA AITRDIFTDS PNVRWEDIAG LDSAKRLIKE AVVMPIKYPQ LFTGLLAPWK
TbKat2      PTVELQELAM TIQRDILDSN PNVRWGSIAA LDEVKRLLKE AVVMPVKYPE LFAGIVRPWK
TcKat2      LSVEQQELAM TIQRDILDVN PNVRWSAIAE LDQAKQLLKE AVVMPVKYPE LFSGILRPWK
LmKat2      PTSELSELAA TILREILDVD PSVRWRDIAD LENAKHLLRE AVVMPVKYPG LFQGILRPWK
TtKat2      DVQELKELAA YLQRDILVEN PNVKFKDIVG LDDAKRLLKE AVQIPLKYPH FFTGILEPWR
PtKat2      DVPEFQQLAA YLQRDICSEN PNVKFSDIAG LDQAKRLLKE AVLVPLKYPH FFQGILEPWK
DmKat2      SSLEWQSLAE LVKTSILQEN IKIKWSDVCG NQRAIELIKE AVLTPIEFPQ LFAHGLKPWR
TtKat1      GQGPDSDLIA MLERDVVDQN PNISFDQIAE LDKAKDMLQE AVLLPILIPQ YFRGIRRPLK
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Figure 3.4 **Disruption of *KAT2* (encoding p60-like protein) and *SPA1* (spastin) does not affect gross morphology of *Tetrahymena*.** Confocal images of cells subjected to immunofluorescence using the 12G10 anti-a-tubulin antibody. All scale bars represent 20 μm.

Figure 3.4

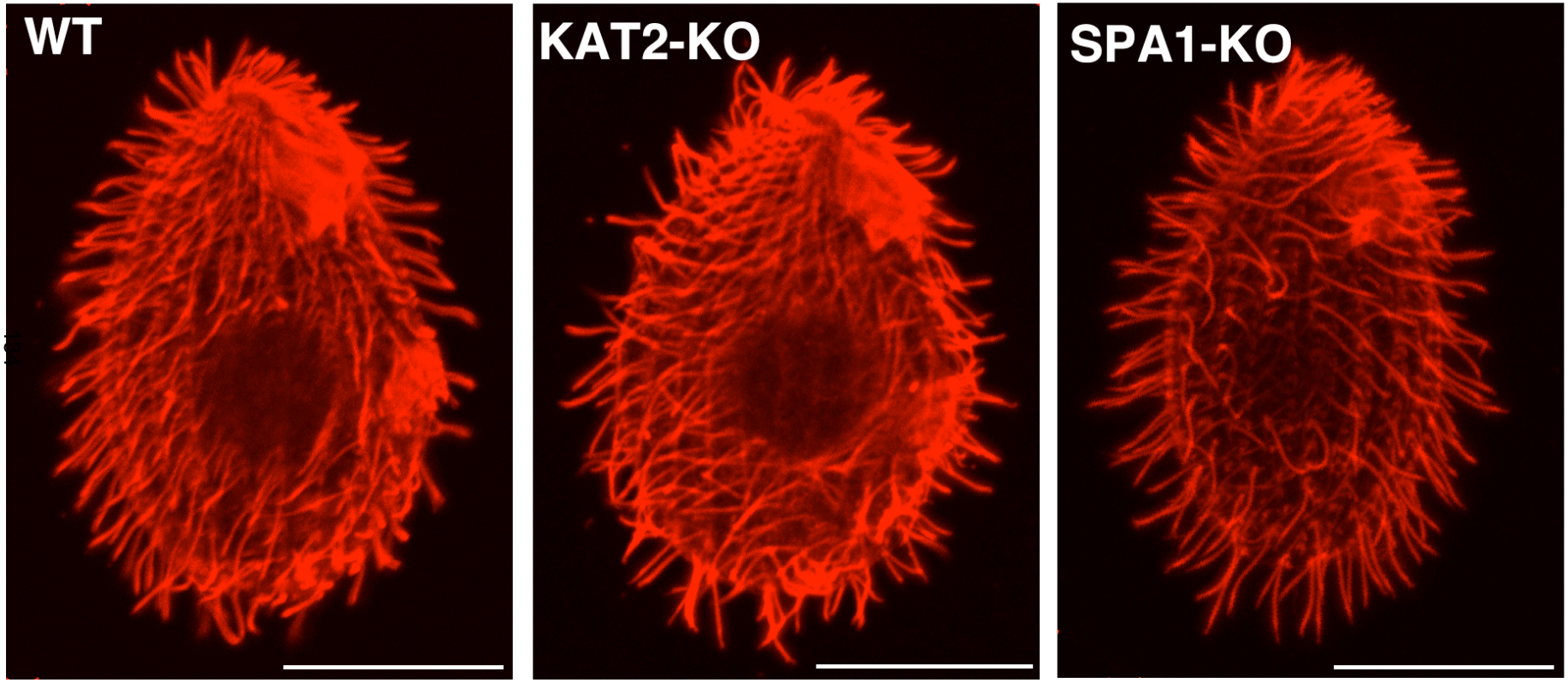


Figure 3.5. **The katanin subunit null mutations phenocopy the βDDDE$_{440}$ mutation on β-tubulin that inactivates sites of polymodifications.** Representative *Tetrahymena* mutant cells were immunolabeled with an anti-centrin antibody and co-stained with DAPI. (A) A mutant cell lacking zygotic Kat1p (p60). (B) A mutant cell lacking zygotic Kat3p (p80). (C) A βDDDE440 mutant cell-chain. Abbreviations: *ma*, macronucleus; *mi*, micronucleus; *oa*, oral apparatus; bb, basal body. Note that nuclei divided and segregate but cells fail cytokinesis multiple times. All scale bars represent 50 μm.

Figure 3.5

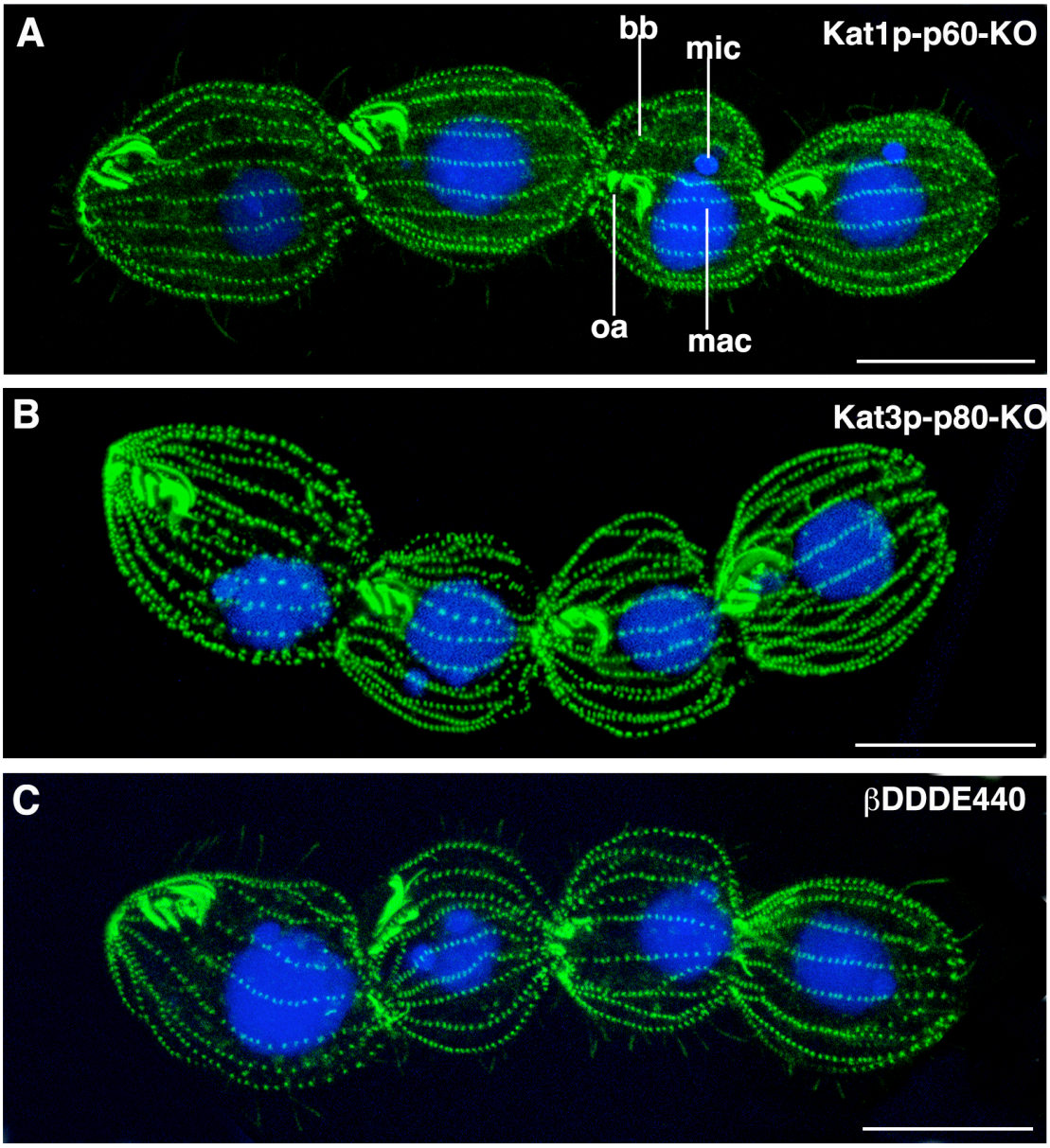


Figure 3.6. **Cells lacking zygotic Kat1p assemble excessively short cilia and have thickened cortical microtubule bundles (LMs).** Cells were labeled for α-tubulin using the 12G10 antibody. (A) Wildtype. (B) An early stage cell-chain lacking zygotic Kat1p composed of 2 subcells, (C) A Kat1p null cell-chain composed of 4 subcells. Note an increase in the thickness of LM bundles as the mutant cell continues to grow while failing cytokinesis (compare with B). While some LMs are broken in the cleavage furrow area, some LMs (arrows) appear to be continuous across subcells indicating a failure in their depolymerization during cytokinesis. Abbreviations: *nc*, newly assembled short cilia; *oc*, pre-existing (old) cilia; *lm*, longitudinal microtubule bundles. All scale bars represent 20 μm.

