

Posttranslational Modifications of Tubulin and Cilia

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Tubulin undergoes several highly conserved posttranslational modifications (PTMs) including acetylation, deetyrosination, glutamylation, and glycylation. These PTMs accumulate on a subset of microtubules that are long-lived, including those in the basal bodies and axonemes. Tubulin PTMs are distributed nonuniformly. In the outer doublet microtubules of the axoneme, the B-tubules are highly enriched in the deetyrosinated, polyglutamylated, and polyglycylation tubulin, whereas the A-tubules contain mostly unmodified tubulin. The non-uniform patterns of tubulin PTMs may functionalize microtubules in a position-dependent manner. Recent studies indicate that tubulin PTMs contribute to the assembly, disassembly, maintenance, and motility of cilia. In particular, tubulin glutamylation has emerged as a key PTM that affects ciliary motility through regulation of axonemal dynein arms and controls the stability and length of the axoneme.

TYPES OF CONSERVED TUBULIN PTMs

Tubulin undergoes several conserved posttranslational modifications (PTMs) (Janke 2014; Song and Brady 2015; Yu et al. 2015). The most studied tubulin PTMs and their responsible enzymes are summarized in Figure 1. These PTMs are enriched on the long-lived microtubules, including those of cilia and centrioles. Some tubulin PTMs appear to have co-evolved with cilia (Janke et al. 2005; Shida et al. 2010). Although antibodies that recognize PTMs have been widely used to detect cilia (Magiera and Janke 2013), the functions of tubulin PTMs have started to emerge only recently. Within the cilium, there are striking differences in the levels of tubulin PTM among the specific microtu-

bules and along the length or even around the circumference of the same microtubule (Fig. 2). According to the “tubulin code” model (Verhey and Gaertig 2007), tubulin PTMs form patterns of marks on the microtubules that locally influence various activities, such as the motility of motor proteins or severing factors. Although we focus on cilia, we will discuss key findings on the nonciliary microtubules as well, as it is likely that tubulin PTMs provide related if not identical functions inside and outside of cilia.

ACETYLATION OF K40 OF α -TUBULIN

This highly conserved PTM was discovered in cilia of *Chlamydomonas reinhardtii* (L'Hernault

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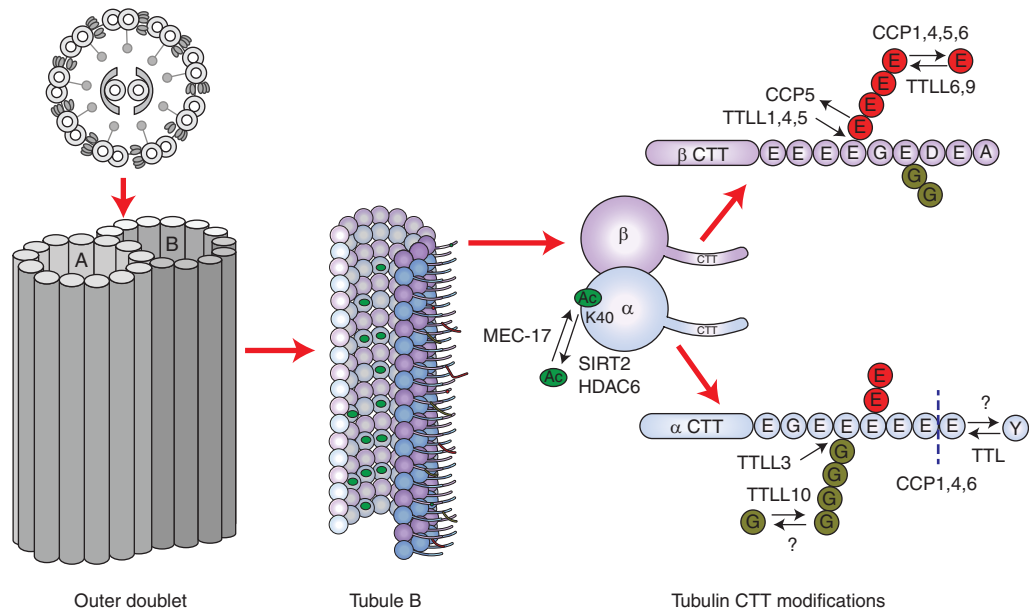


Figure 1. Conserved and widely studied types of tubulin posttranslational modifications (PTMs) and the responsible enzymes that act in cilia. CTT, Carboxy-terminal tail; TTL(L), tubulin tyrosine ligase(-like); CCP, cytosolic carboxypeptidase.

