Review

Tubulin polymodifications in *Tetrahymena*

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Ciliates have a microtubule-rich cytoskeleton. *Tetrahymena thermophila* assembles at least 18 types of distinct microtubules that are located in cilia, cell cortex, nuclei and cytoplasm of the cell body. Specific microtubules differ in the filament length and curvature, degree of bundling, level of subunit turnover and levels of microtubule-interacting proteins. How diverse microtubules assemble and function is a largely unanswered question, to which ciliate studies have recently offered important insights. Commonly, multiple variants of the microtubule building blocks, dimers of α-/β-tubulin, are expressed in the same cell. Often tubulin variants are spatially segregated, and therefore could be involved in the functional adaptations of specific microtubules. Tubulin variants arise by: 1) expression of distinct tubulin isotypes (products of distinct genes) and 2) production of tubulin isoforms by post-translational modifications.

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Tubulin isotypes in *Tetrahymena*

Early studies suggested that *T. thermophila* contains only single isotypes of α- and β-tubulin (Gaertig et al., 1993; McGrath et al., 1994). This view has been revised on the basis of the sequence of the macronuclear genome (Eisen et al., 2006). *Tetrahymena* has only a single gene, *ATU1*, encoding a conventional α-tubulin, Atu1p (McGrath et al., 1994) and two genes for a conventional β-tubulin (*BTU1* and *BTU2*) that encode exactly the same protein, Btu1/2p (Gaertig et al., 1993). The amino acid sequences of Atu1p and Btu1/2p are over 90% identical to orthologs in most eukaryotes. Not surprisingly, *Tetrahymena* needs conventional α- and β-tubulin for survival. *ATU1* is essential (Hai et al., 1999). While the expression patterns of *BTU1* and *BTU2* are distinct (Gu et al., 1995), *Tetrahymena* needs only one of them for survival (Xia et al., 2000). In addition, *Tetrahymena* has single genes encoding highly conserved tubulins that are associated with the sites of microtubule nucleation, including γ- δ-, η- and ε-tubulin (Eisen et al., 2006; Shang et al., 2002). The γ-tubulin of *Tetrahymena* is essential and required for duplication and maintenance of basal bodies (Shang et al., 2002). The δ, η- and ε-tubulin have

not been studied in *Tetrahymena*. However, these tubulins are essential in *Paramecium.* δ-tubulin, as shown earlier in *Chlamydomonas* (Dutcher and Trabuco, 1998), is required for the assembly of the C-tubule in triplet microtubules of the basal body (Garreau De Loubresse et al., 2001). The η- and ε-tubulin are required for duplication of basal bodies (Dupuis-Williams et al., 2002; Ruiz et al., 2000). Thus ciliates have a set of conserved conventional tubulins, most if not all of which are essential. However, the genome of *Tetrahymena* also contains predicted genes encoding 3 extremely divergent α-tubulin-like proteins (*ALT1* to 3) and 9 equally divergent β-tubulin-like proteins (*BLT1* to 6, and 3 so called ι-tubulins) that appear to be ciliate-specific (Aury et al., 2006; Eisen et al., 2006). Due to their similarity to α- and β-tubulins, the α-like and β-like tubulins could be used as building blocks of microtubules. Thus, contrary to the earlier assumptions (Gaertig et al., 1993; McGrath et al., 1994), isotypic diversity could play a major role in ciliates. In vegetative cells, Atu1p and Btu1/2p, are incorporated into all types of microtubules (Thazhath et al., 2004 and our unpublished data). It is likely that the divergent tubulins (α-like, β-like and ι-) do not form separate microtubules, but instead co-assemble with conventional tubulin. Since the divergent tubulin isotypes may be specific to ciliates, these proteins could be involved in assembly of types of microtubules that are unique to ciliates. Indeed, in *Tetrahymena*, an epitope tagged Blt1p, is not targeted to axonemes (K. Clark, M. Gorovsky, personal communication), organelles that contain mostly if not entirely conventional tubulin dimers (Gaertig et al., 1995). The divergent α- and β-tubulins could co-assemble with conventional tubulin in microtubules with unusual properties such as the cortical bundles, microtubules that form during amitosis of the macronucleus (Fujiu and Numata, 2000) or those forming the contractile vacuole pore (CVP). CVP contains a small ring of microtubules twisted into a right-handed helix (Elliott and Bak, 1964; McKanna, 1973). This is a rare example of microtubules with an extreme angle of curvature. The CVP microtubules may require an unusual degree of flexibility, which could involve unusual bonds between tubulin subunits. In the future, it will be of interest to determine whether divergent tubulins of ciliates have amino acid substitutions at domains that are known to form inter- and intradimer surfaces and thus affect polymer flexibility (Nogales et al., 1999; Nogales et al., 1998).