Figure 3.6

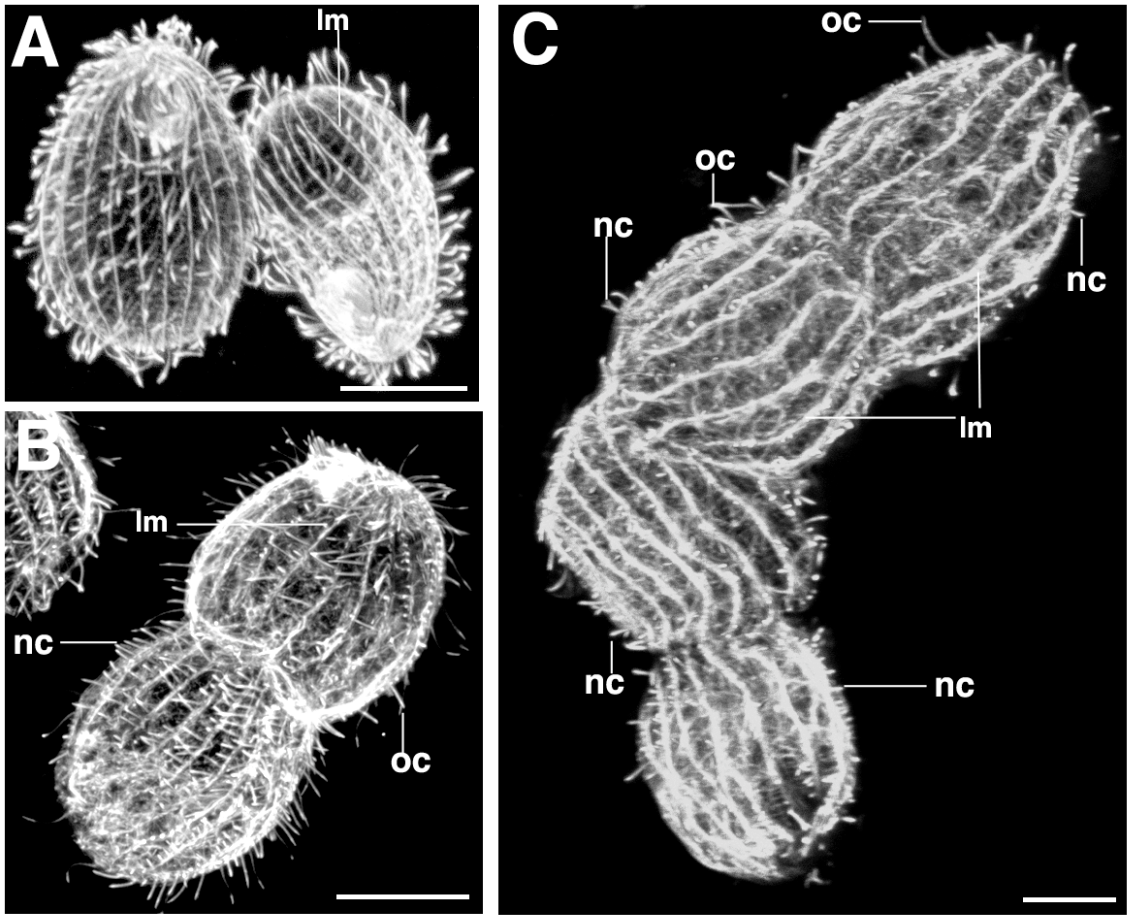
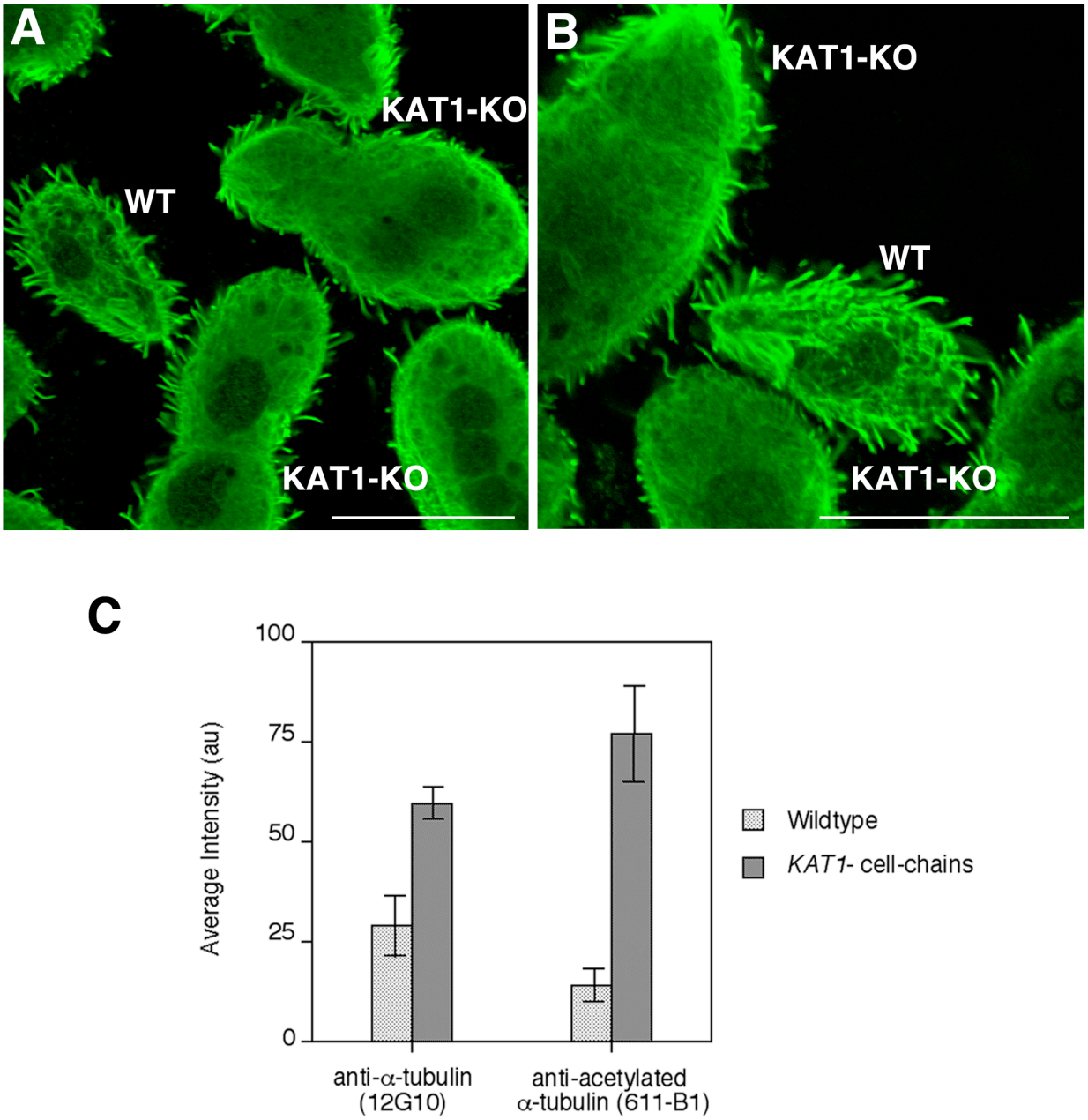


Figure 3.7. **Cells lacking zygotic Kat1p have increased levels of polymerized tubulin in the cell body.** (A-B) Mutant and wildtype cells were mixed, detergent extracted and processed for immunofluorescence using 12G10 anti-a-tubulin. Internal confocal sections are shown. Note increased in tubulin signal in the mutant cell-chains. (C) Results of quantitative immunofluorescence of using a general anti-a-tubulin antibody (12G10) and 6-11 B-1, an anti-acetylated Lys-40 a-tubulin antibody. Wildtype and mutant cell-chains that were lying side-by-side on the same confocal optical sections were imaged. Internal sections were chosen and 100 average pixel values were determined for randomly chosen rectangular areas for 10 different mutant and adjacent wildtype cells. Scale bars represent 50 mm.

Figure 3.7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KAT1-KO
B
KAT1-KO
WT
KAT1-KO
C
Average Intensity (au)
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75
50
25
0
anti-α-tubulin (12G10)
anti-acetylated α-tubulin (611-B1)
Wildtype
KAT1- cell-chains

Figure 3.8. **Kat1p is required for assembly of 9+0 cilia and overexpression of Kat1p disassembles cilia.** (A-C) Lack of Kat1p katanin p60 induces 9+0 cilia. Cross-sections of wildtype (A) and Kat1p null mutant (B and C) cilia. C contains a section of multiple cilia of a developing membranelle of an oral apparatus. Note that mutant cilia lack the central pair of microtubules while the doublet microtubules with dynein arms and radial spokes are present. (D-P) Excessive Kat1p nicks and severs microtubules *in vivo*. TEM images obtained for *Tetrahymena* cells that overproduced GFP-Kat1p after induction with cadmium chloride for 2-6 hrs. (D-G) Longitudinal sections of axonemes in different stages of breakage and translocation of the distal part. Note that the transitional zone (arrowhead) does not show any signs of severing. (H-M) Cross-sections of cilia showing two axoneme profiles that positioned side by side. For k-m note that one axoneme fragment (probably distal in origin, see G) often shows more extensive depolymerization of doublets, but the central pair remains stable. M shows a case where the distal axoneme fragment that translocates lacks all doublets except one but still has a central pair. (N-O) Cross-sections of axonemes showing doublets missing the B-tubule (arrows). (P) A cross-section of an oral undulating membrane at the level of basal bodies. Note that some triplet microtubules are partly depolymerized to doublets or singlets (arrows in inset). Note that the affected triplets are severed at precisely the same circumferential positions in different basal bodies. All scale bars represent 0.2 μm except in panel P where scale bar is 0.25 μm.

Figure 3.8

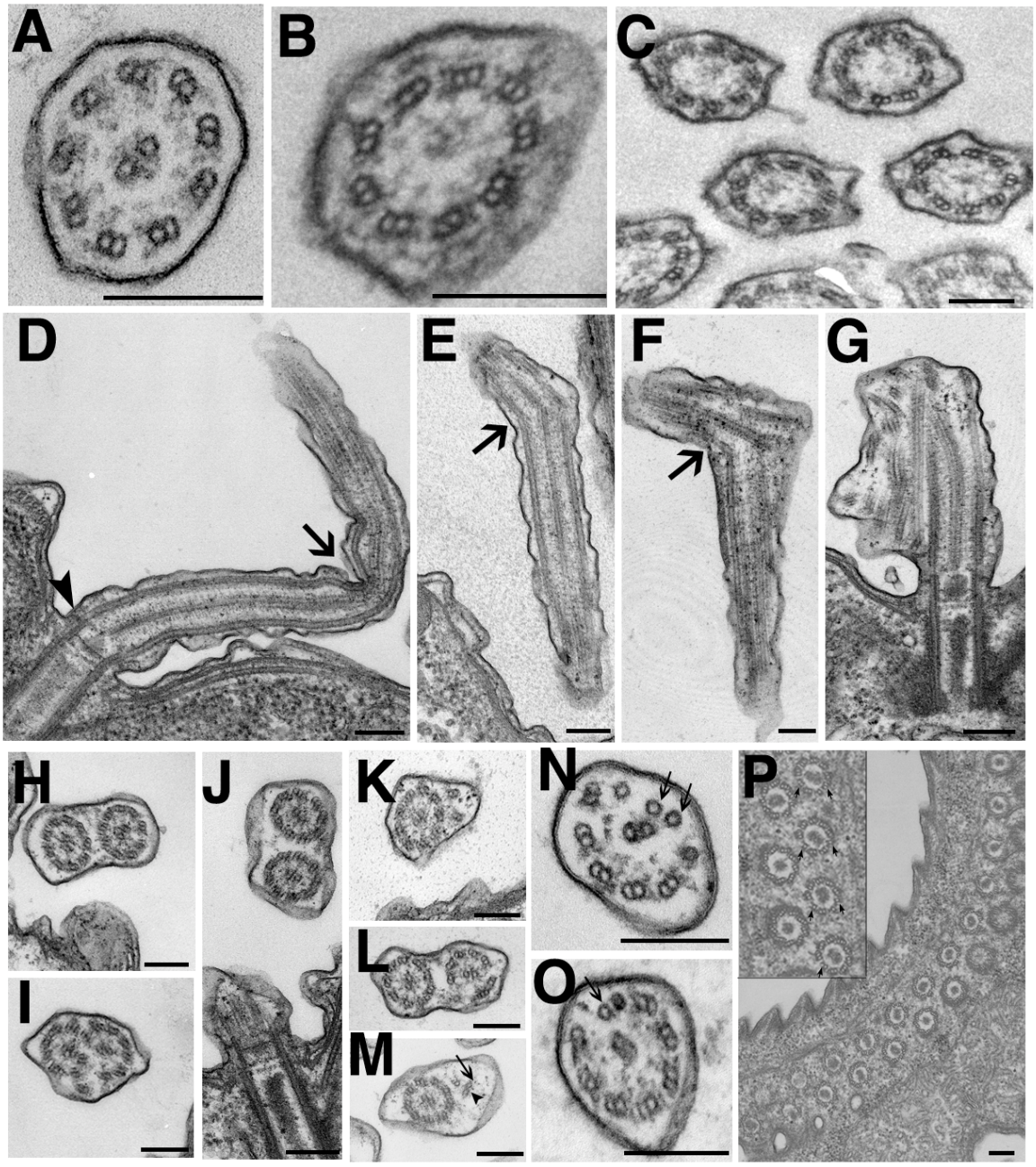
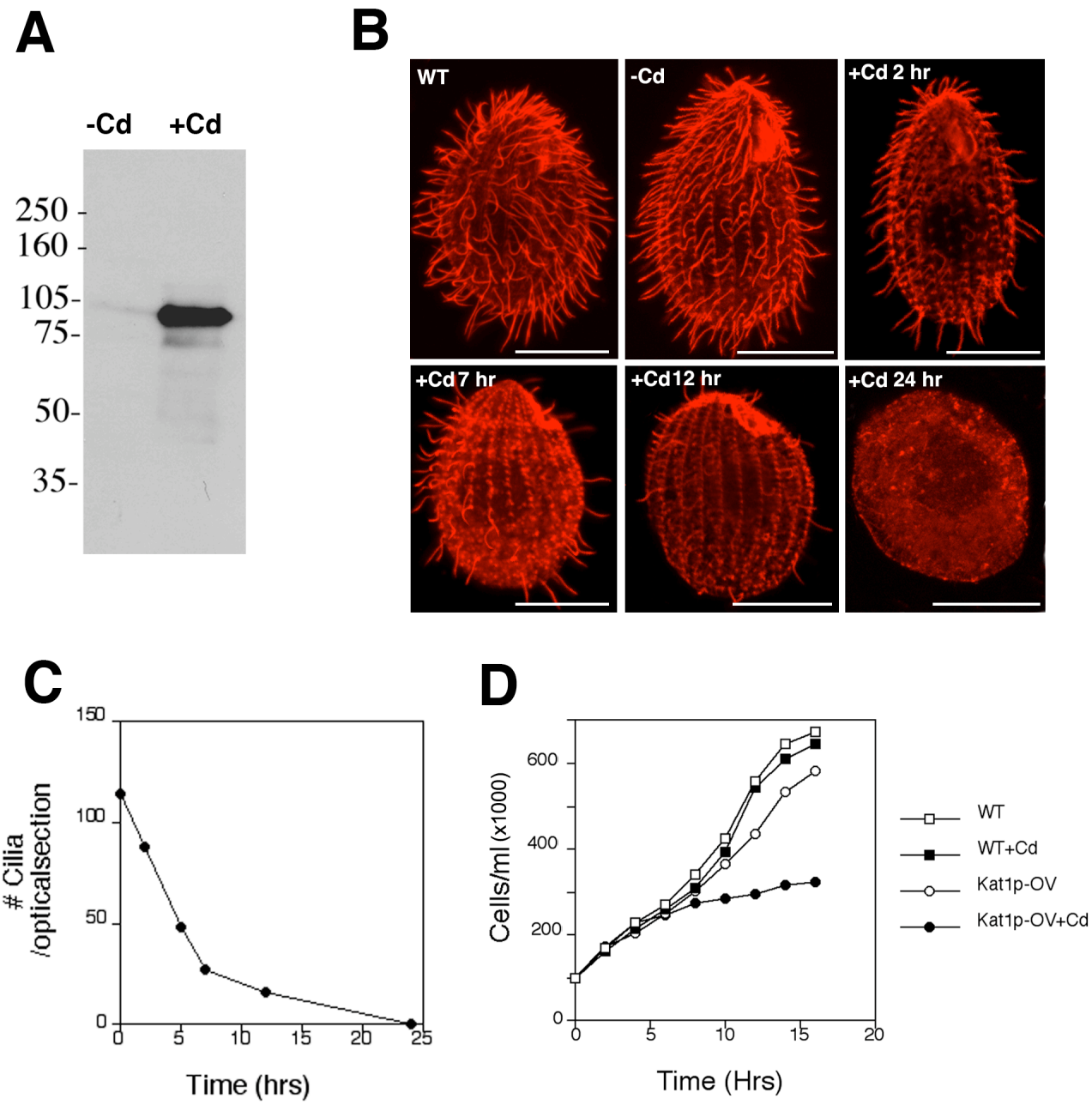


