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Tubulin modifications and their cellular functions

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All microtubules are built from a basic α/β -tubulin building block, yet subpopulations of microtubules can be differentially marked by a number of post-translational modifications. These modifications, conserved throughout evolution, are thought to act individually or in combination to control specific microtubule-based functions, analogous to how histone modifications regulate chromatin functions. Here we review recent studies demonstrating that tubulin modifications influence microtubule-associated proteins such as severing proteins, plus-end tracking proteins, and molecular motors. In this way, tubulin modifications play an important role in regulating microtubule properties, such as stability and structure, as well as microtubule-based functions, such as ciliary beating, cell division, and intracellular trafficking.

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Introduction

Microtubules are polar cytoskeletal filaments assembled from head-to-tail and lateral associations of α/β -tubulin heterodimers. Most microtubules occur as single tubes and form cellular structures such as the mitotic spindle and the interphase network (Figure 1). A subset of microtubules exist as fused structures where a complete microtubule (A tubule) is fused with one or two incomplete tubules (B and C tubules) to comprise ciliary axonemes (doublet microtubules) or centrioles and basal bodies (triplet microtubules) (Figure 1). How the basic α/β -tubulin building block is used to generate a variety of microtubule structures with organelle-specific properties and functions is not clear. One hypothesis that has gained experimental support recently is that post-translational modifications (PTMs) of the tubulin building block generate functional diversity of microtubules. These modifications may act individually and/or in a combinatorial fashion to recruit specific protein complexes and thus regulate organelle-specific properties of microtubules.

Many tubulin PTMs have been known for decades – detyrosination and the related Δ^2 modification, glutamylation, glycylation, acetylation, phosphorylation, and palmitoylation (Figure 2) (for reviews, see [1,2]). Yet their functional roles in different microtubule structures are just beginning to be discovered. Most PTMs occur on microtubules rather than on unpolymerized tubulin and it has long been known that stable microtubules, as compared to dynamic microtubules, accumulate more modifications. The PTMs are postulated to play a role in specific functions of stable microtubules as differences in tubulin PTM patterns can be seen between stable microtubules. For example, in axonemes, although all microtubules are highly stable, the A tubule of the doublet microtubules and the central singlet microtubules are mostly unmodified, whereas the B tubule is highly detyrosinated, polyglycylated, and polyglutamylated (Figure 1). This review will focus on the four best-studied PTMs of tubulin, with a particular focus on recent studies indicating specific functional roles for these modifications in regulating microtubule-based functions *in vivo*.