Tubulin isoforms in *Tetrahymena*

Tubulin of most eukaryotes undergoes a number of conserved post-translational modifications (PTMs). *Tetrahymena* tubulin is highly heterogeneous and extensively modified post-translationally (Gaertig et al., 1995; Redeker et al., 2005; Suprenant et al., 1985). The most conserved and well studied tubulin PTMs that are also present in ciliates (Fig. 1) include: acetylation of α-tubulin (at K40) (Greer et al., 1985), proteolytic removal of the terminal Y residue from α-tubulin (detyrosination) (Argarana et al., 1978), and two types of so-called polymodifications, glutamylation (Eddé et al., 1990) and glycylation (Redeker et al., 1994) (on α- and β-tubulin). With exception of acetylation at K40 of α-tubulin that occurs inside the microtubule lumen (Nogales et al., 1999), the remaining PTMs occur on the outside surface of microtubules. More precisely, detyrosination, glutamylation and glycylation occur on the C-terminal tails (CTTs) of tubulin, flexible domains (Luchko et al., 2008) that interact with motor proteins and other types of microtubule-associated proteins (MAPs) (Roll-Mecak and Vale, 2008; Skiniotis et al., 2004; Wang and Sheetz, 2000). *Tetrahymena* studies showed that the CTT on both α- and β-tubulin is essential (Duan and Gorovsky, 2002).

Here we will focus on PTMs that are based on ligations of amino acids to tubulin, and in particu-

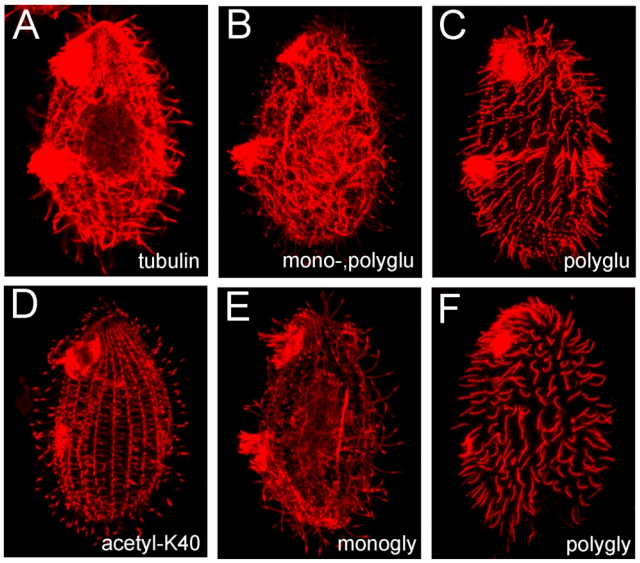

Fig. 1. Immunofluorescence images of a dividing *Tetrahymena* labeled with antibodies that recognize either the tubulin primary sequence (A) or various post-translational form of tubulin. The following antibodies were used: (B) GT335, a monoclonal antibody (mAb) that recognizes mono- and polyglutamyl side chain (Wolff et al., 1992), (C) ID5, a mAb that in *Tetrahymena* is specific to polyglutamylation (Rüdiger et al., 1999; Wloga et al., 2008), (D) 6-11 B-1, a mAb against acetyl-K40 on α-tubulin (Piperno and Fuller, 1985), (E), TAP952, a mAb against monoglycylated tubulin (Bré et al., 1998) and (F), AXO49, a mAb that recognizes polyglycylated tubulin (Bré et al., 1998).