Figure 3.9. **Excessive GFP-Kat1p causes massive loss of microtubules *in vivo*.** (A) A western blot shows accumulation of GFP-Kat1p in overproducing cells. Total cell protein was obtained from transformed cells carrying a GFP-Kat1p encoding gene under control of the *MTT1* promoter at high copy number that either were treated or not with 2.5 mg/ml cadmium chloride for 3 hrs. The blot was reacted with anti-GFP antibodies. (B) Confocal images of cells labeled by immunofluorescence using 12G10 anti-a-tubulin. The cell shown in the top left corner is a wildtype control treated with cadmium. The cell in the middle in the top row is a GFP-Kat1p transgene carrying cell, which was not exposed to exogenous cadmium. The remaining cells were treated with cadmium chloride for 2-24 hrs. Note progressive loss of microtubular structures including cilia and basal bodies. All scale bars represent 20 μm. (C) A graph that illustrates the rate of loss of cilia in response to accumulation of GFP-Kat1p. (D) A graph that shows the deleterious effect of GFP-Kat1p overproduction on multiplication of *Tetrahymena* Either wild type of transgenic cells carrying *KAT1* coding region under *MTT1* promoter at high copy number (Kat1p-OV) were grown with and without exogenous cadmium. Note that cadmium alone does not affect normal cells.

Figure 3.9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160
105
75
50
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+Cd12 hr
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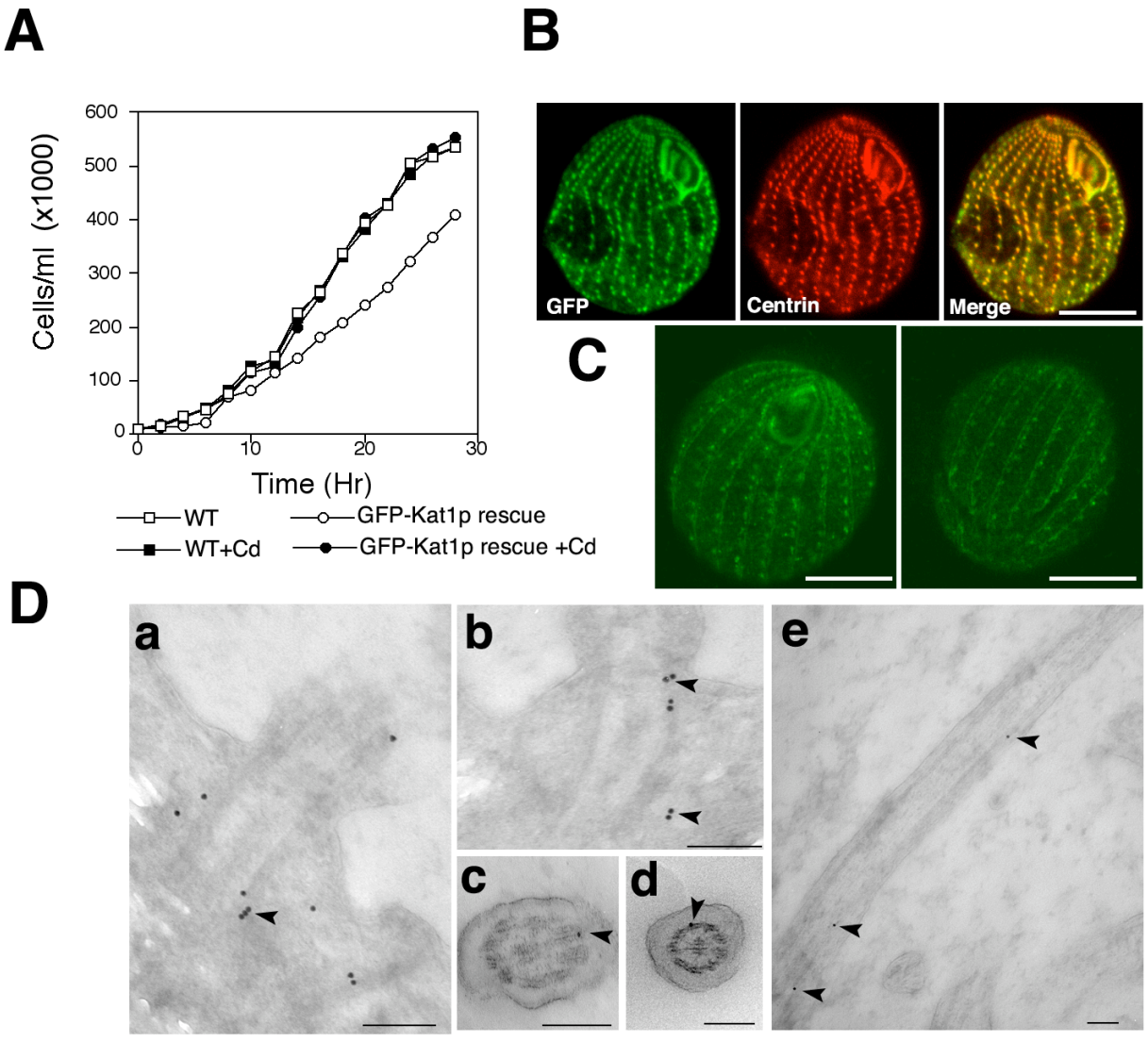
C
Cilia /opticalsection
150
100
50
0
0
5
10
15
20
25
Time (hrs)
D
Cells/ml (x1000)
600
400
200
0
0
5
10
15
20
Time (Hrs)
WT
WT+Cd
Kat1p-OV
Kat1p-OV+Cd

Figure 3.10. **GFP-Kat1p overexpressed at a moderate level does not affect cell multiplication and localizes to a subset of microtubules.** (A) A graph shows the rates of cell multiplication of a wild type and GFP-Kat1p (low copy) rescue strain with and without exogenous cadmium. Note that the rescue strains grow more slowly without cadmium presumably because an uninduced *MTT1* promoter is not expressed at a level that support normal growth rate. However, the rescue strain grows normally with cadmium. (B) Confocal images showing that GFP-Kat1p (direct GFP signal) co-localizes with basal bodies that were co-labeled with anti-centrin antibodies. (C) Confocal images of GFP-Kat1p rescue cells that were fixed with paraformaldehyde. Note that in addition to dots of fluorescence that correspond to the basal bodies, GFP-Kat1p is present as a line on one side of dots, consistent with the location of LM cortical bundles (see Fig. 1B). (D) Immunogold (post-embedding) study reveals the presence of GFP-Kat1p in association with basal bodies (a-b) and outer doublets in cilia (c-e). All scale bars represent 0.25 μm except in D c-e where scale bars are 0.2 mm.

Figure 3.10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B
C
D
Cells/ml (x1000)
600
500
400
300
200
100
0
0
10
20
30
Time (Hr)
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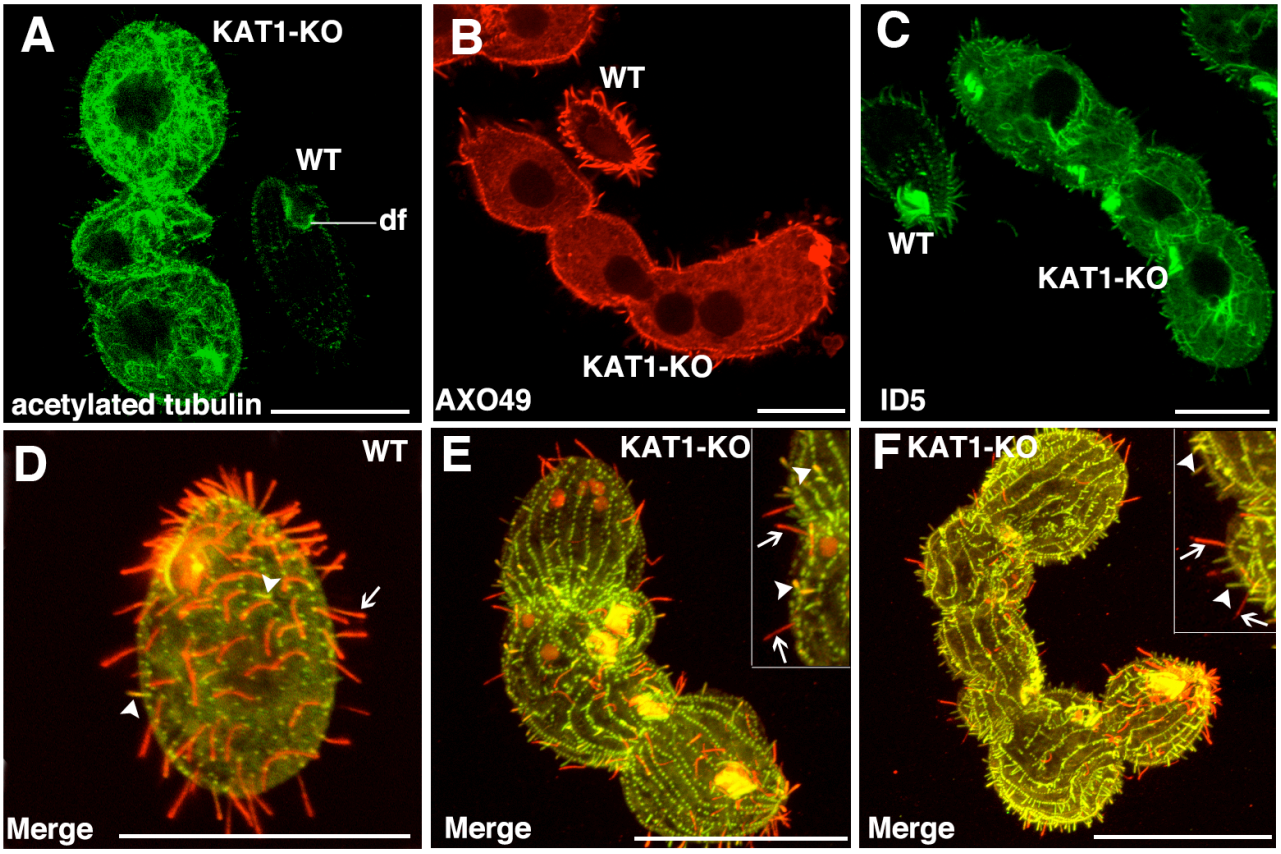
WT+Cd
GFP-Kat1p rescue
GFP-Kat1p rescue +Cd
GFP
Centrin
Merge
a
b
c
d
e

