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Posttranslational Modifications of Tubulin: Pathways to Functional Diversity of Microtubules

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Abstract

Tubulin and microtubules are subject to a remarkable number of posttranslational modifications. Understanding the roles these modifications play in determining functions and properties of microtubules has presented a major challenge that is only now being met. Many of these modifications are found concurrently, leading to considerable diversity in cellular microtubules, which varies with development, differentiation, cell compartment and cell cycle. We now know that posttranslational modifications of tubulin affect not only the dynamics of the microtubules, but also their organization and interaction with other cellular components. Many early suggestions of how posttranslational modifications affect microtubules have been replaced with new ideas and even new modifications as our understanding of cellular microtubule diversity comes into focus.

Keywords

Tubulin; microtubule; detyrosination; acetylation; polyglutamylation; polyamination

Microtubule Diversity and Posttranslational Modifications

Forty years ago, the first tubulin posttranslational modification (PTM) was described: RNA-independent enzymatic incorporation of tyrosine [1]. A remarkable number of modifications to tubulin and microtubules (MTs) have since been identified (Table 1), including the most recent, polyamination [2]. Tubulin PTMs are found in all cells with MTs [3, 4] and they are particularly diverse in neurons [3-5], but many questions remain, such as the fraction of tubulins with a given modification, the distribution of modifications along a MT or between MTs, and the functional consequences of many modifications. Although regional differences in MT dynamics and stability are important for all cells [6], the significance of PTMs goes beyond MT dynamics.

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Tubulin may be modified as a soluble dimer or in a MT (Table 1), and some PTMs occur on both. The variable C-terminal domains of α-tubulin represent a hot spot for modification, while fewer modifications appear to associate with the C-termini of β-tubulin (Fig. 2). In addition, some modifications map to other regions of the tubulin dimer. Studies have revealed that MTs are modified in a heterogeneous manner, with PTMs being coextensive or concentrated in distinct domains on MTs, thereby adding an additional level of complexity. Indeed, assigning specific functions to a given modification in vivo is complicated by the heterogeneity of modifications found in the MT as well as the multiple modifications that may be present on the individual tubulin dimers themselves (Fig. 1).

In this review, we examine recent studies that have provided insights into the roles that PTMs play in specifying MT function. Given the diversity of PTMs in neurons and their importance in neuronal function, we emphasize PTMs in the nervous system while citing examples from other cell types, keeping in mind that PTMs may play different roles in different cellular contexts, such as cell division.

Detyrosination and Tyrosination of Tubulin

Most, but not all, α-tubulins contain a terminal tyrosine. Exceptions include human TUBA4A with a terminal glutamate and TUBA8 with a terminal phenylalanine [7]. Detyrosination was the first modification identified that preferentially affected MTs, rather than tubulin dimers [8]. The terminal tyrosine is removed by an unidentified cytosolic carboxypeptidase (CCP) to expose a glutamate, but surprisingly little is known about the regulation of this activity. The development of methods to prepare pure fractions of polymerizable tyrosinated and detyrosinated tubulin [9] may provide a basis for identifying this elusive carboxypeptidase.

In contrast, the tubulin tyrosine ligase (TTL) that rapidly tyrosinates detyrosinated tubulins released from MTs is well characterized [3, 4], completing a detyrosination/tyrosination cycle. Structural studies indicate that TTL has multiple interactions with both α- and β-tubulin in the dimer [10]. TTL binding to dimer interferes with polymerization and TTL overexpression inhibits MT polymerization in vivo [11]. Stathmin, a protein that interacts with tubulin dimers, and regulates MT polymerization [12] may also regulate TTL function. TTL and stathmin compete for binding on tubulin dimers, due either to partial overlap in binding sites or altered conformation of stathmin–tubulin complexes; and stathmin inhibits tyrosination of tubulin [13].

Both detyrosinated and tyrosinated tubulins [14-16] are distributed in patches along axonal MTs with enrichment of detyrosinated tubulin in proximal segments of axon shafts and enrichment of tyrosination at growth cones [15, 16]. This distribution is critical for development as revealed by the TTL-null mouse, which dies within 24h after birth with respiratory problems, ataxia, and profoundly altered neuronal morphologies [17]. Lethality may be associated with changes in neurite formation, axon guidance, and increased Rac1 signaling [18]. Detyrosination has been associated with MT longevity because many detyrosinated MTs turn over more slowly than tyrosinated MTs in vivo, but MTs enriched in detyrosinated tubulin are not intrinsically more stable [19]. Enrichment of detyrosinated

tubulin in stable MTs reflects preferential action of the carboxypeptidase on MTs, creating composite MTs with newly formed plus ends rich in tyrosinated tubulin and older domains enriched in detyrosinated tubulin.

The differential interactions of proteins with tyrosinated and detyrosinated tubulins are well documented (Fig.4). The reciprocal reactions of detyrosination/tyrosination of tubulin help define distinct subcellular compartments and generate a scaffold for cellular signaling by regulating protein interactions with MTs [20, 21]. For example, although EB1 binds plus ends of MTs independent of tubulin tyrosination, CAP-Gly domain proteins such as CLIP170 or P150 Glued need both EB1 and tyrosinated tubulin to localize to MT plus ends [22]. MT tyrosination also affects the activity of the MT severing protein spastin, which preferentially cleaves detyrosinated MTs [23], despite the association of detyrosination with long-lived MTs.

Detyrosination/tyrosination dependent changes in MT dynamics and interactions can occur in different cell types at various developmental stages. For example, detyrosination affects MT interactions with intermediate filaments [24] and microfilaments in vivo [18]. In addition, detyrosination is associated with differentiation and polarization of some cells [25, 26] (Fig. 3). In these studies, subconfluent cells have moderate levels of detyrosination, which increases with confluency and declines as confluent cells become polarized [25, 26]. Local reversibility of detyrosination generates tubulin and MTs with distinct properties more efficiently than protein synthesis and degradation. .

Tubulin detyrosination and tyrosination may play a role in motor protein function, although the effects may depend on the model and assays used. Kinesin-13 binds preferentially to tyrosinated plus ends and depolymerizes MTs both in vivo and in vitro [27, 28], suggesting that detyrosination would protect MTs. In addition, detyrosination enhanced nucleotide-free binding of kinesin-1 to MTs in vitro with a K_d of 29 nM (deTyr) and 81 nM (Tyr) [29], but the physiological significance of this observation is uncertain, because kinesin concentrations in neurons (500 nM) are an order of magnitude higher. In cultured neurons, some kinesins (i.e. kinesin-2 and -3) accumulate in both axons and dendrites, while others (kinesin-1) enter axons preferentially, raising the possibility that differences in MT tracks might explain differential targeting [30]. Increasing detyrosinated MT levels in neurons led to kinesin-1 accumulation in all neurites [30] and GFP-kinesin-1 preferentially associated with detyrosinated MTs and exhibited a slower rate of movement [31]. Similarly, mutations in a region proposed to be sensitive to tyrosination convert kinesin-1 from an axon-only to an all-neurite motor [30]. However, a subsequent study failed to find a direct correlation between detyrosinated MT content of a neurite and targeting of kinesin-1 [32]. Extensive analysis of in vitro motor interactions with modified tubulin C-terminals using chimeric yeast tubulins [33] found that detyrosination modestly decreased kinesin-1 processivity without affecting its velocity or dynein movement. In contrast, detyrosination increased both processivity and velocity of kinesin-2, suggesting that tyrosination effects on motor function are complex.

