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REVIEW

The emerging role of tubulin posttranslational modifications in cilia and ciliopathies

Kai He[3], Kun Ling[3], Jinghua Hu[1,2,3]✉

[1] Division of Nephrology and Hypertension, Mayo Clinic, Rochester, MN, USA
[2] Mayo Translational Polycystic Kidney Diseases Center, Mayo Clinic, Rochester, MN, USA
[3] Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN, USA

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Abstract Tubulin posttranslational modifications (PTMs) add "tubulin code" to generate functional diversities of microtubules. Several types of tubulin PTMs accumulate on axonemes and basal bodies of cilia, including acetylation, glutamylation, glycylation and detyrosination. Among them, glutamylation, glycylation and detyrosination are mostly enriched in the B-tubules, whereas acetylation occurs on both A- and B-tubule of the microtubule doublets in a similar level. Recent studies indicate that tubulin PTMs are critical for the fine tuning of assembly/disassembly, maintenance, motility, and signaling of cilia. Dysregulated tubulin PTMs are strongly implicated in human disorders including ciliopathies and neuron degeneration. Here, we review the current understanding how tubulin PTMs regulate cilia formation and function, and their relevance to human health.

Keywords Tubulin posttranslational modifications (PTMs), Cilia, Ciliopathies, Acetylation, Glutamylation, Glycylation

INTRODUCTION

Microtubules, assembled from highly conserved α/β-tubulin heterodimers, are the key cytoskeletal elements for constructing various subcellular organelles. Despite their uniformed structure, microtubules can adapt to a large diversity of functions through spatial–temporal generation of specialized identities. The 'tubulin code', which is generated by the expression of different tubulin isotypes and posttranslational modifications (PTMs), confers the dynamic, functional diversity on microtubules (Gadadhar *et al.* 2017a; Janke 2014; Magiera *et al.* 2018a, b; Song and Brady 2015). The PTMs not only accumulate on a subset of long-lived microtubules, including those found in the centrosomes, cilia, and axons of neurons, but also on the highly dynamic ones found in mitotic spindle and marginal bands of blood platelets (Gadadhar *et al.* 2017a; Magiera *et al.* 2018a, b). In living cells, microtubules can interact with a variety set of microtubule-associated proteins (MAPs), such as microtubule motor proteins, microtubule plus end tracking proteins (+TIPs) and severing enzymes. Mechanistically, PTMs control microtubule functions either by direct alteration of their mechanical properties or by modulating their interactions with other proteins. Many types of PTMs have been discovered on tubulin. Some of them, including acetylation, phosphorylation, and methylation, also occur on non-tubulin substrates. While the others, such as (poly) glutamylation, (poly) glycylation and tyrosination/detyrosination, are mostly abundant in tubulin, thus allowing the generation of locally restricted and specialized functions on microtubules.

The cilium is a hair-like protrusion on cell surface of most eukaryotic cells, which is mainly compose of microtubule-cored axoneme anchored by the basal body transformed from the mother centriole. There are two main types of cilia: motile cilia and the primary cilia. In

✉ Correspondence: hu.jinghua@mayo.edu (J. Hu)

general, motile cilia are characterized by the 9 + 2 arrangement of microtubule doublets: 9 pairs of outer microtubule doublets and a central pair. The dynein arms and radial spokes connect with the outer doublets to generate force for cilia beating. With this kinetic capability, motile cilia tightly control cell locomotion, sexual reproduction and fluid flow generation. The primary cilia lack of central pair and motility and present a 9 + 0 arrangement. All cilia are built and maintained by a microtubule-based intraflagellar transport (IFT) (Rosenbaum and Witman 2002). The IFT particle is composed of two multimeric subcomplexes (IFT-A and IFT-B). In a simple model, anterograde transport is regulated by kinesin-2, whereas dynein regulates retrograde transport. IFT machinery mediates the bidirectional movement of IFT cargos that are required for the biogenesis, maintenance, and signaling of all cilia (Berbari *et al.* 2009; Pazour and Rosenbaum 2002; Pedersen and Rosenbaum 2008; Rosenbaum and Witman 2002; Scholey 2008; Scholey and Anderson 2006).

Sensory transduction capabilities of cilia are highly conserved across species. Polarized cells utilize primary cilia to receive environmental stimuli that are converted into physiological responses (Nauli *et al.* 2003; Praetorius and Spring 2001, 2003a, b). Once overlooked as an evolutionary vestige, it have recently been identified as the ubiquitous sensory antenna of many pivotal signaling pathways, such as Hedgehog, Polycystin, GPCR, platelet-derived growth factor receptor (PDGFR), planar cell polarity (PCP) and TGF-β signaling (Goetz and Anderson 2010; Nishimura *et al.* 2019). With rapid advancements in the positional cloning of human disease genes, 35 disorders (∼187 causal loci), such as polycystic kidney disease (ADPKD and ARPKD), Bardet-Biedl syndrome (BBS), Joubert syndrome (JBTS), nephronophthisis (NPHP), and Meckel–Gruber syndrome (MKS), have been characterized molecularly as cilia-related diseases, or ciliopathies (Adams *et al.* 2008; Badano *et al.* 2006). Consistent with the presence of cilia on most cell surfaces in human body, most ciliopathies occur as syndromic disorders that affect many organs during development, including the kidneys, limbs, central nervous system (CNS), liver, eyes, and fat storage tissue. Despite the physiological and clinical relevance of cilia, the molecular mechanisms that regulate cilia formation and function and the connections between disease gene functions and pathology remain largely elusive.

The microtubule doublets of the axoneme consist of A-tubule and B-tubule that attached to the A-tubule. A-tubule forms a complete microtubule with 13 protofilaments, whereas B-tubule forms an incomplete microtubule structure with 10 protofilaments (Ichikawa *et al.* 2017; Ma *et al.* 2019). Unlike the highly dynamic non-axonemal MTs, the axonemal MTs are long-lived and endow cilia with stability, long-range transport, and structural basis for sensory function (Orbach and Howard 2019). Tubulin PTMs, including acetylation, (poly) glutamylation, (poly) glycylation and tyrosination/detyrosination, occur predominantly along the axoneme. Dysregulated tubulin PTMs are closely linked to a variety of human diseases. Here, we review current understanding of tubulin PTMs in cilia and related human disorders (Fig. 1, Table 1).