Figure 3.11. (A-C) **Lack of zygotic Kat1p leads to accumulation of acetylated and polymodified microtubules in the cell body of *Tetrahymena*.** Cell-chains lacking zygotic Kat1p were mixed with wild type cells and process for immunofluorescence using the 6-11 B-1 anti-acetylated Lys40 a-tubulin (A), AXO49 anti-polyglycylated tubulin (B) and ID5 anti-polyglutamylated tubulin (C) antibody. A single internal confocal optical section of a mutant and wildtype cell. Note that wildtype cells lack internal acetylated, polyglycylated and polyglutamylated microtubules in the cell body except for the acetylated oral deep fiber (df). (D-F) Composite confocal images of wildtype (D) and Kat1p-null (E, F) cells double labeled for polyglycylation (red) and polyglutamylation (green). Note that in a normal cell, short developing cilia (arrowhead) show a low signal of polyglycylation and a strong signal of polyglutamylation while a reverse relationship is observed for the majority of cilia that are mature and full length (arrow). In the mutants (E, F) the majorities of cilia are short (arrowheads) and maintain an apparent high level of polyglutamylation and low level of polyglycylation as compared to normal length parental cilia (arrows). (G) A western blot analysis of either wildtype of a population highly enriched in cells lacking zygotic Kat1p. To prepare the mutant population, we mated *KAT1* heterokaryons *en mass*, refed cells after 24 hrs and allowed the progeny to develop the mutant phenotype. We prevented the population from being overgrown by cells that failed to mate by adding paromomycin (progeny cells but not cells that aborted conjugation, are resistant to paromomycin due to the expression of the *neo3* cassette). Either total cell (total) or soluble protein (sup) or cytoskeletons (cyt) after Triton X-100 extraction were loaded and probed with either 12G10 antibody (anti-a-tubulin) or 6-11 B1 (anti-acetylated Lys-40 on a-tubulin). Soluble (sup) and cytoskeletal protein fractions (cyt) were loaded in an equivalent amount to the number of total cells used in other lanes (note that for mutants we loaded the same total number of

subcells). The blot was first probed with anti-tubulin antibody (upper blot), stripped off and reprobed with an anti-acetylated tubulin antibody (lower blot). Note that the levels of soluble tubulin are similar in wildtype and mutant cells, indicating that katanin does not affect the size of the soluble tubulin pool. Also, the levels of acetylated tubulin in microtubules are similar. Given that mutant cells have excessively short, cilia, there must have been an increase in the levels of acetylation in the cell body, consistent with immunofluorescence shown in panel A. All scale bars are 50 μm

Figure 3.11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WT
df
acetylated tubulin
B
WT
KAT1-KO
AXO49
C
WT
KAT1-KO
ID5
D
WT
Merge
E
KAT1-KO
Merge
F KAT1-KO
Merge

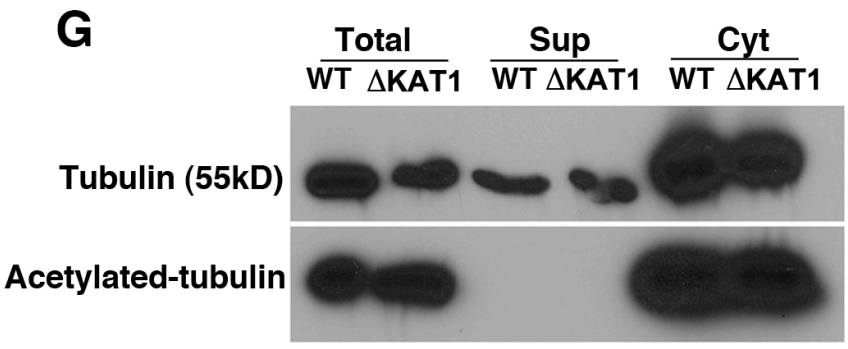

G
Total
WT ΔKAT1
Sup
WT ΔKAT1
Cyt
WT ΔKAT1
Tubulin (55kD)
Acetylated-tubulin

Figure 3.12. **Cytoplasmic microtubules in cells lacking zygotic katanin are hyperstable.** (A-D) Internal confocal optical sections of either wildtype cells (A,C) or cell-chains lacking zygotic Kat1p (B,D) that were treated with DMSO as a solvent control (A-B) or 40 mM nocodazole (C-D) and processed for immunofluorescence using 12G10 anti-a-tubulin. Note that cytoplasmic microtubules depolymerized in the wildtype cell but are still abundant in the mutant. All scale bars represent 20 μm. (E) The effect of either paclitaxel or oryzalin on the rate of multiplication in wildtype cells. (F) Cell multiplication in a population enriched in mutant cells following mass mating of *KAT1* heterokaryons, treated either with 2 mM oryzalin, 10 mM paclitaxel or DMSO as a solvent control.

Figure 3.12

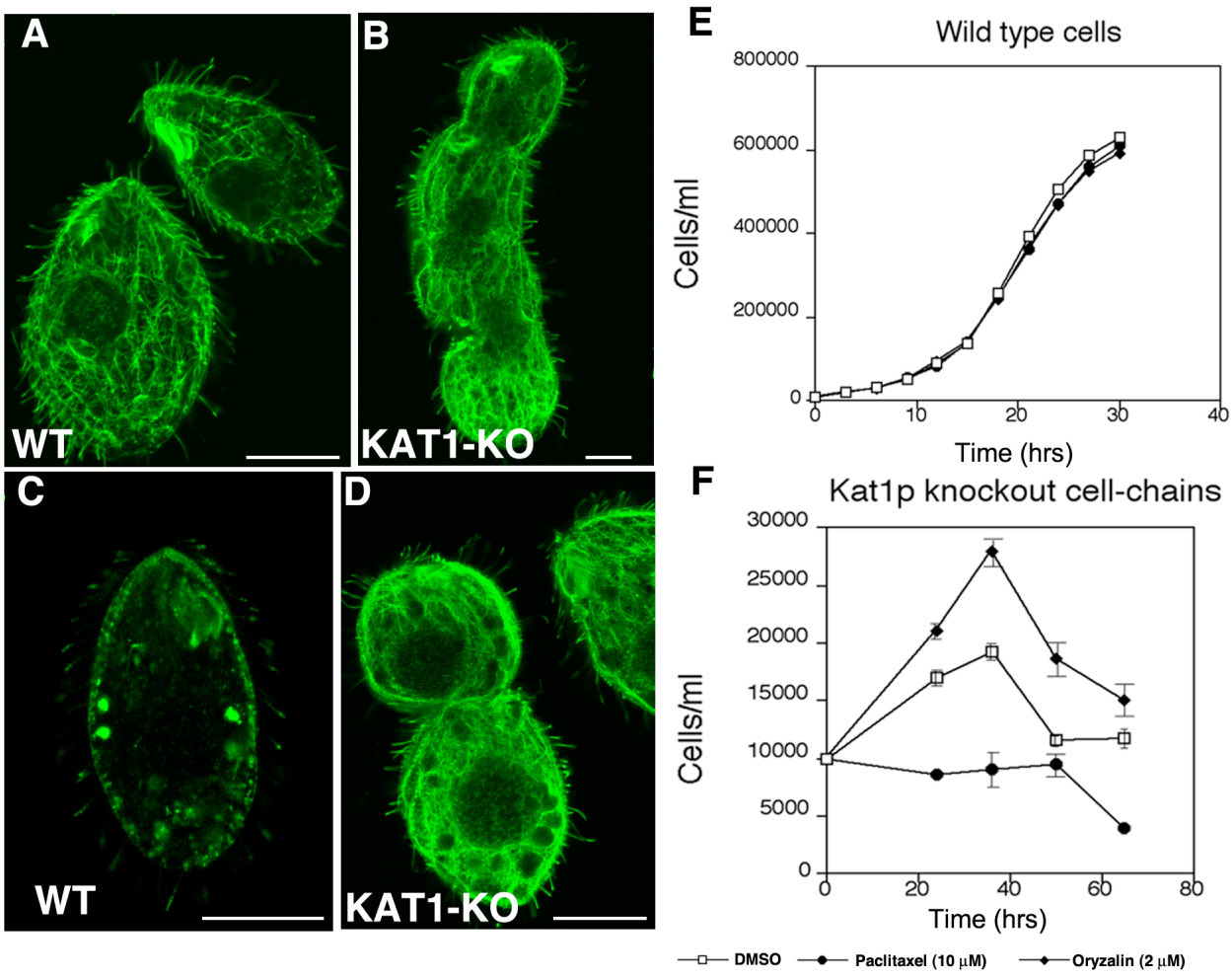

A
B
WT
KAT1-KO
C
D
WT
KAT1-KO
E
Wild type cells
800000
600000
400000
200000
0
Cells/ml
0
10
20
30
40
Time (hrs)
F
Kat1p knockout cell-chains
30000
25000
20000
15000
10000
5000
0
Cells/ml
0
20
40
60
80
Time (hrs)
DMSO
Paclitaxel (10 μM)
Oryzalin (2 μM)

Figure 3.13**. Inhibition of cytokinesis is not responsible for accumulation of hyperacetylated microtubules in cells lacking zygotic Kat1p.** A. Confocal images of cells that underwent a block in cytokinesis due to either lack of zygotic Kat1p B. Cell blocked in cytokinesis due to complete lack of cilia caused by a mutation in the IFT52 gene (Brown et al., 2003). In the complete absence of cilia, *Tetrahymena* cells are unable to undergo the process of rotokinesis, a rotational movement of daughter cells that produces torque required for breaking of the cytoplasmic bridge at the termination of cytokinesis. Note that unlike cells lacking Kat1p, *IFT52* mutants do not display acetylated microtubules in the cell body (only sections of cell cortex are stained). Scale bars in panel A and B are 50 and 20 μm respectively.

Figure 3.13

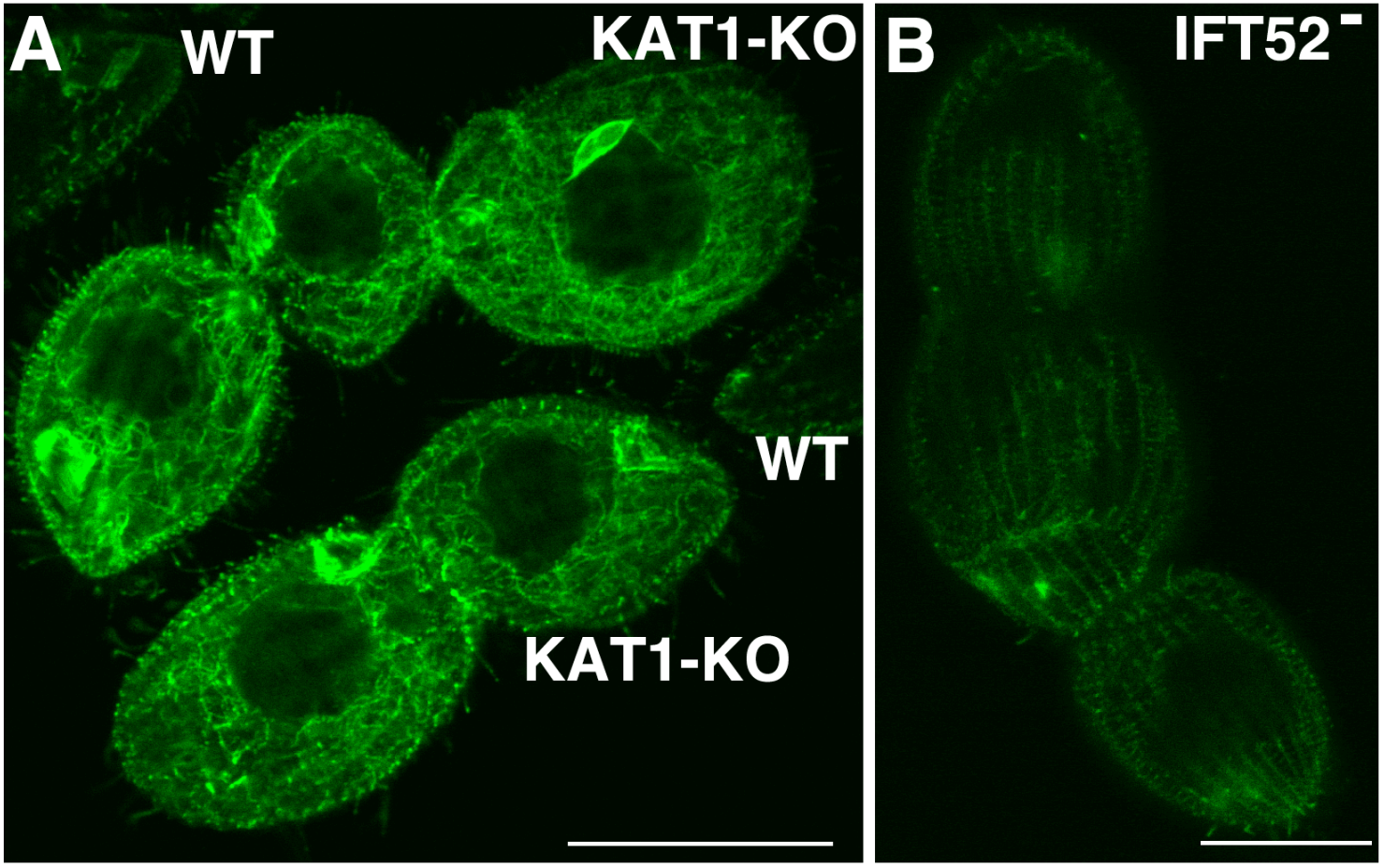


Figure 3.14. **Cells lacking zygotic Kat1p accumulate polyglutamylation in the cell cortex.** Wildtype and Kat1p-null cell chains were labeled with ID5, an anti-polyglutamylated tubulin antibody. Note that in wildtype, ID5 labels only cilia and basal body microtubules. In mutant cells, portions of LMs were also labeled with ID5 (arrowhead inset). Scale bars are 20 μm.