and Rosenbaum 1983; LeDizet and Piperno 1987). Acetyl-K40 is the only known PTM that is located inside the microtubule lumen (Nogales et al. 1999; Soppina et al. 2012). Although mass spectrometry detected additional acetyl-Ks on tubulin (Choudhary et al. 2009; Chu et al. 2011; Liu et al. 2015), acetyl-K40 is the most abundant, if not exclusive in cilia (Akella et al. 2010). Acetyl-K40 marks long-lived microtubules, including those in the axonemes and basal bodies (Piperno and Fuller 1985). The enzyme that generates acetyl-K40, MEC-17/ATAT1 (Akella et al. 2010; Shida et al. 2010), has a catalytic domain homologous to that of the histone acetyltransferases (Friedmann et al. 2012; Kormendi et al. 2012; Li et al. 2012; Taschner et al. 2012; Davenport et al. 2014; Yuzawa et al. 2015), but its enzymatic rate is relatively slow because of “suboptimal” positions of the catalytic residues. Consequently, only long-lived microtubules carry acetyl-K40 (Szyk et al. 2014). Most of the axonemal α -tubulin has acetyl-K40 (Gaertig et al. 1995), and the PTM is present on both the central and outer microtubules (Piperno and Fuller 1985; Satish Tammanna et al. 2013).

Tetrahymena mutants lacking MEC-17/ATAT1 or expressing a K40R nonacetylatable α -tubulin have a normal gross phenotype but display altered sensitivities to tubulin-binding compounds (Gaertig et al. 1995; Akella et al. 2010). In *Chlamydomonas*, expression of a K40R α -tubulin did not detectably affect the gross phenotype (Kozminski et al. 1993). Surprisingly, in the apicomplexan *Toxoplasma gondii*, K40R α -tubulin-expressing parasites are viable only if a second mutation that confers oryzalin resistance (and likely changes the dynamics of microtubules) is cointroduced into α -tubulin. Furthermore, a depletion of MEC-17/ATAT1 in *Toxoplasma* inhibits mitosis and changes cell shape, indicating that the lack of acetylation affects both the nuclear and subpellicular microtubules (Varberg et al. 2016). In *Caenorhabditis elegans*, the acetyl-K40 α -tubulin is expressed exclusively in the mechanosensory (ciliated and nonciliated) neurons (Fukushige et al. 1999; Shida et al. 2010). Without MEC-17/ATAT1, the animals become touch-insensitive (Chalfie and Au 1989; Zhang et al. 2002; Akella et al. 2010; Shida et al. 2010), and