ACETYLATION

Acetylation at residue lysine 40 (K40) of α-tubulin, the predominant form of tubulin acetylation and the only tubulin PTM that occurs inside the microtubule lumen, is associated with long-lived subsets of microtubule structures including cilia axoneme (Gadadhar *et al.* 2017a; Janke and Bulinski 2011; LeDizet and Piperno 1987; Lhernault and Rosenbaum 1985; Soppina *et al.* 2012). The level of αK40 acetylation in A-tubule and B-tubule is similar (Orbach and Howard 2019). Of note, recent studies also revealed the existence of a novel acetylation modification of lysine 252 (K252) of β-tubulin (Choudhary *et al.* 2009; Chu *et al.* 2011; Liu *et al.* 2015), but with its distribution and physiological importance awaiting further characterization.

αK40 acetylation is majorly catalyzed by the highly conserved tubulin acetyl transferase αTAT1 and removed by the deacetylases histone deacetylase 6 (HDAC6) and sirtuin 2 (SIRT2) (Akella *et al.* 2010; Hubbert *et al.* 2002; Kalebic *et al.* 2013b; North *et al.* 2003). HDAC6 and SIRT2 are both enriched in cilia proper and at cilia base (de Diego *et al.* 2014; Pugacheva *et al.* 2007; Zhou *et al.* 2014). Strong biochemical evidences suggest that acetyl-K40 only takes place on microtubule lattice but not the cytosolic tubulin heterodimers (Bulinski *et al.* 1988; Lhernault and Rosenbaum 1983; Maruta *et al.* 1986; Piperno *et al.* 1987). Accordingly, αTAT1 preferentially modifies polymeric tubulin *in vitro* (Kalebic *et al.* 2013a). Therefore, αTAT1 must access to the narrow lumen of microtubules to acetylate αK40. Two proposed models attempt to elucidate the entry mechanism of αTAT1 (Coombes *et al.* 2016). The first model is based on the observation that lattice defects and protofilament switches have been observed *in vitro* (Chretien *et al.* 1992; Schaedel *et al.* 2015). Although it has not been formally visualized in microtubule lumen, αTAT1 may locally and transiently enters into the microtubule lumen through these cracks. Accordingly, αTAT1 preferentially acetylates the highly

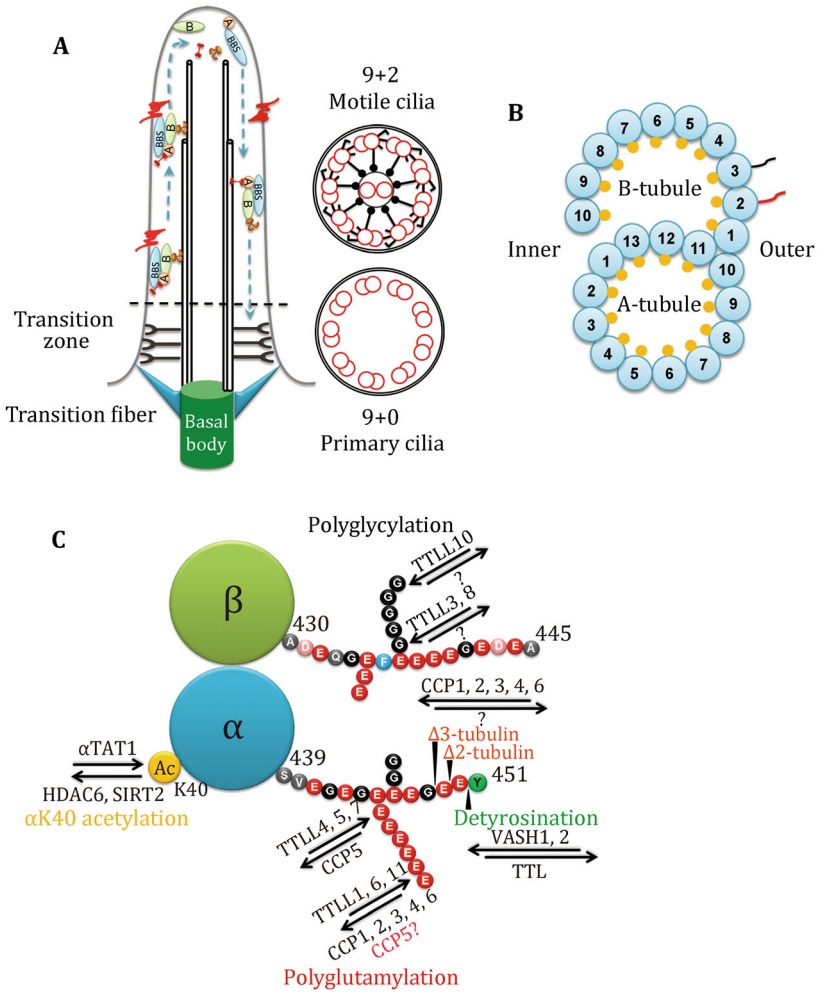

Fig. 1 Tubulin posttranscriptional modifications on cilia axoneme. **A** Basic structure and microtubule doublets arrangement patterns of cilia. Cilium is a membrane-bound structure and composed of microtubule-cored axoneme running from the basal body, diffusion barrier and so on. The ciliary membrane is continuous with the cell membrane, but specialized by distinct lipid compositions and membrane proteins. The motile cilia have 9 + 2 axoneme compositions. The primary cilia have 9 + 0 axoneme compositions and lack of inner and outer dynein arms, radial spokes, and central sheath that compose of motile cilia. Driven by microtubule motors dynein and kinesin, the intraflagellar transport (IFT) machineries bidirectionally transport along the axoneme that is essential for the formation, maintenance of cilia and the proper ciliary translocation of signaling proteins. **B** Cartoon depicting the cross section of microtubule doublet with PTMs (αK40 acetylation: *yellow*; polyglycylation chain: *black*; polyglutamylation chain: *red*). **C** Schematic representation of the distribution of PTMs on α/β tubulin heterodimer in cilia and their corresponding involved enzymes