Figure 3.14

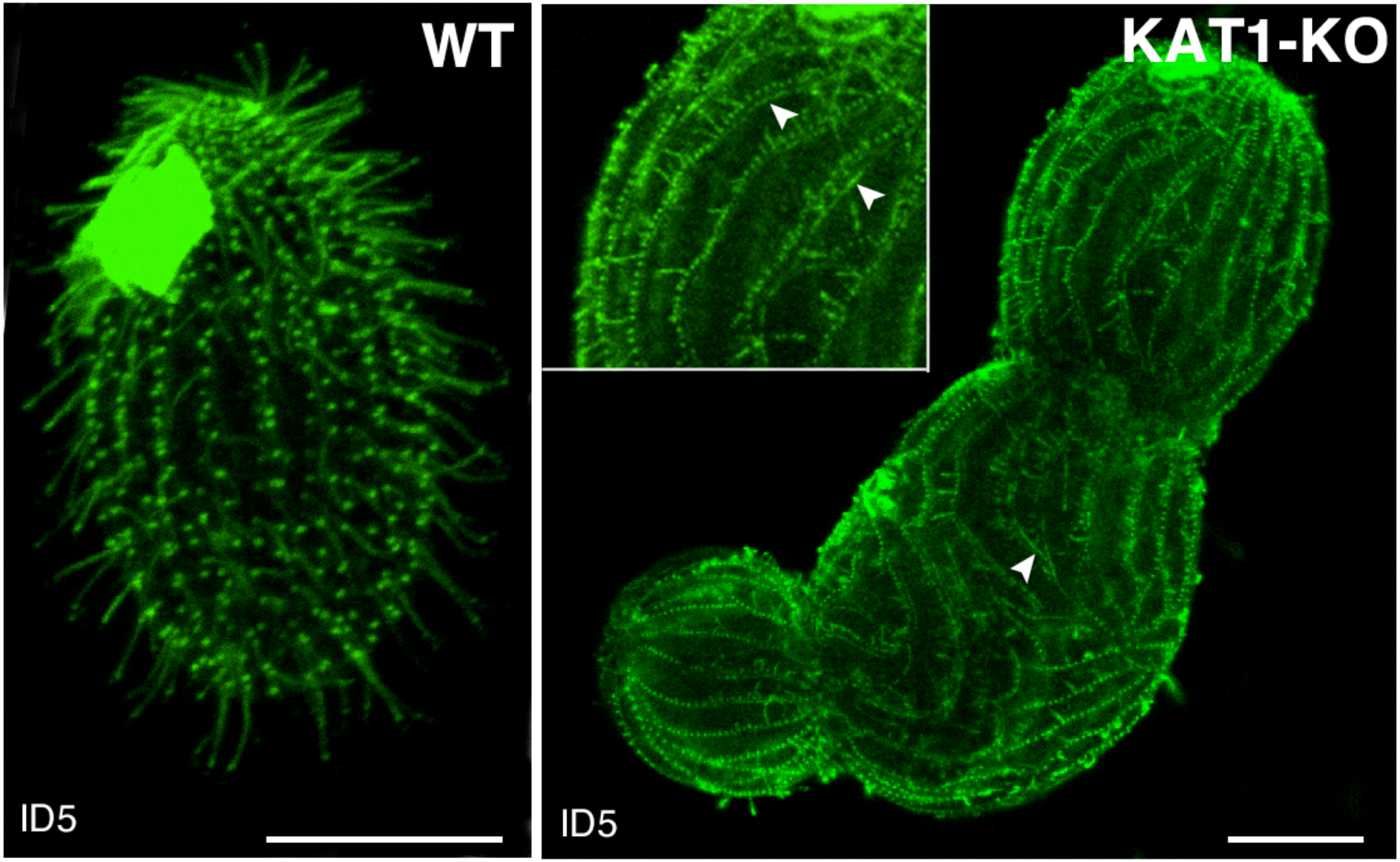
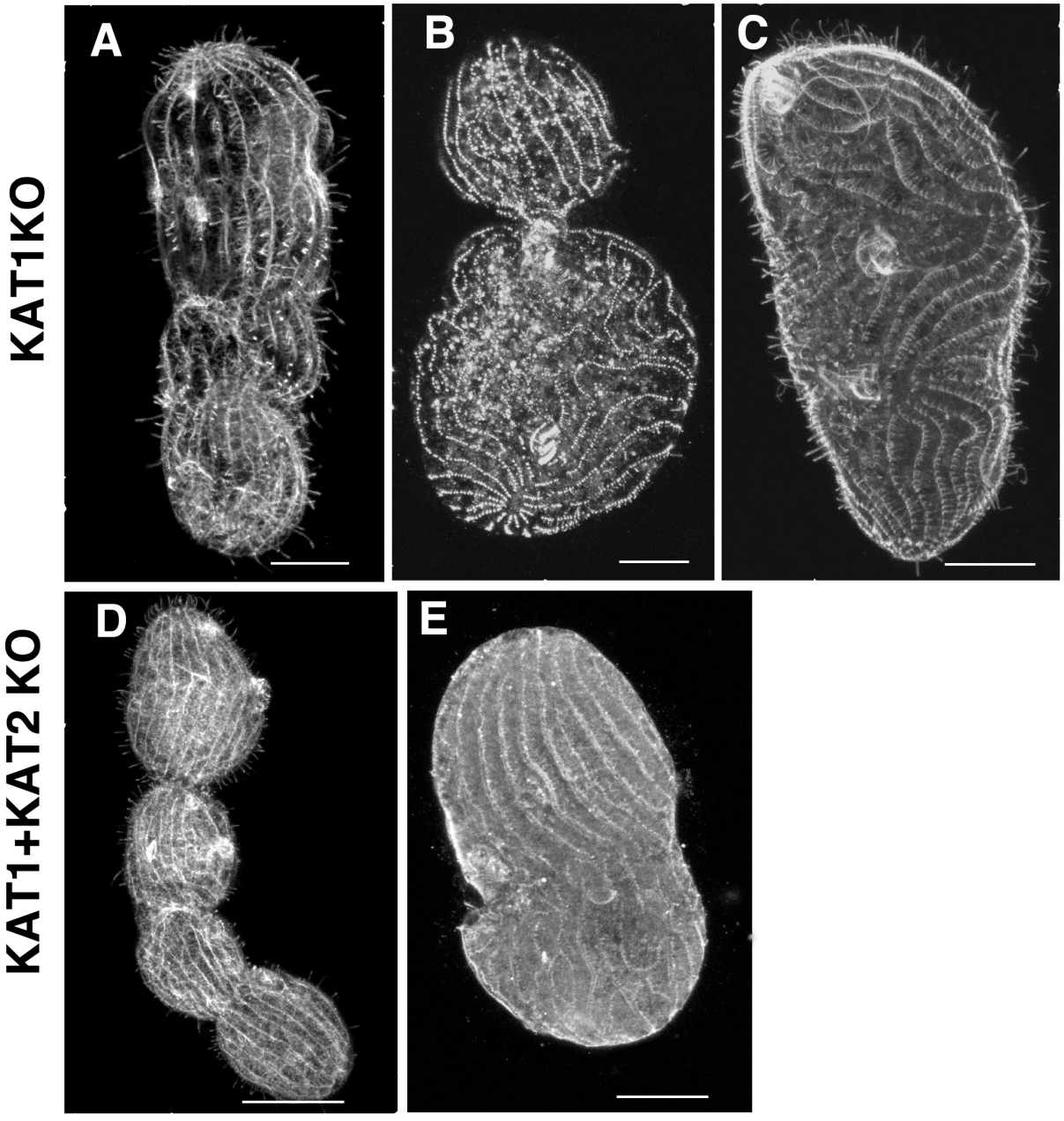


Figure 3.15. **Cells lacking zygotic Kat1p and Kat2p undergo deciliation.** A-C. Cell-chains lacking zygotic Kat1p were subjected to deciliation using a pH shock method, and allowed to regenerate cilia in growth medium. A Kat1p-null cell before deciliation procedure (A), shortly after deciliation (B) and 2 hrs later (C) is shown. Note a nearly complete loss of cilia in B, and that mutants regenerate uniformly short cilia (C). D-E. Cell chains lacking both Kat1p and Kat2p also undergo deciliation when subjected to deciliation by pH shock. Scale bar in panel A is 20 μm while scale bars in B-E represent 100 μm.

Figure 3.15



CHAPTER 4

CONCLUSION

The main question, I have addressed throughout my dissertation project is what is the significance of microtubule severing and how this process is regulated *in vivo*. In particular, I have focused on establishing whether a functional relationship exists between MT-severing proteins and microtubule PTMs. As discussed in the introduction, the properties of MTs are strongly affected by accumulation of PTMs but little is known about their *in vivo* functions. PTMs could potentially be a major mechanism that can generate structurally and functionally diverse MTs. Importantly, the types of PTMs as well as modification sites for PTMs on α- and β-tubulin are evolutionarily conserved from protists to humans, indicating their biological significance. In recent years, our lab has been primarily focused on the *in vivo* function of tubulin polyglycylation and polyglutamylation, PTMs known collectively as polymodifications.

Previous research indicates that the sites of polymodifications are required for cytokinesis and assembly of cilia

Initial studies from our lab tested the significance of polymodification sites on α- and β-tubulin. Polymodifications occur on the CCT of both α- and β-tubulin, on overlapping or adjacent sites provided by glutamic acids of the primary sequence. Polymodifications on α-tubulin are not essential but the sites of polymodifications on β-tubulin are essential for cell growth and multiplication (Xia et al., 2000). Five adjacent glutamic acids of CCT of β-tubulin that correspond to the sites of polyglycylation identified in *Paramecium* (Redeker et al., 1994; Vinh et al., 1999) were studied in detail by creating (nonmodifiable) aspartate substitutions in various combinations. None of the sites was essential but certain combinations of multiple substitutions

were lethal or affected the phenotype. A hypomorphic mutant, $\beta EDDD_{440}$, showed slow motility, multinucleation (indicating arrests in cytokinesis) and reduced growth rate and widespread defects in cilia including lack of closure of the B-tubule in doublet microtubules (Xia et al., 2000). The mutant heterokaryons that carried the $\beta DDDE_{440}$ in the MIC and WT alleles in the MAC were used to study the lethal phenotype. In this mutant, the conserved sites for polyglycylation at Glu 437-439 were replaced by Asp residues (in a charge conserved manner), which can not be postranslationally modified. The progeny of these mutants showed a complete loss of cell motility and all cells were arrested in cytokinesis that led to formation of chains of subcells (Thazhath et al., 2002).

Electron microscopic analysis of $\beta DDDE_{440}$ mutant cells revealed that cortical LM bundles were comprised of an abnormally large number of MTs (Thazhath et al., 2004). In WT cells, these bundles contain several overlapping MTs and the individual LMs are maintained in such a way that each LM cross-section possesses approximately 7-9 MTs (Huang and Pitelka, 1973). However, in mutant cells, LMs consists of almost twice the normal number of MTs. This abnormal elongation of LMs is believed to physically obstruct the ingressing contractile ring and block the separation of daughter cells.

The phenotype of polymodification site mutants provided a starting point for my doctoral studies. The hypertrophy of cortical MTs suggested that sites of polyglycylation are a positive regulator of an activity that depolymerizes microtubules in the cell cortex. Specifically, the presence of polymodifications could stimulate activity of a MT depolymerizer. Three possible mechanisms of depolymerization of MTs could be considered: 1) plus end depolymerization by kinesin-13, (or kinesin-8 or -14), 2) destabilization of MTs by a dimer-sequestering protein, stathmin, and 3) microtubule severing by katanin or spastin. The potential role of dimer

sequestration could not be studied because *Tetrahymena* lacks an obvious homolog of stathmin. However, *Tetrahymena* genome contains sequences encoding both depolymerizing kinesins and MT severing proteins. A clue that polymodifications could be regulating a microtubule severing factor came from a study published more recently by the Elizabeth Smith laboratory using *Chlamydomonas*. An insertional mutation in the PF15 gene encoding p80, a putative non-catalytic subunit of katanin, led to assembly of paralyzed flagella that lacked the central pair MTs (Dymek et al., 2004). Thus, lack of microtubule severing could potentially explain both the cortical phenotype (hypergrowth of LM bundle MTs) and ciliary phenotype (short 9+0 cilia) of the $\beta DDDE_{440}$ polymodification site *Tetrahymena* mutant. However, there were three important differences between the *Chlamydomonas* p80 mutants and the *Tetrahymena* $\beta DDDE_{440}$ mutants. First, the p80 mutation in *Chlamydomonas* did not affect the cell body function and did not block cytokinesis. Second, the length of cilia in *Chlamydomonas* mutants was normal and therefore the outer doublets could elongate to full size. Third, in *Tetrahymena*, a frequent loss of the B-tubule of doublets was observed while doublets in *Chlamydomonas* were normal. These differences could to some extent reflect the differences in the organization of the microtubular cytoskeleton between the two species. For example, *Chlamydomonas* lacks cortical microtubules that are perpendicular to the cleavage furrow. Regarding the ciliary phenotype, it is possible that the *Chlamydomonas* mutation was not a complete loss of function mutation. Furthermore, it is possible that polyglycylation regulates MT severing factors as well as a number of other effectors of MTs and therefore a polymodification site mutation gives a stronger phenotype that also includes consequences of lack of MT severing. However, when both phenotypes are considered together, an intriguing possibility has emerged that polymodifications regulate

microtubule severing activity and that somehow this severing activity is also required for assembly of motile 9+2 cilia and in particular for assembly of the central pair of microtubules.