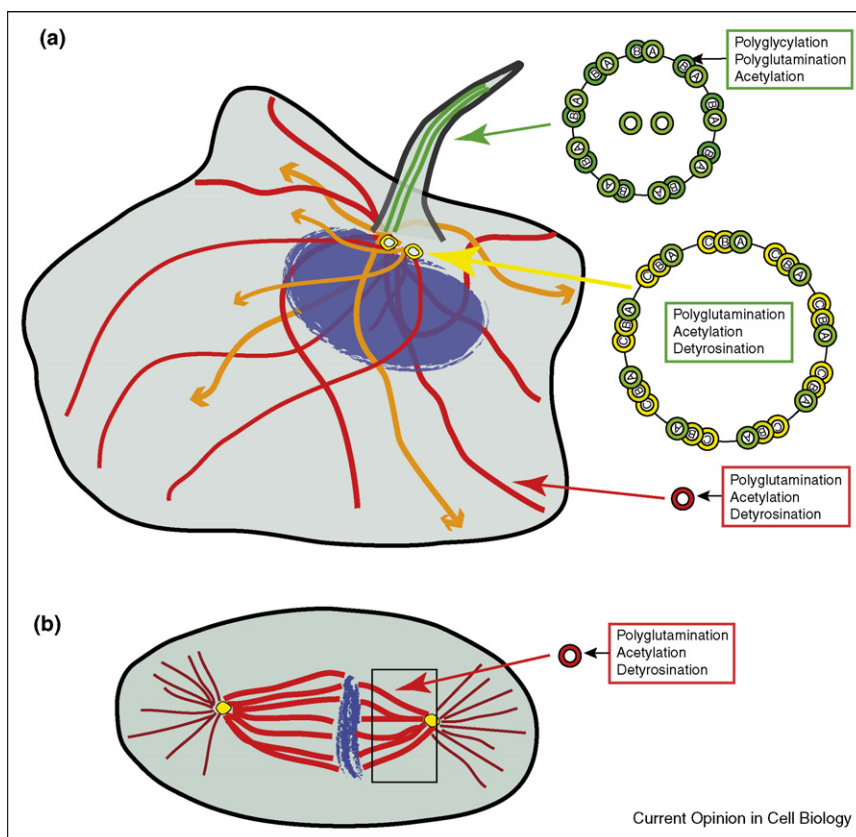
Detyrosination/tyrosination

Detyrosination involves the removal of the gene-encoded C-terminal tyrosine of α -tubulin in microtubule polymers by an unidentified carboxypeptidase (Figure 2) [1,2]. A novel family of cytosolic carboxypeptidases was recently identified whose founding member, Nna1/CCP1, shares some characteristics with the known properties of tubulin carboxypeptidase [3,4]. The reverse tyrosination reaction, or addition of a tyrosine residue to the now C-terminal glutamate residue of α -tubulin, occurs on soluble tubulin heterodimers and is catalyzed by tubulin tyrosine ligase (TTL) [1,2].

The detyrosination/tyrosination cycle differentially recruits two types of microtubule-binding proteins, molecular motors and plus-end tracking proteins (+TIPs). The motor protein Kinesin-1 binds preferentially to detyrosinated microtubules rather than tyrosinated microtubules [5,6]. This could enable Kinesin-1 to preferentially move various cargoes, including vimentin filaments and transferrin, along detyrosinated microtubules [6–8]. Tyrosinated microtubules have recently been shown to play a direct role in the recruitment of +TIP proteins that contain a CAP-Gly domain (e.g. CLIP-170, CLIP-115, and p150Glued/dynactin). +TIPs are proteins that localize to and track with the plus-ends of growing microtubules. A pioneering study in *Saccharomyces cerevisiae* showed that expression of an α -tubulin gene that lacks the C-terminal phenylalanine (thus resembling

2 Cell structure and dynamics

Figure 1



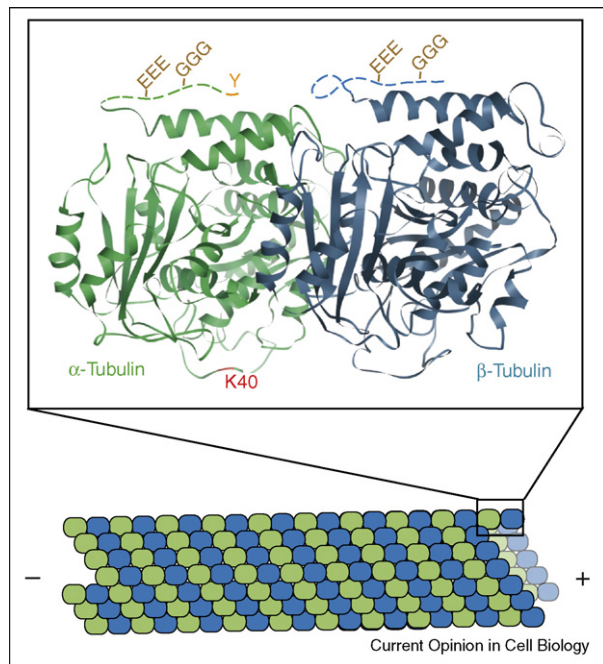
Localization of microtubule structures and modified tubulin subunits in mammalian cells. **(a)** In interphase cells, dynamic microtubules (orange) lack PTMs whereas stable microtubules (red) accumulate PTMs in a time-dependent manner. Ciliary microtubules (green) contain PTMs primarily on the B tubule of the outer doublets. Note that motile cilia contain the 9 + 2 arrangement of nine doublet microtubules around a central pair (shown) whereas primary cilia lack the central pair (9 + 0, not shown). PTMs are also prevalent in centriolar/basal body triplet microtubules (yellow). **(b)** In mitotic cells, spindle microtubules (kinetochore and/or interpoles) microtubules are highly modified whereas astral microtubules are mostly unmodified.