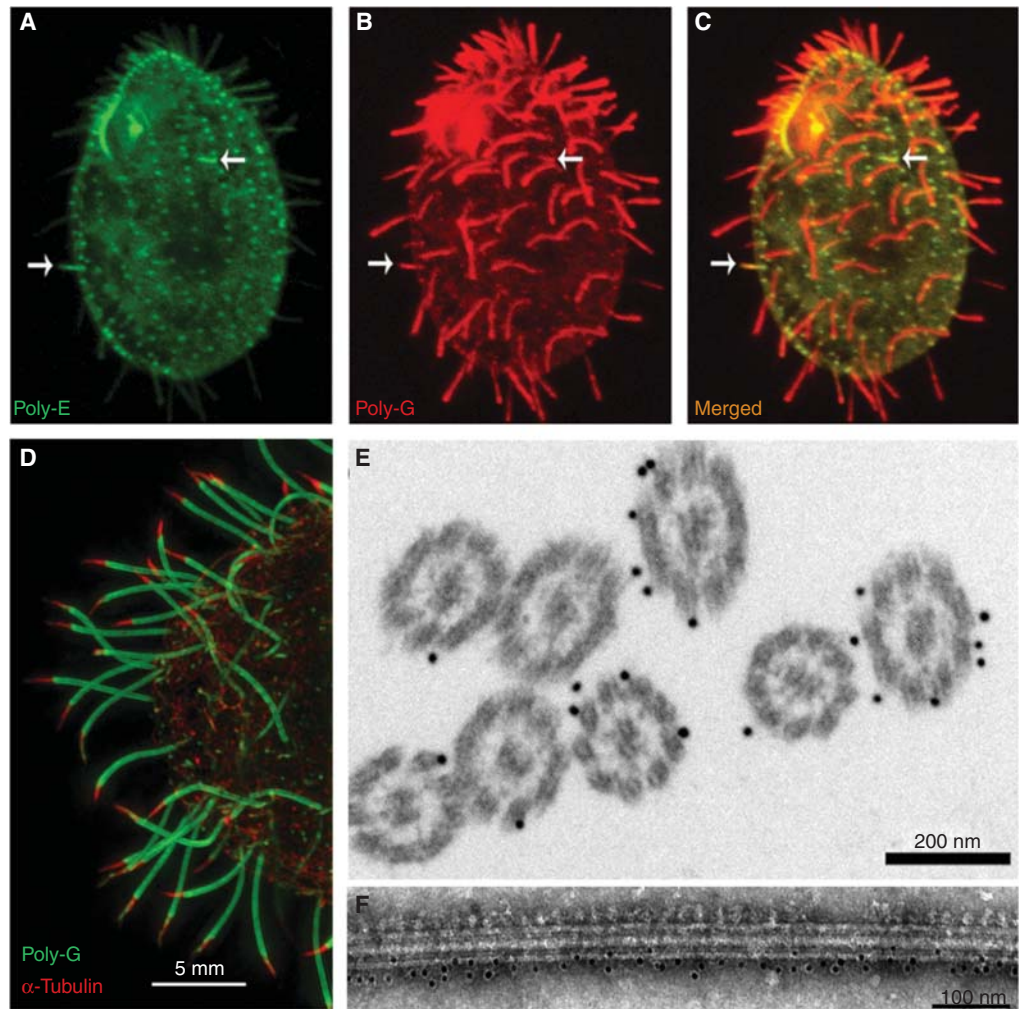


Figure 2. The nonuniform patterns of tubulin PTMs in cilia. (A-C) A *Tetrahymena* cell labeled with antibodies that recognize either polyglutamate (poly-E, green) (Shang et al. 2002) or polyglycine side chains (AXO49) (Bré et al. 1998). Note that the shorter assembling cilia (arrows) have a higher signal of polyglutamylation and a lower signal of polyglycylation as compared to the longer mature cilia on the same cell. These data suggest that during their assembly, cilia undergo remodeling of tubulin PTMs. (Figure adapted from Sharma et al. 2007; originally published in *Journal of Cell Biology*.) (D) A superresolution structured illumination microscopy (SR-SIM) image of the posterior end of a *Tetrahymena* cell, with cilia labeled with poly-G (green) (Duan and Gorovsky 2002) and anti- α -tubulin primary sequence antibodies 12G10 (red) (Jerka-Dziadosz et al. 1995). Note an absence of a signal of poly-G near the tips of cilia (distal segment) where the B-tubules are absent. (E) A postembedding immunogold image of cross sections of *Tetrahymena* cilia labeled with antipolyglycine AXO49 antibodies. Note the absence of a signal near the central microtubules. (From Wloga et al. 2009; adapted, with permission.) (F) An isolated doublet microtubule of *Chlamydomonas* labeled by poly-E antibodies with 10 nm colloidal gold and negatively stained with uranyl acetate. The A-tubule side can be identified by the presence of dynein arms (*top*), whereas the B-tubule has a smoother surface (*bottom*). Note that the gold particles are enriched along the B-tubule. (From Kubo et al. 2010; reprinted, with permission, from Elsevier © 2010.)

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the neurites of the nonciliated touch receptor neurons contain fewer microtubules that have reduced diameters and lattice defects and are prone to buckling as the animal body bends (Cueva et al. 2012; Topalidou et al. 2012). A molecular dynamics simulation suggests that acetylation of K40 promotes the cohesion between protofilaments of microtubules (Cueva et al. 2012). Thus, it appears that acetyl-K40 has a microtubule lattice-stabilizing activity. Importantly, MEC-17/ATAT1 has additional functions beyond its acetyltransferase activity. The loss of touch sensation in the *C. elegans* mec-17 mutant animals (but not the defects in microtubule organization) can be rescued by expression of a catalytically inactive MEC17/ATAT1 (Topalidou et al. 2012). Also, the mammalian MEC-17/ATAT1 has a microtubule-destabilizing activity that is independent of its acetyltransferase activity (Kalebic et al. 2013a,b). Although siRNA depletion of MEC-17/ATAT1 delays the assembly of primary cilia (Shida et al. 2010), the mice lacking MEC-17/ATAT1 have not been reported to have cilia that are structurally defective or of improper length (Kalebic et al. 2013b; Kim et al. 2013).

ROLE OF ACETYL-K40 AND ASSOCIATED ENZYMES IN THE DISASSEMBLY OF CILIA

HDAC6 (Hubbert et al. 2003; Zhang et al. 2003) and SIRT2 (North et al. 2003) are two acetyl-K40 deacetylases that promote disassembly of primary cilia in mammalian cells. Knockdowns of either HDAC6 or SIRT2 increase the frequency of primary cilia or make them longer, whereas overexpressions lead to fewer or shorter cilia (Pugacheva et al. 2007; Yang et al. 2014; Zhou et al. 2014; Bangs et al. 2015; Ran et al. 2015). The cilia-shortening effect of overexpressed HDAC6 is counteracted by overexpression of a K40Q (acetyl-K mimic) α -tubulin indicating that HDAC6 acts through deacetylation of acetyl-K40. Thus, by deacetylating acetyl-K40, HDAC6 and SIRT2 could have a microtubule lattice-destabilizing effect (Cueva et al. 2012). Alternatively, deacetylation could enable another PTM on K40. In *Chlamydomonas*, α -tubulin (but not β -tubulin) undergoes lysine poly-

ubiquitination when the cilium disassembles (Huang et al. 2009). However, the effects of HDAC6 and SIRT2 are unlikely to be mediated solely by K40 ubiquitination, because polyubiquitinated α -tubulin has acetyl-K40 (Huang et al. 2009). HDAC6 also deacetylates cortactin, and this activity also shortens the cilium, by promoting actin polymerization around the base of the primary cilium (Ran et al. 2015); it is well documented that actin dynamics affects the length of cilia (discussed in Liang et al. 2016). The HDAC6 knockout mouse lacks ciliary phenotypes (Zhang et al. 2008), but the loss of HDAC6 could be covered by SIRT2.

