The tubulin code and its role in controlling microtubule properties and functions

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Abstract | Microtubules are core components of the eukaryotic cytoskeleton with essential roles in cell division, shaping, motility and intracellular transport. Despite their functional heterogeneity, microtubules have a highly conserved structure made from almost identical molecular building blocks: the tubulin proteins. Alternative tubulin isotypes and a variety of post-translational modifications control the properties and functions of the microtubule cytoskeleton, a concept known as the 'tubulin code'. Here we review the current understanding of the molecular components of the tubulin code and how they impact microtubule properties and functions. We discuss how tubulin isotypes and post-translational modifications control microtubule behaviour at the molecular level and how this translates into physiological functions at the cellular and organism levels. We then go on to show how fine-tuning of microtubule function by some tubulin modifications can affect homeostasis and how perturbation of this fine-tuning can lead to a range of dysfunctions, many of which are linked to human disease.

Axonemes
A tubular structure built from microtubules and associated proteins at the core of all eukaryotic cilia and flagella. In motile cilia and flagella, the axoneme consists of nine microtubule doublets arranged around a central microtubule pair, accessory proteins and axonemal dynein motors that ensure the beating of cilia. Primary cilia lack the motor protein and central-pair microtubules.

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Microtubules are cytoskeletal filaments with an outer diameter of approximately 25 nm, and are composed of heterodimers of globular α-tubulin and β-tubulin molecules. As they are hollow cylinders, microtubules are mechanically rigid[1], thus allowing their assembly into large intracellular structures. These structures are essential for cell function and include mitotic and meiotic spindles, which ensure the correct division of cells[2–4], axonemes, which are the central molecular machines of cilia and flagella[5–7], and the neuronal cytoskeleton, which controls the connectivity and function of neurons[8–10]. Microtubules are intrinsically dynamic as they can alternate between phases of polymerization and spontaneous depolymerization in a process known as dynamic instability[11]. How the cell tames this fluctuating system into highly ordered and controlled structures has been studied by a large scientific community for more than half a century[12].

Since the early ultrastructural analyses of microtubules by electron microscopy[13,14], advances have been made towards understanding how nearly identical microtubules made of highly conserved α–β-tubulin heterodimers can carry out such diverse cellular functions[15–19]. Microtubules do not function alone; they interact with a diverse array of microtubule-associated proteins (MAPs) that influence their assembly and dynamics. Some MAPs specifically bind to the plus or the minus ends of microtubules to control microtubule dynamics and the attachment of microtubules to other cellular structures[20]. Other MAPs bind to the microtubule lattice along its entire length and are thus considered to regulate microtubule dynamics and stability, but might also have additional roles that in many cases remain to be explored[21]. In addition, active molecular motors either carry cargos along microtubules or generate forces within microtubule assemblies. Specific combinations of non-motile MAPs and molecular motors can therefore explain the mechanisms underlying self-organizing assemblies such as mitotic and meiotic spindles[22,23], or the highly controlled wave-like beating of flagellar axonemes[24,25].

By contrast, how the incorporation of specific α-tubulin or β-tubulin variants (known as tubulin isotypes) or the post-translational modification (PTM) of tubulins can functionally modulate microtubules remained mostly unexplained until the beginning of the twenty-first century. The concept that molecular patterns generated by combinations of tubulin isotypes and PTMs, termed the 'tubulin code'[26], could control microtubule functions was proposed as early as the 1970s[27,28], but resisted a thorough functional characterization for many years. Recent advances revealed that the tubulin code acts in many instances as a fine regulator, and not as a binary switch, of microtubule functions. While this might have only a minor impact at the level of single cells, dysfunction of these fine-tuning mechanisms can lead to massive perturbations of homeostasis and eventually result in disease. In this Review we summarize the

Marginal band
A microtubule coil of precisely 12 turns at the outer rim of blood platelets.

current understanding of the tubulin code, its elements and their regulation and discuss the functional implications of the tubulin code for the cell and for the organism as a whole.

Elements of the tubulin code

Microtubules exist in every eukaryotic cell. The striking sequence conservation of tubulins throughout evolution is reflected by their near identical structure across virtually every species in which tubulin structure has been investigated so far[29,30]. Tubulin across all eukaryotic organisms assembles into microtubules, hollow tubes that are most often built of 13 chains of stacked α–β-tubulin dimers known as protofilaments. Despite their high degree of evolutionary conservation, microtubules can show different behaviours, properties or even structures between species and cell types or even within single cells owing to the incorporation of different tubulin isotypes and their PTM.

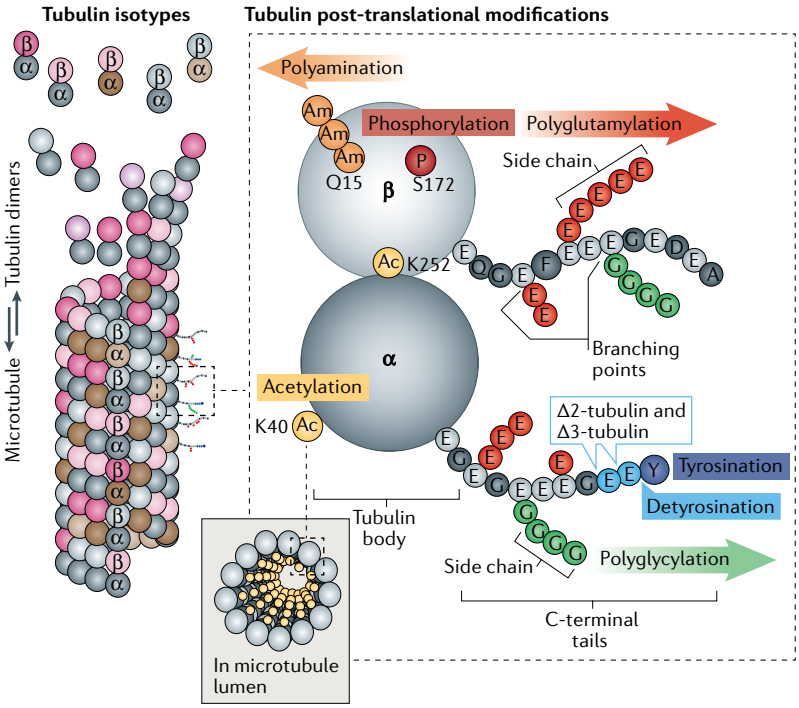


Fig. 1 | **The elements of the tubulin code.** Microtubules dynamically assemble from dimers of α-tubulins and β-tubulins. Tubulins are highly structured proteins, forming 'tubulin bodies', whereas their C-terminal amino acids form unstructured tails that protrude from the microtubule surface. The tubulin code refers to the concept that different tubulin gene products, together with a variety of post-translational modifications (PTMs), modulate the composition of individual microtubules. Tubulin isotypes (α-tubulins, dark grey and brown; β-tubulins, light grey and pink) are encoded by different tubulin genes and can mix during microtubule assembly. Tubulin PTMs are catalysed by a range of enzymes (TABLE 1) and are located either at the globular, highly structured tubulin bodies (for example, acetylation (Ac), phosphorylation (P) and polyamination (Am)), or at the unstructured C-terminal tails of tubulin (for example, glutamylation, glycylation, tyrosination, detyrosination and removal of glutamate residues to produce Δ2-tubulin and Δ3-tubulin). Tubulin PTMs can generate binary switches (on–off signals) by adding or removing single functional residues (acetylation, phosphorylation or detyrosination) or can gradually modulate the strength of their signals by adding different numbers of residues (polyamination, polyglutamylation and polyglycylation). Polymodifications are initiated by the generation of a branching point, from which side chains are further elongated. Figure adapted with permission from REF.[304], Elsevier.

Tubulin isotypes

Tubulin isotypes arise from the expression of alternative tubulin genes, and their numbers differ largely between species and phyla. In yeast, for example, two genes encode α-tubulin[31], and only one gene encodes β-tubulin[32], whereas the human genome contains nine genes for each[33]. There is no clear evolutionary trajectory of these tubulin genes, which is why orthologues can be identified only in evolutionarily close species. This fact is reflected in the confusing nomenclature of the tubulin genes[34]. 'Generic' α-tubulin and β-tubulin isotypes are highly conserved between evolutionarily distant species, whereas less common isotypes appear to have evolved as novel microtubule functions arose. For example, β1-tubulin (encoded by *TUBB1*) has co-evolved[35] with platelets, small cell fragments essential for blood coagulation that are unique to mammals. Platelets assemble a specialized microtubule array called the 'marginal band', which requires β1-tubulin[36], a highly divergent isotype in the vertebrate phylum.

In *Drosophila melanogaster*, β3-tubulin (encoded by *βTub60D*) is expressed in subsets of cells during development[37]. Gene knock-in experiments demonstrated that this isotype cannot replace the generic β2-tubulin (encoded by *βTub85D*) in key microtubule functions in the testes, such as axoneme assembly or spindle formation[38], suggesting that β3-tubulin had evolved to regulate specific microtubule functions in the fly.

Although these examples show that some isotypes are essential to form functionally specialized microtubule arrays, it is unclear why so many tubulin isotypes are almost identical across many species, including mammals. We consider this question in this Review.

Tubulin PTMs

Tubulin is subjected to a large number of PTMs (FIG. 1; TABLE 1). Some of these modifications, such as phosphorylation[39–55], acetylation[56], methylation[57], palmitoylation[58], ubiquitylation[59,60] and polyamination[61] are found on a broad range of proteins; others were initially discovered on tubulin. Examples of such PTMs are the enzymatic, ribosome-independent incorporation of tyrosine (tyrosination)[62,63], glutamate (glutamylation and polyglutamylation)[64–66] and glycine (glycylation and polyglycylation)[67]. Moreover, enzymatic removal of single amino acids, such as tyrosine from the C terminus of α-tubulin (detyrosination)[68,69], or generation of Δ2-tubulin[70,71] or Δ3-tubulin[72] through subsequent removal of glutamate residues from the detyrosinated α-tubulin C terminus (BOX 1; FIG. 1; TABLE 1) was also first discovered in tubulin. Although tubulin is the main substrate for glutamylation and glycylation in a variety of species, other substrates have also been described (Supplementary Box 1).

Most PTMs label distinct microtubule subpopulations in cells and are expected to programme these microtubules for specific functions. Enzymes catalysing detyrosination[73], acetylation[74] and polyglutamylation[75] preferentially modify microtubules over soluble tubulin dimers, indicating that targeted modification of selected microtubules in cells is mechanistically feasible. Reverse enzymes such as deglutamylases, in contrast, appear to work equally well on soluble tubulin dimers and microtubules[76].