Δ2-Tubulin

Δ2-tubulin is a further modification of detyrosinated tubulin where the penultimate glutamate is also removed [5].All six selective CCPs (CCP1 [34], CCP2, CCP3 [35], CCP5 [36], CCP4 and CCP6 [37]) modify α-tubulin C-terminals by removing terminal glutamates or shortening polyglutamate chains (Table 1). Like detyrosination, Δ2-tubulin accumulates in long-lived MTs and is enriched in differentiated neurons as well as in centrioles and cilia [38]. Unlike detyrosination, Δ2-tubulin is a terminal modification whereby the removal of the penultimate glutamate produces tubulins that cannot be tyrosinated [39]. Removal of the penultimate glutamate affects availability of tubulin for modifications, such as tyrosination and polyglutamylation [4]. Although Δ2-tubulin may represent ≥35% of tubulin in the brain [38], the functional role of Δ2-MTs remains poorly understood.

Polyglutamylation and Polyglycylation of tubulin in MTs

Polyglutamylation is abundant in neurons [40] as well as in cilia and centrioles [41, 42]. Enzymes containing a domain related to TTL (i.e. TTL-like (TTLL) proteins) add amino acids to tubulins through an RNA-independent enzymatic reaction. Different TTLLs add glutamate or glycine to side chains initiated on one or more of the glutamates in the C-terminal domains of both α and β-tubulins [3, 4, 43, 44]. Some TTLL glutamylases (TTLL4, 5, and 7) add a single glutamate by forming a γ-linked isopeptide bond, while others (TTLL1, 6, 11 and 13) add glutamate side chains to the γ-linked glutamate through standard peptide linkages to form polyglutamate side chains [3]. Glutamates may be added differentially to specific tubulin isotypes or at different sites. For example, TTLL7 preferentially modifies β-tubulin, while TTLL5 and 6 prefer α-tubulin as a substrate[44]. Some TTLLs also modify other cytoplasmic and nuclear proteins [44].

The number of combinatorials for polyglutamylation with variable chain lengths and modifications on different glutamates in the C-terminal domain as well as on both α and β tubulin isotypes make it difficult to determine its functions. One common element is that the adaptor PGs1 is required to localize the polyglutamylase complex to MTs in cilia and neurons [45], and loss of PGs1 function in the ROSA22 mouse significantly reduces MT polyglutamylation [46]. This mouse is viable but exhibits abnormal sperm motility, behavioral changes, reduced body fat [47] and altered synaptic composition and function [46]. Polyglutamylation effects may depend on cellular context and associated proteins.

As with other tubulin PTMs, polyglutamylation may alter protein interactions with MTs. For example, it differentially affects MAP1 and MAP2 binding to tubulin [48] and stimulates spastin-mediated MT severing [49] (Fig. 4). In contrast, the effects of polyglutamylation on MT motors are less clear. One study reported that reducing tubulin polyglutamylation in the brain altered distribution of kinesin-3, but not kinesin-1 [46]. However, a second study reported that increases in MT polyglutamylation following inhibition of synaptic activity correlated with inhibition of kinesin-1, but not kinesin-3 mobility [50]. The connection with synaptic activity suggested that local changes in polyglutamylation might serve to target selected cargoes [50]. However, these finding are in contrast with studies using chimeric yeast tubulin C-terminal domains for assays of in vitro motility of kinesins and cytoplasmic

dynein [33]. The addition of polyglutamate to the C-terminus of β3-tubulin affected various kinesins and dynein differentially: Chains of Glu_{10} reduced processivity but not velocity of kinesin-1, while Glu_3 chains had no effect. Kinesin-2 also appeared unaffected by polyglutamylation, while dynein showed increased processivity. However, these polyglutamatylations were linked to tubulin by maleimide through an engineered Cys residue [33] rather than through the normal glutamate γ-carboxyl isopeptide bond. The structure and effects of these artificial constructs could differ from endogenous polyglutamylations.

CCP1 (Nna1) is upregulated during motor neuron development and regeneration suggesting a role in neurite growth [51] (Fig. 5). The pcd mouse, which results from a loss of function mutation in CCP1, exhibits degeneration of certain neurons (including Purkinje cells) and increased polyglutamylation of tubulin in adults [37]. When CCP1 was identified as the mutant gene, the neuropathology in pcd mice was presumed to result from decreased Δ2-tubulin, but Δ2-tubulin levels are normal in adult pcd mice and polyglutamylated tubulin continues to accumulate. Consistent with increased polyglutamylation causing neuropathology, suppressing TTLL1 protected Purkinje cells from degeneration in pcd mice [34, 37]. Other CCPs may compensate for loss of CCP1 in spared tissues [37], while severely affected neurons (Purkinje and mitral cells) express lower levels of other CCPs, showing the greatest differences in tubulin modification.

In contrast to polyglutamylation, polyglycylation is restricted to ciliary tubulins [42, 52] and may be important in ciliary assembly in some organisms [53]. Since glutamylation and glycylation target the same glutamates next to the C-terminus, a reciprocal pathway whereby glycylation modulates polyglutamylation may exist [4]. Both TTLL3 and 8 add a single glycine to glutamates in the C-terminal domain through a γ-linked isopeptide bond [53, 54], which is extended in polyglycylation by TTLL10 [55]. Since TTLL10 is inactive in humans, human sperm flagellar tubulins are monoglycylated [54], and the absence of long chain polyglycylation does not affect ciliary motility [56], so the functional significance of extended polyglycine chains is uncertain. However, a knockout of TTLL3 eliminates all tubulin glycylation in the colon, where TTLL3 is the only glycylase expressed [57]. This absence of glycylation led to reductions in primary cilia and enhanced carcinogenesis in the colon, suggesting that glycylation of tubulin may play a role in the maintenance of primary cilia and cell proliferation.

Acetylation and Deacetylation of Tubulins

Acetylation

Tubulin is the major cytoplasmic target for acetylation [58]. Unlike detyrosination and glutamylation, the canonical site for tubulin acetylation is α-tubulin Lys40 [59] located on the MT lumenal surface [60]. How the acetylase gains access to Lys40 has been unclear, but recent studies suggest that tubulin acetyltransferase (TAT) efficiently scans MTs bidirectionally within the lumen using surface diffusion, acetylating Lys40 stochastically [61]. This activity is not limited by constraints to lumenal diffusion, consistent with observations that rates of tubulin acetylation are similar for short and long MTs [62]; therefore, acetylation is not restricted to MT ends but is distributed along MTs.

Ultrastructural studies also show acetylase binding to MT surfaces in vitro, independent of acetylation state [63].