lar on the mechanism and function of polymodifications (glycylation and glutamylation). Tubulin undergoes post-translational ligations of amino acids to the primary polypeptide that utilize protein translation-independent mechanisms. These reactions are mediated by ATPase enzymes that function as amino acid ligases (see below). Arce, Argarana and colleagues first reported that α-tubulin is modified by post-translational ligation of tyrosine to the C-terminal glutamic acid (Arce et al., 1975; Argarana et al., 1977). Tyrosination is a reverse reaction for a post-translational modification based on proteolytic removal of the genome-encoded C-terminal tyrosine by a carboxypeptidase (Argarana et al., 1978; Hallak et al., 1977). Eddé and colleagues discovered that murine brain tubulin undergoes post-translational addition of one or multiple glutamic acids (glutamylation) (Eddé et al., 1990). Redeker and colleagues showed that axonemal tubulin of *Paramecium* is extensively modified by ligation of glycines (glycylation) (Redeker et al., 1994). Unlike tubulin tyrosination that is based on a standard peptide bond, glycylation and glutamylation involve isopeptide bonds that utilize the γ-carboxyl group in the side chain of an internal glutamic acid of CTT. The first added glycine or glutamate can be extended into a side chain by standard isopeptide bonds (Redeker et al., 1991; Regnard et al., 1998; Rogowski et al., 2009; Wolff et al., 1994). To some extent, polymodifications resemble PTMs based on post-translational ligation of peptides, (e.g. ubiquitination or neddylation), except that the unit of polymodifications is a single amino acid. Due to the polymeric nature, glycylation and glutamylation generate a large number of tubulin isoforms as a consequence of utilization of multiple modification sites (glutamic acids within the CTTs), variable length of side

chains and the fact that both polymodifications often co-exist on the same tubulin subunits in various combinations (reviewed in (Gaertig and Wloga, 2008). It came as a surprise, that despite major differences between the biochemical mechanisms of tyrosination and polymodifications, all 3 PTMs are generated by structurally related enzymes (see below).

Tubulin glutamylation

Tubulin glutamylation is present in most eukaryotes with a possible exception of fungi. The phylogenic patterns suggest that this PTM co-evolves with cilia and centrioles/basal bodies (Janke et al., 2005). While, axonemes and centrioles/basal bodies are highly enriched in glutamylated tubulin, typically in the same cell, this PTM is also present on other types of microtubules. For example, mammalian fibroblasts have high levels of tubulin glutamylation in primary cilia and the centrosome, but the PTM is weakly detectable on cytoplasmic and spindle microtubules (Bobinnec et al., 1998; van Dijk et al., 2007). Bré and colleagues have documented the distribution of glutamylated tubulin in *Paramecium* and *Tetrahymena* by immunofluorescence with a glutamylation-specific antibody (Bré et al., 1994) and later confirmed by mass spectrometry of purified tubulin of *Tetrahymena* (Redeker et al., 2005). In both *Paramecium* and *Tetrahymena*, glutamylation is present in most if not all types of microtubules. However, the extent of glutamylation is spatially regulated. The formation of the glutamyl side chain consists of two distinct steps: 1) initiation based on a isopeptide peptide bond that involves the γ-carboxyl group of the glutamic acid in the primary sequence and 2) elongation based on a standard isopeptide bond (Redeker et al., 1991; Regnard et al., 1998; Wolff et al., 1994). A number of mono- and polyclonal antibodies have been developed that recognize glutamyl side chains made of a specific number of E residues. Perhaps the most complete picture of glutamyl side chain distribution emerged from our recent studies in *Tetrahymena* (Wloga et al., 2008). It appears that every type of a microtubule in *Tetrahymena* contains at least some subunits that have glutamyl side chains. However, the upper limit of the glutamyl side chain is microtubule type-specific. Intracytoplasmic, nuclear microtubules and a subset of cortical microtubules have side chains limited to monoglutamylation. Side chains with an upper limit of 2 Es (biglutamylation) are present in the postoral fiber and CVP. Basal bodies and cilia are the only locations containing side chains composed of 3 or more Es (Wloga et al., 2008). However, in locations that contain the longest side chains there is a whole range of tubulin subunits having from 0 to 20 Es per tubulin (Redeker et al., 2005). Moreover, the distribution of glutamylated subunits within the microtubule is far from random. Immunofluorescence studies in several ciliated models showed that in the axoneme, the density of glutamyl side chains changes, forming an increasing gradient from the tip of cilia toward the basal body (Fouquet et al., 1994; Huitorel et al., 2002; Kann et al., 1995; Lechtreck and Geimer, 2000). Strikingly, within the peripheral doublet microtubule, most of glutamylation is located in the B-tubule ((Lechtreck and Geimer, 2000; Multigner et al., 1996) and our unpublished results for *Tetrahymena*). Thus, enzymes that generate glutamylation (E-ligases, see below) may be selectively binding to specific microtubule surfaces.

