POST-TRANSLATIONAL MODIFICATIONS REGULATE MICROTUBULE FUNCTION

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The αβ-tubulin heterodimer, the building block of microtubules, is subject to a large number of post-translational modifications, comparable in diversity to the intensively studied histone modifications. Although these unusual modifications are conserved throughout evolution, their functions have remained almost completely elusive. Recently, however, important advances in the understanding of how tubulin modifications regulate function and organization have been made.

MITOTIC SPINDLE
A bipolar array of microtubules that functions to move the duplicated chromosomes during mitosis and meiosis.

AXONEME
A bundle of microtubules and associated proteins that form the core of a flagellum or cilium.

CILIA
Hair-like extensions of cells, which contain a microtubular axoneme. Beating movements of cilia are responsible for swimming.

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Microtubules are an important component of the cytoskeleton and carry out a variety of essential functions. During cell division, microtubules form the MITOTIC SPINDLE, the structure that is required to faithfully segregate replicated sister chromatids. Together with accessory proteins, they constitute the AXONEME of CILIA and FLAGELLA and so contribute to cell motility. In addition, they are important factors in the generation of cell polarity and also function as tracks along which — with the help of motor proteins — organelles and vesicles are transported through the cell. This diversity of roles raises the question of how individual microtubules, which are formed by polymerization of αβ-tubulin heterodimers (BOX 1), are assigned to these specific functions. In other words, what distinguishes a spindle microtubule from one that is part of a cilium? One answer is that the cell generates microtubule diversity on two different levels[1]. First, various different α- and β-tubulin isotypes are expressed within the cell. However, although some tubulin isotypes clearly have specific functions, in most cases that have been studied, the isoforms seem functionally interchangeable. Second, microtubule diversity is also generated by an extensive array of reversible post-translational modifications[2], such as acetylation, polyglycylation and polyglutamylation, tyrosination/detyrosination, phosphorylation and palmitoylation (TABLE 1). In fact, for some PROTISTS that express only a single set of αβ-tubulin genes, this is the only source of tubulin diversity. Most of these modifications affect the carboxy-terminal domain of αβ-tubulins, which is located on the outside of the microtubule[3] where it is well positioned to influence interactions with other proteins.

Here, we review the literature on the occurrence of these modifications, the enzymes that are involved in generating them, and their potential functions.

The role of the tubulin tails

Electron crystallographic studies of the αβ-tubulin dimer have provided important insights into its structure. However, they failed to resolve the carboxy-terminal ten residues of α-tubulin and the carboxy-terminal 18 residues of β-tubulin[4]. These residues immediately follow helices H11 and H12 in the dimeric tubulin structure and are thought therefore to be located on the outer surface of the microtubule (FIG. 1). The failure to resolve these residues in the crystal structure could be due to the fact that they are disordered, as indicated by some structural studies on synthetic carboxy-terminal peptides[5]. Not only are the carboxy-terminal sequences of α- and β-tubulin highly acidic, but they also constitute the 'isotype defining regions'[6], where the various tubulin isotypes differ most strongly from each other.

Several studies indicate that αβ-tubulin that lacks the carboxy-terminal domains can still polymerize into microtubules, and that these microtubules are, in fact, more stable than wild-type microtubules[7,8]. However, it is possible that these domains might be important for the generation of higher-order microtubule structures — for example, a testis-specific β2-tubulin variant that

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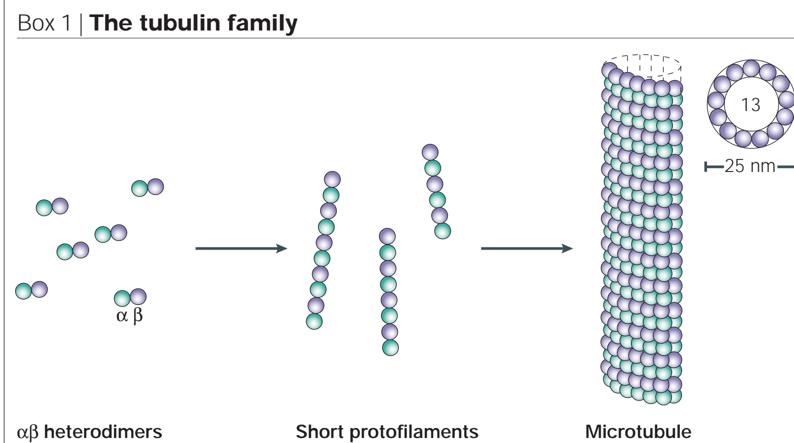
Box 1 | **The tubulin family**