ROLE OF ACETYL-K40 IN MOTOR-DRIVEN MOTILITY

Acetyl-K40 may influence motor proteins, also in cilia. Mice lacking MEC-17/ATAT1 are healthy, but the males have defective sperm cells whose flagella beat with a reduced bend amplitude (Kalebic et al. 2013b; Kim et al. 2013). Enzymatic acetylation of K40 increases, whereas deacetylation by SIRT2 decreases the rate of motility of axonemal outer arm dynein on microtubules in vitro, respectively (Alper et al. 2014). The effect of acetyl-K40 on the motility of axonemal dynein is surprising because K40 is located inside the microtubule lumen (Nogales et al. 1999; Soppina et al. 2012). However, changes in the microtubule lumen can affect the functionality of the external surface (Raff et al. 2008). Cryoelectron tomography failed to detect a difference between the tubulin dimers inside microtubules that are assembled of either acetylated or nonacetylated tubulin, but perhaps the resolution achieved (8 Å) was insufficient to detect a change that affects dynein (Howes et al. 2014).

In vitro, kinesin-1 binds more weakly and moves more slowly on mutant axonemes containing K40R α -tubulin, as compared to wild-type axonemes carrying acetyl-K40 (Reed et al. 2006). However, more recent studies that used enzymatically in vitro acetylated or deacetylated microtubules failed to detect an effect of acetyl-K40 on kinesin-1 motility in vitro (Walter et al. 2012; Kaul et al. 2014). In the study that reported a positive result for kinesin-1 (Reed et al.



2006), the nonacetylated microtubules were produced genetically and used in the form of isolated axonemes. The K40R mutation could have altered the composition of other axonemal proteins or the levels of other tubulin PTMs. One potential “reader” of the acetyl-K40 mark is a protein with a bromodomain, found in the flagellum of *Trypanosoma* (Alonso et al. 2014). Also, acetyl-K40 could affect the components of the lumen of outer doublets, microtubule inner proteins (MIPs) (Nicastro et al. 2011; Yanagisawa et al. 2014). Although the effects of acetyl-K40 on other tubulin PTMs have not been studied, a reverse relationship exists: The levels of acetyl-K40 increase in the axonemes of *Tetrahymena* mutants that are deficient in tubulin glycylation (Wloga et al. 2009). Such a PTM cross talk could compensate for an absence of acetyl-K40 and could explain the mild consequences of the loss of this PTM in some models.

DETYROSINATION OF α -TUBULIN

The carboxy-terminal Y on α -tubulin is removed by an unidentified carboxypeptidase resulting in detyrosination that occurs preferentially on microtubules (Kumar and Flavin 1981; Arce and Barra 1983). The tubulin tyrosine ligase (TTL) restores the carboxy-terminal Y on the unpolymerized tubulin (Schroder et al. 1985; Ersfeld et al. 1993; Szyk et al. 2011; Prota et al. 2013). Mice lacking TTL die shortly after birth, with defects in the organization of neurons (Erck et al. 2005). In cilia, detyrosinated tubulin is enriched on the B-tubules of outer doublets (Johnson 1998). The anterograde intraflagellar transport (IFT) trains that are driven by kinesin-2 move on the B-tubule, whereas the retrograde trains driven by IFT dynein move on the A-tubule (Kozminski et al. 1995; Pigino et al. 2009; Stepanek and Pigino 2016). In vitro, detyrosination increases the velocity and processivity of kinesin-2, suggesting that this PTM could stimulate the anterograde IFT (Sirajuddin et al. 2014). Detyrosination increases the landing rate of kinesin-1 on microtubules (Kaul et al. 2014). A combination of effects on the landing rate and processivity could explain the bias of kinesin-2-driven anterograde IFT trains for the B-tubule,

which in turn could mediate the collision-free trafficking between the base and the tip of the cilium (Stepanek and Pigino 2016). However, the B-tubule is also modified by polymodifications (see below). How the retrograde IFT trains could be biased for the A-tubule is less clear. The A-tubule has low levels of PTMs and its α -tubulin is mostly tyrosinated (Johnson 1998). The yeast cytoplasmic dynein, a motor similar to IFT dynein, is not sensitive to the status of the carboxy-terminal Y (Sirajuddin et al. 2014).