One straightforward mechanism that could control the side chain density and length is the age of the microtubule polymer. There are major differences in the rate of subunit exchange among specific types of microtubules. In *Tetrahymena*, “pulse-chase” type experiments with an epitope-tagged tubulin showed that cytoplasmic, nuclear, and longitudinal cortical (LM) microtubules turnover rapidly, while basal body, transverse, post-ciliary and axonemal microtubules turn over very

slowly (Thazhath et al., 2004). The types of microtubules that have short glutamyl side chains are mostly the dynamic cell body microtubules that have a short life span. Thus, these microtubules may not exist for a sufficient time to acquire long glutamyl side chains. On the other side, most microtubules that exchange subunits very slowly, such as basal bodies and cilia, also have the longest glutamyl side chains. While the microtubule age could play some role, additional mechanisms are needed for generation the precise pattern of glutamylation, including restrictions to specific microtubule surfaces. Furthermore, in *Tetrahymena,* artificial stabilization of dynamic monoglutamylated microtubules by paclitaxel does not lead to accumulation of long glutamyl side chains (our unpublished data). This argues that the length of glutamyl side chains is primarily regulated at the level of activity of tubulin modifying or demodifying enzymes.

Tubulin glycylation

The first hint of the existence of tubulin glycylation came from studies that utilized antibodies generated against axonemal tubulin of *Paramecium tetraurelia*. These antibodies recognized ciliary tubulin in a variety of species but often failed to cross-react with non-ciliary cytoplasmic microtubules in the same cells (Adoutte et al., 1985). Mass spectrometry showed that these axoneme-tubulin specific antibodies recognize a polyglycine side chain attached to either α- or β-tubulin. While generally, tubulin glycylation is found only in cells with cilia (Levilliers et al., 1995), in ciliates, this PTM is also present on non-ciliary microtubules (Adoutte et al., 1991; Iftode et al., 2000; Thazhath et al., 2002). In *Tetrahymena* the spatial distribution of glycylation resembles that of glutamylation. Thus, the long side glycyl chains are present in cilia, basal bodies and cortical microtubules, while cytoplasmic and nuclear microtubules have side chains limited to monoglycylation (Thazhath et al., 2004; Thazhath et al., 2002; Xia et al., 2000). Mass spectrometry studies on ciliary tubulin detected from 0-42 G residues per tubulin (Redeker et al., 2005). However, as is the case of glutamylation, within the organelle, or possibly a single microtubule, glycylation is distributed non-uniformly. Within the axoneme of *Tetrahymena*, both mono- and polyglycylation could not be detected on the central pair microtubules, but were abundant on the peripheral doublets (Wloga et al., 2009). It is not known yet whether, like glutamylation, glycylation is also confined to the B-tubule of outer doublets in *Tetrahymena*, but this is the case of axonemes of sea urchin sperm (Multigner et al., 1996). Multiple adjacent glutamic acids in the tail domains of both α- and β-tubulin are used for glycylation, based on mass spectrometry studies in *Paramecium* and mutational studies that located homologous sites in *Tetrahymena* (Vinh et al., 1999; Xia et al., 2000).

Significance of sites of polymodifications on tubulin

Our laboratory has studied the significance of E residues on CTTs of α- and β-tubulin in *Tetrahymena* that either are homologous to residues known to serve as sites of polymodifications in other organisms or are adjacent to these sites. Charge-conserving amino acid substitutions of CTT (E to D) were used to assess how important the polymodification sites are *in vivo*. These studies have benefited greatly from the use of heterokaryon strains that have disruptions of *ATU1* or *BTU1* and *BTU2* genes in the micronucleus. When tubulin heterokaryons mate, their progeny dies but could be rescued by introduction of a corresponding tubulin gene (Hai et al., 1999; Xia et al., 2000). The rescue method was used to rapidly assess the significance of multiple sites of polymodifications. Sites of polymodifications on the Atu1p α-tubulin