Although several enzymes can acetylate tubulin, including ELP3, ARD1-NAT1 and GCN5 [58], the major tubulin acetylase is αTAT1/MEC17 [64-67]. Elimination of αTAT1/MEC17 activity effectively abolishes MT acetylation in various models, but regulation and substrate specificity of αTAT1 is poorly understood. The major substrate for αTAT1 is α-tubulin, but αTAT1 also auto-acetylates [68]. Eliminating αTAT1 auto-acetylation sites does not affect MT binding, but does reduce tubulin acetylation [68], suggesting it is regulated by auto-acetylation. αTAT1 is also enriched near active clathrin-mediated endocytosis, due to its interaction with clathrin assembly protein, AP2 [69]. Furthermore, αTAT1 associates with acetylated MTs in axons and cilia in vivo [65, 66]. Indeed, male mice lacking αTAT1 are less fertile due to altered sperm morphology and motility [65]; however, they do not exhibit obvious behavioral or neurological defects beyond subtle changes in brain architecture [70]. However, in mice without αTAT1 or acetylated MTs, the MTs were more resistant to nocodazole, suggesting increased MT stability [65]. MEC17 may also have other functions because the expression of enzyme-dead MEC17 restores touch sensitivity in the absence of tubulin acetylation [67].

Although Lys40 is highly conserved in most mammalian α-tubulins isotypes (an exception is human TUBA8), not all isotypes are acetylated to the same extent. In Physarum [71] and Trypanosoma [72], only TUBA3 is acetylated, while proteomic analysis of acetylation in human cells [73] suggests preferential acetylation of the widely expressed TUBA1 and TUBA4 [7]. Tubulin acetylation also varies in different cells and tissues [58], being lower in rapidly dividing cells than in postmitotic cells. However, some cells and organisms lack acetylated tubulin [58], indicating that tubulin acetylation is not essential for cell survival. For example, MEC12 is the only α-tubulin gene in C. elegans with a Lys40, but a MEC12 Lys40Gln mutant eliminates acetylation without affecting viability [67]. Additional acetylation sites on tubulin have also been identified in proteomic screens [73] and in studies of other acetyltransferases [74]. The prevalence and function of these sites are not well understood, but may affect MT polymerization [74].

Despite being a major PTM of tubulin, there is little consensus on the functions for tubulin acetylation [58]. The association of acetylation with long-lived MTs suggested a role in the stabilization of MTs. While this idea persists, direct tests showed no effect of acetylation on MT dynamics [3]. Acetylation is enriched in long-lived MTs, because soluble tubulin dimers are rapidly deacetylated and newly formed MTs are deficient in acetylation. The dependence of αTAT1 on lattice geometry of MTs and its slow catalytic rate effectively limit acetylation of dynamic MTs [61]. Further, both active and acetylase-dead forms of mouse αTAT1 can bind to MTs and destabilize them in vitro [68].

Acetylation may also affect MT structure. Wild-type MTs in C. elegans touch sensitive neurons have 15 protofilaments, and mutations in MEC17 or in MEC12 result in a reduced number of MTs in touch neurons, while the remaining MTs have a variable protofilament number [67, 75]. However, the effect of acetylation on MT protofilaments is not observed in other systems where acetylated MTs have the standard 13 protofilaments. Futhermore, cryo-

electron microscopy failed to find structural differences between acetylated and deacetylated MTs [63].

MT acetylation affects protein interactions with MTs. Indeed, the binding of Hsp90 to MTs is enhanced by acetylation, and recruiting Hsp90 activates Akt and p53 [76]. Increasing MT acetylation can also induce IL10 (an anti-inflammatory cytokine) and activate p38 MAP kinase in macrophages [77]. Acetylated tubulin is the preferred site of action for the severing protein katanin [78] (Fig. 4) and overexpressing katanin produced MT cleavage in nonneuronal cells and somatodendritic regions of neurons [78]. In contrast, axonal MTs are less sensitive to katanin, because axonal tau protects acetylated MTs from katanin [79, 80]. The question of how katanin senses lumenal acetylation of MTs remains to be answered.

MT acetylation was also reported to enhance kinesin-based transport [81], because inhibition of tubulin deacetylase modestly enhanced AMP-PNP mediated kinesin binding to MTs and redistributed a putative kinesin cargo. However, acetylation and kinesin motility were not correlated in vivo [32], and in vitro analysis of kinesin velocity and run length found no effect of MT acetylation [82], leaving the role of acetylation on motor function unresolved.

Deacetylation

While acetylation preferentially occurs on tubulin in MTs, tubulin dimers are rapidly deacetylated when released from MTs [58]. Eukaryotic protein deacetylases comprise two families: 11 classical histone deacetylases (HDACs) [83] and 7 sirtuins [84]. Although HDAC6 is the primary α-tubulin deacetylase in non-dividing cells [85], it has other substrates that may not be related to its effects on tubulin, such as Hsp90, cortactin, β-catenin, and periredoxins. HDAC6 also interacts with ubiquitin independent of its acetylase activity [86], suggesting a role in responding to protein aggregates and other stressors [87]. Thus, manipulations of HDAC6 levels or activity may affect multiple pathways [83-85]. However, while nuclear HDAC knockouts are embryonic or perinatal lethal [83], HDAC6 null mice are viable and develop normally, albeit with hyperacetylated tubulin [88]. The mild phenotype of HDAC6 null mice suggests that compensatory pathways exist for HDAC6 functions. These may include HDACs that are normally nuclear, such as HDAC5, which is exported from the nucleus of sensory neurons after injury and transported into the axon where it can deacetylate tubulin [89, 90].

Nonclassical HDACs or sirtuins (SIRT) are NAD^+-dependent deacetylases and ADP-Ribosyltransferases [84] that are structurally unrelated to classical HDACs [83]. SIRTs have multiple substrates [91], but only SIRT2 deacetylates α-tubulin [92]. SIRT2 is primarily cytoplasmic, but may be shuttled to the nucleus [93]. Although α-tubulins are a preferred cytoplasmic substrate, SIRT2 has nuclear substrates [84] and may play a role in cell cycle regulation by deacetylating histone-4 and/or α-tubulin [94].

While overexpressed SIRT2 and HDAC6 interact and co-immunoprecipitate, either can individually deacetylate α-tubulin [92]. Treating cells with siRNA for SIRT2 or HDAC6 increases tubulin acetylation, but HDAC6 silencing is more effective. SIRT2 deacetylase is regulated by cell cycle dependent kinases including Cdk1, Cdk2 and neuronal Cdk5 [95].

Since Cdk5 is a major neurofilament kinase [96], whose activity is high in neurons [97], SIRT2 deacetylation of tubulin in neurons may be limited due to phosphorylation-dependent inhibition by Cdk5 [98]. However, SIRT2-dependent acetylation of tubulin plays a key role in dividing cells and cancer [91]. Both SIRT2 knockout and SIRT2/HDAC6 double knockout mice are also viable and normal [98], despite tubulin hyperacetylation. The minimal phenotype is puzzling given that tubulin deacetylases have multiple substrates in addition to α-tubulins. A phenotype for these mice may emerge late in life or in response to specific stressors, but neither hypo- nor hyperacetylation of tubulin significantly disrupts cell or organismal function, therefore physiological roles of α-tubulin acetylation remain enigmatic.

Polyamination of tubulin in microtubules

MTs in mature axons are unusually stable, remaining intact after depolymerizing treatments such as cold, Ca^{2+} and antimitotic drugs [2, 99]. This tubulin fraction contains biochemically distinct tubulins significantly more basic than the sequence predicts [99], suggesting a novel posttranslational modification that adds positive charges. Polyamination, in which a transglutaminase (TG) covalently links a polyamine to intracellular proteins is such a modification.

