The emerging role of tubulin posttranslational modifications in cilia and ciliopathies

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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 cillum 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 cillum and the primary cillum. In
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 cilium 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 has recently been identified as the ubiquitous sensory antenna of many pivotal signal-transduction 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. 2017a; 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; Huhb 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
curved areas of microtubules, the same regions that accumulate lattice openings or cracks, suggesting that $\alpha$TAT1 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. $\alpha$TAT1 has shown a higher affinity for microtubule ends. Alternatively, the high density of exposed luminal sites at the tapered extremities could be captured by $\alpha$TAT1. This proposed mechanism is supported by studies showing the preferential entry of $\alpha$TAT1 at open microtubule ends (Coombes et al. 2016; Ly et al. 2016; Szyk et al. 2014). It is conceivable that the entry mechanism of $\alpha$TAT1 may depend on the way by which microtubules were assembled and the mechanical stresses they were experiencing. In contrast to $\alpha$TAT1, 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).
| Modification | Tubulin | Enzyme | Ciliary functions | Related diseases and disorders |
|--------------|---------|--------|-------------------|-------------------------------|
| Acetylation  | α       | TAT1   | Regulate cilia assembly/disassembly (Pugacheva et al. 2007, Ran et al. 2015) | Spermatozoa abnormalities in αTAT1−/− mice (Kalebic et al. 2013b) |
|              |         | HDAC6  | Render microtubules resistant to mechanical stress (Portran et al. 2017, Xu et al. 2017) | Decreased axonemal acetylation in Joubert syndrome patients with ARMC9 or TOGARAM1 mutation (Latour et al. 2019) |
| Deacetylation|         | SIRT2  |                                 | Bardet-Biedl syndrome protein BBIP10 promotes microtubule acetylation (Loktev et al. 2008) |
| Glutamylation| α/β     | TTLL4, 5, 7 | Biphasically regulate microtubule severing (Valenstein and Roll-Mecak 2016) | Neurodegeneration in Purkinje cell degeneration (CCP1 mutant) mice (Rogowski et al. 2010, Shashi et al. 2018) |
| Polyglutamylation | TTLL1, 6, 11, 13 | CCP5 | Stabilize microtubules by interacting with CASP (Backer et al. 2012, Ohta et al. 2015) | 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) |
| Deglutamylation of branch point Glu | CCP1, 2, 3, 4, 6, CCP5? | | 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) | Respiratory disorders in TTLL1 mutated mice (Ikegami et al. 2010) |
| Deglutamylation of shorten polyGlu chain | CCP1, 2, 3, 4, 6, CCP5? | | Modulate the length of primary cilium (He et al. 2018) | Retinal degenerative diseases in mice and human with abnormal glutamylation (Grau et al. 2017, Marchena et al. 2011) |
| Glycylation  | α/β     | TTLL3, 8 | Control ciliary targeting of signaling molecules (He et al. 2018, Hong et al. 2018) | Decreased axonemal polyglutamylation in Joubert syndrome patients with certain gene mutation (He et al. 2018, Latour et al. 2019, Lee et al. 2012) |
| Polyglycylation | TTLL10 | Unknown | Modulate the length of primary cilium (Gadadhar et al. 2017b) | Retinal degeneration in TTLL3−/− mice (Grau et al. 2017) |
| Deglycylation |         |        | Reciprocal or competition of glutamylation (Gadadhar et al. 2017b, Rogowski et al. 2009, Wloga et al. 2009) | Suppression of TTLL3 in colon carcinogenesis (Rocha et al. 2014) |
| Detyrosination | α       | VASH1, 2 | Regulate the stability and maintenance of motile cilia (Grau et al. 2013, Pathak et al. 2011, Rogowski et al. 2009, Wloga et al. 2009) | Brain development defects in TTL−/− mice (Erck et al. 2005) |
| Δ2/Δ3-tubulin Tyrosination | CCP1, 2, 3, 4, 6 | TTL | Modulate the length of primary cilium (Gadadhar et al. 2017b) | Muscle and cardiac dysfunctions (Kerr et al. 2015, Robison et al. 2016) |
|              |         |        | Reciprocal or competition of glutamylation (Gadadhar et al. 2017b, Rogowski et al. 2009, Wloga et al. 2009) | 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 glycolysis 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 interprotofilament 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 infertility, 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 HDAC6 (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 polyplody.
(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; Bobiniec 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 acceptor site, 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 induce 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 complexes 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).
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 CCP5-1 (CCP1 homolog) is a real deglutamylase in C. elegans, the cilia of ccp5-1 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; Ikekami 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; Ikekami 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 CCP-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 several human Joubert syndrome genes, including CEP41, 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<sup>−/−</sup> (Tort et al. 2014), Ccp3<sup>−/−</sup> (Tort et al. 2014), and Ccp5<sup>−/−</sup> (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<sup>−/−</sup> 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<sup>ORF15</sup>), the product of the major causal gene of retinal dystrophy, localizes to the connecting cilium and can be glutamylated by TTLL5<sup>in vivo</sup> (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).

**GLYCATION**

Tubulin glycation 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). Glycation has been extensively studied by using specific antibodies that can detect glycation modification in different length: with the antibody TAP952 detecting monoglycation and AXO49 detecting chains with three or more glycine residues (polyglycation) (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). Glycation modification occurs predominately 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 monoo or polyglycation (Pathak et al. 2011). Whether this is caused by species specificity of anti-glycation antibodies need to be further examined.

Tubulin glycation is generated by a subset of enzymes that belong to the same protein family as that of glutamylation, the tubulin tyrosine ligase-like proteins (TTLs) (Ikegami and Setou 2009; Rogowski et al. 2009). The enzymes catalyzing deglycation process remain unidentified, though. In mammals, TTLL3 and TTLL8 catalyze the initiation whereas TTLL10 catalyzes the elongation steps of polyglycation (Ikegami and Setou 2009). The enzymes catalyzing deglycation process remain unidentified, though. In mammals, TTLL3 and TTLL8 catalyze the initiation whereas TTLL10 catalyzes the elongation steps of polyglycation (Ikegami and Setou 2009). In *Drosophila melanogaster*, no TTLL10 gene has been found (Rogowski et al. 2009). The polyglycation in *Drosophila* is generated by bifunctional initiating/elongating glycyrases dmtTTL3A and dmtTTL3B (Rogowski et al. 2009). Interestingly, unlike other mammalian species, the axoneme of humans cells only carry monoglycation, which is caused by a mutation in *TTLL10* that inactive its elongating activity (Rogowski et al. 2009). This suggests that the function of microtubule glycation might be sufficiently fulfilled by monoglycation.

Tubulin glycation 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 polyglycation causes either shortening or loss of motile cilia in several organs (Wloga et al. 2009). In mammalian cilia, glycation 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 glycation antibody (gly-pep) was generated recently (Gadadhar et al. 2017b). The glycation in primary cilium is unevenly distributed, prominent at the proximal part of the cilium but not confined to the transition zone. Deletion or overexpression of glycylases modulates the length of primary cilium 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 glutamatases, 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 glutamatases 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/Δ2-2-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-Lafanche 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. 2010).
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. 2001), 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/A2-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 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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