detyrosinated tubulin) resulted in mislocalization of Bik1p (CLIP-170 homologue) but not Bim1p (EB1-type +TIP that lacks a CAP-Gly domain), as well as defects in nuclear positioning and spindle dynamics [9^{••}]. Importantly, recruitment of CAP-Gly containing +TIPS by tyrosinated α -tubulin occurs in mammalian cells as well. Fibroblasts isolated from TTL null mice showed a severe reduction in tyrosinated microtubules, mislocalized CLIP170 protein (but not EB1), and misoriented mitotic spindles [10^{••}]. The latter effect may be a direct consequence of the ability of highly tyrosinated astral microtubules to recruit CAP-Gly-containing +TIPs, such as CLIP-170 and p150Glued/dynactin, and promote attachment of microtubule plus-ends to the cell cortex [10^{••}, 11–13]. Recent structural work has shown that a conserved basic region in the CAP-Gly domain is required for targeting to the acidic EEY/F sequence present at the C-terminus of α -tubulin [14, 15]. CAP-Gly domains and C-terminal EEY/F motifs also contribute to binding interactions between +TIP proteins ([14–17] and

references therein). Thus, multiple interactions between tyrosinated α -tubulin, +TIPs, and the dynein/dynactin complex likely contribute to microtubule-based functions during mitosis and interphase.

Proper functioning of the detyrosination/tyrosination cycle has also been shown to influence tumorigenesis and neuronal organization. Multiple studies have shown that TTL is downregulated in animal and human cancers. Low levels of TTL protein and tyrosinated tubulin correlate with increased tumorigenesis, tumor invasiveness, and poor prognosis [18–21], suggesting that detyrosinated tubulin provides a growth advantage. The role of tyrosinated tubulin in neuronal organization was highlighted by the fact that TTL null mice undergo normal embryonic development but die perinatally because of neuronal disorganization. Analysis of TTL^{-/-} neurons in culture suggested that TTL activity, and the relative amounts of detyrosinated/tyrosinated tubulin, are important for the regulation of neurite extension and axon

Figure 2



Localization of tubulin PTMs on the α/β -tubulin heterodimer. Microtubules are formed by the end-on-end and lateral associations of α - (green) and β - (blue) tubulin heterodimers. The crystal structure (PDB file 1TUB) of one tubulin heterodimer is shown. The CTTs of both α - and β -tubulin, disordered in the structure, are represented in this schematic as dashed lines. Detyrosination entails the removal of the C-terminal tyrosine (Y) from α -tubulin. The resulting detyrosinated α -tubulin is often referred to as Glu tubulin because of the exposed glutamate residue. Polyglutamylation (EEE) and polyglycylation (GGG) occur on the CTTs of α - and β -tubulin. Acetylation of α -tubulin occurs on lysine 40 (K40) which is predicated to lie on the luminal face of the microtubule.

specification, perhaps because of the recruitment of CLIP-170 and other +TIPs in growth cones [22[•]].

Polymodifications—glutamylation and glycylation

Glutamylation and glycylation involve the addition of variable numbers of glutamate or glycine residues, respectively, onto glutamate residues in the C-terminal tails (CTTs) of both α - and β -tubulin (Figure 2) [1,2]. Glycylation is mainly limited to tubulin incorporated into axonemes (cilia and flagella) whereas glutamylation is prevalent in neuronal cells, centrioles, axonemes, and the mitotic spindle. Both modifications have been found on the same tubulin CTT and there is cross-talk between the α - and β -tubulin tails regulating the type and level of each modification [23,24].

An important breakthrough in the study of tubulin PTMs was made with the recent identification of the enzymes that catalyze tubulin glutamylation [25^{••}]. The tubulin glutamylases belong to a family of enzymes that contain a

tubulin tyrosine ligase-like (TTLL) domain. Eight mammalian TTLL domain-containing proteins (TTLLs 1,4–7,9,11,13) have been shown to be tubulin glutamylases that differ in their specificity for α - or β -tubulin and in preferential chain-initiating or chain-elongating activity ([25^{••},26^{••},27[•]] and J. Gaertig, personal communication). Other TTLL domain-containing proteins (TTLLs 3,8,10,12) are suspected (but not yet shown) to facilitate ligation of glutamates or other amino acids such as glycine to tubulin or other proteins. Both glycylation and glutamylation are reversible reactions, however the enzymes responsible for this reverse reaction are still elusive.