GLUTAMYLATION

Glutamylation is an addition of a side-chain peptide composed of Es to the γ -carboxyl group of an E in the protein primary sequence (Eddé et al. 1990). Glutamylation occurs on multiple E sites within the carboxy-terminal tail domains (CTTs) of α - and β -tubulin (Redeker et al. 1991, 1992). The CTTs are flexible domains that are likely to form a negatively charged “polymer brush” on the microtubule (Roll-Mecak 2015). A related PTM, tubulin glycylation, occurs within the same cluster of Es (Redeker et al. 1994) and the two polymodifications are in a competition (Rogowski et al. 2009; Wloga et al. 2009). In *Tetrahymena*, a substitution of multiple Es (to charge-conserving but nonmodifiable Ds) on the CCT of β -tubulin, causes a failure in the axoneme assembly, including its shortness, loss of the central microtubules, and loss of the B-tubules (Xia et al. 2000; Thazhath et al. 2002). A substitution of all Es on the CTT of α -tubulin is well tolerated (Wloga et al. 2008). The exact placement of the modifiable Es on tubulin is not important; for example, the severe axoneme assembly defect caused by E to D substitutions on β -tubulin is rescued by replacement of the CTT of α -tubulin with the corresponding sequence of the β -tubulin CTT (Xia et al. 2000; Duan and Gorovsky 2002).

Glutamylation is generated by a subset of enzymes related to TTL, TTL-like proteins (or TTLLs) (Janke et al. 2005). The glutamylating (glutamylase) TTLLs use a region adjacent to the enzymatic core as a microtubule-binding domain and preferably modify microtubules over unpolymerized tubulin (Garnham et al.

2015). There are several glutamylase TTLL subtypes, which differ in the levels of chain-initiating and elongating activities, on either α - or β -tubulin, but the exact enzymatic profiles vary greatly among the TTLL orthologs or even paralogs (van Dijk et al. 2007; Wloga et al. 2008; Suryavanshi et al. 2010). The catalytic core of the mammalian TTLL7 is flexible enough to allow the microtubule-docked enzyme to perform both chain initiation and elongation reactions on β -tubulin (Mukai et al. 2009; Garnham et al. 2015), but other TTLLs are active mostly in either initiation or elongation (van Dijk et al. 2007; Wloga et al. 2008; Suryavanshi et al. 2010). Some TTLLs have an autonomous activity, whereas others need associated activators (Janke et al. 2005). TTLLs are enriched in cilia and basal bodies (van Dijk et al. 2007; Wloga et al. 2008; Suryavanshi et al. 2010). In mammalian and zebrafish cells, a centrosomal protein, CEP41, is required for targeting of TTLL6 to cilia (Lee et al. 2012). In *Chlamydomonas* cilia, TTLL9 is transported by IFT, in a complex with an adapter, FAP234 (Kubo et al. 2014).

Trimming of the polyglutamate side chains on tubulin (deglutamylation) is accomplished by the zinc carboxypeptidases: CCP1, -4, -5, and -6 (Kimura et al. 2010; Rogowski et al. 2010; Tort et al. 2014). CCPs also remove the terminal E from the end of deetyrosinated α -tubulin, creating the “ $\Delta 2$ α -tubulin” (Rogowski et al. 2010) also in cilia (Paturie-Lafanechère et al. 1994). Polyglutamylation also occurs on nontubulin proteins (van Dijk et al. 2008), and the same TTLL can glutamylate both a tubulin and a nontubulin substrate (van Dijk et al. 2007; Xia et al. 2016). CCPs also deglutamylate nontubulin proteins (Rogowski et al. 2010; Ye et al. 2014). Thus, caution is needed in the interpretation of the effects of manipulations of TTLLs or CCPs. In *Tetrahymena*, glutamylated tubulin is present on the outer doublets but absent from the central pair (Suryavanshi et al. 2010). However, the central microtubules are glutamylated in the sperm of *Drosophila melanogaster* and *Lytechinus pictus* (Hoyle et al. 2008). In motile cilia, within the outer doublet, glutamylation is enriched on the B-tubule (Fig. 2F) (Lechtreck and Geimer 2000; Kubo et al. 2010;

Suryavanshi et al. 2010). It is not clear to what extent this enrichment is a result of targeting of TTLLs or CCPs or a consequence of the saturation of most of the A-tubule surface with protein complexes such as dynein arms and radial spokes (Ishikawa 2015).