α- and β-tubulin form a heterodimer of 110 kDa, and head-to-tail association of these dimers leads to the formation of linear protofilaments. The lateral association of protofilaments — usually 13 — forms a cylindrical microtubule with an outer diameter of 25 nm. Microtubules are inherently polar structures with a dynamic plus end and a minus end that can be stabilized by embedding the filament into a microtubule-organizing centre (MTOC). The dynamic behaviour of individual microtubules *in vivo* is described by the term 'dynamic instability', as a microtubule can frequently switch between phases of growth and rapid depolymerization. The chemical basis for this behaviour is thought to be a delayed hydrolysis of GTP at the β-subunit, which creates a GTP-cap that promotes further microtubule growth.

Structural similarities between tubulin and the bacterial cell-division protein FtsZ indicate that these proteins have a common ancestor[98]. Further members of the tubulin family include γ-tubulin, a protein for which a role in nucleating microtubules is well established. Four 'new' tubulins — delta (Δ)-, epsilon (ε)-, zeta (ζ)- and eta (η)-tubulin — have been discovered recently[99]. The conspicuous absence of these proteins from organisms that lack bona fide centrioles, together with a functional characterization of ε-tubulin[100], strongly indicate that the new tubulins have functions that are associated with the eukaryotic centriole and/or basal body. So far, the post-translational modifications discussed in this review have only been described for α- and β-tubulins. γ-tubulin, as well as the new tubulins, generally lacks the highly acidic carboxy-terminal target sequences.

Many eukaryotic cells express various α- and β-tubulin isotypes. In vertebrates, six different genes for each α- and β-tubulin form a multigene family. Within one species, the greatest sequence variability between tubulin isotypes is found in the carboxy-terminal tails (isotype-defining sequences).

FLAGELLA
Long protrusions that contain a microtubular axoneme, the beating of which can drive a cell through liquid media. Note that bacterial flagella are constructed very differently from eukaryotic flagella.

PROTISTS
Single-celled eukaryotic organisms that are either free living or parasitic.

TETRAHYMENA THERMOPHILA
Unicellular ciliated eukaryote.

CHLAMYDOMONAS REINHARDTII
Flagellated green algae that are often used as a model organism to study flagellar assembly and architecture.

lacks the carboxy-terminal domain fails to assemble into a functional axoneme in *Drosophila melanogaster*[9]. In addition, elegant genetic studies in TETRAHYMENA have recently addressed the role of the tubulin tails[10]. Tail deletions of either α- or β-tubulin proved to be lethal. However, the essential role of both tails is redundant because, in chimeric tubulins, the carboxy-terminal β-tubulin sequence can compensate for the loss of the α-tubulin sequence, and vice versa. Furthermore, an α-tubulin variant with a duplicated α-tubulin carboxyl terminus could rescue a mutant β-tubulin tail in which all potential post-translational modification sites had been mutated. However, the duplicated α-tail failed to rescue a complete deletion of the β-tubulin tail, which shows that the presence of a tail itself, in addition to the function carried out by its post-translational modifications, is essential.

With the exception of acetylation, the post-translational modifications occur in the carboxy-terminal tubulin tails. Notably, their spatial arrangement is reminiscent of post-translational modifications of the amino-terminal histone tails[11], which also lie on the outside of a polymer (in this case, chromatin), where they are accessible to modifying enzymes and can interact with other proteins to alter the properties of the polymer.

Acetylation/deacetylation of α-tubulin

When does it occur? Most α-tubulins are known to be acetylated on the ε-amino group of a conserved lysine residue at position 40 in the amino terminus[12,13]. Acetylation is mostly associated with stable microtubular structures such as axonemes, and it occurs after microtubule assembly[14]. On the basis of the electron-crystallographic structure, it has been predicted that the modified residue points towards the lumen of the microtubule[3]. Acetylation occurs in the primitive eukaryotes *Giardia lamblia*[15], *Tritrichomonas mobilensis*[16] and *Trypanosoma brucei*[17], and therefore arose early during eukaryotic evolution.