Table 1 | **Enzymes catalysing tubulin post-translational modifications**

Modification sites	Forward enzymes	Reverse enzymes
Acetylation		
α-Tubulin Lys40 (REF.[102])	α-Tubulin acetyltransferase 1 (ATAT1)[74,236]	Histone deacetylase 6 (HDAC6)[244]; sirtuin 2 (SIRT2)[306]
β-Tubulin Lys252 (REF.[117])	San acetyltransferase[117]	Not known
Methylation		
α-Tubulin Lys40 (REF.[57])	SET domain-containing protein 2 (SETD2)[57]	Not known
Detyrosination		
α-Tubulin C-terminal Tyr residue	Vasohibin (VASH) proteins[228,229] in complex with small vasohibin-binding protein (encoded by *SVBP*)[307–311]	Tubulin–tyrosine ligase (TTL)[312]
Generation of Δ2-tubulin and Δ3-tubulin (removal of C-terminal Glu residues from detyrosinated α-tubulin[71,72])		
α-Tubulin penultimate C-terminal Glu residues	Cytosolic carboxypeptidases (CCPs) (encoded by *AGTPBP1*, *AGBL1*, *AGBL2*, *AGBL3*, *AGBL4* and *AGBL5*)[248,313,314]	No reverse reaction known to date. Tyrosination of Δ2-tubulin by TTL is not possible[71,78]
Glutamylation or polyglutamylation (addition of Glu to γ-carboxy group of Glu side chains and chain elongation by further addition of Glu residues)		
α-Tubulin and β-tubulin C-terminal tails (multiple Glu residues can be modified)[64–66]	Tubulin–tyrosine ligase-like (TTLL) proteins, multiple members in most organisms (9 glutamylases in mammals)[152,249,315]	CCPs, multiple members in most organisms (6 deglutamylases in mammals)[248,313,314]
Glycylation or polyglycylation (addition of Gly to γ-carboxy group of Glu side chains and chain elongation by further addition of Gly residues)		
α-Tubulin and β-tubulin C-terminal tails (multiple Glu residues can be modified)[67,209]	TTLL proteins, multiple members in most organisms (3 glycylases in mammals)[196,300,316]	No reverse reaction or enzymes known
Polyamination (addition of polyamines to the γ-carboxamide group of Gln side chains)		
α-Tubulin and β-tubulin, major modification site β-tubulin Gln15 (REF.[61])	Transglutaminases[61]	No reverse reaction or enzymes known
Phosphorylation (addition of phosphate group to Ser, Thr or Tyr)		
β-Tubulin Ser172 (REF.[53])	Cyclin-dependent kinase 1 (CDK1)[53]	Not known
β-Tubulin Ser172 (REF.[55])	Dual-specificity tyrosine-regulated kinases (DYRK1A, Minibrain)[55]	Not known
β3-Tubulin Ser444 (REF.[47])	Not known	Not known
α-Tubulin Tyr432 (REF.[51])	Spleen tyrosine kinase (SYK)[51]	Not known
α-Tubulin and β-tubulin Tyr residues (not identified)[42,49]	Neuronal proto-oncogene tyrosine-protein kinase SRC[42,49]	Not known
Ubiquitinylation (addition of ubiquitin to Lys residues of tubulin[59,317])		
α-Tubulin, major modification site Lys304 (REF.[60])	Not known	No reverse reaction or enzymes known
Sumoylation (addition of SUMO to Lys residues of tubulin[318])		
α-Tubulin (modification site unknown)[318]	Not known	No reverse reaction or enzymes known
Palmitoylation (addition of long-chain fatty acid palmitate to Lys residues of tubulin)		
α-Tubulin, major modification site Lys376 (REF.[58])	Not known	No reverse reaction or enzymes known

Some reverse enzymes, such as tubulin–tyrosine ligase (TTL), exclusively modify tubulin dimers[77–79], thus ensuring that the non-polymerized tubulin pool in cells can be 'reset' to the unmodified state before reincorporation into newly polymerizing microtubules.

In the past decade, great advances in our understanding of the biological roles of tubulin acetylation, tyrosination and detyrosination, polyglutamylation and polyglycylation have been made, which is why we focus on these PTMs in this Review.

Regulation of microtubule properties

The concept that the incorporation of different tubulin variants can affect the intrinsic properties of microtubules, such as flexibility or assembly–disassembly dynamics, is as old as the discovery of tubulin isotypes[27]. However, mechanistic insights into how tubulin isotypes and PTMs control microtubule properties have mostly been obtained in recent years.

Control of mechanical properties

The tubulin code determines structural features of microtubules. Recent advances in cryo-electron microscopy have provided high-resolution structures of entire microtubules[16,17,29,80,81] that directly visualize the amino acid residues of α-tubulins and β-tubulins involved in the formation of the microtubule lattice. These structures make it possible to model how different tubulin isotypes, which often differ by only a few amino acids, could alter the properties of microtubules. Indeed, the

A tubules
Components of the microtubule doublets of axonemes. A tubules are generic microtubules made of 13 protofilaments.

evolutionarily distant mammalian and yeast tubulins assemble into highly similar 13-protofilament microtubules, but show some differences at the ultrastructural and mechanical levels[29,82]. Novel approaches to generating recombinant mammalian tubulin[83–85] have further demonstrated the strong impact that mammalian β-tubulin isotypes have on the structural features of microtubules. For example, dimers of α1B-tubulin and β2B-tubulin (encoded by *TUBA1B* and *TUBB2B*) preferentially assemble into 14-protofilament microtubules in vitro, while dimers of α1B-tubulin and β3-tubulin (encoded by *TUBA1B* and *TUBB3*) mostly form 13-protofilament microtubules[85].

In *Caenorhabditis elegans*, a worm composed of only ~1,000 somatic cells, the structure of microtubules differs between cell types. Most somatic cells in *C. elegans* contain 11-protofilament microtubules; however, some neurons assemble hyperstable 15-protofilament tubes (FIG. 2a), and microtubule doublets in cilia have A tubules of 13 protofilaments[86]. This diversity in microtubule structure is mirrored by the large sequence variability between *C. elegans* tubulin isotypes. Indeed, specific α-tubulin (MEC-12 (REF.[87])) and β-tubulin (MEC-7 (REF.[88])) isotypes are required for the assembly of the neuronal 15-protofilament microtubules, whereas the α-tubulins TBA-6 and TBA-9 and the β-tubulin TBB-4 are essential for ciliary microtubule functions[89,90] in this organism. The concept that tubulin isotypes are determinants of protofilament number was further corroborated by a cross-species study showing that the formation of 16-protofilament accessory microtubules, which are normally found in the sperm tails of the moth *Heliothis virescens* but not in those of *D. melanogaster*, could be induced by expressing the testis-specific β2-tubulin isotype from *H. virescens*[91] in *D. melanogaster*.

Direct evidence for the intrinsic capacity of tubulin isotypes to determine microtubule structure was recently provided by assembling microtubules from purified *C. elegans* and bovine brain tubulin in vitro. Similarly to observations made in the cells of these organisms, *C. elegans* tubulin preferentially assembled into 11-protofilament microtubules, whereas bovine brain tubulin formed 13-protofilament and 14-protofilament microtubules[30]. Although it cannot be excluded that PTMs present on these purified tubulins influenced protofilament numbers, these in vitro experiments provide strong evidence that tubulin isotypes directly determine microtubule structure. In cells, however, microtubules assemble in the presence of MAPs such as the neuronal migration protein doublecortin[92], or Bim1p, the yeast orthologue of the microtubule plus end-binding (EB) protein EB1 (REF.[82]), which may further influence protofilament numbers.

Finally, emerging evidence suggests that tubulin PTMs can also influence the structure of microtubules. The assembly of the characteristic 15-protofilament microtubules in *C. elegans* touch-receptor neurons (FIG. 2a), for instance, is dependent on tubulin acetylation mediated by α-tubulin *N*-acetyltransferase 1 (ATAT1: encoded by *mec-17*), and the absence of this enzyme routinely results in microtubules with lower protofilament numbers[93,94]. Moreover, mice lacking the glutamylase tubulin–tyrosine ligase-like 9 (TTLL9) show defects in the characteristic structure of ciliary axonemes, such as missing doublets[95].

Tubulin isotypes determine the mechanical features of microtubules. Mechanical bending of microtubules requires adjacent protofilaments to slide against each other, a process that is affected by non-covalent interactions between tubulin molecules of neighbouring protofilaments[96,97]. No direct evidence for the involvement of isotypes in microtubule flexibility has been reported so far, but studies of blood platelets have implicated isotype choice as a factor in regulating microtubule flexibility. Platelets attain their round shape and defined diameter from the assembly of a microtubule coil of precisely

Box 1 | Complex PTMs on the C-terminal tails of tubulin

The complexity of post-translational modifications (PTMs) is particularly high on the C-terminal tails of tubulins. The majority of α-tubulin genes in most organisms encode a C-terminal tyrosine or phenylalanine, which can be enzymatically removed[69] and readded[63]. It was surprising that the initial PTM is the removal, and not the addition, of a functional group. The discovery of tyrosination was the first observation of the enzymatic incorporation of an amino acid into a peptide chain without mRNA and a ribosome[62,63]. Although the tubulin PTM became known as tubulin tyrosination, the enzymatic detyrosination is the actual modification for most α-tubulin isotypes, apart from those missing the C-terminal tyrosine, such as the mammalian α4A-tubulin[299].

The enzymatic removal of C-terminal tyrosine can be followed by further amino acid cleavages that on mammalian α-tubulin give rise to Δ2-tubulins and Δ3-tubulins, which lack the first and second glutamate residues before the C-terminal tyrosine, respectively[71,72] (FIG. 1). Other amino acid residues in tubulin C-terminal tails might also be edited; an antibody specific to Δ3-tubulin also labelled β-tubulin, implying that four C-terminal amino acids of β-tubulin must have been removed to generate the specific epitope for this antibody[72]. Tyrosination, on the other hand, can occur only on detyrosinated tubulin. Structural data show that the enzyme that adds tyrosine to tubulin, tubulin–tyrosine ligase (TTL), binds detyrosinated tubulin C-terminal tails so specifically that even Δ2-tubulin cannot be retyrosinated[78].

Polyglutamylation and polyglycylation were initially discovered on tubulin. Both PTMs consist of secondary peptide chains that branch from the main peptide chain, using the γ-carboxy group of glutamate residues as modification sites (FIG. 1). As tubulin C-terminal tails are rich in glutamate, there are many sites on which these two PTMs could be added. Theoretically, this could give rise to a large variety of combinatory signals on both α-tubulins and β-tubulins; however, little insight has been gained into the complexity of these PTMs in living cells. Both PTMs were discovered by mass spectrometry approaches designed to analyse the highly acidic C-terminal tails of tubulin, which are usually lost in proteomic analyses. Through analysis of purified brain tubulin — for a long time the gold standard in tubulin biochemistry — the main modification sites found were Glu445 on α1-tubulin (encoded by *TUBA1*)[64], Glu435 on β2-tubulin (encoded by *TUBB2*)[66] and Glu438 on β3-tubulin (encoded by *TUBB3*)[65]. With use of a similar approach, polyglycylation was discovered on ciliary tubulin isolated from *Paramecium tetraurelia*, and accumulation of up to 34 glycine residues per tubulin molecule was observed[67].