GLUTAMYLATION AND CILIARY MOTILITY

Loss of glutamylating TTLL subtypes (TTLL1, TTLL5, TTLL6, TTLL9) either disturbs the waveform or reduces the beat frequency or causes stalling of motile cilia (Ikegami et al. 2010; Kubo et al. 2010; Suryavanshi et al. 2010; Pathak et al. 2011; Bosch Grau et al. 2013; Konno et al. 2016); in most of these studies, the motility defects are not associated with gross structural defects in the axoneme (with some exceptions, see below). In the mouse, loss of function of TTLL1 phenocopies primary ciliary dyskinesia associated with reduced motility of respiratory cilia (Ikegami et al. 2010; Vogel et al. 2010). Among the axonemal dynein arms that drive ciliary motility, the inner dynein arms (IDAs) are mainly involved with the ciliary waveform, whereas the outer dynein arms (ODAs) control the beat frequency (Kamiya 2002). The reduction in the beat frequency in the absence of TTLL6, a β -tubulin elongase (Suryavanshi et al. 2010; Pathak et al. 2011; Bosch Grau et al. 2013), indicates that glutamylation regulates ODAs. Of key importance is that in *Chlamydomonas*, a mutant lacking ODAs has residual ciliary motility (supported by the IDAs alone), but a double mutant lacking TTLL9 glutamylase and ODAs is completely paralyzed. This indicates that tubulin glutamylation is essential for the waveform control by IDAs (Kubo et al. 2010). A depletion of CCP5 in zebrafish leads to hyperglutamylation of ciliary microtubules and a reduction in the beat amplitude of pronephric cilia (Pathak et al. 2014). Surprisingly, in vitro, in the axonemes lacking ODAs that were treated with ATP, the velocity of sliding of microtubules increases in the absence of either TTLL9 or TTLL6 (Kubo et al. 2010; Suryavanshi et al. 2010). This leads to a counterintuitive model that the IDA activity on microtubules is inhibited by tubulin glutamylation. There are several subtypes of IDAs,

each occupying a different position within the 96-nm repeat on the A-tubule (Bui et al. 2009; Barber et al. 2012). In *Chlamydomonas*, a loss of TTLL9 specifically affects the function of dynein ϵ , an IDA subspecies (Kubo et al. 2012). This effect could involve positive charge patches in the microtubule-binding domain of dynein ϵ that could interact with the negatively charged polyglutamate side chains (Kubo et al. 2012). Glutamylation of tubulin on the B-tubule could increase the time spent by dynein ϵ in a microtubule-bound state. This could increase the cohesion between the adjacent microtubule doublets and affect the engagement of other dyneins with the B-tubule (Kubo et al. 2012). Alternatively, the dynein ϵ /B-tubule links could increase the microtubule curvature by generating a drag force on the microtubules that are simultaneously pushed by other “faster” dyneins (Kotani et al. 2007; Suryavanshi et al. 2010). In addition to the effects on dynein ϵ , TTLL9 also affects the axoneme integrity by influencing the DRC-nexin/B-tubule links between the adjacent doublets (Kubo et al. 2012; Alford et al. 2016).

GLUTAMYLATION IN CILIARY ASSEMBLY AND MICROTUBULE STABILITY

Glutamylating TTLLs are important for the assembly or stability of ciliary microtubules, most notably in the mammalian sperm. The males of mice lacking TTLL1 or its partner subunit Pgs1 are infertile and produce sperm with truncated axonemes (Campbell et al. 2002; Regnard et al. 2003; Vogel et al. 2010). In the TTLL5-null mouse, the sperm axonemes frequently lack a single doublet, number 4 (Lee et al. 2013). Similarly, a deletion of TTLL9 leads to a loss of the distal portion of doublet 7 in the sperm axoneme (Konno et al. 2016).

The sperm axoneme defects could result from either a lack of proper assembly or instability. In the *Tetrahymena* cells overproducing TTLL6, the cytoplasmic microtubules undergo hyperglutamylation and excessive stabilization, associated with increased K40-acetylation and resistance to depolymerization (Wloga et al. 2010). Tubulin glutamylation could increase

the microtubule stability by promoting the binding of stabilizing factors. Centriole and spindle-associated protein (CSAP) colocalizes with the polyglutamylated microtubules, including those in cilia, centrioles, and the mitotic spindle (Backer et al. 2012). Depletion of CSAP destabilizes microtubules of the mitotic spindle (Ohta et al. 2015). In zebrafish, a depletion of CSAP causes defects in the left–right asymmetry in association with impaired beating of cilia inside Kupffer’s vesicle (Backer et al. 2012). Thus, CSAP could be a tubulin code reader that recognizes and stabilizes polyglutamylated microtubules, including those present in motile cilia.