TGs mainly catalyze two reactions on proteins in vivo: 1) Covalent cross-linking of peptides and proteins through γ-glutamyl-ε-lysyl bonds; 2) Polyamination, i.e. covalent formation of γ-glutamyl amine bonds to poly/di/monoamines such as putrescine, spermine and spermidine [2]. When polyamine levels are high, as in the nervous system, polyamination of glutamine predominates and is likely to be irreversible under physiological conditions. TG crosslinking activity is dependent on calcium and can be inhibited by free GTP/GDP [100]. Deamidation may occur in low pH conditions in vitro, where polyamines can be removed. The major cytoplasmic TG (TG2) may also function as a kinase [101], GTPase [100, 102] and protein disulfide isomerase [103].

Modification of proteins by TG2 may play a role in apoptosis [104]. TG2 may stabilize misfolded protein aggregates and promote inflammation in diseases such as Alzheimer's, Huntington's, Parkinson's and amyotrophic lateral sclerosis (ALS) [105, 106]. TG2 knockout mice are viable and grossly normal despite some abnormalities in insulin secretion, wound healing, autoimmune reaction and inflammation [107, 108].

Neuronal tubulin, polymerized MTs and taxol-stabilized MTs are all substrates for TG-catalyzed polyamination in vitro and this modification conferred resistance of MTs against cold and Ca^{2+} [2]. Inhibiting polyamine synthesis by difluoromethylornithine (DFMO) or genetically knocking out TG2 reduced MT stability in vivo [2]. Some TG activity and stable MTs remain in brain homogenates from TG2 null mice, suggesting that multiple TGs contribute to the stabilization of axonal MTs [2].

Polyaminated tubulin facilitated MT nucleation and polymerization in vitro, preventing MT dissociation, consistent with polyamination regulating MT dynamics [109]. TG protein levels and enzymatic activity increase in axons as neurons develop and mature, correlating with increased stable MTs. Inhibiting TG2 activity in culture reduces neurite outgrowth and

maturation, while neurons in a demyelinating mouse model exhibit lower TG2 activity and decreased MT stability [109, 110].

Neuronal structure, axonal guidance, growth and retraction among other parameters require a balance between MT stability and plasticity. Both polyamines and TG levels correlate with MT stability during neuronal development and maturation. Questions remain about the distribution and functions of tubulin polyamination in neurons, but altered MT stability and plasticity via this pathway may affect many aspects of brain development, aging, regeneration and degeneration. Changes in tubulin polyamination may be neuroprotective when MT hyperstability helps maintain axonal structures, but may exacerbate pathology by reducing plasticity and altering synaptic function.

Antibodies against polyaminated tubulins based on identified polyamination sites along MTs [2] will facilitate studies of tubulin polyamination. Similarly, fluorescent TG-inhibitors [111] will help track TG2 distribution and activity at various stages of neurons, defining roles for TG2 in axonal health and disease. Combining these reagents with the TG2 knockout mouse [112] will further our understanding of tubulin polyamination.

Other Modifications of Tubulin

The tubulin PTMs discussed so far affect a large fraction of the tubulin/MTs in the brain and/or other tissues. However, a number of additional modifications have been reported, which are less abundant or less common. These PTMs may affect minor fractions of tubulin or MTs, may be restricted to specific cell types or subcellular compartments, or may be transient. Nonetheless, they appear to play an important role in the functional diversity of MTs.

Phosphorylation of tubulin

Phosphorylation is one of the most common posttranslational modifications. Although phosphorylation of microtubule-associated proteins receives the most attention, tubulins can also be phosphorylated. Phosphorylation of β-tubulin was initially reported in MTs of differentiated neuroblastoma cells [113]. Subsequently, α and β-tubulins were shown to be phosphorylated by serine/threonine kinases like Cdk1, CK2, and CamKII as well as tyrosine kinases like Jak2, Syk, Fes, and Src [4]. Most of these kinases phosphorylate tubulin in MTs [4], but the cell cycle kinase Cdk1 phosphorylates S172 on soluble β-tubulin and inhibits polymerization [114]. Tubulin phosphorylation may also be associated with MT interactions with membranes [4] or the regulation of polymerization [114, 115].

Glycosylation and Glycation of tubulin

Carbohydrates can be added to tubulin through either O-linked glycosylation [116, 117] or non-enzymatic glycation [118]. O-linked glycosylation often occurs on phosphorylation sites and may regulate protein-protein interactions and activity. Tubulin O-linked glycosylation appears heterogeneous and cell specific, but our knowledge regarding its cellular functions is limited. It is known that glycosylated tubulin can assemble into MTs and MTs near the nucleus are positive for a sialyloligosaccharide antibody [117]. The differentiation of a neuron-like cell line by retinoic acid alters O-GlcNAcylation of α and β-

tubulins and inhibits tubulin polymerization [119]. Furthermore, glycosylation of TUBB3 may be associated with resistance to chemotherapeutic drugs [116].

Whereas O-linked glycosylation is a normal physiological event, non-enzymatic glycation is pathological and associated with diabetes [118]. Proteins are glycated when high levels of glucose associated with hyperglycemia react with amino acids [120]. Tubulin glycation in axons is significant in diabetes and may be a factor in diabetic neuropathy [118] as glycation may denature tubulin and lead to depolymerization.

Palmitoylation of Tubulin

Low levels of tubulin in membrane fractions are consistently observed [121], but poorly understood. Palmitoylation can affect protein interactions with membranes and subcellular trafficking [122]. While multiple mechanisms may mediate tubulin interactions with membranes, palmitate can be reversibly linked to α-tubulin Cys376 [123]. Palmitoylation of tubulin may affect membrane microviscosity or alter tubulin interaction with membrane proteins [121]; however, palmitoylated tubulin is not restricted to membranes. For example, C376S mutant α-tubulin affects organization of yeast astral MTs and nuclear positioning [123]. In addition, palmitoylated tubulin does not cycle efficiently, but can incorporate into MTs [124]. The antimitotic vinblastine inhibits tubulin palmitoylation [125], but it is not known whether this plays a role in the action of vinblastine on MT polymerization.

Ubiquitylation and Sumoylation of Tubulin

Ubiquitin and SUMO are small proteins added covalently to proteins by selective ligases [126, 127]. The addition of polymeric ubiquitin or SUMO is typically associated with proteasomal degradation, but adding a single ubiquitin or SUMO may affect protein interactions, enzyme activity, protein transport or localization [126, 128].

The ubiquitin E3 ligase parkin, which is mutated in some cases of familial Parkinson's disease [129, 130], binds and ubiquitylates tubulin dimers, increasing tubulin degradation or recycling [131]. Parkin and ubiquitinylated α-tubulin can accumulate in aggregates of Parkinson's disease and ALS [132, 133], which is enhanced by proteasome inhibition [134]. Ubiquitylation of α-tubulin, but not β-tubulin was essential for the disassembly and resorption of flagella in Chlamydomonas [135], but the responsible E3 ligase was not identified. In addition, sumoylated tubulin was identified in proteomic screens in yeast [136] and then mammalian cells [137], where TUBA1, TUBA6 and TUBB6 were modified. Sumoylation is associated with cellular functions that involve MTs such as intracellular trafficking and plasticity and may be altered in neurological diseases [126]. Thus, these modifications play a role in the degradation of tubulin, but may have additional effects that are less well understood.