Which enzymes are required? A partial purification of a tubulin acetyltransferase activity from CHLAMYDOMONAS flagella has been reported[18], but the enzyme that is responsible has not been identified. By contrast, two enzymes that catalyse the opposing reaction — that is, deacetylation — have been recently described[19–21]: HDAC6 (histone deacetylase 6) is a member of the histone deacetylase family that is predominantly cytoplasmic and colocalizes with microtubules. It can deacetylate tubulin and microtubules *in vitro,* and suppression of HDAC6 function by pharmacological inhibitors or by small interfering RNA (siRNA) *in vivo* leads to a marked increase in tubulin acetylation. Conversely, overexpression of HDAC6 greatly decreased the level of tubulin acetylation. A second histone deacetylase, SIRT2 (the human Sir2 orthologue), which is NAD^+ dependent, has also been identified as a tubulin deacetylase[22], and shows preferential activity towards a tubulin peptide substrate in comparison to a histone peptide substrate. SIRT2 co-immunoprecipitates with HDAC6 from cell extracts, which raises the possibility that these enzymes cooperate to deacetylate tubulin *in vivo.*

Functions of microtubule acetylation. Microtubules acetylated with partially purified *Chlamydomonas* tubulin acetyltransferase showed normal assembly/disassembly characteristics *in vitro*[18]. *In vivo,* overexpression of a non-acetylatable α-tubulin variant in *Chlamydomonas*[23], or complete elimination of tubulin acetylation by site-directed mutagenesis of the usually acetylated lysine residue to arginine in *Tetrahymena*[24], had no observable phenotype. Also, disruption of the HDAC6 gene in embryonic stem cells, which led to highly increased tubulin acetylation levels, did not significantly affect cell proliferation or differentiation[21]. Although these results indicate that tubulin acetylation/deacetylation is not generally essential for cell survival, it has been reported to affect specialized functions. A role for tubulin acetylation in cell motility has been proposed on the basis that HDAC6 overexpression

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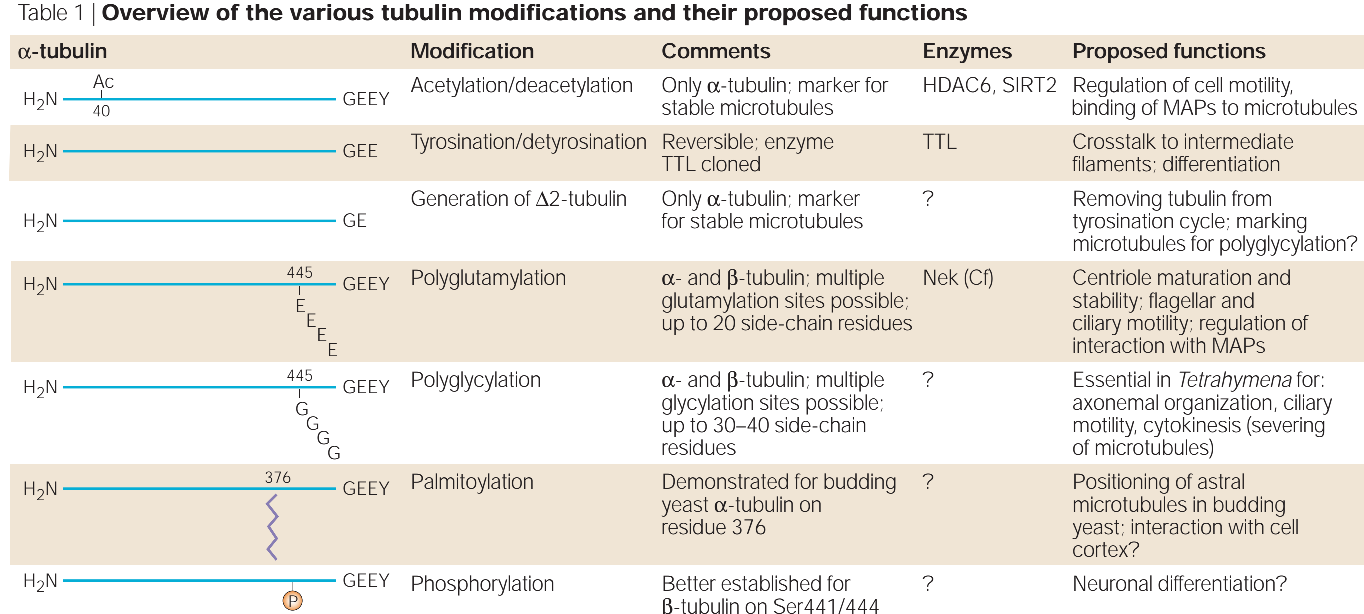
Table 1 | **Overview of the various tubulin modifications and their proposed functions**