The enzymes catalysing the glutamylation and glycylation reactions, both members of the tubulin–tyrosine ligase-like (TTLL) family (TABLE 1), show enzymatic preferences for either α-tubulin or β-tubulin, or for the generation of short or long glutamate or glycine chains[152,300]. To what extent these enzymes also modify specific positions among the many possible modification sites within the tubulin tails remains an open question. Nevertheless, the existing selectivity of the modifying enzymes indicates that these two PTMs can generate highly controlled patterns on cellular microtubules.
To generate these patterns, TTLL enzymes must be selectively activated or localized by as-yet-unknown regulatory circuits. Evidence that such control mechanisms exist includes the fact that centriole, cilia and spindle-associated protein (encoded by *CCSAP*) directly activates TTLL enzymes[301], and some other proteins interact with TTLLs, such as centrosomal protein of 41 kDa (encoded by *CEP41*) localizing TTLL6 to cilia[208], or tubulin polyglutamylase complex subunit 1 (TPGS1), which may position the TTLL1 protein complex at centrosomes[302].

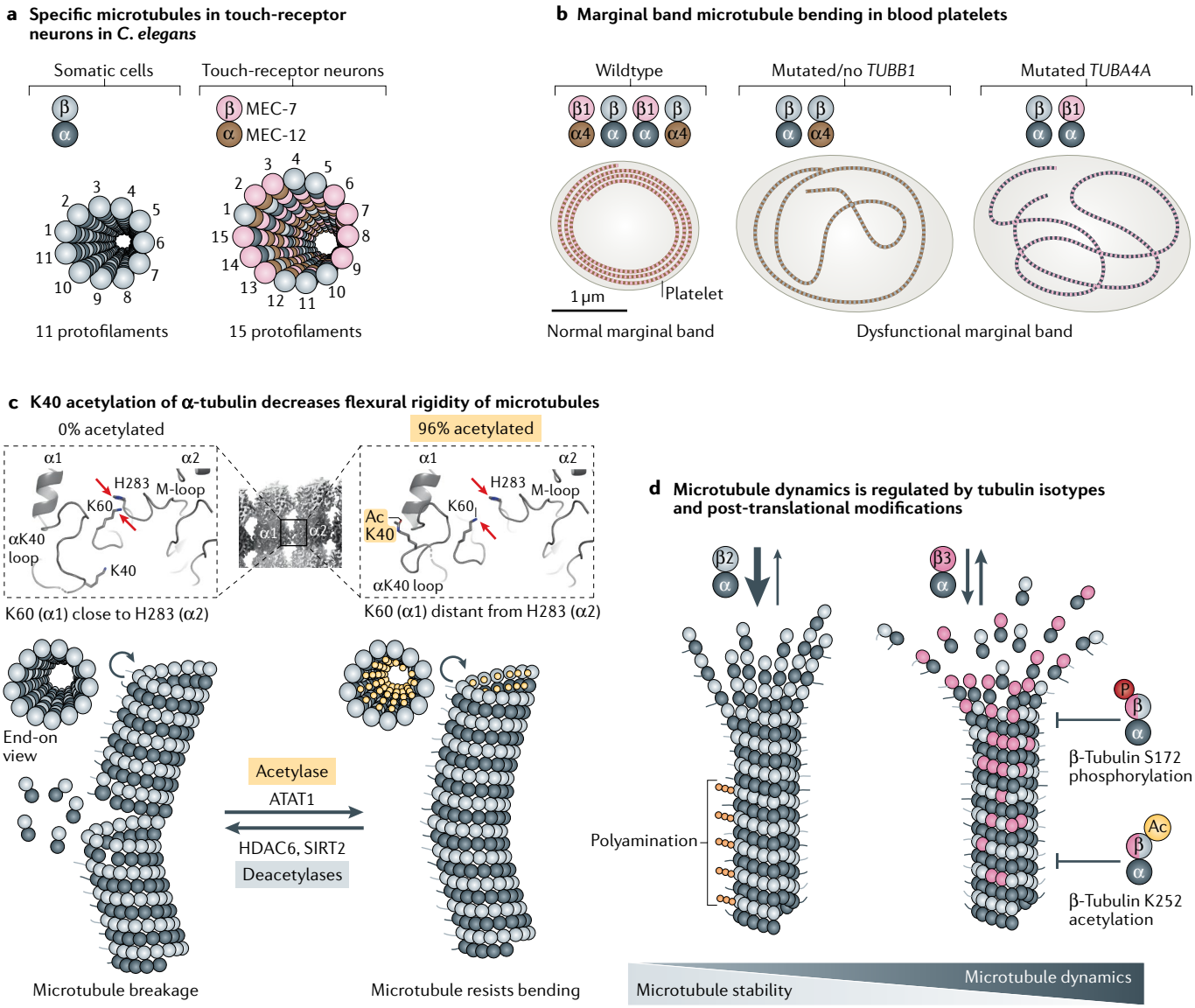


Fig. 2 | **The tubulin code impacts microtubule properties. a** | Tubulin isotypes can determine protofilament numbers. In *Caenorhabditis elegans*, two isotypes specific to touch-receptor neurons (MEC-7 and MEC-12) determine the 15-protofilament microtubule architecture in these cells[86–88]. **b** | In mammals, tubulin isotypes guide the formation of a geometrically defined microtubule array known as the marginal band. This band assembles from microtubules along the outer rim of blood platelets and is essential for the shape and correct function of the platelets. Two tubulin isotypes, α4A-tubulin[107] and β1-tubulin[36], drive the correct assembly of the marginal band, and mutation or absence of the genes encoding either of these isotypes leads to defects in this microtubule structure and to dysfunctional blood platelets associated with bleeding disorders. **c** | Tubulin post-translational modifications can change the mechanical properties of microtubules. Acetylation (Ac) of α-tubulin at Lys40 (K40) changes the structure of the αK40 loop of α-tubulin, which disrupts the interaction of Lys60 with His283 (H283; dashed boxes, red arrows) from α-tubulins of the neighbouring protofilaments[106]. The loss of this interaction weakens interprotofilament contacts, which reduces the flexural rigidity of microtubules, making them more resistant to mechanical bending-induced breakage and disassembly[104,105]. **d** | Tubulin isotypes and post-translational modifications can control microtubule dynamics. Microtubules containing β3-tubulin are more dynamic than those containing β2B-tubulin[85,115]. Phosphorylation (P) of any β-tubulin isotype at Ser172 (S172) by cyclin-dependent kinase 1 (CDK1)[53] or dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A)[55], or acetylation of Lys252 (K252) by San[117], impedes the incorporation of tubulin dimers into microtubules, thus reducing overall dimer availability and favouring microtubule depolymerization. Tubulin polyamination, in contrast, renders microtubules resistant to depolymerization[61]. The structural panels in part **c** of the figure are adapted with permission from REF.[106], PNAS.

12 turns known as the marginal band[98]. The extreme bending of microtubules in the marginal band depends on β1-tubulin[35], as the mutation or absence of *TUBB1* leads to severe defects in the architecture of this microtubule array[36,99] (FIG. 2b). β1-Tubulin is the most divergent tubulin isotype in mammals (~25% sequence divergence from all other β-tubulin isotypes) and does not have close homologues in phyla that do not have platelets. Thus, β1-tubulin may have specifically evolved to sustain the high degree of microtubule bending required for platelet function[100,101]. However, direct biophysical evidence that β1-tubulin-containing microtubules are more flexible than microtubules containing other β-tubulin isotypes is still elusive.

Possible impact of PTMs on microtubule mechanics. Acetylation of α-tubulin at Lys40 (REFS[56,102]) was for many years the most enigmatic PTM of tubulin as it occurs inside the lumen of microtubules (FIG. 1) (reviewed in REF.[103]). It was unclear whether acetylation stabilized microtubules or labelled stable microtubules. Recent work suggests that acetylation of α-tubulin at Lys40 protects microtubules from a process known as mechanical ageing, in which microtubules lose their flexural rigidity following repetitive bending[104]. Consequently, acetylation prevents microtubule breakage, thus prolonging microtubule lifespan inside the cell[105] (FIG. 2c). A structural study showed that acetylation of Lys40, which is located in an unstructured loop of α-tubulin, reduces interprotofilament interactions[106] (FIG. 2c) and therefore might facilitate protofilament sliding and increase microtubule flexibility. Intriguingly, the loop containing Lys40 is one of the hotspots of sequence variation between tubulin isotypes and can adopt different conformations as shown in α-tubulin from budding yeast[82] and *C. elegans*[30]. Thus, Lys40 acetylation and expression of different α-tubulin isotypes could cooperate to adjust the mechanical features of microtubules in cells.

Little is known about how other tubulin PTMs affect microtubule mechanics. A potential role for detyrosination in regulating microtubule flexibility can be inferred from the role of a specific α-tubulin isotype, α4A-tubulin (encoded *TUBA4A*). Loss of this isotype in blood platelets affects the architecture of the microtubule marginal band[107] (FIG. 2b), indicating that α4A-tubulin plays an essential role in the assembly of this coiled microtubule structure. However, as α4A-tubulin is a conserved, 'generic' α-tubulin, it is unlikely to contain unique structural features that change the mechanics of microtubules. A distinct feature of α4A-tubulin, however, is the lack of the gene-encoded C-terminal tyrosine residue, which is present in all other α-tubulin isotypes. Expression of α4A-tubulin therefore mimics α-tubulin detyrosination, and its functions might thus be identical to those of enzymatic detyrosination. Although it has not yet been tested whether tubulin detyrosination directly affects microtubule flexibility, the essential role of this PTM in microtubule flexing during heart and skeletal muscle contraction[108,109] implicates the presence or absence of tyrosine as a regulator of microtubule flexibility. It remains unclear whether detyrosination directly renders microtubules more flexible or rather attracts proteins to the microtubules, which then change microtubule mechanical behaviour.

Control of microtubule dynamics

Tubulin isotypes can control microtubule polymerization dynamics. 'Microtubule dynamics' describes the speed and persistence of microtubule growth, as well as their propensity to spontaneously depolymerize, a process known as microtubule catastrophe[11]. Structural work has shown that the contacts between tubulin molecules within the microtubule lattice determine microtubule dynamics[19]. Experiments using β-tubulin isotype-specific monoclonal antibodies to fractionate tubulin isotypes from bovine brain[110] demonstrated that different β-tubulin isotypes directly affect the dynamic properties of microtubules[111–114]. The use of recombinant human tubulin with a defined isotype composition confirmed these early experimental findings by demonstrating that microtubules assembled from pure α1B–β2B-tubulin dimers were more resistant to spontaneous or catalysed depolymerization than microtubules assembled from α1B–β3-tubulin[85,115] (FIG. 2d). Considering that β3-tubulin is predominantly expressed in neurons[116], this suggests that neuronal microtubules are more dynamic, a concept that was initially suggested after the observation that brain tubulin biochemically depleted of β3-tubulin showed an increased assembly speed[111].

An even more striking difference was found between species with divergent tubulin isotype pools. Tubulin purified from *C. elegans*, for example, assembled more than three times faster than bovine brain tubulin in vitro[30]. Together, these observations provided direct evidence that the isotype composition of tubulin pools controls the dynamic instability of microtubules.

Regulation of microtubule dynamics by tubulin PTMs. There are few examples of PTMs that can directly modulate microtubule dynamics. Some PTMs, such as phosphorylation of β-tubulin at Ser172 by cyclin-dependent kinase 1 (CDK1)[53] or by dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A)[55], as well as the acetylation of β-tubulin at Lys252 by San[117], preclude the incorporation of tubulin dimers into microtubules (FIG. 2d), thus downregulating microtubule polymerization. Conversely, polyamination of tubulin stabilizes microtubules and prevents their depolymerization[61] (FIG. 2d).