curved areas of microtubules, the same regions that accumulate lattice openings or cracks, suggesting that αTAT1 can locally modify the mechanical properties of the microtubule to protect it against mechanical stresses. However, in view of the low frequency of microtubule defects, this model may not explain how the acetylation spread over the entire microtubule. αTAT1 has shown a higher affinity for microtubule ends. Alternatively, the high density of exposed luminal sites at the tapered extremities could be captured by αTAT1. This proposed mechanism is supported by studies showing the preferential entry of αTAT1 at open microtubule ends (Coombes *et al.* 2016; Ly *et al.* 2016; Szyk *et al.* 2014). It is conceivable that the entry mechanism of αTAT1 may depend on the way by which microtubules were assembled and the mechanical stresses they were experiencing. In contrast to αTAT1, how deacetylase HDAC6 and SIRT2 act *in vivo* is less defined. HDAC6 can act on both free tubulin dimers and polymerized microtubules *in vitro* (Hubbert *et al.* 2002; Matsuyama *et al.* 2002; Miyake *et al.* 2016; Zhao *et al.* 2010). It also can interact with the microtubule plus end protein EB1, suggesting the end of the microtubules might be the important entry site for HDAC6 (Zilberman *et al.* 2009).

Table 1 Tubulin PTMs in cilia and related diseases and disorders

Modification	Tubulin	Enzyme	Ciliary functions	Related diseases and disorders
Acetylation Deacetylation	α	αTAT1 HDAC6 SIRT2	Regulate cilia assembly/disassembly (Pugacheva *et al.* 2007, Ran *et al.* 2015) Render microtubules resistant to mechanical stress (Portran *et al.* 2017, Xu *et al.* 2017)	Spermatozoa abnormalities in $\alpha TAT1^{-/-}$ mice (Kalebic *et al.* 2013b) Decreased axonemal acetylation in Joubert syndrome patients with *ARMC9* or *TOGARAM1* mutation (Latour *et al.* 2019) Bardet-Biedl syndrome protein BBIP10 promotes microtubule acetylation (Loktev *et al.* 2008) Loss of ADPKD gene *PKD1* leads to decreased level of tubulin acetylation (Zhou *et al.* 2014) Tubulin hyperacetylation is evident in ARPKD patients (Berbari *et al.* 2013) Abnormal maturation of megakaryocyte and platelets spreading (Iancu-Rubin *et al.* 2012, Sadoul *et al.* 2012) Neurodegenerative disorders (Dompierre *et al.* 2007, Benoy *et al.* 2017, d'Ydewalle *et al.* 2011, Lazo-Gomez *et al.* 2013, Godena *et al.* 2014)
Glutamylation Polyglutamylation Deglutamylation of branch point Glu Deglutamylation of shorten polyGlu chain	α/β	TTLL4, 5, 7 TTLL1, 6, 11, 13 CCP5 CCP1, 2, 3, 4, 6, CCP5?	Biphasically regulate microtubule severing (Valenstein and Roll-Mecak 2016) Stabilize microtubules by interacting with CASP (Backer *et al.* 2012, Ohta *et al.* 2015) Differentially regulate cilia motility in different organisms (Bosch-Grau *et al.* 2012, Ikegami *et al.* 2010, Kubo *et al.* 2010, Suryavanshi *et al.* 2010, Janke *et al.* 2005, Pathak *et al.* 2014) Modulate the length of primary cilium (He *et al.* 2018) Control ciliary targeting of signaling molecules (He *et al.* 2018, Hong *et al.* 2018)	Neurodegeneration in Purkinje cell degeneration (*CCP1* mutant) mice (Rogowski *et al.* 2010, Shashi *et al.* 2018) Male infertility in glutamylation/deglutamylation enzymes mutated mice (Campbell *et al.* 2002, Giordano *et al.* 2019, Konno *et al.* 2016, Mullen *et al.* 1976, Vogel *et al.* 2010, Wu *et al.* 2017) Respiratory disorders in *TTLL1* mutated mice (Ikegami *et al.* 2010) Retinal degenerative diseases in mice and human with abnormal glutamylation (Grau *et al.* 2017, Marchena *et al.* 2011) Decreased axonemal polyglutamylation in Joubert syndrome patients with certain gene mutation (He *et al.* 2018, Latour *et al.* 2019, Lee *et al.* 2012)
Glycylation Polyglycylation Deglycylation	α/β	TTLL3, 8 TTLL10 Unknown	Regulate the stability and maintenance of motile cilia Bosch (Grau *et al.* 2013, Pathak *et al.* 2011, Rogowski *et al.* 2009, Wloga *et al.* 2009) Modulate the length of primary cilium (Gadadhar *et al.* 2017b) Reciprocal or competition of glutamylation (Gadadhar *et al.* 2017b, Rogowski *et al.* 2009, Wloga *et al.* 2009)	Retinal degeneration in $TTLL3^{-/-}$ mice (Grau *et al.* 2017) Suppression of TTLL3 in colon carcinogenesis (Rocha *et al.* 2014)
Detyrosination Δ2/Δ3-tubulin Tyrosination	α	VASH1, 2 CCP1, 2, 3, 4, 6 TTL	Unclear	Brain development defects in $TTL^{-/-}$ mice (Erck *et al.* 2005) Muscle and cardiac dysfunctions (Kerr *et al.* 2015, Robison *et al.* 2016) Development of several types of cancer (Kato *et al.* 2004, Mialhe *et al.* 2001, Du *et al.* 2017)

Although the αK40 acetylation of tubulin has been discovered over 30 years, its biological importance in cilia only emerges recently. Cells lacking of αTAT1 lost acetylation but preserve unaffected cilia morphology (Kalebic *et al.* 2013b; Shida *et al.* 2010). Deletion of HDAC6 results in tubulin hyperacetylation but shows no impact on cilia biogenesis and morphology, too (Pugacheva *et al.* 2007; Ran *et al.* 2015; Zhang *et al.* 2008). Loss of αTAT1 appears to delay the assembly rate of primary cilia (Ran *et al.* 2015) while pharmacological and genetic suppression of HDAC6 protects primary cilia from disassembly (Pugacheva *et al.* 2007; Ran *et al.* 2015). Intriguingly, inhibition of SIRT2 not only blocks cilia disassembly but also increase ciliation ratio and cilia length (Zhou *et al.* 2014). Overexpression of HDAC6 or SIRT2 decreases cilia number and length (Ran *et al.* 2015; Zhou *et al.* 2014). The discrepancies of the effects of αTAT1, HDAC6 and SIRT2 on ciliogenesis and cilia length may be attributable to non-tubulin acetylation activities of those enzymes (Drazic *et al.* 2016; Narita *et al.* 2019).