α-tubulin	Modification	Comments	Enzymes	Proposed functions
H_2N —Ac 40— GEEY	Acetylation/deacetylation	Only α-tubulin; marker for stable microtubules	HDAC6, SIRT2	Regulation of cell motility, binding of MAPs to microtubules
H_2N — GEE	Tyrosination/detyrosination	Reversible; enzyme TTL cloned	TTL	Crosstalk to intermediate filaments; differentiation
H_2N — GE	Generation of Δ2-tubulin	Only α-tubulin; marker for stable microtubules	?	Removing tubulin from tyrosination cycle; marking microtubules for polyglycylation?
H_2N — 445 (E E E E) — GEEY	Polyglutamylation	α- and β-tubulin; multiple glutamylation sites possible; up to 20 side-chain residues	Nek (Cf)	Centriole maturation and stability; flagellar and ciliary motility; regulation of interaction with MAPs
H_2N — 445 (G G G G) — GEEY	Polyglycylation	α- and β-tubulin; multiple glycylation sites possible; up to 30–40 side-chain residues	?	Essential in *Tetrahymena* for: axonemal organization, ciliary motility, cytokinesis (severing of microtubules)
H_2N — 376 — GEEY	Palmitoylation	Demonstrated for budding yeast α-tubulin on residue 376	?	Positioning of astral microtubules in budding yeast; interaction with cell cortex?
H_2N — (P) — GEEY	Phosphorylation	Better established for β-tubulin on Ser441/444	?	Neuronal differentiation?

Ac, acetate; Cf, *Crithidia fasciculata*; E, glutamic acid; G, glycine; Nek, NIMA (never in mitosis gene A)-related kinase; HDAC6, histone deacetylase 6; MAP, microtubule-associated protein; P, phosphate; SIRT, Sir2 homologue; TTL, tubulin tyrosine ligase; Y, tyrosine.

increased the chemotactic movement of NIH-3T3 cells[19], whereas inhibition of HDAC6 inhibited cell migration[25]. Whether this reflects a direct effect of acetylation on microtubule stability and dynamics remains unsolved. Pallazzo *et al.* reported that the acetylated microtubules generated by HDAC6 inhibition do not show an increased resistance to the depolymerizing drug nocodazole[26]. By contrast, Matsuyama *et al.* find that a certain subpopulation of microtubules that are normally highly dynamic shows an increased stability after HDAC6 inhibition[20]. Clearly, further work is needed to dissect the mechanism by which tubulin acetylation influences cell motility.

Polyglycylation of microtubules

The occurrence of polyglycylation. Polyglycylation is the covalent attachment of a polyglycine side chain through an isopeptide bond to the carboxyl group of conserved glutamate residues that are in the carboxyl terminus of α- and β-tubulin. This prominent modification of axonemal microtubules was first detected using mass spectrometry (BOX 2) of carboxy-terminal tubulin peptides that were isolated from the cilia of *Paramecium*[27]. Polyglycylation can be very extensive — involving the addition of up to 34 extra residues — and it is the predominant modification in mammalian sperm tubulin[28,29]. Multiple neighbouring glutamate residues can be polyglycylated[30]. The use of monoclonal antibodies that differentially recognize either highly glycylated or only monoglycylated tubulin variants showed that in the ciliate *Paramecium*, polyglycylation occurred extensively in the axonemal tubulins, whereas the cytoplasmic pool of tubulin had only a moderate level of polyglycylation, in which most tubulin variants had side-chain lengths of one or two glycine residues[31].

siRNA
Small interfering RNA that is used to specifically reduce protein expression by degradation of the corresponding messenger RNA.