Loss of katanin function to large extent phenocopy the βDDDE$_{440}$ polymodification site mutation

Katanin was identified as a microtubule-severing factor in mitotic extracts of *Xenopus* oocytes (McNally and Vale, 1993). McNally and Vale observed higher levels of katanin activity in M-phase compared to interphase in *Xenopus* egg extracts, which pointed towards a mitotic function of katanin. Katanin is a heterodimer of p60 catalytic and p80 non-catalytic subunit. p60 is an AAA type ATPase and it requires ATP binding and hydrolysis for its severing function (Confalonieri and Duguet, 1995; Hartman and Vale, 1999). Orthologs of p60 katanin are present in human, *Chlamydomonas, C. elegans, Drosophila, Arabidopsis* and *Tetrahymena*, but not in fungi. Interestingly, yeast also lacks polymodifications (based on the absence of tubulin glutamylase enzymes TTLL6, TTLL1, TTLL4) (Janke et al., 2005). This suggests that both katanin and polymodifications are important for the function of complex types of microtubules such as cilia, centrioles or cortical arrays in plants. Katanin assembles on surface of a MT polymer lattice and subsequent conformational changes upon ATP hydrolysis produce a torque that breaks a MT probably by localized removal of tubulin dimers from the lattice (Hartman and Vale, 1999). Due to the lack of a 3D structure of katanin it is not clear how katanin binds to MTs. One clue regarding the nature of katanin/MT interaction came from *in vitro* severing assays. The CTTs of α- and β-tubulin can be cleaved by a limited proteolysis with subtilisin (Paschal et al., 1989). CTTs are not essential for *in vitro* polymerization of MTs. Katanin was able to bind to the MTs lacking CTTs and hydrolyze ATP but could not disassemble MTs

(McNally and Vale, 1993). This observation indicates that CTTs regulate the conformation of katanin and are important for the force production. Interestingly, spastin also requires CTTs for its severing activity (Roll-Mecak and Vale, 2005). A recent study showed that tubulin tail peptide of CTT containing 104 amino acid residues acts as a competitive inhibitor of spastin activity upon polymerized MTs *in vitro* (White et al., 2007).

CTTs are important for interactions with multiple types of MT effectors and this is probably why CTT are essential *in vivo* despite the fact that they are not required for polymerization of MTs (Duan and Gorovsky, 2002). It is also highly relevant to our study that most PTMs occur on CTTs, including detyrosination, polyglycylation, polyglutamylation and phosphorylation. Thus, all these studies provided an additional support to a model that polymodifications regulate katanin-mediated severing of microtubules.

Recent studies have uncovered a number of functions for PTMs that all appears to amount to regulation of interactions with various MT effectors. For instance, the binding of kinesin-1 to MTs is stimulated by acetylation of Lys-40 on α-tubulin (Reed et al., 2006). Tyrosinated MTs are a preferred substrate for +TIPs with a Cap-Gly domain including p150 Glued and CLIP170 implicating the PTM, detyrosination as a negative regulator of +TIPs, proteins that accumulate at the plus ends of microtubules and regulate interactions with the plasma membrane (Peris et al., 2006). These observations encouraged me to look for potential functional interactions between katanin and tubulin polymodifications.

The macronuclear genome of *Tetrahymena thermophila* was recently sequenced and assembled producing a high quality sequence (Eisen et al., 2006). Using searches with established katanin sequences from other organisms, we found two genomic sequences that encode p60 subunit of katanin, *KAT1* and *KAT2* and a p80 katanin subunit encoding sequence,

KAT3. Both predicted p60 subunit protein sequences contain an AAA domain sequence. A homologue of related AAA ATPase, spastin, Spa1p is also present in *Tetrahymena*. This ciliate also has another poorly characterized AAA protein, fidgetin, whose mutation leads to a developmental defect in mice (Yang et al., 2006).

Using a germline knockout heterokaryon strategy, we created cells completely lacking either katanin or spastin subunits. To our knowledge, this is the first systematic study that included creation of gene knockouts for all predicted subunits of MT severing factors in one organism. Our ability to create all knockouts in the germline demonstrates the power of the *Tetrahymena* experimental model. Kat2p and Spa1p deletions did not reveal any obvious mutant phenotype in my study. At the light microscopy level, we did not see any noticeable defects in these mutant strains. Despite the fact that Kat2p contains all features of a p60-like katanin subunit, its overproduction did not show signs of microtubule severing *in vivo* (data not shown). Because knockouts of both *KAT2* and *SPA1* did not produce an obvious phenotype, we considered that there is a functional redundancy between Kat2p and Spa1p, and we created double knockout strains lacking *KAT2* and *SPA1* genes. Double knockout strains showed normal morphology and also appeared to grow normally (data not shown). Thus, the *in vivo* function of Kat2p and Spa1p in *Tetrahymena* remains unclear. The results are puzzling because both proteins are highly conserved. Kat2p belongs to a separate evolutionarily conserved clade of p60 like proteins. Because most organisms have both p60 (similar to Kat1p) and p60-like subunit (similar to Kat2p), it is reasonable to assume that p60 and p60-like proteins have evolved non-identical functions, and that both proteins have a common evolutionary origin from an ancient gene duplication that have occurred before the emergence of the main subgroups of contemporary eukaryotes. We should, however, point out that we have not investigated in detail

the phenotypes of cells lacking Spa1p and Kat2p and functions of these proteins may be discovered in the future. There is no doubt, however, that these two proteins are dispensable for cell survival under laboratory conditions and their functions are either subtle or important only under specific environmental conditions that can not be recreated in the lab. Furthermore, there is also a possibility that one or both of these proteins play a role during meiosis or nuclear events of conjugation of *Tetrahymena* and this can also be tested in the future.

On the other hand, deletion of *KAT1* gene by the knockout heterokaryon approach resulted in two dramatic defects. The initial phenotypic changes that were observed in the progenies of *KAT1* knockout heterokaryons involved a gradual loss of cell motility and an arrest in cytokinesis. Such an incomplete cytokinesis defect led to formation of chains with subcells. Like $\beta DDDE_{440}$ mutants, Kat1p-null progenies formed cell chains with 2-5 subcells and died in approximately 90-100 hrs. The heterokaryon progeny lacking a zygotic Kat1p showed a similar life span on SPP, regular medium for *Tetrahymena*, as well as on MEPP, a specialized medium that is used for propagation of ciliary mutants (Brown et al., 1999b). In the regular SPP media, cells use phagocytosis as primary route for uptake of nutrients. However, mutants lacking an ability to phagocytose can be propagated on MEPP, probably because on this medium cells increase the rate of micropinocytosis for the intake of nutrients (Basmussen and Orias, 1975). In *Tetrahymena*, phagocytosis requires the function of motile cilia of the oral apparatus. In our earlier study, cilia-less kinesin-2 mutants did not grow in SPP but successfully grew on MEPP (Brown et al., 1999b). Thus, the requirement for cilia-dependent phagocytosis can be bypassed by the use of MEPP medium. However, the Kat1p null cell-chains showed a similar life span on both media (data not shown). These observations indicated that Kat1p-null cell-chains do not die from starvation but most likely due to an abnormal cytoskeleton organization, in particular

excessive polymerization and stability of internal microtubules and likely secondary effects on the intracellular trafficking that is dependent on microtubules.

Initially we suspected that the lack of ciliary motility in Kat1p-null cells could be the cause of the cytokinesis failure as earlier reported for IFT cilia-less mutants in *Tetrahymena* (Brown et al., 1999b). *Tetrahymena* cells undergo an unusual process called rotokinesis before the completion of cytokinesis (Brown et al., 1999a). At the final stage of cell division, only a thin cytoplasmic bridge connects two future daughter cells. At the end of cytokinesis, *Tetrahymena* cells exhibit a ciliary motility-based unidirectional rotational movement of the posterior daughter cell and the two daughters briefly pull apart before the cytoplasmic bridge is ruptured (Brown et al., 1999a). It is believed that the rotations introduce a strain within the thin cytoplasmic bridge to facilitate subsequent scission by pulling apart. However, it appears that the block in cytokinesis in *KAT1* knockout progenies occurred by a distinct mechanism earlier observed in $\beta DDDE_{440}$ mutant cell chains. In both cases ($\beta DDDE_{440}$ mutants and Kat1p-null progenies), cytokinesis was impeded at an early stage, when a relatively wide cytoplasmic connection still existed and daughter cells. Furthermore, the arrest in cytokinesis in IFT mutants can be rescued by rapid shaking presumably because the mechanical force produced by shaking substitutes for the absence of rotokinesis (Brown et al., 2003). However, our attempts at rescuing Kat1p null cell-chains by shaking were not successful (data not shown). Furthermore, unlike IFT mutants, Kat1p-null subcells did not integrate rapidly to form multinucleated cells. This is probably because in the Kat1p cell chains there is cortical continuity due to the lack of depolymerization of LM bundles.

Lack of katanin affects the organization of cortical MTs

Immunofluorescence analysis showed abnormally thick longitudinal MTs in Kat1p-null cells, which therefore could have impeded the cleavage furrow progression. As I earlier mentioned that in βDDDE$_{440}$ mutants that LMs show hypertrophy. LMs are bundles of short overlapping MTs of uniform polarity. Our immunofluorescence data showed that LMs undergo hypertrophy in the absence of Kat1p. We showed that GFP-Kat1p localizes near LMs (but not other types of cortical MTs such as TMs or PCs). We propose that Kat1p associates with the LMs and its severing activity controls the length of microtubules in the bundle. As a result, the bundle thickness (number of microtubules per bundle profile) can be controlled. However, it makes sense to postulate that Kat1p severs LM microtubules in a nonrandom fashion so that segments of MTs have a uniform length. The simplest possibility is that Kat1p severs near the minus end. Furthermore, we can propose that accumulation of polymodifications near the minus ends triggers Kat1p activity. When cells are deficient in tubulin polymodifications, either katanin cannot localize to LMs or the katanin cannot sever unmodified MTs (this will need to be tested in the future by localization of Kat1p in the background of the βDDDE$_{440}$ mutation). The preferential localization of Kat1p to the minus ends of MTs could be achieved by a simple mechanism. The accumulation of polymodifications is a time-dependent process. As a result, the longest chains of polymodifications could be present near the minus ends of MTs that also represent the older part of the polymer. Thus, polymodifications could activate katanin as a function of maturation of MTs and katanin could be removing older parts of the polymer. Coupled with addition of tubulin subunits at the plus end, preferential severing by katanin at the minus end could establish a subunit flux that keeps MTs dynamic but at the same time allows for control of their length. When either polymodification sites or katanin are absent or

downregulated, the LM segment microtubules could grow excessively and the LM bundle could be too thick to be broken by the ingressing contractile ring.

Studies in *C. elegans* are consistent with the model that katanin preferentially severs polymodified MTs. In *C. elegans,* MEI-1 and MEI-2 are homologs of p60 and p80 subunits of the katanin complex (Srayko et al., 2000). Both subunits are essential for the assembly of meiotic spindle MTs in the *C. elegans* embryo. Out of six β-tubulins in *C. elegans*, only TBB-1 and TBB-2 β-tubulins are expressed in the embryo. Genetic studies indicate that katanin prefers TBB-2 to TBB-1 for its function. An extragenic suppressor, which is an E/K substitution in TBB-2 sequence, rescued the lethality caused by MEI-1 overexpression (Lu et al., 2004). Importantly, this critical E residue, whose mutation rescues MEI-1 overexpression lethality, is located in the CTT domain at the position homologous to the strongest site of glycylation (E437) mapped for *Tetrahymena* and *Paramecium*. While glycylation may not be present in C. elegans (K. Rogowski and J. Gaertig unpublished data), glutamylation sites have been mapped to the same or adjacent E residues. It is possible that the susceptibility of TBB-2 to modification at E_{441} makes it a better substrate for katanin because the same position in TBB-1 contains alanine that cannot undergo a modification. The composition of tubulin polymodifications in *C. elegans* has not been studied in detail but polyglutamylated tubulin was detected during spermatogenesis in another nematode (Mansir and Justine, 1998). These observations indicate that E_{441} in TBB-2 in *C. elegans* undergoes glutamylation and that the modification affects katanin. Mutated TBB-2 tubulin is less glutamylated that prevents its interaction with MEI-1 katanin and may rescue the katanin overexpression phenotype.