Other tubulin PTMs can control microtubule dynamics indirectly by regulating MAPs. Detyrosination, for instance, can reduce the binding of cytoplasmic linker protein CLIP170 or dynactin subunit 1 (DCTN1) to microtubules, which in turn negatively impacts microtubule growth speed and persistence[118–120] (FIG. 3a). Detyrosination also reduces the active disassembly of microtubules by the depolymerizing motors of the kinesin-13 family, as they require tyrosinated microtubules as substrates[121] (FIG. 3a). Polyglutamylation promotes the enzymatic severing of microtubules by spastin and katanin[122–124] in a biphasic fashion[123] (FIG. 3b) and could thus modulate microtubule mass and dynamics in cells[125]. Additionally, polyglutamylation might control the binding of a variety of MAPs to microtubules[126,127], which could eventually stabilize them[21] (FIG. 3b).

Microtubule–MAP interactions

Although the term 'microtubule-associated protein' is often used for non-motile proteins that bind microtubules with high affinity, all proteins that interact with microtubules, including molecular motors, plus end-tracking and minus end-tracking proteins, and even microtubule-depolymerizing proteins, could be considered MAPs. One of the central concepts of the tubulin code is that it regulates interactions between MAPs and microtubules in a specific and selective manner. Intuitively, PTMs are perfectly suited as dynamic, rapidly adjustable regulators of such interactions, as they can be generated on tubulin dimers within existing

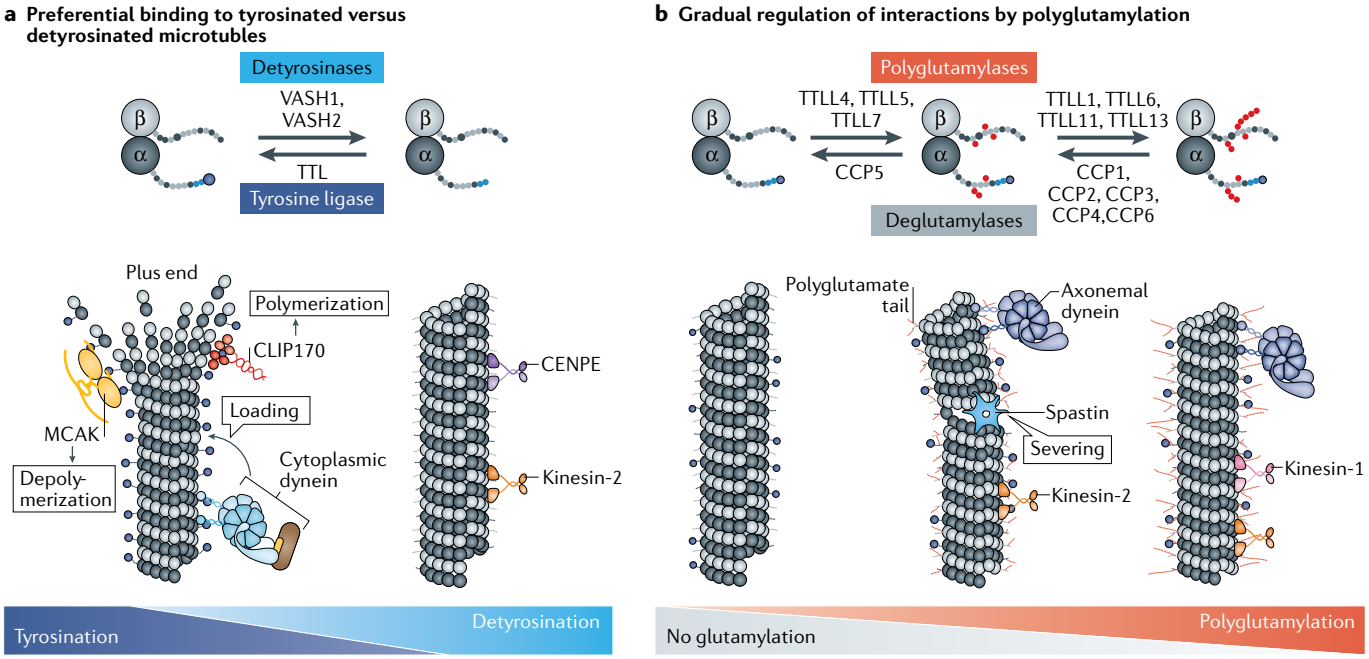


Fig. 3 | **The tubulin code impacts MAP–microtubule interactions. a** | Both tyrosinated and detyrosinated microtubules can attract specific subsets of microtubule-associated proteins (MAPs). Mitotic centromere-associated kinesin (MCAK)[121], CLIP170 (REFS[139,140]) as well as a complex of dynein, dynactin and the adapter protein bicaudal D homologue 2 (BICD2)[148] are attracted to tyrosinated microtubules, whereas the kinesin motors centromere-associated protein E (CENPE)[146] and kinesin-2 (REF.[145]) preferentially associate with detyrosinated microtubules. **b** | Different levels and patterns of tubulin polyglutamylation, catalysed by different modifying enzymes, can fine-tune the interactions and thus change the behaviour of MAPs. The activity of the microtubule-severing enzyme spastin is upregulated by the initial polyglutamylation of substrate microtubules[122,123]; however, further accumulation of this post-translational modification inhibits spastin activity[123]. The activity of molecular motors, such as kinesin-1 and kinesin-2 (REF.[145]), or axonemal dynein[181], can also be differentially regulated by various degrees of polyglutamylation. The velocity and processivity of kinesin-2 are induced by moderate levels of polyglutamylation, whereas kinesin-1 requires higher levels of this post-translational modification to stimulate its processivity[145].

microtubules. Tubulin isotypes can also control MAP–microtubule interactions, although this type of regulation might be less dynamic, as newly synthesized isotypes need to be incorporated into microtubules via de novo polymerization.

Tubulin isotypes and MAPs

In the past 10 years, mechanistic details of interactions between several MAPs and the microtubule lattice have been elucidated in structural studies (for examples, see REFS[78,81,128–134]). As these studies reveal the precise sites of interaction between MAPs and tubulin to the resolution of individual amino acid residues, it can now be deduced how sequence differences between tubulin isotypes could affect these interactions.

The unfolded C-terminal tubulin tail is a tubulin domain that is involved in many, but not all, microtubule–MAP interactions. Direct experimental evidence with chimeric yeast tubulins demonstrated that a single amino acid difference, such as the presence of a lysine residue in the tail of human β3-tubulin, is sufficient to substantially reduce the run length of kinesin-1 on microtubules.

Strikingly, this effect could be counteracted by the addition of glutamate residues in the form of a side chain similar to polyglutamylation onto the β3-tubulin tail. This illustrates the potential crosstalk between tubulin isotypes and PTMs. Similarly, α4A-tubulin, which mimics the detyrosinated form of α-tubulin as it lacks a genetically encoded C-terminal tyrosine, can be enzymatically tyrosinated[135,136]. Furthermore, the distribution of glutamate residues within the C-terminal tubulin tails (the modification sites for glutamylation and glycylation) might affect the patterns of these two PTMs, as the modifying enzymes are likely to preferentially modify some of these sites. However, the exact nature of these preferences is still not fully understood[137] (BOX 1).

Tubulin PTMs and MAPs

The detyrosination–retyrosination cycle. The idea that tubulin PTMs could dynamically regulate the interaction landscape of microtubules emerged with the discovery of these modifications. Experiments in the 1980s suggested that there are differences in MAP–microtubule interactions depending on the tyrosination state of microtubules[138]. More recently it was demonstrated that the C-terminal tyrosine of α-tubulin plays an essential role in the localization of CAP-Gly domain-containing proteins to +TIP complexes[139,140]. Structural work showed that CAP-Gly domains specifically recognize C-terminal EEY/F sequence motifs, which are characteristic of the tyrosinated form of α-tubulin[141].

CAP-Gly domain-containing proteins
Cytoskeleton-associated proteins (CAP) containing a glycine (Gly)-rich domain. These proteins contain a well-conserved GKNDG sequence motif that specifically recognizes EEY/F sequences, which targets them to the plus ends of tyrosinated microtubules.

+TIP complexes
A group of microtubule-interacting proteins localized to the plus ends of microtubules. For most of these proteins, plus-end localization is mediated by end-binding (EB) proteins, such as mammalian EB1, EB2 and EB3 or yeast Bim1p.

Kinetochores
Multiprotein structures associated with the centromeres of duplicated chromosomes in eukaryotic cells. Kinetochores are the docking sites for spindle microtubules to pull sister chromatids apart. Kinetochores further control correct sister chromatid attachment via checkpoints.

Another molecular mechanism that depends on the presence of tyrosinated tubulin in the microtubule lattice is kinesin-13-mediated microtubule disassembly. Complete detyrosination can protect microtubules from active depolymerization by motor proteins of this family, such as mitotic centromere-associated kinesin (MCAK) and KIF2A[121] (FIG. 3a). This discovery provided a mechanistic rationale for the established notion that detyrosinated microtubules are more stable, which was mostly derived from observations in cells[142–144].

Other microtubule interactors have a greater affinity for detyrosinated microtubules than their tyrosinated counterparts. Studies using chimeric yeast tubulin revealed that kinesin-2, but not kinesin-1, has increased motility and processivity when microtubules are detyrosinated[145] (FIG. 3a). Similarly, centromere-associated protein E (CENPE), a kinesin-7 motor associated with kinetochores, shows stronger interactions with, and thus has greater processivity on, detyrosinated rather than fully tyrosinated microtubules purified from HeLa cells[146,147] (FIG. 3a).

The binding and motility of the minus end-directed motor protein dynein, in contrast, were not affected by the tyrosination status of microtubules[145], whereas a complex of dynein, dynactin and the adapter protein bicaudal D homologue 2 (BICD2) required tyrosination for its initial loading onto microtubules in vitro (FIG. 3a). This dependency on tyrosination is mediated by dynactin subunit 1, a CAP-Gly protein. Strikingly, once the complex is loaded on microtubules, it can walk through patches of detyrosinated microtubules without changes in motility[148].

Fine-tuning microtubule–MAP interactions. Polyglutamylation and polyglycylation of tubulins generate lateral glutamate or glycine peptide chains of differing lengths at different glutamate residues within the C-terminal tails of α-tubulins and β-tubulins. A study using chimeras of yeast tubulin bodies with mammalian C-terminal tails, on which controlled patterns of polyglutamylation were generated by chemical addition of glutamate chains of defined length, showed that kinesin motors have a differential sensitivity to glutamylation patterns. Kinesin-2 motility was induced by polyglutamylation of tubulin with chains of three or ten glutamate residues, whereas chains of ten glutamate residues were required for activation of kinesin-1 (REF.[145]) (FIG. 3b). By contrast, neither the motility of dynein nor the depolymerizing activity of kinesin-13 was affected by the presence of glutamate chains of any length[145]. These observations have far-reaching functional implications in the light of polyglutamylation levels found in cells. In the brain, the majority of α-tubulin carries approximately three glutamate residues; chains of ten glutamate residues are not detected[64]. This fact implies that kinesin-2, but not kinesin-1, might be directly regulated by tubulin polyglutamylation in neurons.