There are evidences suggesting that K40 acetylation may be implicated in regulation of motility of molecular motors. αK40 acetylation has been shown to increase axonemal dynein motility (Alper *et al.* 2014). However, there is controversial views on whether kinesins could be affected acetylation or not, with an *in vitro* study showing that kinesin-1 shows less binding and motility along the axoneme with α-tubulin K40R mutant (Reed *et al.* 2006), while the others reported acetylation state of tubulin alone did not affect the motility of kinesin-1 on the microtubule track by using enzymatically generated acetylated or deacetylated microtubules (Kaul *et al.* 2014; Walter *et al.* 2012). Given that tubulin acetylation is located in the inaccessible lumen of microtubules, the finding that this modification can control motility of molecular motors indeed came as a surprise. It remains as an open question that acetylation modification might influence other characteristics of the microtubules to indirectly regulate motor motility. For example, tubulin detyrosination is significantly decreased in αTAT1-deleted cells (Xu *et al.* 2017) and tubulin glycylation deficiency is associated increased levels of acetyl-K40 in *Tetrahymena* mutants (Wloga *et al.* 2009). Despite its elusive role in regulating motor motility, acetyl-K40 could be the key protecting mechanism for cilia to cope with mechanical stresses. Cilia are frequently exposed to mechanical forces that can cause microtubules breakage on axoneme, such as the fluid flow in kidney. αK40 acetylation has been shown to soften the microtubules by weakening inter-protofilament interactions, thus enhances its flexibility and confers resilience against mechanical stresses to ensure the persistence of long-lived microtubules (Portran *et al.* 2017; Xu *et al.* 2017).

In neurons, tubulin acetylation contributes to axon branching (Dan *et al.* 2018), cortical neurons migration and morphological development (Li *et al.* 2012). Links between decreased tubulin acetylation and axonal transport defects have been found in a range of neurodegenerative disorders, such as Huntington's disease (Dompierre *et al.* 2007), Charcot-Marie-Tooth disease (Benoy *et al.* 2017; d'Ydewalle *et al.* 2011), amyotrophic lateral sclerosis (ALS) (Lazo-Gomez *et al.* 2013), and Parkinson's disease (Godena *et al.* 2014). However, the contribution and mechanism of abnormal tubulin acetylation in neurodegenerative disorders remains to be elucidated, as the mice lacking αTAT1 merely showed defects in touch sensation but did not develop any of the expected degenerative phenotypes (Morley *et al.* 2016).

Tubulin acetylation also regulates blood clotting. The discoidal shape of blood platelets in the fast bloodstream is maintained by a closed ring of microtubules, called the marginal band (MB) (White and Rao 1998). Platelets are activated after vessel injury and undergo a major shape change known as disc to sphere transition that result from the contraction of MB, which leads to blood clotting (Johnson *et al.* 2007). MB microtubules in resting platelets are heavily acetylated (Diagouraga *et al.* 2014; Patel-Hett *et al.* 2008), and defects in tubulin acetylation of microtubules in marginal band affect the maturation of the precursors of platelets and the following platelet formation (Iancu-Rubin *et al.* 2012). Tight regulation of acetylation/deacetylation process is critical during platelet spreading after activation (Sadoul *et al.* 2012). Abnormal tubulin acetylation of sperm flagella has been directly linked to male subfertility, as the mice lacking of αTAT1 show abnormal sperm morphology and motility (Kalebic *et al.* 2013b). Recently, a study found that mutations in Joubert syndrome genes *ARMC9* or *TOGARAM1* results in short cilia with decreased axonemal acetylation (Latour *et al.* 2019).

Interestingly, in the context of ciliopathies, dysfunction of several ciliopathy proteins is correlated with globally aberrant tubulin acetylation, in either the axoneme or cytoplasmic microtubules. Bardet-Biedl syndrome protein BBIP10 is required for cytoplasmic microtubule polymerization and acetylation, which is likely through inhibiting HADC6 (Loktev *et al.* 2008). Deletion of Joubert syndrome gene *KIF7* results in decreased level of acetylated microtubule in the cytoplasm (Dafinger *et al.* 2011). In addition, deletion of the ADPKD gene *Pkd1* increases SIRT2 protein levels and decreases total tubulin acetylation levels, which lead to abnormal centrosome amplification and polyploidy

(Zhou *et al.* 2014). It was shown that cilia ablation by depleting structural gene *Ift88* and *Kif3a* leads to increased αTAT1 activities and hyperacetylation of cytosolic microtubules, with similar impact evident in the kidneys of ARPKD patients (Berbari *et al.* 2013). These observations raise an interesting perspective that cilia may orchestrate activities of tubulin acetylases/deacetylases to impact global microtubule acetylation.