Katanin is required for assembly of motile 9+2 cilia

We also observed that Kat1p-null cells assemble two types of cilia; normal length cilia present near the ends of the cell chains and short cilia present throughout the surface of mutants. Electron microscopic analysis showed that short cilia lack a central pair but had normal peripheral doublet MTs and associated dynein arms. The lack of a central pair is the most probable cause of lack of ciliary motility. This phenotype was also found in the $\beta DDDE_{440}$ mutant, making it likely that polymodifications regulate katanin also in the context of ciliary functions. The complete absence of cilia from some basal bodies indicated that assembly of cilia was affected at nucleation stage as well.

The abnormally thick LMs and accumulation of internal MTs (see below) are two phenotype components that can be explained by a lack of MT severing function, but the defective assembly of ciliary MTs suggested a counterintituitive function for katanin. We first suspected that Kat1p may not be a true MT severing protein and it may have a role in the assembly or stabilization of MTs inside the axoneme. If this is the case, the observed hypertrophy of LMs and internal MTs may be an indirect consequence of lack of Kat1p. To test this possibility, I overproduced a GFP-tagged Kat1p in wild-type cells. Immunofluorescence studies showed a rapid loss of MTs of all types, consistent with a severing activity. Interestingly even ciliary MTs were lost. Ultrastructural analyses revealed that doublet MTs inside the axoneme underwent breakage that further led to complete disassembly of cilia from the surface of cells. Therefore Kat1p is a genuine MT severing protein. Still the mechanism of katanin involvement in the assembly of the central pair and elongation of doublet MTs was unclear. Two non-mutually exclusive models can be considered. First, katanin could have a function inside cilia. It is possible that the ciliary function of katanin is a novel type and it does not require its

severing activity. Alternatively the severing action of katanin could generate precursor tubulin, which can be further utilized for the formation of central pair. It appears that katanin may be involved in the assembly of the central pair but not doublets MTs. The arrest in elongation of the doublet MTs could be a consequence of a failure to assemble the central pair. The doublet lengths were not affected by the p80 mutation in *Chlamydomonas*, indicating that the coupling between the assembly of the central pair and elongation of doublets is specific to *Tetrahymena*. In support of our model, in sea urchin eggs, the cilium initially assembles as a so-called procilium lacking the central pair and the central pair MTs assemble with a delay (Morris and Scholey, 1997). It is therefore possible that in the absence of katanin activity (as well as polymodifications) *Tetrahymena* can only assemble 9+0 procilia and that the lack of elongation of doublets could reflect a general defect that does not allow cells to enter the late stages of ciliary assembly. If this is the case, it is reasonable to predict that severing of doublet MTs may generate MTs specifically for the central pair assembly. The main question arises whether Kat1p is targeted to cilia, if it does, then to what compartment of axoneme.

Katanin localizes to peripheral doublet microtubules

Overexpression of GFP-Kat1p clearly showed the potential of katanin to be targeted to cilia but this experiment could not prove that Kat1p is targeted to cilia at its normal level of expression. However, previous studies done in *Chlamydomonas* indicate that katanin subunits are present inside cilia. Antibodies directed against human p60 detected epitopes at the transition zone of cilia (Lohret et al., 1999). These results raised some doubts about the specificity of antibodies against *Chlamydomonas* katanin and its localization pattern. However, more recent biochemical fractionation experiments showed that the p80 subunit of katanin is present in the

Chalymdomonas cilia and ciliary-basal body complex (Dymek et al., 2004). Furthermore, fractionation studies that utilized cilia lacking specific structures indicated that p80 is associated with the outer doublets (Dymek et al., 2004). Dymek et al could not detect p80 inside the flagella using immunofluorescence. Our attempts of generating specific polyclonal antibodies against Kat1p were also unsuccessful. We therefore constructed *KAT1* knockout rescue strains, which express GFP-Kat1p at the native level. Unfortunately, the native level of GFP-Kat1p expression was below the detection limit by fluorescence and immunofluorescence. We then turned to knockout rescue strains, which expressed GFP-Kat1p under an inducible promoter but at the lowest possible level that supported growth without causing massive depolymerization of MTs as observed in the overexpressing strains. In contrast to overproducing strains, the induction of GFP-Kat1p in rescue strains did not affect the cell growth and architecture of MTs. Uninduced rescue strains showed slightly slow growth rate than wildtype cells, most likely due to suboptimal Kat1p activity. Immunofluorescence analysis of uninduced and induced rescue cells did not reveal a ciliary localization of GFP-Kat1p. However, using immunogold electron microscopy, we found that Kat1p associates mainly with the outer doublet MTs in the axoneme and little if any Kat1p is associated with the central pair MTs. These data argue that katanin generates precursors from doublet MTs for the assembly of central pair.

Katanin preferentially severs polymodified B tubule

Indeed, it appears that katanin may prefer to sever the B-tubule over the A-tubule. The clue came from the studies on cells overproducing katanin. In numerous cross sections of the axoneme, the B tubule appeared to be more affected by Kat1p overexpression. Such cross-sections frequently lack B tubules while central pair MTs consistently appeared to be stable to

Kat1p overexpression. Very interestingly, few axoneme profiles consisted of central pair MTs while lacking all peripheral doublet MTs. The delayed disappearance of A tubule can be explained by overall instability of doublet structure due to loss of one of the partner component. These results are consistent with my model that peripheral doublets act as a substrate for katanin severing and provide precursors for assembly of central pair MTs.

Such preferential severing of B tubule provided another important piece of information. The A and B tubules of axonemal outer doublet microtubules differ in their molecular organization and their thermal stabilities (Stephens, 1970; Sui and Downing, 2006). This allowed the separation α-and β-tubulins associated with corresponding A and B tubules. Biochemical characterization of these isolated tubulins revealed that α-and β-tubulins are extensively polyglycylated and detyrosinated (α-tubulin) (Multigner et al., 1996). On the other hand, α-and β-tubulins from A tubules remain largely unmodified, lacking polyglyclation and detyrosination. The mechanism restricting polymodifications and detyrosination to B tubules is unknown. *Drosophila* spermatogenesis studies indicate that polyglycylation is a late event, which takes place well after the assembly of axonemal structure (Bressac et al., 1995) whereas detyrosination seems to be an early step in axoneme formation in sea urchin cilia formation (Stephens, 1992). Similar results were observed in *Chlamydomonas* flagella (Westermann and Weber, 2003). The segregation of tubulin variants inside cilia poses some interesting questions regarding the specialized functions of both tubules. In my study, I found that B tubules are more prone to severing by Kat1p, which suggest that longer side chains of polymodifications in B tubules act as a signal for katanin localization and or severing. Altogether, our first model is still in preliminary stage showing that Kat1p preferentially associates with doublet MTs and B tubules serve as

favorable sites for severing. While I believe that the above hypothesis is best-supported, other hypotheses that assume the presence of katanin inside cilia can also be considered. I will not exclude the possibility that katanin can be transported to the doublet MTs by IFT motors which in turn require polymodified B tubules as molecular tracks. In polymodification-deficient mutants, it is also quite possible that katanin cannot be transported to outer doublet MTs (or B tubule) due to inefficient transport of IFT cargo by virtue of unmodified B tubules. Absence of katanin-mediated severing of doublet MTs could in turn affect the assembly of central pair. It should also be mentioned here that central pair formation is more dependent on the process of IFT compared to elongation of doublets. IFT mutants either lacking anterograde motor, kinesin-II or IFT cargo particles resulted in assembly of short cilia with no central pair (Brown et al., 2003). However, in Kat1p-null cells, IFT may be functional but the overall absence of katanin may not allow the assembly of central pair.

Katanin is required for proper dynamics of microtubules

Our second model proposes that the role of katanin in assembly of ciliary MTs could be indirect and based on a requirement for proper dynamics of microtubules in the cell body. Katanin could generate tubulin dimers or short pieces of MTs in the cell body, which can be further transported by IFT process for the assembly of ciliary MTs. This model is proposed in analogy to what has been suggested as a mechanism for assembly of axonal microtubules in neurons (Baas et al., 2005). Like cilia, axons do not possess protein synthesis machinery, and proteins synthesized in the cell body are transported into axons. There has been a considerable debate regarding the form in which tubulin required for assembly of axonal microtubules is transported. Several studies support the "oligomer transport model" in which tubulin is

transported to growing ends of MTs inside the neurons in a dimeric or oligomeric form (Kim and Chang, 2006; Terada et al., 2000). Such analogous observations in neurons encouraged me to assume that precursors for the assembly of ciliary MTs could be tubulin dimers or oligomers. Alternatively, research primarily from the Baas laboratory, led to a hypothesis that the precursor tubulin is transported in the form of short microtubules along tracks of long microtubules (Baas et al., 2005; Baas et al., 2006). Either way, both hypotheses bring a possibility that a polymerized form of tubulin (oligomer or MT) needs to be transported into cytoplasmic extensions such as axons. Furthermore, several studies have implicated katanin in the generation of tubulin cargo for transport into the axon (Ahmad et al., 1999; Karabay et al., 2004). Katanin is required for axonal extension. Furthermore, injection of katanin antibodies led to accumulation of long microtubules around the centrosome in the cell body of neurons (Ahmad et al., 1999). Based on these observations, Baas and colleagues proposed the cut and run model: katanin could generate tubulin precursors (short MTs) by severing cell body MTs near the centrosomes (Baas et al., 2005). These MTs could be transported down the axon using a cytoplasmic dynein motor. By analogy, we can postulate that in ciliated cells katanin operates inside the cell body and generates tubulin cargo destined to cilia to be transported by IFT.

Based on our data, katanin could use cytoplasmic microtubules for generation of tubulin cargo for transport into cilia. In the simplest form, this hypothesis could be reduced to a model in which the role of katanin is completely indirect and based on maintaining a proper level of unpolymerized tubulin in the cell body. Our data indeed indicate that katanin is required to maintain the normal level of dynamics of cytoplasmic microtubules (see below). Thus, katanin severing of cytoplasmic and perhaps also cortical microtubules could release a tubulin dimer as a product and therefore could affect the size of unpolymerized tubulin pool in the cell body.

However, this hypothesis is not well supported because I found that the levels of unpolymerized (detergent-soluble) tubulin were similar between Kat1p-null and wildtype cells. Furthermore, deciliated Kat1p-null cell-chains could regenerate immotile cilia, indicating that there is no general deficiency in the precursor tubulin. If anything, the cell body could lack a subpool of precursors required for the central pair formation. Thus, if katanin acts in the cell body, it is probably involved in generation of a specialized tubulin cargo, perhaps in the oligomeric form, and probably destined for assembly of the central pair.

We thus postulate that katanin generate required precursors for the assembly of axonemal MTs by severing cytoplasmic MTs. The cytoplasmic MTs originate mostly from the basal bodies and penetrate the cell body. Remarkably, there is an excessive polymerization of these MTs in Kat1p-null cells. Normally, internal cytoplasmic MTs are labile and highly dynamic in *Tetrahymena* based on their sensitivity to MT-depolymerizing compounds (Stargell et al., 1992). In agreement with their short half-life, in normal cells these MTs are not detectably acetylated of Lys40 on α-tubulin, a PTM that is known to accumulate on MTs that are stable and drug-resistant (Gaertig et al., 1995). In contrast, this PTM significantly occurs on relatively stable and less dynamic MTs present in the cilia and cell cortex. Strikingly, the Kat1p-null cell chains accumulated a strong signal of acetylation on the cytoplasmic MTs. However, the level of acetylation on ciliary MTs remained unchanged. Quantitative measurements were highly consistent with the immunofluorescence data indicating that acetylation on cell body MTs is at least five fold higher as compared to acetylation in normal cells. While the western blot of total cell-chains showed levels of tubulin acetylation similar to normal cells, it should be pointed out here that Kat1p-null cell-chains have excessively short and less number of cilia and therefore lack a major source of acetylated tubulin (Gaertig et al., 1995) that wild-type cells normally

posses. Therefore increase in cell body MTs acetylation compensate for the loss of ciliary acetylation in our western blot analysis. To interpret my results correctly, I wanted to be sure that the increase in the levels of acetylation on internal MTs is not an indirect consequence of an arrest in cytokinesis caused by loss of Kat1p. In order to rule out this possibility, I took advantage of IFT mutants, which display cytokinesis arrest and form cell-chains owing to the lack of ciliary motility. IFT mutant cells chains did not accumulate acetylation on cytoplasmic MTs showing that the phenomenon is not an indirect consequence of absence of cytokinesis and therefore is specific to the absence of katanin. Furthermore, βDDDE mutants also showed increased acetylation on internal MTs, providing another common phenotype with the katanin mutants (Thazhath and Gaertig, unpublished data). I should mention here that acetylation occur on assembled MTs but not on unpolymerized tubulin dimers and does not lead to stabilization of MTs (Maruta et al., 1986). Generally it is considered that the (yet to be identified) tubulin acetyltransferase modifies MTs slowly and that its ability to modify polymers is primarily dependent on the polymer longevity. My results show that the loss of katanin leads to hyperacetylation of cytoplasmic MTs and suggest a potential *in vivo* function of katanin in regulating MT dynamics. These data are also supported by pharmacological approaches. Previous studies showed that cytoplasmic MTs of *Tetrahymena* undergo rapid depolymerization when treated with nocodazole whereas ciliary and cortical MTs are drug-resistant (Stargell et al., 1992). The accumulation of acetylation indicates that cytoplasmic MTs are less dynamic and more stable in Kat1p-null cell chains. Remarkably, treatment of Kat1p cell chains with a relatively high concentration of nocodazole did not depolymerize cytoplasmic MTs confirming that these MTs are hyperstable. While the mechanism of nocodazole-induced MT disassembly is not well understood, one well accepted model suggests that nocodazole promotes GTPase

activity of free tubulin dimer and convert it to GDP-tubulin (Lin and Hamel, 1981). Increase in the level of GDP-tubulin, which cannot be incorporated into growing ends of MTs, causes cessation of MT elongation and increases catastrophe rate of polymerized MTs. The stability of MTs in the absence of Kat1p, against nocodazole raises the possibility of a general deficiency of GTP-tubulin dimer in cytoplasm. The unavailability of soluble GTP-tubulin pool may disregard the nocodazole treatment and does not depolymerize the MTs. However, this does not appear to be the situation in our mutants because mutant cell-chains because the levels of unpolymerized tubulin were normal. It appears that in the absence of katanin, some binding proteins stabilize cytoplasmic MTs, which makes them insensitive to nocodazole treatment because GDP-tubulin dimers can not be incorporated into MTs.