Microtubule severing, an enzymatic process which causes the disassembly of microtubules for the control of microtubule mass in cells[149], is regulated by polyglutamylation. Comparison of virtually non-glutamylated and differentially glutamylated microtubules showed that spastin is activated by microtubule polyglutamylation[122]. Use of tubulin polyglutamylase TTLL7 to generate microtubules with controlled polyglutamylation patterns in vitro further revealed a biphasic relationship between the extent of polyglutamylation and spastin activity, where an initial increase in tubulin polyglutamylation induced the severing activity of spastin, but further accumulation of the PTM reversed this effect[123] (FIG. 3b). These experiments demonstrated that the length of glutamate chains, and/or the accumulation of glutamylation at different sites within a single tubulin molecule, could fine-tune the functional readout of this PTM. A similar concept had been proposed earlier for several other MAPs that showed binding differences from differentially glutamylated tubulin in blot-overlay assays[126,127,150,151]; however, more direct evidence is required to confirm those conclusions. Ultimately, the discovery that different polyglutamylases can specifically determine the length and distribution of glutamate chains on α-tubulin or β-tubulin[152] suggests that microtubule interactors may be coordinated by different degrees and patterns of polyglutamylation. Expressed in a cell-specific and tissue-specific manner, the large variety of modifying and demodifying enzymes could cooperate to generate defined glutamylation patterns (BOX 1) that control the intracellular distribution of MAPs and organelles.

Regulatory mechanisms of tubulin PTMs in cells. In cells, tubulin PTMs can have a range of effects on the behaviour, function and interaction landscape of microtubules. Some of these effects initially described in cells, such as tubulin detyrosination controlling CAP-Gly protein–microtubule interactions[139] and kinesin-13-mediated microtubule depolymerization[121], have been readily confirmed in vitro. Indeed, these functions are essentially dependent on the presence of tyrosinated tubulin, and complete detyrosination abolishes them[121,140]. In other cases, however, it has been harder to link observations in cells to a direct impact of PTMs on a single MAP–microtubule interaction. In neurons, excessive tubulin detyrosination abolished the preference of kinesin-1 motors to move into axons, which suggests that detyrosination might occur to a greater degree in axons than in dendrites to help targeting kinesin-1[153]. Preferences of kinesin-1 for detyrosinated microtubules were also reported in non-differentiated cells[154]. Analyses using super-resolution microscopy further showed that lysosomes accumulate on detyrosinated stretches of microtubules in a kinesin-1-dependent manner, and preferentially fuse with autophagosomes at these stretches[155]. All these experiments suggest a preference of kinesin-1 for detyrosinated microtubule tracks, but subsequent in vitro experiments with single kinesin-1 motor proteins did not confirm this notion[145].

Manipulation of acetylation levels in cells also altered cargo transport[156], particularly in neurons[157–161]. While the evidence for transport regulation in most of these studies is compelling, it is still an open question whether acetylation alone leads to this effect. Indeed, neither mice lacking the tubulin-lysine deacetylase HDAC6 (REF.[162]) nor mice lacking the acetyltransferase ATAT1

Ependymal cells
Glial cells lining the ventricles of the mammalian brain and the central canal of the spinal cord. Ependymal cells have multiple motile cilia, whose coordinated beating determines the direction of flow of cerebrospinal fluid. They are also called 'ependymocytes'.

B tubules
Components of the microtubule doublets of axonemes. Partial microtubules made of ten protofilaments that partly share the wall of the A tubules.

Basal bodies
A microtubule-based multiprotein structure at the base of cilia and flagella. The core microtubule structure of the basal body, the centriole, is the same as that which constitutes the core of the centrosomes of dividing cells.

(REFS[163,164]) show obvious defects in neuronal functions, which would be expected when neuronal transport is perturbed. Moreover, in vitro assays with purified components have shown that the motility of single kinesin-1 motor proteins is not affected by the acetylation status of the tubulin tracks[165,166].

Discrepancies between cell-based and in vitro experiments might be explained by other factors that influence transport processes in cells; for instance, the combined effect of multiple PTMs and/or isotypes present at the microtubule tracks[167]. Indeed, a recent in vitro study comparing microtubules assembled from brain tubulin containing many PTMs with tubulin from HeLa cells lacking any PTMs found that the kinesin-3 motor protein KIF1A makes fewer pauses on microtubules made from HeLa tubulin compared with those from brain tissue. So far it is not clear whether this is caused by a single PTM, a single tubulin isotype or a combination of both[168].

Another difference between single-molecule experiments and measurements in cells is that motor proteins are accompanied by adapter and helper proteins on transported vesicles and organelles[169–171] in cells, but not in vitro. These adapters could, in combination with motor proteins, sense the PTM status of the microtubules[172]. Indeed, entire vesicles purified from brain tissue move faster on normally acetylated, wild-type microtubules when compared with non-acetylated microtubules from *Atat1*-knockout brains in a cell-free assay[173]. Moreover, the transported vesicles themselves carry the enzyme ATAT1, and are therefore able to modify their own tracks. This suggests transport complexes can carry specific subsets of tubulin-modifying enzymes, thus optimizing their own transport paths in cells.

Finally, MAPs that directly bind the microtubule tracks can affect the use of these tracks, either by facilitating or by blocking specific motor proteins[174]. The recent observation of tau islands that hinder the advance of motor proteins[175,176] provides a novel model of how MAPs can form locally constrained road blocks for active transport. Considering that tubulin PTMs might regulate the binding of several MAPs, this suggests that a complex interplay between the tubulin code and a hypothetical 'MAP code'[177] could control microtubule-based functions, which is an exciting concept to be explored soon.

Box 2 | **An evolutionary link between tubulin PTMs and cilia and flagella**

Tubulin post-translational modifications (PTMs) are enriched on axonemal microtubules and have essential ciliary functions[191] (FIG. 4a). Strikingly, most of the known tubulin PTMs appear to be evolutionarily linked to cilia and flagella. Tubulin–tyrosine ligase-like (TTLL) enzymes, which catalyse tubulin glutamylation[152] and glycylation[196,300], for instance, can be easily identified in different organisms on the basis of their highly conserved TTL domain[152,196,249,300]. Homologues of *TTLL* are absent from eukaryotes without cilia, such as the yeasts *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*, as well as from many plants, but can always be identified in organisms with ciliated cells if a well-annotated genome sequence is available. For example, *Batrachochytrium dendrobatidis* is a fungus that, in contrast to the aforementioned yeasts, can grow cilia and consequently assembles basal bodies and axonemes[303]. A BLAST search with mouse TTLL1, a polyglutamylase, reveals the presence of highly homologous proteins in this fungus. Although no systematic evolutionary study has been published so far, the presence of TTLL homologues could be considered a strong indication for the presence of glutamylation and/or glycylation in any eukaryotic organism and could be used as a starting point for subsequent functional characterization.

Cellular and physiological roles

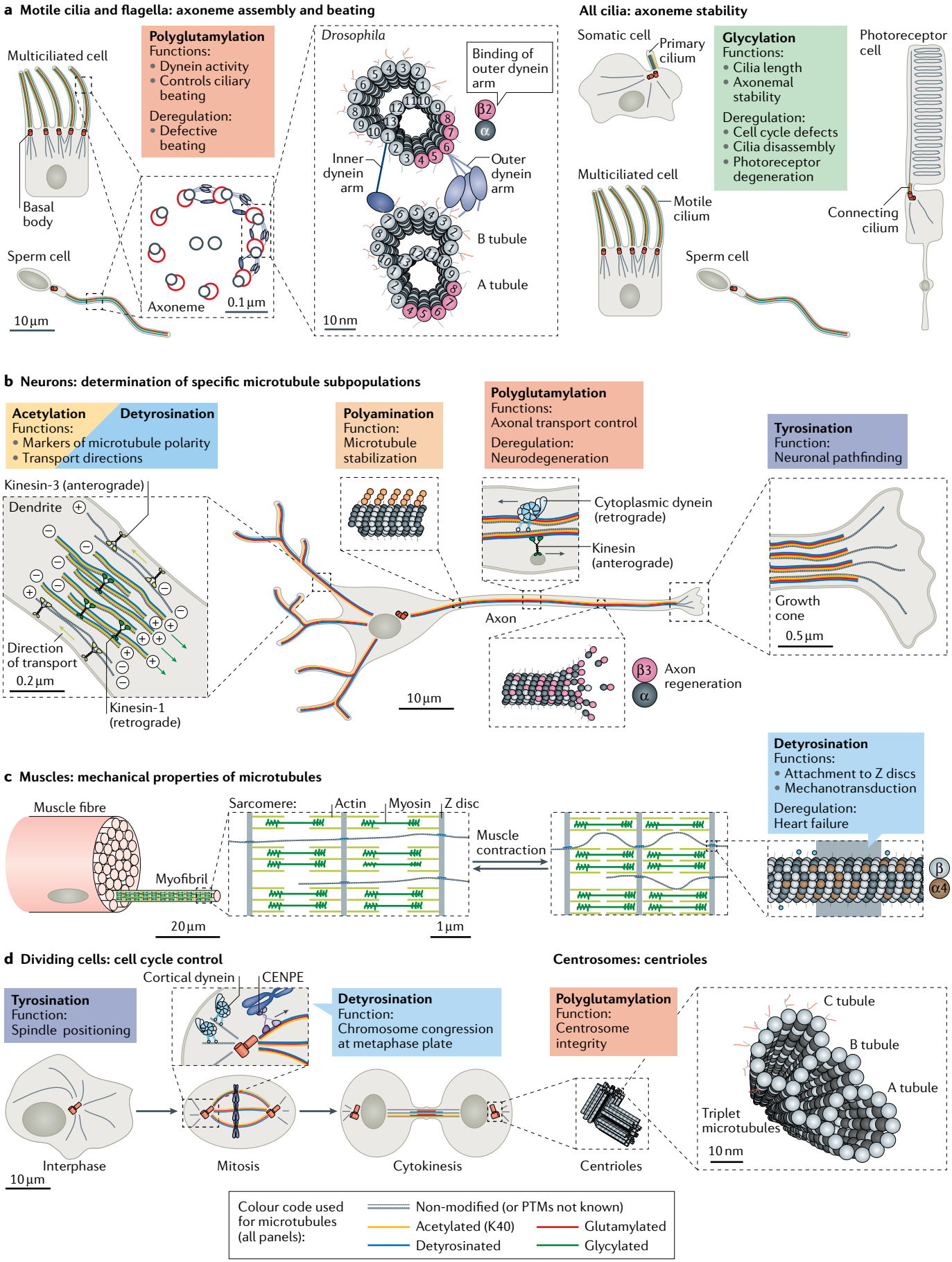
Microtubules adopt an amazing variety of structures and behaviours. Tubulin isotypes and tubulin PTMs contribute to the assembly of microtubule arrays by modulating their intrinsic properties, as well as their interactions with a multitude of associated proteins. On the organism scale, the tubulin code can help microtubules adapt to changing physiological requirements, ensuring homeostasis. Indeed, a growing number of studies show that perturbations of the tubulin code can have devastating consequences at the organism level.