The axoneme structure defects could result from a suboptimal IFT (Hou et al. 2007; Dave et al. 2009). The anterograde IFT trains driven by kinesin-2 move on the B-tubule of the outer doublets (Kozminski et al. 1995; Pigino et al. 2009; Stepanek and Pigino 2016), which is enriched in tubulin glutamylation (Lechtreck and Geimer 2000; Kubo et al. 2010; Suryavanshi et al. 2010). Polyglutamylation of β -tubulin increases the processivity and speed of kinesin-2 on microtubules in vitro (Sirajuddin et al. 2014). The velocity of Osm3 kinesin-2 increases on the hyperglutamylated axonemes in cilia of *C. elegans* lacking CCP1 (O’Hagan et al. 2011). In zebrafish, defects in ciliogenesis caused by depletions of either flier/DYF1/IFT70 or IFT88 IFT train subunits are partially rescued by a depletion of CCP5 (Pathak et al. 2014), arguing that increased tubulin glutamylation can improve a suboptimal IFT. However, the axoneme assembly defects caused by deficiencies in TTLLs could also be caused by a lack of glutamylation of non-tubulin proteins. In addition to the sperm defects (Lee et al. 2013), a loss of TTLL5 also causes retinal dystrophy (Sergouniotis et al. 2014), but this phenotype is mainly, if not entirely, caused by a lack of glutamylation of RPGR, a protein that functions in the connecting cilium of the photoreceptor (Sun et al. 2016).

ROLE OF GLUTAMYLATION IN THE DISASSEMBLY AND NEGATIVE LENGTH REGULATION OF CILIA

Excessive tubulin glutamylation may destabilize and shorten cilia. In *C. elegans*, despite a stim-

ulatory effect on the anterograde IFT velocity described above, a loss of CCP1 causes an age-dependent degeneration of cilia, including a loss of the B-tubules (O'Hagan et al. 2011). A loss of CCP1 in the (*pcd*) mouse causes degeneration of the Purkinje neurons, bulb mitral cells, and retina photoreceptors and male sterility (Greer and Shepherd 1982; Fernandez-Gonzalez et al. 2002; Rogowski et al. 2010). Because all of the cell types affected in the *pcd* mouse are ciliated, these phenotypes can at least in part be caused by a degeneration of cilia. Consistently, the mammalian CCP1 is a positive regulator of the length of the primary cilium (Kim et al. 2010). In *Tetrahymena*, overexpression of TTLL6 glutamylase causes rapid shortening and fragmentation of axonemes and a loss of B-tubules (Wloga et al. 2010), which phenocopies overexpression of katanin (Sharma et al. 2007). The depolymerizing effect of TTLL6 is axoneme-specific; in the same cells, nonciliary microtubules undergo stabilization (Wloga et al. 2010). This argues that hyperglutamylolation does not have a microtubule disassembly-promoting effect per se but rather that it activates axoneme-associated microtubule depolymerizers. Specifically, hyperglutamylolation could activate katanin to sever the axoneme (O'Hagan and Barr 2012). There is substantial evidence that tubulin glutamylolation stimulates katanin and the structurally similar spastin (Lu et al. 2004; Sharma et al. 2007; Roll-Mecak and Vale 2008; Lacroix et al. 2010; Valenstein and Roll-Mecak 2016). Katanin subunits are present inside cilia where they associate with the outer doublets (Dymek et al. 2004; Sharma et al. 2007).

Recent observations in *Chlamydomonas* provide further evidence that links tubulin glutamylolation to axoneme stability and depolymerization and ultimately to the length of the cilium. It has been puzzling that *Chlamydomonas* mutants that lack repeated components on the A-tubule (IDAs, ODAs, DRC-nexin) often have short cilia (Huang et al. 1979; LeDizet and Piperno 1995; Yamamoto et al. 2010). A loss of function of either TTLL9 or the associated FAP234 reduces tubulin glutamylolation in cilia and, surprisingly, partially rescues the length defect of the IDA, ODA, and DRC-nexin mu-

tants (Kubo et al. 2015). Thus, tubulin glutamylolation has a strong shortening effect on an axoneme that has incomplete A-tubule-associated protein complexes. Importantly, a loss of either FAP234 or TTLL9 alone mildly elongates cilia and decreases the rate of ciliary disassembly (Kubo et al. 2015). In the absence of TTLL9, other TTLLs could provide the IFT-promoting function of tubulin glutamylolation (discussed above). It therefore appears that the repeated protein complexes bound to the A-tubule stabilize the axoneme and are in competition with tubulin glutamylolation that destabilizes the axoneme. It is tempting to speculate that the shortening of the axoneme is mediated by tubulin glutamylolation that activates ciliary katanin.

Surprisingly, a loss of katanin causes shortening of cilia and a failure to assemble the central pair (Dymek et al. 2004; Sharma et al. 2007; Dymek and Smith 2012), a counterintuitive phenotype for a microtubule-severing factor. However, in vitro, the severing activity of another microtubule depolymerizer, spastin, is dependent on the number of glutamates per tubulin, with a small number stimulating spastin and a large number inhibiting spastin (Valenstein and Roll-Mecak 2016). The levels of tubulin polyglutamylolation detected by antibodies are higher in the assembling cilia than in the steady-state cilia, which also have a higher level of polyglycylation (Fig. 1A–C) (Adoutte et al. 1991; Sharma et al. 2007; Bosch Grau et al. 2013). It is possible that the polyglutamate side chains undergo remodeling as the cilium matures, including their shortening (by deglutamylases) and replacement by the polyglycine chains (see below). Such a PTM remodeling could modulate the possible multiple activities of katanin at different stages of ciliogenesis.