Concluding remarks

Relating specific tubulin modifications to specific cellular functions remains a major challenge. Initial correlations often broke down with additional study due to the overlap of different modifications. Nonetheless, common themes are emerging and questions continue to arise (Outstanding questions box). First, the regulation of MT dynamics remains a key

aspect of tubulin PTMs, but new mechanisms have been identified and new functions are emerging. Second, MTs are intrinsically heterogeneous. There are not only tyrosinated plus ends, but other PTMs may be concentrated in specific domains along the MT and these domains may overlap. Individual subunits within the MT may be unmodified or have multiple modifications. Third, PTMs affect the interaction of specific proteins with MTs, such as the association of some +TIP proteins with tyrosinated tubulin or the targeting of severing proteins by acetylation and polyglutamylation. As a result, PTMs may play a critical role in defining MTs as a cellular compartment for organizing signaling cascades within the cell. Fourth, the cellular contexts of PTMs remain critical for proper cell function, including enrichment of polyamination in axons and polyglycylation in cilia. MTs in these cellular domains require specific properties, such as the need for unusual stability in axonal domains that may extend a meter or more from the primary site of tubulin synthesis. Finally, tubulin PTMs are dynamic and must be regulated during development and differentiation and in response to injury and stress. Just as MTs can be dynamic or long-lived, both the distribution and levels of a given PTM may be changed in response to physiological and pathological cues.

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Highlights

- Tubulin modifications contribute to functional diversity of MTs
- PTMs affect MT dynamics, organization and interactions with other cellular components
- Various PTMs overlap or localize to specific MT domains demonstrating heterogeneity
- PTMs vary with time, cell type, subcellular compartment and physiological state

Outstanding Questions Box

Many substantive questions remain about tubulin PTMs:

- How can we reconcile in vitro and in vivo effects of tubulin PTMs?
- Which PTMs regulate MT dynamics and which ones change as a consequence of altered dynamics?
- What are the enzymes responsible for specific tubulin PTMs in vivo, i.e. what is the carboxypeptidase responsible for detyrosination and the removal of glycylation?
- Why are there multiple enzymes to make the same modification and how do their physiological roles differ?
- What is the role of modified MTs as scaffolds for localization of signaling complexes?
- What are the implications of PTMs in neurodevelopment and neurological disorders?

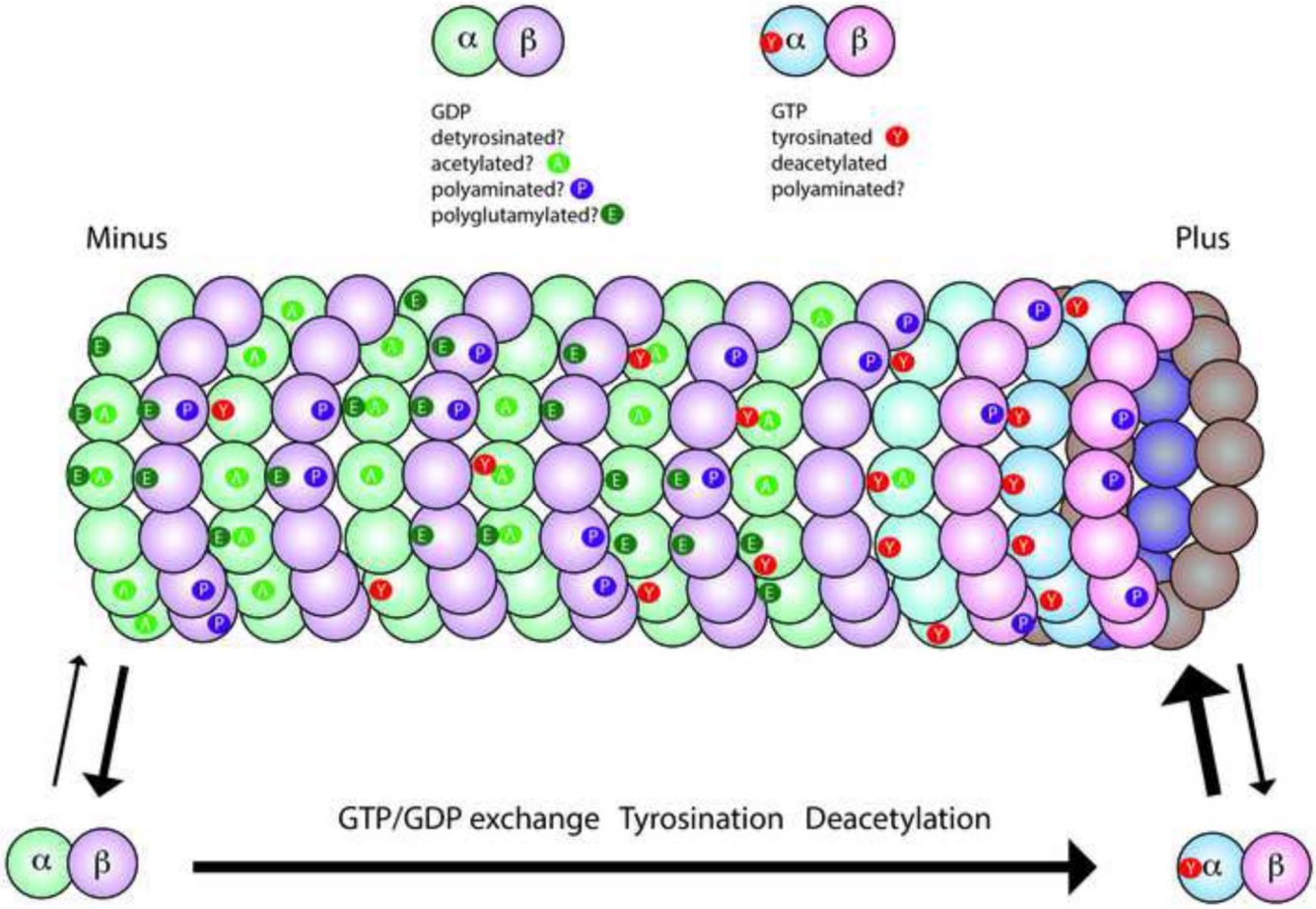

Figure 1. Microtubules and tubulins are subject to a variety of posttranslational modifications

This diagram illustrates the major modifications in axonal microtubules. Tubulin dimers are GTP-rich (blue/pink shading) in the soluble pool and on the plus end of the microtubule, but polymeric tubulin gradually hydrolyzes GTP and becomes GDP-rich (green/purple shading). Some modifications are associated specifically with tubulin polymerized in microtubules including acetylation (A), detyrosination, and polyglutamylation (E), while others may occur only on soluble tubulin like tyrosination (Y), or on either soluble or polymerized tubulin like polyamination (P). Some tubulins will have a single modification, while others have multiple modifications and some have no modification. However, the exact distribution along a microtubule and the fraction of the tubulin modified is highly variable, even within a single cell. A few rules have emerged such as tyrosinated tubulin is enriched at the plus ends of growing microtubules, while acetylation and detyrosination are more likely to be seen in the middle or minus end of the microtubule.