Katanin regulates the levels of tubulin polymodifications in vivo

An idea of an indirect involvement of katanin in the assembly of the central pair and doublet MTs may appear to be convoluted, but our results provide some reasonable clues. As I stated earlier, normally cytoplasmic MTs lack detectable acetylation due to their highly dynamic nature. Similarly, polymodifications are present on the cytoplasmic MTs but the side chains are limited to single glycine or glutamate residue per side chain. Strikingly, lack of severing in Kat1p-null cells induces the accumulation of longer side chains of glycine and glutamate residues, detected by specific antibody labeling. These observations suggest possible mechanisms that could restrict the polymodification to monoglycylation and monoglutamylation on cytoplasmic MTs. MTs often undergo treadmilling which causes rapid addition and removal of tubulin subunits at respective plus and minus ends. *In vivo* capping of MT ends inhibits the treadmilling process. However, severing by katanin can generate free ends, which allows for

rapid turnover of tubulin from polymerized MTs. Katanin could be continuously active near the minus end of MTs, which are older segments of polymerized MTs. The aged regions of MTs (minus ends of MTs) posses longer side chains of polymodifications as evident from study in sea urchin and *Paramecium* cilia. Polyglycylation and polyglutamylation occur in gradient along the length of cilia with the highest level near the proximal region (transition zone lacks both polymodifications) and lowest labeling at distal end of cilia (Bre et al., 1996; Pechart et al., 1999). On the other hand, monoglycylation display a reverse gradient in *Paramecium* cilia (Pechart et al., 1999) with the highest level at the tip and lowest level at the proximal part of the cilia. For stable microtubular structures like axonemes, occurrence of such gradient can be explained by the process where single glycine or glutamate is added at newer segment of MTs (plus end of MTs), which is subsequently replaced by addition of more glycine or glutamate units (older minus end of MTs). However, dynamic MTs may not possess such gradient of polymodification most likely caused by severing of polymodified segments at the minus end of MTs by katanin. This phenomenon seems more legitimate since katanin has been detected near the minus end of MTs at the centrosome and spindle poles (Hartman et al., 1998; McNally et al., 2000). In the light of our available data, I speculate that katanin actively severs polymodified regions of cytoplasmic MTs (this could explain why long chain polymodified MTs are never detected by immunofluorescence when katanin is present) and these smaller pieces of MTs are transported by IFT process to the growing outer doublet MTs.

Katanin reduces the MT polymer mass in the cell body

Another novel aspect of my study is the finding that lack of katanin leads to increased density and stability of cytoplasmic microtubules. In contrast to the observations made in *C.*

elegans, (Srayko et al., 2006) we demonstrate that katanin acts as a negative regulator of MT polymerization for intracytoplasmic locations. However as above-mentioned, katanin is also required for the assembly of MTs in ciliary locations (either directly or indirectly). In *C. elegans*, oocytes meiotic spindles and blastomere mitotic spindles function in chromosome segregation, there are substantial differences in their organization. Mitotic spindles of blastomere possess one pair of spherical centrosomes at each pole, which are involved in nucleation of astral array of MTs (catalyzed by γ-TuRc). These MTs interact with chromosomes to form the spindles and also required for correct positioning of assembled spindles. On the other hand, meiotic spindles in oocytes lack centrosomes at the poles. Using electron tomography, Srayko et al analyzed wildtype and MEI-1 p60 katanin mutant meiotic spindle MTs (Srayko et al., 2006). In contrast to our results, they found that in the absence of katanin, there is a decrease in the amount of MT polymer as well as number of individual MTs in the meiotic spindles. They suggest that in the case of *C. elegans*, severing products of katanin do not immediately depolymerize but act as seeds for further polymerization resulting in increase in the number and polymer mass of the MTs. Such results make more sense for the special cases where anastral spindles lack the MT nucleating centre. Cells lacking centrosome can use the katanin-mediated process of MT production. Subsequently, another study from McNally lab supported the katanin-based mechanism for the generation of MTs (McNally et al., 2006). Srayko et al also pointed out that ectopic expression of katanin led to generation of short MTs in astral mitotic which can not undergo further elongation and does not result in increase in polymer mass. This could be explained by the existence of a mechanism that could stabilize the free ends of newly generated short MTs. Here, our studies reveal a function of katanin that could explain the novel phenotypes

observed in ciliary and non-ciliary locations. In my case, increased density of intracytoplasmic MTs in the absence of severing activity can be explained by the supposition that sole role of katanin is in severing MTs and resulting short pieces of MTs can not be further elongated inside the cell body. Lack of severing activity led to increased polymerization of MTs that further causes increase in total polymer mass of MTs inside the cell body. Excessive polymerization of internal MTs intrigued us to test MT-depolymerizing drug, oryzalin. Oryzalin disassemble MTs by disrupting the association between protofilaments and subsequent disassembly of MTs. To our surprise, oryzalin partially rescued the cytokinesis phenotype indicated by increased number of cell population compared to control experiment. Such observation indicates that katanin negatively regulate the MT polymerization *in vivo* and absence of katanin causes abnormal polymerization of MTs. Also the treatment of Kat1p-null cell-chains with taxol further arrested the cell growth and inhibited the cell chain formation, suggesting that already suppressed MT depolymerization is supplemented by taxol-derived stability. Such effects of MT-stabilizing and depolymerizing drugs suggest that *in vivo* katanin decreases MT longevity and polymer mass. It should, however be, mentioned that the consequences of the loss of spastin, another AAA protein involved in microtubule severing, in other organisms were remarkably similar to what we observed for katanin. A deletion of the murine spastin gene led to the formation of swellings in axons that were enriched in detyrosinated, stable microtubules (Tarrade et al., 2006). RNAi-based knockdown of the spastin mRNA in Drosophila also caused excessive stabilization of microtubules, and remarkably, the phenotype was ameliorated by treatment with microtubule depolymerizers (Orso et al., 2005; Trotta et al., 2004). The function of spastin in *Tetrahymena* is unclear because the deletion of this gene did not change the gross phenotype, but functional

redundancy with katanin genes could be responsible and future work could reveal subtle phenotypic changes associated with lack of spastin in a ciliate.

Katanin (Kat1p and Kat2p) and spastin are not required for deciliation

We also seeked to reevaluate the role of MT-severing proteins in deciliation in *Tetrahymena*. Deciliation or deflagellation is a process in which cells shed their cilia in response to stress (Quarmby, 2004). Deflagellation has been extensively studied in *Chlamydomonas* and most of the knowledge is generated from this organism. Studies in *Chlamydomonas* indicated that katanin might have a role in deciliation. Microtubule severing is believed to play a key role in initiating breakage of outer doublet MTs at a site in the distal region of transition zone. The Quarmby lab has shown that katanin may have a role in deciliation. Their speculations were mainly based on localization of katanin to the transition zone and antibody-inhibited deciliation in *Chlamydomonas*. I generated *Tetrahymena* strains lacking both homologs of katanin (KAT1 and KAT2) as well as spastin. Using a standard method of deciliation, mutant strains lacking either of Kat1p, Kat2p or spastin, shed their cilia and successfully regenerate their cilia. We thought there could be a redundancy of Kat1p and Kat2p in the deciliation process so we generated strains lacking both genes in the same cell. To our surprise, none of the katanin p60 gene products was essential for deciliation in *Tetrahymena*. We generated additional mutant lacking Kat2p and Spa1p to test the possibility that these two proteins together provide the severing function during shedding of cilia. Currently, we are generating mutant cells strains, which will lack all three severing subunits (KAT1, KAT2 and SPA1). If these mutant cells also shed their cilia, we will conclude none of the known MT-severing proteins is involved in deciliation process. However, our conclusion that Kat1p is not required for deciliation is fully

supported by ultrastructural defects present in cell strains overexpressing Kat1p. We did not find any signs of severing in the transition zone, which is the well-documented site of deciliation in *Chlamydomonas*. In *Chlamydomonas*, katanin localizes to outer doublet MTs in the transition zone. Also, my immunogold localization studies of Kat1p demonstrated its localization to outer doublet MTs along the axoneme and near the basal body but we did not observe its localization to transition zone. Taken together my Kat1p studies, it appears that katanin is not directly involved in deciliation in *Tetrahymena*. Earlier subtle mutational studies on centrin, a centrosome/basal body resident protein, suggested that centrin might have a role in deflagellation (Sanders and Salisbury, 1989; Sanders and Salisbury, 1994). However, centrin is essential for cell viability (Susan Dutcher, Personal communication), its function in deflagellation remains uncovered. Future studies need to revisit the role of centrin in deciliation (Stemm-Wolf et al., 2005). Interestingly, *Tetrahymena* has several centrin genes and some but not all of their products are localized to cilia. Thus, in *Tetrahymena* it may be possible to address the role of centrin in deciliation without compromising other essential functions.

Future studies and implications

It should be mentioned here that tubulin tails can be either glycylated and glutamylated because the side chains of both polymodifications occur on adjacent or possibly the same acceptor sites possibly based on competition. Previous mutational studies on α- and β-tubulins established that polymodification sites on β-tubulin are essential in *Tetrahymena* but these studies did not distinguish between the respective roles of glutamylation and glycylation. Based on widespread occurrence of glutamylation I can speculate that glutamylation is a major regulator of katanin. Glutamylation is abundant in the mitotic spindle, neurons, cilia and

centrioles, places where katanin is known to play a role while glycylation is restricted to the cell types with cilia and flagella. Furthermore, there are organisms that lack glycylation while assembles a 9+0 axoneme such as *Trypanosoma*. Based on these evolutionary correlations, we postulate that glutamylation on β-tubulin is a major regulator of katanin locations such as neurons, mitotic spindles and centrosomes. In fact, the polymeric nature of glutamylation could provide an efficient regulator of katanin activity based on the side chain length. One can imagine that the regulation of microtubule stability may be based on elongation of the polyglutamate side chain. When the microtubule grows on the plus end the length of the side chain could increase gradually as the microtubule matures. Our lab has recently identified a β-tubulin specific tubulin glutamylase enzyme that has exclusively a chain elongating activity (D. Wloga unpublished results). As a consequence, the length of the side chain could be maximal near the minus end. I mentioned earlier that antibody labeling revealed the presence of modification gradient along the length of the cilia. One can further propose that the long side chains of polyglutamylation activate katanin. As a result katanin would preferentially trim segments of a microtubule that are closer to the minus end. Such an activity could be useful in regulation of the length of microtubules and in principle could lead to treadmilling. The discovery of tubulin glutamylase family, should in the future allow for testing of the hypothesis that glutamylation regulates katanin activity (Janke et al., 2005). On one side, the availability of purified enzymes will allow for preparation of microtubules with a distinct side chain length for *in vitro* severing assays. On the other side, deletions of tubulin glutamylase genes could shed light on the importance of tubulin glutamylation in vivo. The promising involvement of tubulin polymodifications side chains in marking MTs for the severing may reflect a general mode of how PTMs work and more importantly how MT-severing is regulating in different cellular

environments. My study will open new avenues for understanding the spatial regulation of katanin and spastin mediated severing in neurons. Conserved expression pattern of katanin to basal body (from protists to mammals) may persuade researchers to visit the pathological profile of katanin in cyst development in various organs and Bardet-Biedl syndrome associated with retinal degeneration, obesity, renal and limb malformations and developmental delay, primarily caused by dysfunction of cilia and basal body. It is unknown why there are significant differences in the level of katanin activity during interphase and mitotic stage of the cell cycle. Appreciating and analyzing the function of katanin in cancer cells may be conducive in understanding the mechanism of spindle assembly as it is known that cancer cells use centrosome-dependent as well as centrosome-independent pathway for the assembly of their spindles.

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