Two types of peptide bond are necessary to form a glycyl side chain (the same is true for glutamyl side chains, which will be discussed below). The branching point is formed by an isopeptide bond that involves the γ-carboxylate group of the glutamate. Elongation of the side chain then involves either regular α-peptide bonds or isopeptide bonds, which indicates that different enzymes might be necessary to establish the chemically different types of peptide bond.

Polyglycylation already occurs in the flagellated protist *Giardia lamblia*, which is considered to be the most primitive eukaryote[32] (BOX 3). It is thought to be absent from yeast, although mass-spectrometry analysis of carboxy-terminal tubulin peptides has not yet been conducted. It is important to point out that the evolutionary occurrence of both tubulin polyglutamylation and polyglycylation coincide with the appearance of functional cilia and flagella.

Polyglycylating enzymes. At present, virtually nothing is known about enzymes that catalyse polyglycylation. A deglycylating activity has been described for the *Paramecium* cytosol[31], which indicates that polyglycylation, just like the other tubulin modifications, is reversible. Organisms such as *Paramecium* or *Tetrahymena*, in which polyglycylation is the predominant tubulin modification, seem to be the most suitable starting material for biochemical purification of the glycylation activity in the future.

Possible functions of polyglycylation. Genetic studies using extensive *in vivo* mutagenesis of glycylation sites in *Tetrahymena* have provided the most detailed functional analysis of any tubulin modification so far[33]. Although mutation of all three glycylation sites of α-tubulin (by replacing the modified glutamate residue

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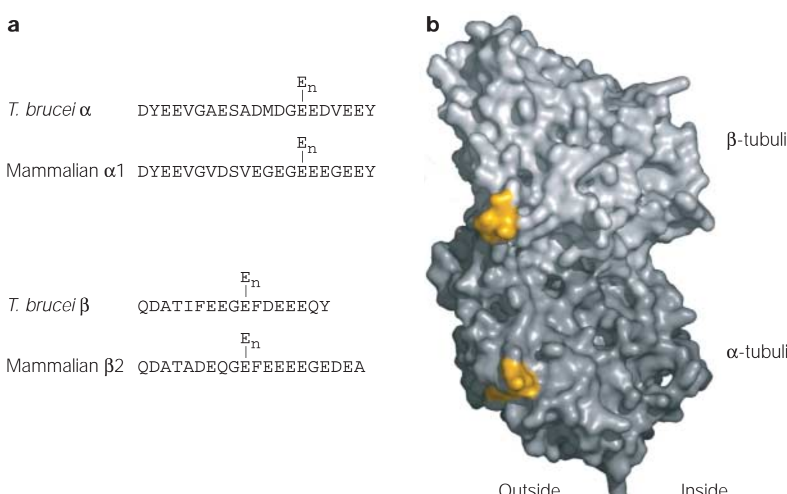

Figure 1 | **Conservation of modification sites and localization of carboxy-terminal domains. a** | Alignment of the carboxy-terminal residues of α- and β-tubulin from *Trypanosoma brucei* and mammalian brain tubulin. Note that the position of the glutamic-acid side chain is conserved in evolutionarily distant organisms. The carboxy-terminal sequences are rich in acidic residues such as glutamate and aspartate, and generally lack basic amino acids. There are examples in which multiple neighbouring residues can carry glutamate side chains. **b** | The position of the carboxy-terminal residues in the tubulin electron-crystallographic model. The extreme carboxy-terminal residues, which could be resolved in the electron-crystallographic structure of the tubulin dimer, are highlighted in yellow. Note that they lie on the outer surface of the microtubule. Part **b** is courtesy of E. Nogales, University of California, Berkeley, USA.

HETEROKARYONS
Multinucleate cells containing nuclei of more than one genotype.