GLUTAMYLATION

The γ-carboxyl groups of glutamate residues in α/β-tubulin and non-tubulin proteins can be added with single (monoglutamylation) or multiple glutamates (polyglutamylation) (Alexander *et al.* 1991; Edde *et al.* 1990; Regnard *et al.* 2000; Rüdiger *et al.* 1992; van Dijk *et al.* 2008). These modifications occur most abundantly on stable microtubule structures such as the ones found in neurons, centrosomes or basal bodies and cilia, whereas it also enriched in the highly dynamic mitotic spindle during mitosis (Audebert *et al.* 1994; Bobinnec *et al.* 1998; Fouquet *et al.* 1994; Mary *et al.* 1996; Regnard *et al.* 1999). Specific antibodies are used for detecting glutamylation: GT335 is specific to the branching point of the glutamate side chain and thus recognizes mono- and all forms of polyglutamylated proteins (Wolff *et al.* 1992); B3 and PolyE antibody recognizes polyglutamylated side chains with a minimum size of two and three glutamate residues, respectively (Gagnon *et al.* 1996; Kann *et al.* 2003; van Dijk *et al.* 2007). The polyglutamylation reaction is initiated by the formation of an isopeptide bond with the γ-carboxyl group of the glutamate acceptor site, and followed by side chain elongation consists of the formation of regular peptide bonds (Janke *et al.* 2008). In cilia, glutamylation is abundant on the B-tubules of the outer-axoneme doublets (Kubo *et al.* 2010; Lechtreck and Geimer 2000; Orbach and Howard 2019; Suryavanshi *et al.* 2010; Wloga *et al.* 2017). Like other protein modifications, polyglutamylation could generate massive microtubule heterogeneity by varying the density of the modification, choice of the tubulin subunit or isotype, choice of specific glutamate acceptor sites within the tubulin tail, and the length of the added side chain, which licensing the elaborate orchestration of the microtubule-associated physiology.

Microtubule polyglutamylation is a reversible process coordinated by tubulin glutamylases and tubulin deglutamylases in a cooperative manner. Enzymes catalyzing glutamylation in mammals belong to the tubulin tyrosine ligase-like (TTLL) protein family, which is characterized by the conserved core TTL domain with ATPase activity. Each of glutamylases shows intrinsic reaction and substrate specificity (Ikegami *et al.* 2006; Janke *et al.* 2005; Regnard *et al.* 1998; van Dijk *et al.* 2007): TTLL4, 5 and 7 preferentially catalyze the initiating step, whereas TTLL1, 6, 11 and 13 show more reaction specificity on the elongation step. As to the substrate specificity, TTLL1, 5, 6, 11, and 13 modify mostly on α-tubulin, while TTLL4 and 7 show a preference toward β-tubulin. Structural studies of glutamylases suggest that their catalytic specificities are determined by binding of the enzymes to the entire microtubule lattice (Garnham *et al.* 2015; Natarajan *et al.* 2017). However, the tubulin subunit preference of glutamylases could be overwritten by saturation of particular enzymes under certain physiological conditions (van Dijk *et al.* 2007). Of note, TTLL1 is active only when complexed with other proteins, whereas the others act in an autonomous manner (Janke *et al.* 2005; van Dijk *et al.* 2007). The enzymes that catalyze deglutamylation (deglutamylases) belong to the cytosolic carboxypeptidase (CCP) family (Kimura *et al.* 2010; Rogowski *et al.* 2010). CCP1, 4 and 6 act as the long-chain deglutamylases which catalyze shortening of glutamate side chains. So far, CCP5 is the only deglutamylase and has been identified that it specifically removes the branching point glutamate. However, CCP5 can also hydrolyze C-terminal glutamate residues from linear peptide chains similar to other members of the CCP family (Berezniuk *et al.* 2013). Both TTLL glutamylases and CCP deglutamylases have been reported to catalyze non-tubulin proteins such as myosin light chain kinase and telokin (Rogowski *et al.* 2010).

TTLLs have been reported to localize to the basal body or the axoneme (He *et al.* 2018; Lee *et al.* 2012; Suryavanshi *et al.* 2010; van Dijk *et al.* 2007; Wloga *et al.* 2008). In mammalian cells, TTLL4, 5, 6 and 7 are detected in both basal bodies and cilia, whereas TTLL1, 9 and 11 specifically label basal bodies. Intriguingly, overexpressed TTLL5 or TTLL6 shows three distinct ciliary localization patterns: densely punctate labeling surrounding the ciliary base, or exclusive cilia localization, or both, suggesting the ciliary import of TTLL5/6 is a transient and dynamic process (He *et al.* 2018). Although considered as cytoplasmic glutamylases, evidences hint that TTLL5/6 are transported to the ciliary base via association with specific group of vesicles, which is regulated by the ARL13B-RAB11-FIP5-mediated trafficking pathway (He *et al.* 2018). Binding of polyglutamylases complex to MTs in cilia, centrioles and neurons require the adaptor PGs1, whose deficiency in the *ROSA22* mouse significantly reduces microtubule polyglutamylation (Campbell *et al.* 2002; Ikegami *et al.* 2007; Janke *et al.* 2005; Regnard *et al.* 2003). In

agreement with the gradient pattern of glutamylation modification in cilia, the ciliary level of TTLL5/6 gradually decreases form the proximal to distal end (He *et al.* 2018; Lee *et al.* 2012; van Dijk *et al.* 2007). For deglutamylases, *ccp2*, *ccp5*, and *ccp6* are expressed in ciliated cells in zebrafish, whereas *ccp1* expression is restricted to the nervous system (Pathak *et al.* 2014). Only *ccp5* knockdown increases cilia tubulin glutamylation, suggesting that ccp5 is the principal tubulin deglutamylase that maintains functional levels of tubulin glutamylation in cilia (Pathak *et al.* 2014). In mammalian cells, CCP5 constitutively and evenly distributes along the whole axoneme (He *et al.* 2018). In consideration of that the careful balance of TTLLs and CCPs is critical for proper level of microtubule polyglutamylation, the difference in ciliary distribution of CCPs and TTLLs generates the gradually tilted deglutamylation/glutamylation balance toward cilia tip. Surprisingly, in hypoglutamylated axoneme that induced by deletion of TTLL5/6 or overexpression of CCP5, residual axonemal glutamylation is always well preserved at the proximal end of primary cilia corresponding the transition zone (He *et al.* 2018; Hong *et al.* 2018), suggesting either unidentified glutamylase/deglutamylases are responsible for TZ polyglutamylation, or this specific region is protected from deglutamylation via a distinct mechanism.