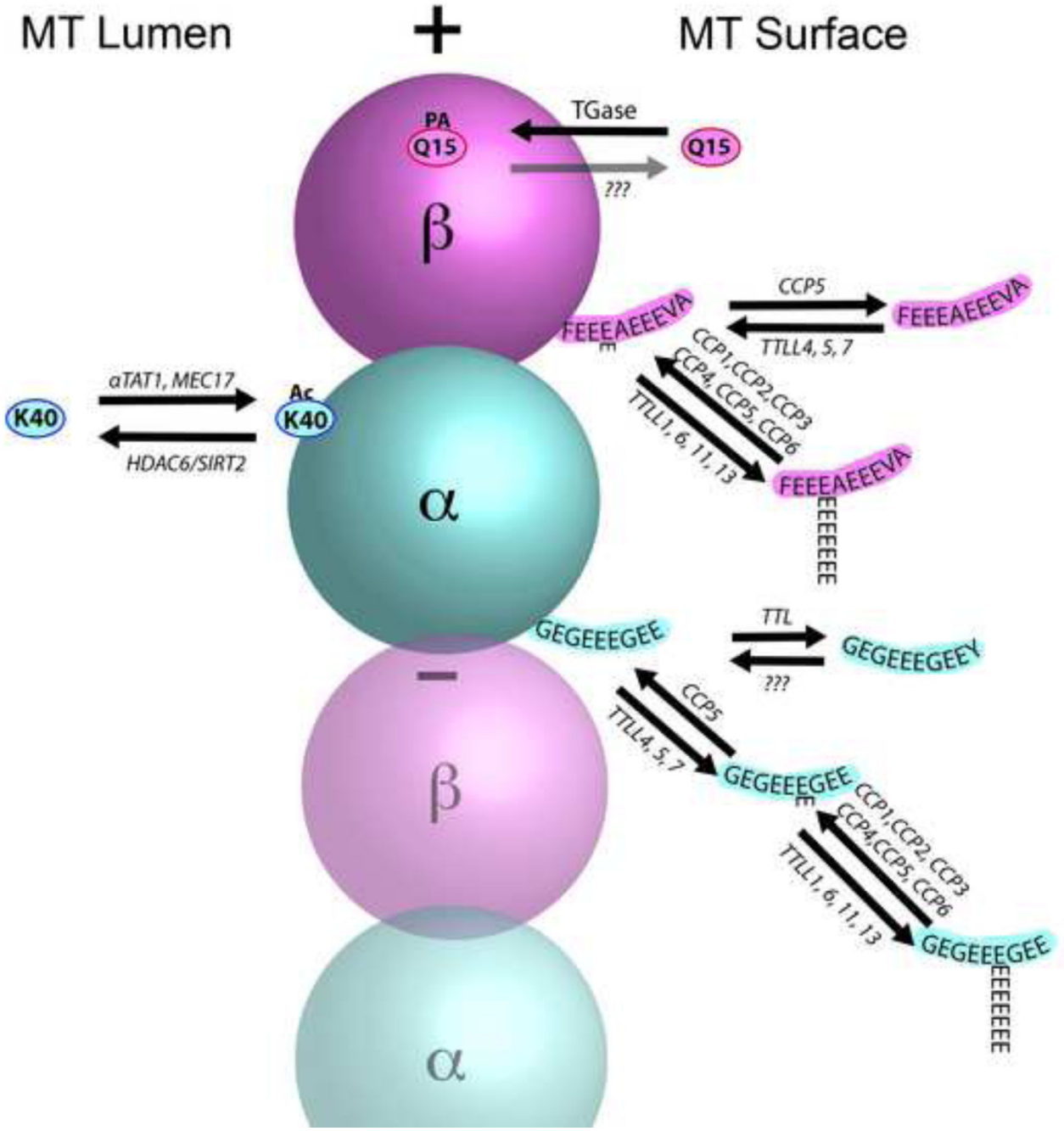

Figure 2. Pathways and sites for the major modifications of tubulin in a microtubule

Acetylation is primarily found on K40 of α-tubulins, which is exposed in the lumen of the microtubule. αTAT1 and Mec17 are the major enzymes responsible for acetylation (Ac), and HDAC6 and SIRT2 are the enzymes involved in deacetylation. The C-terminus of α-tubulin is a hot spot for modification and various combinations are illustrated. Tubulin Tyrosine Ligase (TTL) is the enzyme responsible for tyrosination of tubulin dimers, but the enzyme that catalyzes detyrosination remains to be identified. In contrast, multiple enzymes are capable of removing the penultimate glutamate residue to create Δ2-tubulin, including all

six Cytoplasmic Carboxypeptidases (CCP1-6). These same enzymes can also trim the length of polyglutamylated chains, however, only one enzyme has been shown to remove the final iso-peptide linked glutamate (CCP5). Detyrosination is restricted to α-tubulins, but glutamylation and polyglutamylation can also occur on various glutamates in the C-terminal domain of β-tubulins. Multiple related enzymes have been identified that can add the iso-peptide link for initiating polyglutamylation (Tubulin Tyrosine Ligase Like family members (TTLL4,5,7)). Other members of the same family (TTLL1, 6, 11, 13) can add glycine to the same set of residues in the C-terminal domain of tubulins (not shown), but this reaction is seen only in ciliary microtubules. Curiously, the enzyme responsible for extension of polyglycine chains (TTLL10) is not functional in humans and only monoglycine modifications are seen. Finally, polyamination (PA) is mediated by transglutaminases, particularly TG2 in the nervous system. One putative modification site for polyamination identified by Mass Spectrometry is the conserved Q15 of β-tubulins. However, several other sites on both α- and β-tubulin may also be subject to polyamination. Enzyme(s) responsible for removing polyamines have not been confirmed and the degree to which polyamination is reversible in vivo is uncertain.