are not essential (Wloga et al., 2008; Xia et al., 2000). In fact, all 5 glutamic acids of CTT on α-tubulin could be replaced by aspartates, leading to complete loss of polymodifications from the α-tubulin subunit. The resulting mutant cells (ATU1-5D) are viable but grow more slowly (Wloga et al., 2008). The tail domain of Btu1/2p β-tubulin contains five Es that are homologous to the glycylated residues in *Paramecium* (Vinh et al., 1999; Xia et al., 2000). *Tetrahymena* can tolerate substitutions of 1 or 2 specific polymodifiable Es; however, triple or quadruple mutations are lethal or deleterious (Xia et al., 2000). Mutants with a lethal triple site mutation develop a novel phenotype: undergo 3-4 cell cycles without completing cytokinesis and form "cell-chains". These compound cells can assemble only excessively short disorganized cilia that lack central microtubules and have defects in the peripheral doublet microtubules (Thazhath et al., 2004; Thazhath et al., 2002). The cytokinesis arrest in the lethal triple polymodification site mutant is associated with lack of severing of LM cortical bundles (Thazhath et al., 2004; Thazhath et al., 2002). It is likely that the a failure to sever LMs blocks the ingression of the cleavage furrow (Thazhath et al., 2004). These studies indicate that Es that serve as polymodification sites are essential. The limitation of these studies is that these sites could potentially be used by both glutamylation and glycylation and the sites used by glutamylation have not been mapped for *Tetrahymena*. Moreover, it is not clear to what extent the observed phenotypes of mutations on β-tubulin are loss or gain-of-function. For example, substitutions of polymodification sites on one of the two tubulin subunits, change the composition of polymodifications on the partner (non-mutated) subunit. In a viable β-tubulin mutant lacking 3 adjacent polymodification sites, there is an increase in the level of glycylation and decrease in the level of glutamylation on the non-mutated α-tubulin subunit (Redeker et al., 2005). It is possible that some modifying enzymes, in the absence of high affinity target sites on one tubulin subunit, modify similar sites on the partner subunit. In fact, this has been observed for some tubulin E-ligases (van Dijk et al., 2007). It is not known whether excessive levels of polymodifications on the partner subunit in the tubulin polymodifications site mutants are compensating or contribute to the mutant phenotype. To determine the consequences of loss-of-function of polymodifications sites, future studies are needed to mutate the glutamic acids on both α- and β-tubulin in a single strain of *Tetrahymena*.

Importantly, in *Tetrahymena*, the phenotype of a lethal triple mutation in the polymodification domain on β-tubulin is almost precisely phenocopied by knockouts of genes that encode subunits of katanin, a microtubule-severing protein (Sharma et al., 2007). These observations open a possibility that katanin and polymodifications act in the same pathway. One possibility is that katanin selectively recognizes tubulin subunits that are polymodified. Katanin and related spastin bind to the tubulin CTT (McNally and Vale, 1993; Roll-Mecak and Vale, 2008; White et al., 2007). An antibody that binds to a terminal glutamic acid (to either a detyrosinated end of α-tubulin or glutamyl side chain), blocks spastin-mediated microtubule severing activity *in vitro* (Roll-Mecak and Vale, 2008). A deficiency in katanin activity could lead to lack of severing of LM microtubules during cytokinesis and this could explain the cytokinesis arrest in the triple polymodification site β-tubulin mutants of *Tetrahymena*. Indeed a GFP-katanin fusion protein localizes to LMs in *Tetrahymena* (Sharma et al., 2007). It is more difficult to explain how katanin contributes to assembly of cilia. It appears to katanin acts inside cilia, possibly on the outer doublet microtubules, that are also heavily polymodified (Dymek et al., 2004; Sharma et al., 2007). It remains to be determined whether in the context of ciliary assembly, katanin acts as a microtubule severing factor, or mediates another related activity on the surface of microtubules.