The tubulin code in cilia and flagella

Eukaryotic cilia and flagella assemble on the basis of an evolutionarily conserved microtubule structure known as the axoneme, which consists of nine circularly arranged doublet microtubules, plus two central singlet microtubules for motile cilia and flagella[24]. In motile cilia, the microtubule doublets are interconnected with ciliary dynein motors, thus forming the machinery to generate characteristic ciliary beating[25]. Motile cilia and flagella are important for cell movement, for example for spermatozoids[178] or ciliated microorganisms such as *Tetrahymena* or *Paramecium*[179]. They are also needed to generate liquid flow, for example by the multiciliated ependymal cells in the brain ventricles or ciliated epithelial cells in the trachea[180]. Many tubulin PTMs are strongly enriched on axonemal microtubules and even appear to have evolved together with this organelle (BOX 2).

Tubulin PTMs play key roles in regulating cilia and flagella. Whenever glutamylation is perturbed in different cellular or organism models, motile cilia and flagella are among the most obvious structures showing functional aberrations. Deletion of polyglutamylating enzymes directly perturbed ciliary beating in the unicellular organisms *Chlamydomonas reinhardtii*[181] and *Tetrahymena thermophila*[182] and in the multiciliated ependymal cells in mice[183]. On the ultrastructural level, glutamylation is predominantly found on the B tubules[184,185], which hold the interaction sites of the axonemal dynein heads that control ciliary beating. Therefore, it appears that the polyglutamylation levels of axonemal B tubules directly control dynein activity and beating of the cilia[181,182] (FIG. 4a).

In mice, fine control of glutamylation appears to be important for sperm development and function, as a recurrent phenotype in mouse models of both increased and reduced polyglutamylation is male infertility. The morphological defects range from impaired flagellar motility to erroneous axoneme assembly, which could be related to dysfunctions of either centrioles serving as basal bodies for axoneme assembly or the axonemes themselves[95,186–188]. Even early steps of spermatogenesis can be perturbed; in mice lacking the deglutamylase cytosolic carboxypeptidase-like protein 5 (CCP5), the sperm manchette, a transient microtubule structure essential for the formation of sperm heads, is


a Motile cilia and flagella: axoneme assembly and beating
Multiciliated cell
Basal body
Sperm cell
10 µm
Axoneme
0.1 µm
Polyglutamylation
Functions:
• Dynein activity
• Controls ciliary beating
Deregulation:
• Defective beating
Drosophila
Binding of outer dynein arm
β2
α
Inner dynein arm
Outer dynein arm
B tubule
A tubule
10 nm
All cilia: axoneme stability
Somatic cell
Primary cilium
Glycylation
Functions:
• Cilia length
• Axonemal stability
Deregulation:
• Cell cycle defects
• Cilia disassembly
• Photoreceptor degeneration
Photoreceptor cell
Multiciliated cell
Motile cilium
Connecting cilium
Sperm cell
b Neurons: determination of specific microtubule subpopulations
Acetylation
Detyrosination
Functions:
• Markers of microtubule polarity
• Transport directions
Kinesin-3 (anterograde)
Dendrite
Direction of transport
0.2 µm
Kinesin-1 (retrograde)
Polyamination
Function:
Microtubule stabilization
Polyglutamylation
Functions:
Axonal transport control
Deregulation:
Neurodegeneration
Cytoplasmic dynein (retrograde)
Kinesin (anterograde)
Axon
10 µm
β3
α
Axon regeneration
Tyrosination
Function:
Neuronal pathfinding
Growth cone
0.5 µm
c Muscles: mechanical properties of microtubules
Muscle fibre
Myofibril
20 µm
Sarcomere:
Actin
Myosin
Z disc
1 µm
Muscle contraction
Detyrosination
Functions:
• Attachment to Z discs
• Mechanotransduction
Deregulation:
Heart failure
β
α4
d Dividing cells: cell cycle control
Cortical dynein
CENPE
Tyrosination
Function:
Spindle positioning
Detyrosination
Function:
Chromosome congression at metaphase plate
Interphase
Mitosis
Cytokinesis
10 µm
Centrosomes: centrioles
Polyglutamylation
Function:
Centrosome integrity
Centrioles
C tubule
B tubule
A tubule
Triplet microtubules
10 nm
Colour code used for microtubules (all panels):
Non-modified (or PTMs not known)
Acetylated (K40)
Detyrosinated
Glutamylated
Glycylated

◀ Fig. 4 | **Cellular and physiological roles of the tubulin code.** Known functions of tubulin post-translational modifications (PTMs) and isotypes are depicted. Overview images of cells show all known tubulin PTMs; zoomed-in panels focus on selected PTMs and isotypes and their functions. **a** | Cilia and flagella. Axonemal microtubules are modified by a range of tubulin PTMs, including glutamylation and glycylation, and basal bodies are highly polyglutamylated. In axonemes, glutamylation specifically decorates the B tubules of the microtubule doublets[184,185] and controls dynein activity and ciliary beating[181,182]. In *Drosophila melanogaster*, the β2-tubulin isotype is essential for the fixing of outer dynein arms to the microtubules[212]. Glycylation is a PTM that has so far been found only on axonemes. It controls the length and stability of all types of cilia[183,192,193], and its absence is linked to the degeneration of photoreceptors[193], or cell cycle defects owing to loss of primary cilia[295]. **b** | Neurons. In dendrites, acetylation and detyrosination decorate a subset of microtubule arrays with plus ends pointing towards the cell body, while microtubules of the opposite polarity are tyrosinated. The polarity of these two microtubule arrays controls the direction of cargo transport in dendrites, but it is not known whether the PTMs directly control the motor proteins involved[223]. Polyamination stabilizes unidentified microtubule populations in neurons[61], and the presence of β3-tubulin enhances the overall dynamic instability of neuronal microtubules[85], which is essential for axon regeneration[263]. Polyglutamylation regulates kinesin-driven and dynein-driven bidirectional axonal transport[250,257], and the abnormal accumulation of this PTM leads to neurodegeneration[250,253]. Most neuronal microtubules are highly post-translationally modified, except for a more dynamic, tyrosinated population in growth cones[222] that is essential for neuronal pathfinding[227]. **c** | Muscles. Detyrosinated microtubules in muscle cells, generated by the expression of α4A-tubulin and post-translational detyrosination, are essential for microtubule buckling, which defines their capacity to bear load and influences the viscoelastic behaviour of muscle cells during contraction[109,305]. Aberrant detyrosination is linked to heart failure[266]. **d** | Cell cycle and centrosomes. Microtubules of the mitotic spindle pointing towards the equator and midbody microtubules during cytokinesis are acetylated, glutamylated[122] and detyrosinated[275]. The enrichment of detyrosination on central spindle microtubules[275] guides the kinetochore-associated centromere-associated protein E (CENPE) motor towards the metaphase plate, thus ensuring correct chromosome congression and separation[146]. By contrast, tyrosinated astral microtubules are essential for spindle orientation[279,282] as this PTM is required to load dynein onto the astral microtubules[148] at the cell cortex. Centriolar microtubules have high levels of polyglutamylation[286] specifically located at the C tubules[288]. The high level of polyglutamylation on centrioles is essential for centrosome integrity throughout mitosis[286,291].

dysfunctional. As a result, spermatozoids fail to evacuate their cytoplasm, show supernumerary basal bodies and are unable to assemble functional flagella[189]. Perturbed polyglutamylation in mice also induces defects in other motile cilia, such as airway cilia[95,190], which could lead to respiratory disorders if pathogens cannot be efficiently cleared from the trachea.

Tubulin glycylation was considered highly specific to axonemes of motile cilia and flagella[191] until the recent demonstration of its presence in some primary cilia[192]. Depletion of glycylation led to loss of motile cilia from ependymal cells in mice[183] and to a notable shortening of primary cilia in cultured cells[192]. Photoreceptors of the mammalian retina contain the highly specialized connecting cilia, which progressively shorten in the absence of glycylation. The late-onset retina degeneration observed in mice lacking the tubulin glycylase TTLL3 (REF.[193]) is likely related to suboptimal cargo transport through the connecting cilium, a process essential for photoreceptor homeostasis[194] (FIG. 4a).

Intriguingly, loss of glycylation in murine photoreceptor cells is accompanied by an increase in glutamylation[193], indicating that, as shown in *T. thermophila*[195,196], both PTMs compete for the same modification sites on tubulin and are therefore functionally interconnected. Indeed, patients with mutations in the *AGBL5* gene encoding the deglutamylase CCP5 also develop retina degeneration[197–200]. It is likely that loss of CCP5 expression leads to an accumulation of polyglutamylation, similar to that demonstrated for mice lacking the deglutamylase CCP1 (REFS[193,201]). The concept emerging from these observations is that mutations in genes encoding a range of different tubulin-modifying enzymes can functionally and biochemically lead to similar defects and diseases. Along these lines, mutations in the gene encoding tubulin polyglutamylase TTLL5 also cause retina degeneration in humans[202,203]; however, it appears that in this case it is the perturbation of glutamylation of a non-tubulin substrate (Supplementary Box 1) that causes the loss of photoreceptors in the corresponding mouse model[204].

Other tubulin PTMs such as detyrosination, Δ2-tubulin[205] and acetylation[191] are also enriched on axonemes, but so far little is known of their function. Mice lacking ATAT1 are subfertile[164], suggesting that acetylation is needed for proper axoneme function, perhaps owing to its ability to increase the resistance of microtubules to mechanical fatigue[104,105].

Finally, primary cilia also harbour a range of tubulin PTMs. These non-motile cilia are present on many cells in vertebrates and serve as sensory organelles and signalling hubs. Defective primary cilia can lead to a variety of diseases commonly referred to as ciliopathies[206]. Tubulin PTMs might play similar roles in primary cilia as in their motile counterparts; however, little is known about the direct regulation of their functions by tubulin PTMs. Initial studies showed that acetylation[74], glutamylation[207,208] and glycylation[192] are required for the correct assembly and function of primary cilia (FIG. 4a). Both polyglutamylation and glycylation accumulate towards the proximal part of the primary cilia, whereas acetylation appears to be evenly distributed all along axonemes[183,192,209]. The functional purpose of these distributions is still unclear.

Cilium-specific roles of tubulin isotypes. Early studies demonstrated the presence of distinct tubulin compositions in cilia of different species[210], but it was not clear at the time whether this heterogeneity was related to tubulin isotypes or PTMs. The development of antibodies specific to mammalian isotypes[110,112] revealed β4-tubulin (encoded by *TUBB4*) to be a major β-tubulin isotype in two functionally different types of cilia: the connecting cilia of photoreceptor cells and the motile airway cilia in the trachea[211]. This finding suggests that β4-tubulin possesses properties that are essential for the formation of the axoneme.