TUBULIN GLYCYLATION

Although all ciliated species have tubulin glutamylolation (Janke et al. 2005), most, but not all, have tubulin glycylation (Adoutte et al. 1985; Bré et al. 1996). Glycylation is generated by a subset of TTLLs: TTLL3 is an initiase (Rogowski et al. 2009; Wloga et al. 2009), whereas TTLL10 is an elongase (Ikegami et al. 2008; Ikegami and



Setou 2009; Rogowski et al. 2009). One exception is *D. melanogaster* in which polyglycylated tubulin is generated by TTLL3 alone (Rogowski et al. 2009). Curiously, in the ciliated cells of primates (including humans), the side chain is limited to a single G (monoglycylation), because of a mutation that inactivates the catalytic center of TTLL10 (Kann et al. 1998; Million et al. 1999; Rogowski et al. 2009). *Tetrahymena* cells with a knockout of all six paralogs of TTLL3 grow poorly and have slightly shorter, but ultrastructurally normal cilia that, unlike wild-type cilia, fail to elongate in the presence of paclitaxel, indicating a subtle defect in the tubulin turnover in the axoneme (Wloga et al. 2009). In zebrafish, a morpholino knockdown of TTLL3 glycyllase causes reversals in the direction of ciliary beating in the pronephron. A depletion of TTLL6 glutamylase reduces the beat frequency (Pathak et al. 2011). Surprisingly, a depletion of both TTLL3 and TTLL6 leads to high penetrance ciliary phenotypes, including the body axis curvature and pronephric cysts, associated with reduced ciliary beat amplitude and even paralysis of pronephric cilia (Pathak et al. 2011). The axonemes depleted in both TTLL3 and TTLL6 often lack the B-tubules and have misplaced doublets and ectopic central singlets (Pathak et al. 2011). The severe assembly defects in the zebrafish depleted in TTLL3 and TTLL6 are reminiscent of the profound failure in the assembly of the axoneme in *Tetrahymena* mutants with substitutions in the modifiable Es on the CTT of β -tubulin (Xia et al. 2000; Thazhath et al. 2002). Thus, tubulin glycylation and glutamylation could both compete and cooperate in providing essential functions for axoneme assembly and motility. Polyglycine side chains are predicted to have an extended conformation, whereas the polyglutamate side chains may form swollen coils because of increased charge repulsion (Roll-Mecak 2015), supporting a view that the two polymodifications have different biological effects. In the mouse and *Drosophila*, tubulin glycylation could be important on its own. In *Drosophila*, a depletion of TTLL3 with RNAi causes male sterility associated with a failure in the axoneme assembly (Rogowski et al. 2009). In the murine ependy-

mal cells, a depletion of TTLL3 destabilizes already assembled motile cilia (Bosch Grau et al. 2013) and reduces the frequency of primary cilia in the colon epithelium (Rocha et al. 2014). Because a loss of glycylation leads to an increase in glutamylation (Rogowski et al. 2009; Wloga et al. 2009), it will be of interest to address whether, in the mouse, the defects in cilia caused by depletions of TTLL3 are mediated by tubulin hyperglutamylation.

CONCLUSION

It is increasingly convincing that tubulin PTMs play multiple important roles in cilia. The tubulin code model, originally inspired by the studies on histone tail modifications, seems useful in exploring the functions of microtubule PTMs. Many microtubule interactors, such as the motors, +TIPs, and even the tubulin code generators themselves (MEC-17/ATAT1 [Szyk et al. 2014] and TTLL7 [Garnham et al. 2015]) either walk unidirectionally or undergo a one-dimensional diffusion along the microtubule surface or inside its lumen. Thus, microtubule interactors can sense and respond to the PTM marks in a processive manner and therefore can be seen as genuine readers of the tubulin code. Inside the axoneme, the nonuniform patterns of PTM marks (Fig. 2) may be critical for the confinement of the anterograde and retrograde IFT streams to separate tubules of the outer doublets, and for the generation of the bend curvature in motile cilia. Although the evidence behind the importance of the tubulin code is growing steadily, a major challenge is in linking the phenotypes produced by manipulations of tubulin PTM enzymes to tubulin modifications. This task is difficult because of the complexity of the PTM pathways, which include nontubulin modification targets and nonenzymatic activities of the tubulin PTM enzymes. Despite these challenges, exploring the cilium has proved productive in generating ideas about the mechanisms and the biological effects of the tubulin code. The same studies have greatly added to the knowledge about how cilia assemble, maintain their length, and move.

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