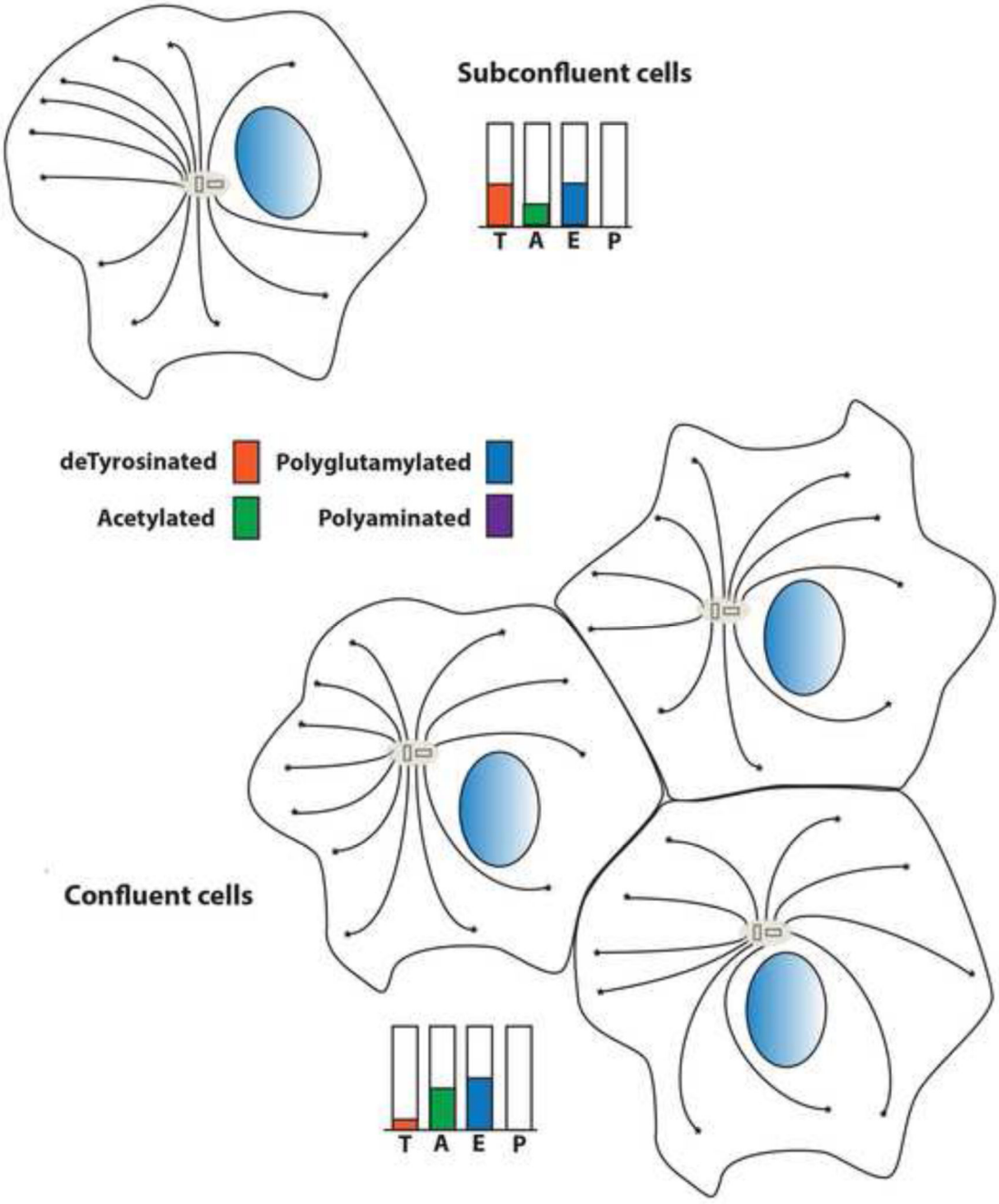

Figure 3. Posttranslational modifications of microtubules are dynamic and vary with cellular context

In some epithelial cells in culture, the degree of cell confluency and polarity of the cells affects the distribution and organization of modified microtubules. At early stages, microtubules are anchored to the microtubule organizing center at their negative ends. In subconfluent cells, microtubules are relatively rich in detyrosinated tubulin, but poor in acetylated tubulins. In confluent cells, the microtubule organization becomes more symmetrical and detryrosinated microtubules are reduced as acetylation of tubulin increases. Polyglutamylation levels appear to decrease slightly between these two states.

Polyamination is generally undetectable in non-neuronal cells and may be specific to neurons.

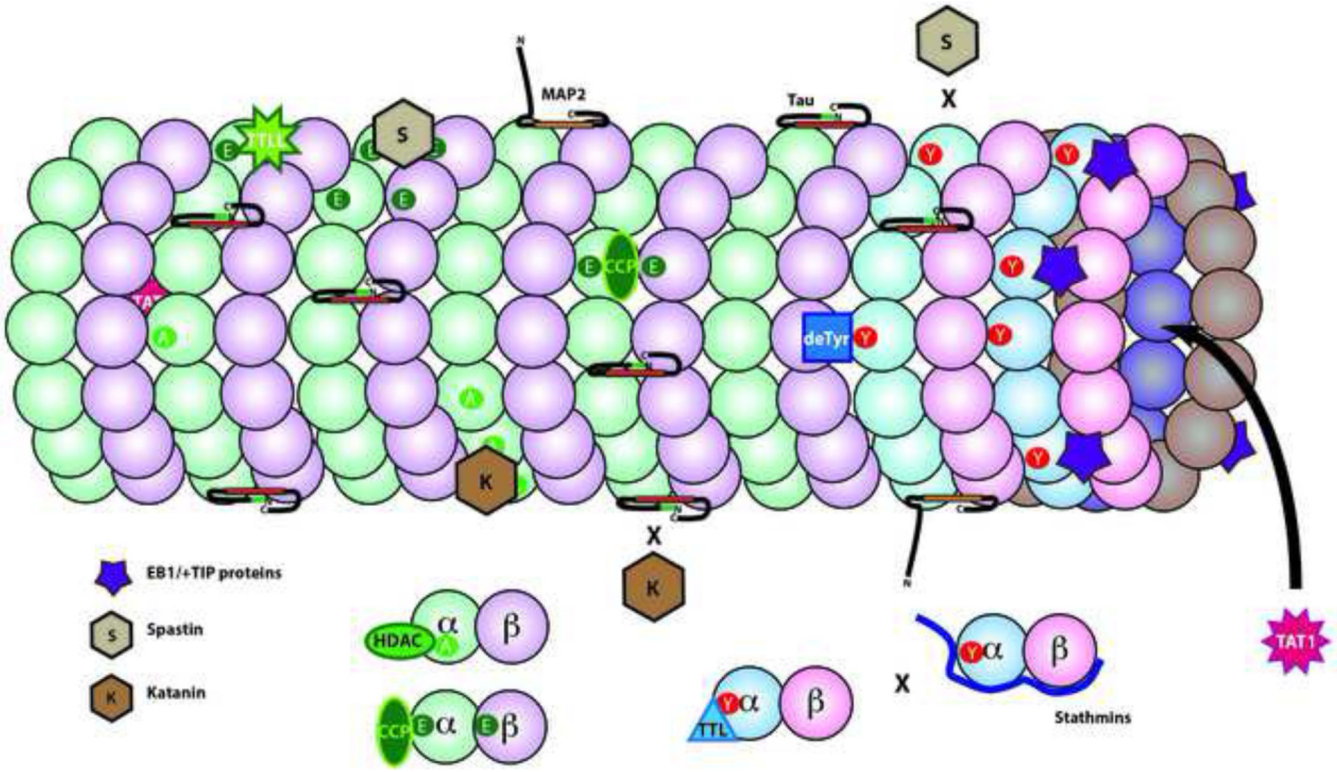

Figure 4. Microtubule Associated Proteins and Posttranslational Modifications of Tubulins

One function of tubulin modifications is to affect the interaction of other proteins and enzymes. In complex with EB1, some +TIP binding proteins (purple 5 point star) bind selectively to tyrosinated tubulin. As a result, these +TIP proteins are enriched on the plus end of dynamic microtubules, which are rich in newly polymerized tyrosinated α-tubulin. Other proteins interact with particular tubulin isotypes like a subset of α-tubulins (deTyr for detyrosination) or to the C-termini of both α- and β-tubulins (TTLLs for polyglutamylation or polyglycylation). Tubulin acetylases (TAT1) are unique in binding to the interior of the microtubule lumen. Deacetylases (HDAC) or Tubulin Tyrosine Ligase (TTL) bind only to tubulin dimers. Stathmins bind to free tubulins as well, but partially overlap and compete with tubulin binding to TTL. The microtubule severing proteins, katanin (K) and spastin (S) are directed to particular microtubule domains by a combination of tubulin modifications and microtubule associated proteins. Katanin preferentially binds and severs acetylated domains of a microtubule, but katanin binding is inhibited by the presence of tau. In contrast, spastin preferentially binds and severs in polyglutamylated domains, but does not sever microtubules enriched in tyrosinated tubulin.