The idea that specific tubulin isotypes convey unique properties on axonemal microtubules was experimentally supported by the observation that purified axonemal tubulin from *Chlamydomonas reinhardtii* displayed a distinct assembly–disassembly behaviour when compared with mammalian brain tubulin[185]. Strikingly, *C. reinhardtii* β-tubulin shares specific sequence motifs with mammalian β4-tubulin, which are absent in other mammalian tubulin isotypes. This fact strongly suggests that the primary peptide sequence of ciliary β-tubulin isotypes determines some of the characteristic features of axonemal microtubules, such as low growth and shrinkage

Primary cilia
A solitary microtubule-based organelle emanating from the cell surface of most mammalian cells. Primary cilia are thought to be environmental sensors and signalling hubs of the cell, and their dysfunction was linked to a variety of ciliopathies and cancers. Primary cilia contain axonemes without dynein motors and are thus non-motile.

Connecting cilia
Modified primary cilia connecting the cell body to the outer segment of photoreceptor cells in the retina.

Growth cone
Dynamic structure at the tip of a growing neurite, able to sense the environment and guide neurite outgrowth and connection. Growth cones are temporal structures in developing neurons.

Microcephaly
A medical condition in which the brain and head of patients are smaller than expected.

rates[185]. Work in *D. melanogaster* further demonstrated that a specific amino acid residue encoded in all axonemal β-tubulins, Gly56, is essential for the attachment of the outer dynein arms to the axonemal microtubules and is thus essential for ciliary motility[212].

In *C. elegans*, an organism without motile cilia, cells with primary cilia express characteristic tubulin genes[89]. Deletion of the α6-tubulin-encoding gene *tba-6* led to a loss of the microtubule doublet structure in the sensory cilia, which instead contained 18 singlet microtubules and displayed defects in intraflagellar transport and vesicle sorting[90]. A unique feature of the *tba-6* gene product is its C-terminal tail, which is longer than that of other α-tubulin isotypes, contains positively charged amino acid residues and, most strikingly, does not contain glutamate residues that could serve as sites for post-translational glutamylation. Because in vitro reconstitution experiments demonstrated that the C-terminal tails of brain tubulin hinder the formation of B tubules[213], it is possible that the unique tail of *C. elegans* α6-tubulin permits doublet formation due to its different biophysical features and perhaps because it cannot be polyglutamylated.

So far, data supporting essential roles of particular tubulin isotypes in axonemal structure and function stem mostly from studies in model organisms. Although the divergence of tubulin isotypes makes it difficult to draw direct parallels to other organisms, these examples show that single amino acid substitutions in the highly structured tubulin body, as well as variations in the peptide sequence of the C-terminal tails of tubulin, can be essential for building and maintaining axonemes. Most excitingly, these sequence variations can influence the PTM of a given isotype, thus directly linking the two core elements of the tubulin code to one single biological function.

The tubulin code in neurons

Differential distribution of tubulin PTMs in neurons. By contrast to most other cell types of multicellular organisms, neurons are peculiar in that their entire microtubule cytoskeleton is highly decorated with PTMs. Neuronal α-tubulin is acetylated at Lys40 (REFS[214,215]), detyrosinated[215,216] and further converted into Δ2-tubulin[71]. Moreover, neuronal microtubules are abundantly polyglutamylated on α-tubulin[64] and β-tubulin[65,66]. All of these PTMs accumulate as neurons differentiate and mature[76,215,217], underpinning the concept that tubulin PTMs are markers of neuronal differentiation. Biochemical analyses of purified brain tubulin have so far provided approximate measures of the levels of individual PTMs[64,70,104,218–220].

Mapping of tubulin tyrosination and acetylation by immunofluorescence and immunoelectron microscopy revealed that acetylation is present all along the axon, but less so at the growing end of the axon, where tyrosinated tubulin is predominant[221]. This pattern fits the expectation of axonal microtubules as long-lived, and thus highly acetylated and detyrosinated, whereas the growing end of the axon, including the growth cone, contains freshly assembled, non-modified microtubules. An elegant approach to separate microtubules of cultured neurons showed that single, continuous microtubules change their PTM status towards the distal end of the axon[222], which might have important implications for growth cone functions (FIG. 4b).

Two decades after this finding, following the advent of super-resolution microscopy, another study described the presence of two different microtubule populations in neurons, one acetylated and barely tyrosinated in the centre of neuronal dendrites, and the other tyrosinated and barely acetylated at the dendrite periphery[223]. These microtubule species showed opposite polarity and thus supported two different types of transport: retrograde, kinesin-1-driven transport occurred on the microtubules in the centre of dendrites that were highly modified, whereas anterograde transport by kinesin-3 occurred on the microtubules at the periphery that were modified at a low level (FIG. 4b). It is still unclear whether the different PTMs are merely markers of different microtubule subtypes, or whether they directly control the motors that walk on them. In axons, where microtubules are uniformly polarized[224,225], all kinesin motors walk towards the distal end.

Functions of tubulin PTMs in neurons. Different PTMs in neurons might play distinct roles in neuronal development and homeostasis. The balance between detyrosination and tyrosination appears to be important in early neuronal development as the unopposed detyrosination of neuronal microtubules in mice lacking the gene for TTL (*Ttl*) leads to perinatal death due to neurodevelopmental defects[226]. Hippocampal neurons cultured from embryos of these mice lack tyrosinated microtubules in axonal growth cones and show abnormalities in neuronal pathfinding[227]. Perturbations of the tubulin detyrosination cycle can also lead to human disease. While mutations of *TTL* might be rare in adult patients owing to the expected massive developmental defects induced by the nearly complete absence of tyrosinated tubulin[226], they are more likely to be found in the vasohibin genes encoding enzymes of the detyrosinase family[228,229]. Indeed, mutations in the gene encoding small vasohibin-binding protein (*SVBP*), a cofactor of vasohibins important for the removal of C-terminal tyrosine residues, have been linked to microcephaly and intellectual disability[230,231], thus confirming the importance of detyrosination in neurodevelopment.

Phosphorylation of β-tubulin Ser172 by the serine/threonine-protein kinase Minibrain (an orthologue of human DYRK1A) controls microtubule dynamics in differentiating neurons in *D. melanogaster*[55]. Perturbations of this PTM in neurons lead to defects in dendrite branching and excitability. As human *DYRK1A* has been linked to Down syndrome[232] and autism-spectrum disorders[233], these findings provide a potential molecular link between tubulin phosphorylation and human neurological disorders.

Acetylation is a prominent tubulin PTM in neurons. However, inhibition of tubulin acetylation in mice induced by knockout of the gene encoding ATAT1 (*Atat1*) caused only mild neurological defects[163], the most remarkable being the loss of touch sensation[234]. This mirrors defects in mechanosensation in acetylation-defective

Purkinje cell
GABAergic neurons located in the cerebellar cortex. Purkinje cells are among the largest neurons in the brain and possess highly branched dendritic trees.

Viscoelasticity
The property of materials that exhibit both viscous and elastic characteristics when undergoing deformation.

Desmin
Muscle-specific intermediate filament assembly essential for the structural integrity and function of muscle fibres.

D. melanogaster[235], as well as in *C. elegans*, in which acetylation by the ATAT1 orthologue mec-17 (REF.[236]) is important for the formation of the characteristic 15-protofilament microtubules[93] that are essential for touch sensation[237]. Mutation of the acetylation site Lys40 in the major neuronal α-tubulin isotype of *D. melanogaster* (encoded by *αTub84B*) further highlighted the importance of acetylation in the dendritic refinement of sensory neurons[238]. A number of reports have indirectly linked tubulin acetylation to neurodegeneration, mostly by inhibiting the activity of the deacetylase HDAC6 (REFS[161,239–243]). The interpretation of these experiments is, however, not straightforward, as HDAC6 deacetylates not only α-tubulin Lys40 (REF.[244]) but also the mitochondrial transport adapter protein mitochondrial Rho GTPase 1 (MIRO1)[245] and the actin regulator cortactin[246] (Supplementary Box 1), which both play important roles in neurons.

Polyglutamylation, however, can cause neurodegeneration directly and cell autonomously as demonstrated by genetic approaches in mouse models. The well-established mouse model for Purkinje cell degeneration, the *pcd* mouse[188], carries a mutation in *Agtpbp1* (REF.[247]), which was later shown to encode the deglutamylase CCP1 (REF.[248]). *Agtpbp1* deficiency causes the accumulation of hyperglutamylated tubulin in the cerebellum, the main brain region undergoing degeneration in *pcd* mice[248]. Rapid degeneration of Purkinje cells can be avoided if the major α-tubulin polyglutamylase in neurons, TTLL1 (REF.[249]), is deleted selectively in Purkinje cells of *pcd* mice. This finding demonstrates that TTLL1-catalysed hyperglutamylation causes the degeneration of these neurons[250] (FIG. 4b). Given the rapid degeneration of Purkinje cells, it was surprising that not all brain regions in *pcd* mice showed signs of neurodegeneration. This was found to be related to another member of the CCP family[251,252], CCP6 (encoded by *Agbl4*), which is expressed specifically in brain regions that do not degenerate in *pcd* mice[248]. Indeed, deletion of *Agtpbp1* and *Agbl4* together induced hyperglutamylation in the entire mouse brain, resulting in the degeneration of neurons that were unaffected in *pcd* mice, such as the pyramidal neurons in the cerebral cortex[250].

The discovery of an infant-onset human condition linked to inactivating mutations in *AGTPBP1* with symptoms remarkably similar to the phenotypes of the *pcd* mouse model[253–255] established deregulated polyglutamylation as a novel cause of human neurodegeneration. It is conceivable that subtler alterations of this PTM, either due to mutations in other, less prominent tubulin deglutamylases, or due to perturbation of upstream regulatory events, could be linked to or even cause other late-onset human diseases. Given that deletion of the tubulin glutamylase gene *Ttll1* can prevent degeneration of CCP1-deficient Purkinje cells in mice[250], small molecules inhibiting tubulin-modifying enzymes could prove to be potential therapies for neurodegenerative disorders linked to perturbed microtubule polyglutamylation.

Exploring the molecular mechanisms by which abnormal polyglutamylation leads to neurodegeneration helps to decipher the physiological role of this PTM in the nervous system. So far, defects in axonal transport have been reported in different types of neurons[250,256,257], and a causative role of the microtubule-severing enzyme spastin in these defects was excluded[250]. However, it is likely that other microtubule-based processes, such as the binding and distribution of neuronal MAPs, could also be affected if polyglutamylation is perturbed.

Tubulin isotypes in the nervous system. β2-Tubulin and β3-tubulin are the predominant isotypes in neuronal microtubules[258]. While β2-tubulin is also expressed in other cell types, β3-tubulin is almost exclusively found in neurons[259,260]. The β3-tubulin encoding gene *TUBB3* is differentially expressed in different types of neurons[261] and upregulated during the regeneration of sensory nerves[262]. Together with the recent observation that β3-tubulin-containing microtubules depolymerize faster than microtubules that do not contain this isotype in presence of depolymerizing factors[85], this suggests that *TUBB3* expression might directly regulate microtubule dynamics in a cell type-dependent and function-dependent context. This concept was confirmed in a *Tubb3*-knockout mouse, which displays defects in axonal regeneration[263] (FIG. 4b). These defects are reminiscent of phenotypes found in *Atat1*-knockout mice[234], indicating once again that different elements of the tubulin code converge to optimize microtubule functions. Indeed, an increase in tubulin acetylation and polyglutamylation was detected in *Tubb3*-knockout mice[263], suggesting that neurons attempt to compensate for the loss of β3-tubulin by adjusting microtubule dynamics, or their interactions with MAPs and molecular motors.