In cilia and flagella, the tubulin CTTs and their polymodifications play a role in the formation and maintenance of axonemal structures. Deletion of the CTTs of either α - or β -tubulin is lethal in *Tetrahymena* [24]. In *Drosophila*, β -tubulin lacking its CTT can support assembly of most types of microtubules but fails to assemble into axonemes of sperm [28]. Thus, the tubulin CTTs can influence the properties of assembled microtubules. The exact sequence of the tails is not important as the CTTs of α - and β -tubulin can substitute for each other [24,29]. In the case of the β -tubulin CTT, it is the polymodifications that are critical for ciliary assembly and motility, as mutation of acceptor glutamate residues in the β -tubulin CTT was either lethal or hypomorphic, depending on the site and extent of the mutations. In particular, a severe loss of polymodifications on the β -tubulin CTT resulted in non-motile, short cilia, perhaps because of structural defects (absence of central pair microtubules and loss of B tubules), as well as effects on intraflagellar transport (see below) [23,29–31]. A role for polymodification of the β -tubulin CTT in ciliary assembly and function is supported by a recent study in which knockdown of the TTLL6 polyglutamylase in zebrafish inhibited the formation of olfactory cilia [32]. For the α -tubulin CTT, the polymodifications appear not to play a major role in ciliary assembly and function in *Tetrahymena* [23,29] but may be critical in mammals, as mice deficient in PGs1, a non-catalytic component of the TTLL1 α -tubulin polyglutamylase complex, have multiple defects including male sterility because of abnormal assembly of sperm flagella [33]. Finally, too much polyglutamylation (specifically chain length) is also detrimental to cilia motility, as overexpression of *Tetrahymena* TTLL6A, an active β -tubulin elongase, resulted in paralyzed cilia [25^{••}].

Polymodification of the α - and β -tubulin CTTs may also influence the transport of structural and membrane components within cilia and flagella, a process known as intraflagellar transport (IFT) [34]. Cells with mutation of the β -tubulin CTT in *Tetrahymena* showed accumulation of dense material, presumably IFT particles, between the outer doublets and the ciliary membrane, reminiscent of cells defective in the molecular motors that drive IFT [23]. Yet the relationship between tubulin modifications

4 Cell structure and dynamics

and IFT is likely complex as illustrated by recent studies on DYF-1. Work in *Caenorhabditis elegans* identified DYF-1 as a component of the IFT particle that functions to positively regulate the homodimeric OSM-3 motor (a kinesin-2 family member), perhaps by docking OSM-3 onto IFT particles [35]. Mutations in the DYF-1 homologue in zebrafish, Fleer, resulted in short cilia with a dramatic loss of polyglutamylation and defects in B-tubule structure [32]. Since polyglutamylation levels were unaffected in *osm-3* mutants [32], it seems unlikely that DYF-1 regulation of OSM-3 transport promotes tubulin polyglutamylation. Rather, DYF-1 may promote tubulin modifications that influence OSM-3 activity. Clearly, more work is needed to resolve the circular connection between polyglutamylation, IFT transport, and axonemal structural defects.

That different kinesin motors are differentially sensitive to the presence of specific PTMs is supported by recent work [1,2]. Kinesin-1 bound effectively to axonemes from wildtype *Tetrahymena* and α -tubulin modification site mutants but was unable to effectively bind axonemes from β -tubulin polymodification site mutants [5**]. A reduction in α -tubulin polyglutamylation, because of mutation in the TLL1 subunit PG1s, resulted in altered distribution of Kif1A (kinesin-3 family) but not Kif3a (kinesin-2 family) or Kif5 (kinesin-1 family) [36**]. Importantly, these mice also displayed decreased density of synaptic vesicles, a Kif1A cargo, in axon terminals [36**]. In a related study, a decrease in β -tubulin glutamylation because of knockdown of TLL7, a β -tubulin initiating glutamylase, resulted in impaired neurite outgrowth [27]. Whether this effect is also because of impaired function of motors or other microtubule associated proteins requires further study.