The enzymes that generate tubulin polymodifications (E- and G-ligases)

Regnard and colleagues developed an *in vitro* assay for tubulin glutamylation and partially purified a tubulin E-ligase activity from murine brains (Regnard et al., 1998; Regnard et al., 1999). Strikingly, one of the proteins in the complex was found to be TTLL1, a protein with a conserved domain related to a tubulin enzyme previously purified by Weber laboratory, tubulin tyrosine ligase (Janke et al., 2005). TTL is responsible for ligation of tyrosine to the terminal glutamic acid that is exposed by detyrosination (Schroder et al., 1985). While TTL (Y-ligase) is a reverse PTM enzyme and E-ligase is a forward PTM enzyme, there are similarities between the reactions catalyzed by these two enzymes. Namely, both enzymes utilize a glutamic acid as a modification site, and in both cases the target residue is present within the CTT of tubulin. Subsequent studies showed that both E-ligase and G-ligase indeed are members of the TTLL superfamily. TTLL1, and several other members of the TTLL superfamily including TTLL4, TTLL6 and TTLL9 are catalytic subunits with tubulin E-ligase activity (Ikegami et al., 2006; Janke et al., 2005; van Dijk et al., 2007; Wloga et al., 2008). Gene knockouts or knockdowns of mRNA encoding specific TTLL E-ligases or associated proteins greatly decrease the levels of tubulin glutamylation on subsets of microtubules in diverse models: mouse, zebrafish and *Tetrahymena* (Ikegami et al., 2007; Janke et al., 2005; Pathak et al., 2007; Wloga et al., 2008). Furthermore, in one case, of the murine TTLL7 enzyme (that belongs to the TTLL6 clade) it was possible to reconstitute the *in vitro* tubulin glutamylation reaction using a recombinant E-ligase (Ikegami et al., 2006; Mukai et al., 2009). (Ikegami et al., 2006; Janke et al., 2005; Pathak et al., 2007. The clades of E-ligases are fairly well conserved across eukaryotes, indicating that subclasses of E-ligases exist that differ in some conserved enzymatic properties. It appears that the major differences among the E-ligases of distinct TTLL classes are based on 1) the preference for α- or β-tubulin and 2) ability to either initiate or elongate the side chains. As an example, TTLL1 proteins (based on studies in *Tetrahymena* and mouse studies) have an E-ligase activity that prefers α-tubulin and in *Tetrahymena* this enzyme has a strong side chain initiating activity (Janke, 2005; van Dijk et al., 2007; Wloga et al., 2008). Deletions of a TTLL1 type enzyme (Ttll1p) and a closely related TTLL9 type enzyme (Ttll9p) in *Tetrahymena* led to a major loss of glutamylation in the basal bodies (Wloga et al., 2008). Double knockout cells grow more slowly and have fewer ciliary rows. Basal bodies with an apparently mature morphology that failed to dock at the plasma membrane were observed in the cell body of *TTLL1* and *TTLL9* double knockout strains. A GFP fusion of Ttll1p localized to basal bodies while Ttll9p was found in both basal bodies and cilia. Both of these enzymes primarily modify α-tubulin (Wloga et al., 2008). Thus, glutamylation on α-tubulin, while clearly not essential, could be important in regulation of the fidelity of basal body maturation. A mutation in PGs1, a protein associated with TTLL1 E-ligase in the mouse causes severe defects in assembly of sperm axonemes (Campbell et al., 2002). In *Tetrahymena*, knockouts of genes encoding of multiple paralogs of TTLL6 type enzyme led to shortening of cilia and loss of ciliary motility (S. Suryavanshi and JG, unpublished data). Among these enzymes, Ttlll6Ap has been well characterized biochemically and found to have an exclusive side chain elongase activity that is largely restricted to β-tubulin (Janke et al., 2005; van Dijk et al., 2007). GFP-Ttll6Ap localizes mainly to cilia (Janke et al., 2005). Thus, selective localization of side chain elongases could be a major mechanism that is responsible for differential side chain length distribution among distinct types of microtubules. A morpholino knockdown of TTLL6 homolog in

zebrafish leads to shortening of olfactory cilia (Pathak et al., 2007). Thus, tubulin glutamylation has emerged as a major factor that contributes to the assembly and function of cilia and basal bodies.