It was proposed that negative charged interfaces generated by polyglutamylation make the microtubule "sticky", and therefore regulates the interaction of other proteins with microtubules (Mitchell 2010). Polyglutamylation regulates activity of microtubule-severing enzymes spastin and katanin (Dymek *et al.* 2004; Lacroix *et al.* 2010; Lu *et al.* 2004; Sharma *et al.* 2007; Shin *et al.* 2019; Valenstein and Roll-Mecak 2016). Interestingly, polyglutamylation biphasically regulates spastin mediated microtubule severing: the spastin microtubule-severing activity increases as the number of glutamates per tubulin rises from one to eight, but decreases beyond this glutamylation threshold (Valenstein and Roll-Mecak 2016). To this end, polyglutamylation may stabilize/assemble or destabilize/disassemble microtubules in different context. The polyglutamylated tubulin in centrioles, spindle, and cilia could be targeted and stabilized by the microtubule stabilizing factor centriole and spindle-associated protein (CASP), which is required for normal brain development and proper left–right asymmetry (Backer *et al.* 2012; Ohta *et al.* 2015). Reduced tubulin polyglutamylation suppresses flagellar shortness in *Chlamydomonas* (Kubo *et al.* 2015). In *Tetrahymena,* hyperglutamylation of tubulin can either stabilize or destabilize microtubules in the same cell (Wloga *et al.* 2010). Although it remains debating that whether CCPP-1 (CCP1 homolog) is a real deglutamylase in *C. elegans,* the cilia of *ccpp-1* worms display a progressive degeneration (Kimura *et al.* 2010; O'Hagan *et al.* 2011). In drastic contrast, disruption of axonemal polyglutamylation in mammalian cilia does not affect cilia biogenesis, but only promotes disassembly induced by deciliation signals (He *et al.* 2018; Hong *et al.* 2018).

Tubulin polyglutamylation also regulates the activity of inner-arm dynein to control the beating behavior of motile cilia (Kubo *et al.* 2010; Suryavanshi *et al.* 2010). Hypoglutamylation induced by mutations in specific TTLLs has been shown to compromise cilia motility in different ciliated organisms (Grau *et al.* 2012, 2013; Ikegami *et al.* 2010; Kubo *et al.* 2010; Pathak *et al.* 2011; Suryavanshi *et al.* 2010). However, hyperglutamylation induced by knockdown of *ccp5* in zebrafish or overexpressing Ttll6Ap in *Tetrahymena* also disrupts cilia motility (Janke *et al.* 2005; Pathak *et al.* 2014). Whether this discrepancy is caused by non-tubulin glutamylation needs to be further examined. In *C. elegans* cilia, *Chlamydomonas* flagella, and mammalian primary cilia, the velocity and processivity of kinesin motors of IFT particles can be promoted by tubulin polyglutamylation (Hong *et al.* 2018; Ikegami *et al.* 2007; Kimura *et al.* 2018; O'Hagan *et al.* 2011; Sirajuddin *et al.* 2014). Emerging evidences also highlight the essential role of axoneme polyglutamylation in controlling ciliary localization of signaling molecules (He *et al.* 2018; Hong *et al.* 2018). Polycystin 1 (PC1) and polycystin 2 (PC2), two major proteins mutated ADPKD, colocalize to the primary cilium and may form a receptor/channel complex to sense environmental cues (Lee and Somlo 2014; Patel and Honore 2010; Nauli *et al.* 2003; Torres and Harris 2006). Depletion of CCPP-1 causes excess PKD-2 accumulation both inside cilia and below ciliary base in *C. elegans* (O'Hagan *et al.* 2011). Consistently, in mammalian primary cilia, axoneme hypoglutamylation compromises the ciliary localization of PC2, which can be restored by concomitant depletion of CCP5 (He *et al.* 2018). This suggests axoneme polyglutamylation likely anchors polycystins on the ciliary surface. Since short chain polyglutamylation alone is sufficient for anchoring ciliary polycystin (He *et al.* 2018), it is very likely that unknown adaptors tether cytoplasmic tails of polycystins with glutamylated tubulins. In addition, transduction of hedgehog (Hh) signaling depends on proper ciliary targeting of signaling molecules such as GLI3 and SMO (Bangs and Anderson 2017). Axonemal hypoglutamylation impairs SAG-induced cilia tip translocation of GLI3 and represses downstream Hh signaling (He *et al.* 2018; Hong *et al.* 2018).

Remarkably, defective polyglutamylation is correlated with a variety of typical ciliopathy phenotypes, such as

male infertility in mice (Campbell *et al.* 2002; Giordano *et al.* 2019; Konno *et al.* 2016; Mullen *et al.* 1976; Vogel *et al.* 2010; Wu *et al.* 2017), respiratory disorders in mice (Ikegami *et al.* 2010), dysfunctional ependymal cilia in the brain ventricles in mice (Grau *et al.* 2013), and axis curvature, pronephric cysts, and abnormal otolith number in zebrafish (Pathak *et al.* 2011). Interestingly, mutations in the several human Joubert syndrome genes, including *CEP41*, *ARL13B*, *ARMC9* and *TOGARAM1*, cause dramatic reduction in axonemal polyglutamylation (He *et al.* 2018; Latour *et al.* 2019; Lee *et al.* 2012), suggesting axoneme hypoglutamylation could be one of the key pathogenic mechanisms for Joubert syndrome.

Polyglutamylation is enriched during neuronal differentiation and is therefore considered a potential key regulator of neuronal microtubules (Audebert *et al.* 1994; Kapitein and Hoogenraad 2015). The extensive studies in Purkinje cell degeneration (*pcd*) mice that carrying *Ccp1* inactivating mutation have directly linked tubulin hyperglutamylation to neurodegeneration (Fernandez-Gonzalez *et al.* 2002; Greer and Shepherd 1982; Mullen *et al.* 1976; Rogowski *et al.* 2010; Shashi *et al.* 2018). Interestingly, for the three CCPs (CCP2, 3, 5) that associated with cilia, $Ccp2^{-/-}$(Tort *et al.* 2014), $Ccp3^{-/-}$(Tort *et al.* 2014), and $Ccp5^{-/-}$(Wu *et al.* 2017; Xia *et al.* 2016) mice are generally healthy without neural degeneration phenotype. This suggests that neuron degeneration observed in *pcd* mice is probably caused by upregulated polyglutamylation of axon microtubules but not the axoneme. Although $Ccp5^{-/-}$ mice with presumably hyperglutamylated cilia are generally healthy without ciliopathy phenotypes, hyperglutamylation was correlated with retinal degeneration in either human (Astuti *et al.* 2016; Branham *et al.* 2016; Kastner *et al.* 2015; Sergouniotis *et al.* 2014) or mouse models (Grau *et al.* 2017; Marchena *et al.* 2011). It is argued that the pathogenesis of hyperglutamylation-associated retinal degenerative diseases may be tubulin-independent. A photoreceptor specific ORF15 variant of retinitis pigmentosa GTPase regulator ($RPGR^{ORF15}$), the product of the major causal gene of retinal dystrophy, localizes to the connecting cilium and can be glutamylated by TTLL5 *in vivo* (Rao *et al.* 2016). *TTLL5* mutations lead to complete loss of RPGR glutamylation and retinal pathology, without marked changes in tubulin glutamylation levels and defects in ultrastructure of microtubule doublets in connecting cilia (Lee *et al.* 2013; Sergouniotis *et al.* 2014; Sun *et al.* 2016).