Recent work has also shown that polymodifications of the tubulin CTTs could mark specific microtubules for severing and thus influence polymer dynamics and density. In *Tetrahymena*, mutations of the β -tubulin polymodification sites resulted in an inability to clear cortical microtubules from the cleavage furrow region, preventing cells from completing cytokinesis [30,31]. Interestingly, loss of katanin, a microtubule severing protein, gives almost an exact phenocopy [37*]. In addition, cells lacking katanin accumulate excessive levels of polymodifications on microtubules [37*]. In *C. elegans*, overexpression of katanin is lethal but can be rescued by mutation of a potential polymodification site on the β -tubulin CTT [38*], suggesting that katanin activity is influenced by modification of β -tubulin. Indeed, the activity of two microtubule severing proteins, spastin and katanin, requires the CTTs of tubulins *in vitro* [39–41].

Acetylation

Acetylation is unique among the known tubulin modifications in that it occurs on lysine 40 of α -tubulin which is

postulated to reside on the luminal face of microtubules (Figure 2). It is unclear how the enzymes that carry out acetylation/deacetylation would have access to this site. It is also unclear how this luminal modification could influence microtubule-based functions that occur on the cytoplasmic face of the microtubule. The acetylation enzyme has not been identified, but two enzymes have been shown to deacetylate α -tubulin *in vitro* and *in vivo*, namely HDAC6 and Sirt2 [42–44]. Knockdown of either HDAC6 or Sirt2 results in hyperacetylated tubulin suggesting that the two enzymes may be interdependent [42,44].

Acetylation of α -tubulin on lysine 40 is fairly common and can be found on stable microtubules in most cell types (Figure 1). However, mutation of lysine 40 in *Chlamydomonas*, *Tetrahymena*, or *C. elegans* had no obvious phenotype (discussed in [1,2]). Although tubulin acetylation is not necessary for cell and organism survival, recent work has suggested that α -tubulin plays a positive role in motor-based trafficking in mammals [5**,45,46**]. Kinesin-1 binds with higher affinity to acetylated microtubules *in vitro* [5**]. Hyperacetylation of microtubules in neuronal cells using small molecule inhibitors of HDAC6 caused Kinesin-1 transport of JIP1 to be redirected from a subset to the majority of neurites and enhanced the anterograde and retrograde transport of BDNF vesicles [5**,46**]. As HDAC6 has recently been shown to deacetylate cytosolic proteins other than tubulin (e.g. cortactin and HSP90) and regulates a variety of other cellular events (e.g. aggresome formation, cell motility, ciliary disassembly, and transcriptional corepression), the role of HDAC6/Sirt2 deacetylation of α -tubulin in regulation of microtubule-based functions remain to be clarified (reviews [1,2,47] and also [48–50]).

Concluding remarks

Although PTMs of tubulin subunits within microtubule structures have been known for many years, the cellular functions of the PTMs have only recently begun to be revealed. So far, tubulin PTMs have been shown to affect primarily two microtubule-based properties. First, tubulin PTMs influence the stability and/or structure of microtubule assemblies. Whether this is a direct effect of tubulin modification on microtubule structure or indirectly because of regulation of microtubule-associated proteins, such as severing proteins, will be a fertile area of future research.

Second, tubulin PTMs can influence recruitment of microtubule-associated proteins such as molecular motors and +TIPs. In the case of motor-dependent transport, it is possible that the PTMs serve as ‘road signs’ to direct polarized trafficking such as axonal/dendritic trafficking in neuronal cells. It is also possible that PTMs simply mark stable microtubules for preferential transport. This would enable molecular motors to avoid the undesirable

situation in which the microtubule track falls apart before the motor/cargo complex has reached its destination. Future research will determine the functional contribution of tubulin PTMs to each of these possibilities and provide important new information about how the tubulin code regulates microtubule-based functions in cells.

Acknowledgements

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530 neuronal cells, the authors used mice that lack functional PGs1, a 590
531 noncatalytic subunit of the TLL1 α -tubulin glutamylase. Decreased 591
532 tubulin polyglutamylation in neurons was correlated with decreased 592
533 levels of Kif1A (kinesin-3 family) in neurites but not Kif3A (kinesin-2 593
534 family) or Kif5 (kinesin-1 family). Deficiencies were also found in the 594
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