Recently, enzymes that catalyze glycylation, G-ligases, were identified among the previously unstudied members of the TTLL protein superfamily. Rogowski and colleagues showed that murine TTLL3 and TTLL10 act as G-ligases on tubulin. In cultured mammalian cells, the murine TTLL3 initiates glycine side chains that are subsequently elongated by TTLL10 (Rogowski et al., 2009). Our laboratory has identified a TTLL3 type protein of *Tetrahymena*, Ttll3Ap, as a tubulin G-ligase with strong initiating activity (Wloga et al., 2009). The fruit fly, *Drosophila melanogaster* is an interesting case, because this species lacks an obvious TTLL10 elongase homolog and TTLL3 type proteins of *Drosophila* have both chain initiation and elongation G-ligase activity on tubulin (Rogowski et al., 2009). In *Tetrahymena* and *Drosophila*, TTLL3 enzymes are required for tubulin glycylation *in vivo* (Rogowski et al., 2009; Wloga et al., 2009).

Tetrahymena has 6 genes encoding TTLL3 type enzymes. When mildly overexpressed as GFP fusions, some TTLL3 paralogs colocalize with cilia, some with basal bodies or remain in the cell body (Wloga et al., 2009). Surprisingly, deletion of all six TTLL3 paralogs resulted in viable cells that are motile and have normal morphology. However cilia in cells lacking TTLL3 activity are on average 15% shorter as compared to wildtype cells. The TTLL3 *Tetrahymena* knockout cells have residual tubulin glycylation estimated to be between 1-8% of wildtype (Wloga et al., 2009). Since all TTLL3 G-ligases were eliminated in these strains, another tubulin G-ligase with an initiating activity must exist. This enzyme could belong to less conserved classes of TTLLs whose members are present in *Tetrahymena* (TTLL14, TTLL15 and TTLL16) or TTLL10 enzyme that acts as an elongase for tubulin, could also have some initiation activity. In addition to a mild reduction in the length of cilia, TTLL3 *Tetrahymena* knockout cells elevated levels of acetyl-K40 on α-tubulin of axonemes (Wloga et al., 2009). It thus appears that TTLL3-mediated tubulin glycylation makes axonemal microtubules more dynamic, which in turn could be a requirement for elongation of axonemes to a proper length. This result fits well with our observation that katanin, a factor that promotes microtubule turnover, is required for ciliogenesis (Sharma et al., 2007). While the impact of TTLL3 on the axoneme length was mild in *Tetrahymena*, this enzyme appears to be important for axoneme assembly in multicellular organisms. In zebrafish, a morpholino knockdown of TTLL3 led to dramatic shortening of multiple classes of cilia (Wloga et al., 2009) and RNAi-mediated knockdown of TTLL3 in *Drosophila* inhibited assembly of sperm axonemes (Rogowski et al., 2009).

Another clue to the function of tubulin glycylation is that Tetrahymena strains lacking TTLL3 genes have greatly increased levels of tubulin glutamylation on axonemal and cortical microtubules (Wloga et al., 2009). Additional experiments have confirmed that the two polymodifications negatively regulate each other. Thus, overexpression of E-ligases increased the levels of tubulin glutamylation (as expected) but decreased the levels of tubulin glycylation on the same microtubules (Wloga et al., 2009). In a reverse experiment, overexpressing a TTLL3 G-ligase increased the levels of tubulin glycylation and decreased the levels of tubulin glutamylation (Wloga et al., 2009). It thus appears that the two modifications compete with each other *in vivo*. The competition could be a result of utilization of the same Es in the CTT domain, or steric inhibition of adjacent polymodification sites. While tubulin glutamylation is highly conserved and present in all eukaryotes that also have cilia and centrioles, a few lineages (e.g. nematodes and trypanosomes) lack tubulin glycylation. It is possi-

ble therefore that the main role of tubulin glycylation is to negatively regulate tubulin glutamylation and that organisms that lack tubulin glycylation could have evolved additional mechanisms for negative regulation of tubulin glutamylation.

SUMMARY

Recent studies brought major advances in elucidation of the mechanisms that diversify microtubules at the subcellular level. Tubulin polymodifications have emerged as a major determinant of assembly and functions of specific microtubules, and in particular, those forming cilia and basal bodies. Studies in ciliates have contributed to identification of enzymes that polymodify tubulin and uncovered complex regulatory interactions among diverse tubulin modifications.

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