GLYCYLATION

Tubulin glycylation was initially discovered on *Paramecium tetraurelia* tubulins that generate side chains of glycine on the γ-carboxyl groups of specific glutamate residues (Redeker *et al.* 1994), which is mostly enriched in the B-tubule of axoneme (Orbach and Howard 2019). Glycylation has been extensively studied by using specific antibodies that can detect glycylation modification in different length: with the antibody TAP952 detecting monoglycylation and AXO49 detecting chains with three or more glycine residues (polyglycylation) (Bre *et al.* 1996, 1998; Levilliers *et al.* 1995), and PolyG detects chains with four or more glycine residues (Tort *et al.* 2014; Xia *et al.* 2000). Glycylation modification occurs predominantly in cilia or flagella (Bre *et al.* 1996; Gadadhar *et al.* 2017b; Redeker *et al.* 1994; Ru *et al.* 1995; Weber *et al.* 1996; Xia *et al.* 2000), suggesting its unique roles in regulating cilia/flagella functions. One exception is that the cilia of Kupffer's vesicle in zebrafish appear to be free of mono- or polyglycylation (Pathak *et al.* 2011). Whether this is caused by species specificity of anti-glycylation antibodies need to be further examined.

Tubulin glycylation is generated by a subset of enzymes that belong to the same protein family as that of glutamylation, the tubulin tyrosine ligase-like proteins (TTLLs) (Ikegami and Setou 2009; Rogowski *et al.* 2009). The enzymes catalyzing deglycylation process remain unidentified, though. In mammals, TTLL3 and TTLL8 catalyze the initiation whereas TTLL10 catalyzes the elongation steps of polyglycylation (Ikegami and Setou 2009; Rogowski *et al.* 2009). In *Drosophila melanogaster*, no TTLL10 gene has been found (Rogowski *et al.* 2009). The polyglycylation in *Drosophila* is generated by bifunctional initiating/elongating glycylases dmTTLL3A and dmTTLL3B (Rogowski *et al.* 2009). Interestingly, unlike other mammalian species, the axoneme of humans cells only carry monoglycylation, which is caused by a mutation in *TTLL10* that inactive its elongating activity (Rogowski *et al.* 2009). This suggests that the function of microtubule glycylation might be sufficiently fulfilled by monoglycylation.

Tubulin glycylation has been linked to stability and maintenance of motile cilia (Grau *et al.* 2013; Pathak *et al.* 2011; Rogowski *et al.* 2009; Wloga *et al.* 2009). In *Tetrahymena* cells, deletion of TTLL3 leads to subtle defect in the tubulin turnover and results in slightly shorter cilia (Wloga *et al.* 2009). In zebrafish, loss of tubulin polyglycylation causes either shortening or loss of motile cilia in several organs (Wloga *et al.* 2009). In mammalian cilia, glycylation is redundantly generated by the enzymes TTLL3 and TTLL8 in most tissues.

Absence or reduction of glycylation along the axoneme destabilizes motile ependymal cilia (Grau *et al.* 2013). The existence of glycylation in primary cilia was not confirmed until a novel glycylation antibody (gly-pep) was generated recently (Gadadhar *et al.* 2017b). The glycylation in primary cilium is unevenly distributed, prominent at the proximal part of the cilia but not confined to the transition zone. Deletion or overexpression of glycylases modulates the length of primary cilia in cultured mammalian cells (Gadadhar *et al.* 2017b).

In agreement with its importance in motile and primary cilia, loss of glycylation results in cilia-related anomalies. Deletion of in *dmTTLL3B* in *Drosophila* causes defects in sperm individualization and axoneme structure (Rogowski *et al.* 2009). In zebrafish, loss of glycylation contributes to randomization of multicilia orientation in embryos (Pathak *et al.* 2011). In mice, absence of glycylation leads to ciliary disassembly in ependymal cells (Grau *et al.* 2013) and shortening of the connecting cilium of photoreceptors and affecting retinal degeneration (Grau *et al.* 2017). Surprisingly, cilia glycylation is also associated with the development of colorectal cancer. TTLL3 is the only glycylase in colon. Suppression of TTLL3 leads to reduced number of primary cilia and strongly enhanced colon carcinogenesis (Rocha *et al.* 2014), suggesting the correlation between primary cilia and cell cycle progression.

It is worth to noting that, glycylation and glutamylation occur within the same cluster of glutamates, indicative of a reciprocal or competition pathway of these two PTMs may exist. It is consistent with the observations that loss of tubulin glutamylation or glycylation alone shows mild defects in ultrastructural axonemal structure, while combined loss of glycylation and glutamylation causes near complete loss of cilia motility and induces a variety of dramatic axonemal ultrastructural defects in zebrafish (Pathak *et al.* 2011). Similarly, *Tetrahymena* mutants with glutamates on the C-terminal tail of β-tubulin mutated to abolish both glycylation and glutamylation are lethal or possess severe axonemal defects (Redeker *et al.* 2005; Thazhath *et al.* 2004; Xia *et al.* 2000). Loss of glycylation in *Tetrahymena, Drosophila* and mouse is accompanied by tubulin hyperglutamylation (Gadadhar *et al.* 2017b; Rogowski *et al.* 2009; Wloga *et al.* 2009). Thus, tubulin glycylation and glutamylation are likely regulated together and may coordinate cilia formation and/or function in certain contexts.