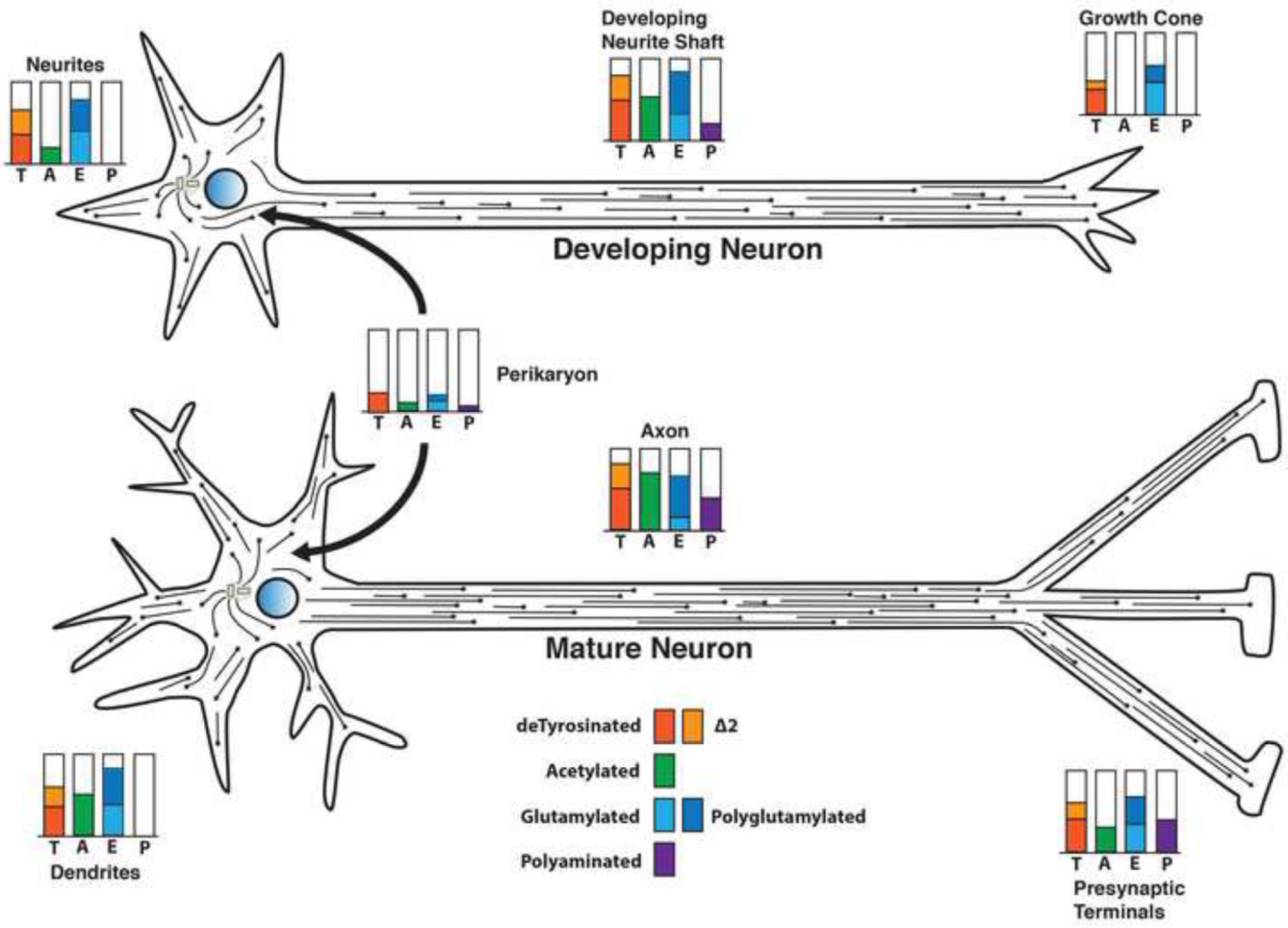

Figure 5. Posttranslational modifications of tubulin and microtubules vary in different regions of a neuron and change during neuronal differentiation

Microtubules in neuronal axons and dendrites are not anchored at the microtubule organizing center. The microtubules vary in length but have both plus and minus ends free. In the developing neuron (upper), when the axon is extending but the dendrites have not yet differentiated, the microtubules in all neurites are plus end out. Modification levels for detyrosination (T), Δ2 tubulin (Δ2), glutamylation (E), and polyamination (P) in the neuronal perikaryon are relatively low. Acetylation, glutamylation and detyrosination are elevated in the growing axon, but all three are reduced in the growth cone. Little or no acetylation is seen in the microtubules of the growth cone, consistent with the presence of highly dynamic microtubules. The minor neurites at this stage are lower in acetylation, but relatively rich in detyrosinated and glutamylated/polyglutamylated microtubules. Polyamination may be detectable in the growing axon at a low level.

In a mature neuron (lower), as both dendrites and synaptic specializations form, tubulin modifications change both quantitatively and qualitatively. Dendritic microtubules exhibit mixed polarity as dendrites form, while axonal microtubules remain plus end out. Polyamination of axonal microtubules increases with maturation, but may be relatively low or absent from dendritic domains. Detyrosinated and Δ2 tubulin levels remain relatively high in differentiated dendrites and axons, while acetylation increases in both axon shafts and

dendrites. Acetylation is also detectable in the distal axon and presynaptic regions, consistent with reduced numbers of highly dynamic microtubules in stable connections. During the differentiation and maturation of neurons, there are also changes in microtubule associated proteins in different neuronal subcellular domains. Adapted from Janke and Kneussel [5].

Table 1

Posttranslational Modifications of tubulin and microtubules.

Modification	α/β	site	enzyme	references
Acetylation Deacetylation	α α	Lys40	TAT (MEC17) HDAC6, SIRT2, HDAC5	[58] [83, 89, 92]
Detyrosination Tyrosination	α α	C-Terminal Tyr Add to C-terminal	Unknown TTL	[3] [10, 11, 17]
Δ2deglutamylation)	α	Removal of C-terminal Glu of detyrosinated MTs	CCP1, CCP2, CCP3, CCP4, CCP5, and CCP6	[35, 37]
Glutamylation Deglutamylation of branch point Glu	α/β α/β	Various C-terminal Glu	TTLL4, 5, and 7 CCP5 & CCP1, CCP4, CCP6	[3, 4] [36, 37]
Polyglutamylation Deglutamylation to shorten polyGlu side chain	α/β	Add γ-linked Glu to C-terminal	TTLL1, 6, 11 and 13 CCP1, CCP4, CCP6	[3, 4] [37]
Glycylation Polyglycylation Deglycylation	α/β	Add γ-linked Gly to C-terminal Glu Addition to γ-linked Gly C-terminal	TTLL3 and 8 TTLL10 Unknown	[54, 55] [54] [3, 4]
Polyamination	α/β	*β* tubulins Q15, others	Transglutaminase	[2]
Glycososylation	α/β	various	Unknown	[4]
Glycation	α/β	various	Nonenzymatic	[118]
Palmitoylation	α	Cys376	Unknown	[121]
Phosphorylation	α/β	β tubulin Ser172, others	Various	[4]
Sumoylation	*α*	multiple	Unknown	[136, 137]
Ubiquitylation	*α*	Multiple	Parkin	[131]

Blue, modifications on tubulin in MTs; red, modifications restricted to soluble tubulin dimers; and black, modifications that are either undetermined or occur on both.