Microtubule functions in muscles

The observations that the enzymatic activity of TTL in muscles is about two times higher than in the brain[264], and reaches a temporal maximum during myofiber development in skeletal muscles[265], indicated early on that the detyrosination–tyrosination cycle could be central in regulating the function of muscle microtubules. The first functional insight, however, only came recently from the observation that mechanotransduction in skeletal and heart muscle is affected by the detyrosination status of muscle microtubules[108] (FIG. 4c). High-speed imaging revealed that microtubules in the heart muscle buckle with every beat — an impressive example of their mechanical resistance. The buckling of microtubules provides a viscous resistance to the actin–myosin force, thus controlling the viscoelasticity of the muscle. This viscoelasticity is directly dependent on tubulin detyrosination, which controls the anchoring of microtubules to the desmin structures of muscle fibres. Absence of detyrosination disrupts microtubule–desmin contacts and consequently perturbs cardiac muscle function[109] (FIG. 4c). Abnormally high levels of microtubule detyrosination lead to overly stiff cardiac muscles, which has been linked to human heart failure[266]. Strikingly, myocardiocytes from patients with heart failure recovered elasticity when treated with the drug parthenolide to reduce tubulin detyrosination[267], or with the microtubule-destabilizing drug colchicine[266]. Genetically, increased detyrosination levels

Astral microtubules
A microtubule population that exists only during mitosis. Astral microtubules connect the centrosomes to the cell cortex and serve to orient the mitotic spindle in the cell.

Meiotic drive
The preferential, non-Mendelian transmission of a particular allele or locus during meiosis.

could originate from two mechanisms: upregulation of the detyrosinating enzymes (encoded by *VASH1* and *VASH2* (REFS[228,229]) or overexpression of α4A-tubulin, which lacks C-terminal tyrosine and mimics the detyrosinated state of α-tubulin. Indeed, it was found that α4A-tubulin is overexpressed in failing hearts[266].

Control of muscle function by microtubule detyrosination revealed an unexpected role of the tubulin code. Other tubulin PTMs and isotypes[268,269] might also play important roles in the regulation of muscle functions, but this is yet to be explored.

The tubulin code in cell division

Regulation of the mitotic and meiotic spindles. The division of eukaryotic cells depends on microtubules as components of mitotic and meiotic spindles. Spindles are complex[270] and highly dynamic assemblies of microtubules[271] that ensure the correct separation of the genetic material into two daughter cells. A huge amount of work has gone into explaining how the self-assembly of different molecular components can give rise to such complex, highly controlled microtubule arrays[272,273], but the potential impact of the tubulin code has rarely been explored.

Direct evidence for a role of tubulin isotypes in controlling spindle behaviour was found in *C. elegans*, where two α-tubulins and two β-tubulins are expressed in the embryo. Despite the high similarity of those two α-tubulin and two β-tubulin isotypes, each isotype confers distinct dynamic properties to mitotic spindle microtubules, and substitution studies have shown that all isotypes are required for proper spindle function[274].

Several tubulin PTMs are present on spindle microtubules. In mammalian cells, detyrosination is enriched on spindle microtubules that point to the cell equator, but is virtually absent from astral microtubules[275]. A similar enrichment on spindle microtubules has been shown for polyglutamylation[122], which together with an increased polyglutamylase activity in mitosis[276] suggests a role for this PTM in cell division (FIG. 4d).

A mechanistic role for detyrosination in cell division is now clear. In mitosis, unaligned chromosomes are transported to the metaphase plate by the kinetochore-associated kinesin-7 motor CENPE[277]. This transport was perturbed by a complete inhibition of detyrosination in dividing cells, suggesting that CENPE can 'read' the detyrosination of spindle microtubules. Indeed, in vitro reconstitution experiments revealed that CENPE motility is enhanced on detyrosinated microtubules[146] (FIG. 4d).

Finally, phosphorylation of β-tubulin Ser172 by CDK1 prevents the incorporation of the tubulin dimer into the microtubule lattice, which might control microtubule dynamics in mitosis[53]. Indeed, mimicking Ser172 phosphorylation in budding yeast perturbed cell division, thus confirming the importance of this PTM for correct spindle function[278].

Generating asymmetries in dividing cells. In mammals, TTL is the sole enzyme that catalyses the retyrosination of tubulin. Absence of TTL leads to the accumulation of detyrosinated tubulin, and *Ttl*-knockout mice die perinatally[226]. Among the dysfunctions observed in these mice was a severe disorganization of neuronal networks, which might be explained by an accelerated, erratic outgrowth of the neurons. Additionally, neurodevelopmental defects could be induced by the failure of spindles to correctly orient themselves during neuronal precursor division in *Ttl*-knockout cells[139,279]. Spindle positioning in cells depends on the interactions of astral microtubules with the cell cortex[280], which are mediated by CAP-Gly proteins[281]. +TIP localization of CAP-Gly proteins depends on tyrosination[140], which is normally enriched on astral microtubules[275] (FIG. 4d). Therefore, the nearly complete absence of tyrosinated tubulin in *Ttl*-knockout cells leads to dysfunctional +TIP complexes and consequently impaired spindle orientation, which in turn determines the fate of daughter cells after neuronal progenitor division[279,282].

During meiosis in mouse oocytes, detyrosination is asymmetrically distributed between the two meiotic half-spindles and thus is involved in non-Mendelian segregation of chromosomes, a process known as meiotic drive[283]. Strikingly, the half-spindle that migrates towards the oocyte cortex progressively accumulates tyrosinated tubulin, which implies that TTL, rather than a detyrosinating enzyme, plays a role in generating this asymmetry[284].

Controlling centrosome functions. Centrosomes are microtubule-organizing centres, which in many different cell types serve as facilitators of mitotic spindle bipolarity and when converted into basal bodies, become the organizing centres of cilia and flagella[285]. Polyglutamylation is particularly enriched on the centrioles[286] — complex microtubule structures at the core of the centrosome[287] (FIG. 4d). Different patterns of polyglutamylation have been mapped to distinct domains of centrioles, suggesting that the modification could serve as a guidance signal for centriole-associated proteins that localize to highly defined positions within these structures[288,289].

So far no experiments have selectively abolished polyglutamylation of centrioles. However, injection of antiglutamylation antibodies into dividing cells[290] led to centriole disassembly and cell cycle defects[286,291], suggesting an important role for polyglutamylation in centriole maintenance and functions. In light of the central role centrosomes play in cell division[4], polyglutamylation, by tightly controlling centriole assembly and perhaps also centriole maturation and duplication, could control cell cycle timing and fidelity. Indeed, spermatozoids from mice lacking *Agbl5*, which encodes the deglutamylase CCP5, show supernumerary centrioles, suggestive of a defect in centriole duplication[189]. Considering the implications of centrosome duplication defects in human diseases[292], it is possible that aberrant centriole polyglutamylation could be one cause of such disorders.

Controlling cell proliferation via the primary cilia. As mentioned earlier, many tubulin PTMs are enriched in ciliary axonemes and basal bodies and might thus affect cell proliferation by controlling the functions of primary cilia. Primary cilia are signalling centres that control cell division and proliferation, and their dysfunction can

lead to cancer[293,294]. The first direct link between tubulin PTMs, cell proliferation and cancer was found for tubulin glycylation. Compared with wild-type mice, mice lacking the gene for the glycylase TTLL3 showed a reduced number of primary cilia in the colon epithelium, which hyperproliferated and showed faster tumour growth in a model of colitis-induced tumorigenesis[295]. Whether perturbed primary cilia are the only cause of this phenotype remains to be established. A remarkable observation in this study was that hyperproliferation of the colon tissue led to no visible morphological changes in non-cancerous colon tissue, and the defect became apparent only after tumour induction by colitis. This example illustrates how subtle effects in defective tubulin PTMs could be overlooked despite their role in key physiological processes and tumorigenesis.

Conclusions and perspectives

In 1976, Fulton and Simpson formulated the first 'multitubulin hypothesis': "The surfaces of a tubulin molecule must interact with many other tubulin surfaces … as well as with associated molecules. … Many of these structural interactions appear to have been conserved throughout evolution, and this probably imposes severe restraints on variations in the amino acid sequence. … On the other hand, subtle changes may have occurred that do not alter the basic topology of tubulin but do provide specialized associative properties or binding sites for particular functions"[27].

This early hypothesis was confirmed by the discovery that tubulin isotypes that are often highly similar, and that tubulin PTMs, despite being found on specific microtubule species in cells, are often not 'essential' in the classical cell-biological sense. At the end of the 1980s, it became clear that most mammalian tubulin isotypes can be interchanged without obvious functional consequences in cells[296], which led to their functional importance being questioned[297,298]. At the same time, research on tubulin PTMs was impeded by the absence of appropriate means of manipulating them, which was overcome in the twenty-first century through the discovery of a number of tubulin-modifying enzymes, some of which were discovered in the past few years[228,229]. Surprisingly, deletion of genes encoding the tubulin-modifying enzymes often causes only mild phenotypic defects, even though in some cases the levels of tubulin PTMs change markedly even when only one modifying enzyme is inactivated. In most cases, only some specific cell types or organs show signs of dysfunction or degeneration when tubulin PTMs are altered, and it is only in rare cases that deletion of a single PTM-modifying enzyme has severe consequences for development and survival, such as deletion of TTL[226].

It thus appears that, in many instances, tubulin isotypes and PTMs have subtle effects on gross microtubule functions but could be critical for the control of complex, long-lasting cellular functions, in some cases by regulating only selected microtubule populations in a cell. Although this confirms the initial predictions of the multitubulin hypothesis[27], it makes the functional analyses of the tubulin code challenging. Detection of subtle alterations of microtubule functions will require more sensitive methods to measure microtubule behaviour in cells and purified systems or to make long-term observations of organisms, including detailed histological and behavioural analyses. Despite the challenges involved, it is also a great opportunity for a conceptual leap in cell biology. Evolution has shown that both tubulin isotypes and tubulin PTMs would be eradicated if they were not needed for cell survival (BOX 2), which strongly suggests that they are functionally important in organisms that have retained them. The fact that so far both elements of the tubulin code have been hard to detect by various analytical approaches indicates the urgent need for more adapted methods, and, more importantly, the need to broaden our concept of biological functions in space and time. Indeed, cellular processes can last over a lifetime, and cells such as neurons can span lengths of up to 1 m in our body. Regulatory processes that can easily be ignored in the cell culture dish might have a key role in controlling such complex systems over a lifetime in vivo. The role of the tubulin code in these processes has recently been proven by the discovery that deregulation of tubulin PTMs can lead to the degeneration of neurons[250,253] or photoreceptors[193], and there is a whole spectrum of neurological disorders linked to mutations in genes encoding tubulin isotypes (Supplementary Box 2). Exploring the role of the tubulin code is a great challenge for the coming years, and will certainly contribute to uncovering novel aspects of the regulation of cellular functions and organism homeostasis.

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