DETYROSINATION/Δ2-TUBULIN

Encoded in α-tubulin genes, the carboxyl-terminal residue of most nascent α-tubulin has a tyrosine which is removed by detyrosination and re-added by tyrosination (Arce and Barra 1983; Kumar and Flavin 1981). In cilia, detyrosinated tubulins are enriched on the B-tubules of outer doublets (Johnson 1998; Orbach and Howard 2019). Tubulin tyrosine ligase (TTL), the first identified tubulin-modifying enzyme, catalyzes the tyrosination modification (Ersfeld *et al.* 1993; Murofushi 1980; Prota *et al.* 2013; Schroder 1985; Szyk *et al.* 2011). TTL works exclusively and efficiently on detyrosinated tubulin heterodimers and thus the newly assembled microtubules are mostly tyrosinated. The enzymes catalyzing detyrosination have just been identified recently (Aillaud *et al.* 2017; Nieuwenhuis *et al.* 2017). When complexed with small vasohibin binding protein (SVBP), vasohibins (VASH1 and VASH2) exhibit robust and specific Tyr/Phe carboxypeptidase activity on polymerized microtubules. Interestingly, pharmacological and genetic suppression of vasohibins didn't abolish the detyrosinated α-tubulin, and the remaining detyrosinated pools were specifically concentrated in neuron axon, implicating the occurrence of other detyrosinases for detyrosinating axonal microtubules (Aillaud *et al.* 2017). Although it hasn't been studied, axonemal microtubules in cilia may be modified by the same detyrosination machinery as mentioned. After detyrosination, the exposed glutamate residues on C-terminal of α-tubulin can further be removed by cytosolic carboxypeptidases (CCPs; CCP1, 2, 3, 4, 6), generating the Δ2- and Δ3-tubulin (Aillaud *et al.* 2016; Berezniuk *et al.* 2012; de la Vega *et al.* 2007; Kalinina *et al.* 2007; Kimura *et al.* 2010; Paturle-Lafanechere *et al.* 1991; Rogowski *et al.* 2010; Tort *et al.* 2014). Δ2-tubulin cannot undergo tyrosination and it accumulates in long-lived microtubules in cilia and axonal microtubules.

Abnormal detyrosinated/Δ2-tubulin has been linked to defects in brain development. *Ttl*-knockout mice die perinatally due to massive defects in brain architecture, particularly in the cortico-thalamic loop, which is very likely due to the aberrant timing and extent of neurite outgrowth (Erck *et al.* 2005). Tubulin detyrosination is also implicated in muscle and cardiac functions. It accumulates at early steps of muscle cell differentiation and affects mechanotransduction (Kerr *et al.* 2015; Robison *et al.* 2016). Excess or diminished tubulin detyrosination changes the stiffness of the cardiomyocytes and may lead to cardiac dysfunction. Upregulated tubulin detyrosination was found in patients diagnosed with hypertrophic and dilated cardiomyopathies (Kerr

et al. 2015; Robison *et al.* 2016). Detyrosination has also been detected on microtubules of the mitotic and meiotic spindles, midbody and centrioles, and implicated in controlling the precision of cell division. Detyrosination facilitates the binding of a kinetochore-associated motor protein CENP-E, helps guide all chromosomes toward the metaphase plate in mitosis, and absence of this tubulin modification leads to misaligned chromosomes (Barisic *et al.* 2015). In female meiosis, detyrosinated microtubules are asymmetrically enriched on one half of the meiotic spindle, which drives non-Mendelian chromosome transmission in mouse oocytes (Akera *et al.* 2017). In light of its essential role in cell division, deregulated detyrosination is linked to several types of cancer. Differential expression of TTL correlates with poor prognosis in neuroblastoma tumors (Kato *et al.* 2004), and detyrosination of tubulin was particularly prominent in aggressive subtypes of breast cancer (Mialhe *et al.* 2001). Moreover, vasohibin is associated with micro-vessel densities, histology grades, invasions, poor clinical features, metastasis, and dissemination in abdominal cavities, as well as EMT (Du *et al.* 2017). The function of detyrosination/Δ2-tubulin in context of cilia remains largely unknown. The anterograde intraflagellar transport (IFT) is driven by kinesin-2 on the B-tubule of axonemal microtubule doublets (Kozminski *et al.* 1995; Pigino et al, 2009; Stepanek and Pigino 2016). It is worth noting that detyrosinated tubulins are strongly enriched on the B-tubules. *In vitro*, detyrosination promotes the motility of kinesin-2 (Sirajuddin *et al.* 2014), suggesting a potential correlation between tubulin detyrosination and selective transportation for anterograde IFT.

CONCLUSION AND PERSPECTIVE

Tubulin PTMs occur on the surface or inside the lumen of microtubules to change their mechanical properties or interactome, by which it fine-tunes the function and confers specialized identities for different microtubule structures. Emerging evidences unveil the physiological importance of tubulin PTMs in both motile and primary cilia. Functional studies in model organisms with mutations in PTMs modifying enzymes and the rapid advances in human genetics strongly suggest the causal role of dysfunctional tubulin PTMs in the pathogenesis of ciliopathies and a wide spectrum of human disorders. However, due to our limited knowledge of tissue specificity, function redundancy, subcellular distribution, and non-tubulin activities for microtubule PTMs modifying enzymes, precisely dissecting the role of tubulin PTMs in the context of cilia and ciliopathies remains challenging. To this end, new tools/reagents/systems are greatly needed to be developed to ensure precise and local manipulation of PTMs modifying enzymes in cilia. Also, as the PTMs modifying enzymes are accessible targets for drug development (Huq and Wei 2010), small-molecule activators/inhibitors of PTM enzymes are desired for basic research and, importantly, may represent promising therapeutic strategies for ciliopathies, neuron degeneration diseases, and other microtubule-associated human disorders.

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Compliance with Ethical Standards

Conflict of interest Kai He, Kun Ling, and Jinghua Hu declare that they have no conflict of interest.

Human and animal rights and informed consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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