9 + 2
The typical organization of microtubules within an axoneme, with 9 outer doublets and one pair of central microtubules.

BASAL BODY
A short cylindrical array of microtubules that is found at the base of cilia and flagella. It is closely related, in structure, to a centriole.

CENTRIOLES
Usually found in the centre of centrosomes in animal cells, the two centrioles contain triplet microtubules and are located orthogonally to each other. Centrioles are closely related to basal bodies.

MIDBODY
The structure formed at the end of animal-cell cytokinesis, which tethers the cells and can persist for some time.

HELA CELLS
A cultured human epithelial cell line derived from a cervical carcinoma.

with aspartate) had no observable effect, mutants lacking all five glycylation sites on β-tubulin were not viable. Importantly, no single site was essential and reduction of the glycylation level by mutating some, but not all, sites produced cells that showed abnormally slow growth and motility, as well as defects in cytokinesis (as shown by the appearance of multinucleate cells). The essential function of β-tubulin glycylation could be rescued by a chimeric tubulin in which the sequence of the wild-type β-tail was joined to the regular α-tubulin sequence. This strongly indicates that a certain threshold level of glycylation has to be reached to keep the cells alive.

Ciliates such as *Tetrahymena* possess two nuclei, which allows the introduction and analysis of potentially lethal mutations by generating HETEROKARYONS[34]. Cells with a lethal β-tubulin polyglycylation mutation — in which three of the five modified glutamates are changed to aspartate to prevent glycylation — failed to complete cytokinesis, whereas nuclear division was unaffected (FIG. 2). The cells had immobile cilia, as the axonemes frequently lacked the central microtubule pair (in the 9 + 2 arrangement) and had incomplete outer pairs that lacked the β-tubules. The axonemes also showed large gaps in the transition zone that normally forms the connection to the basal body. Significantly, the architecture of the BASAL BODY, which is rich in glutamylated, but not glycylated, microtubules, was unaffected. The cytokinesis defect was potentially caused by an incomplete severing of cortical longitudinal microtubules during cleavage. These experiments point to an important role for polyglycylation in axonemal organization and in assigning microtubules for certain cell-cycle regulated events, such as severing during cytokinesis.

Polyglutamylation of tubulin

The occurrence of polyglutamylation. In this unusual modification, a polyglutamate side chain of variable length is attached, through an isopeptide bond, to the γ-carboxylate group of a glutamate in the carboxy-terminal tail. Polyglutamylation was first demonstrated for α-tubulin from mammalian brain using mass spectrometry[35] (BOX 2) and has subsequently been detected in all main α- and β-tubulin isotypes[36–38]. Analysis of α-tubulin from the protist *Tritrichomonas mobilensis*[16], as well as the α4-tubulin isotype from the mammalian brain[39], shows that glutamylation can involve multiple neighbouring residues. Just as for polyglycylation, it already occurs in the protist *Giardia lamblia*[15] (BOX 3), although a lack of reactivity of the GT335 glutamylation-specific antibody indicates that it is probably absent from yeast tubulins. Importantly, the main modification sites are conserved in tubulins from *Trypanosoma* to mammalian brain[17] (FIG. 1). In protists, polyglutamylation is a prominent modification of stable microtubule populations such as the flagellar axoneme, the basal bodies and the submembranous microtubules[40,41]. It is also prominent in axons of mammalian neuronal cells, whereas in non-neuronal cells of vertebrates, GT335 labels the CENTRIOLES, the mitotic spindle and MIDBODY, as well as the primary cilium, as the main sites of tubulin polyglutamylation[42]. This modification is not restricted to tubulins, as it has also been shown for the nucleosomal assembly proteins NAP1 and NAP2 (REF. 43), which raises the possibility that chromatin structural proteins could also be regulated through this modification. Just like the glutamylation sites in tubulin, the modified residues of NAP1 and NAP2 lie in a highly acidic environment. As microtubules and chromatin-structure proteins share acetylation and polyglutamylation, any possible coordination or crosstalk of these modifications should be addressed in future studies.

Polyglutamylating enzymes. Preparations of tubulin polyglutamylase have been isolated from mouse brain and from the trypanosomatid *Crithidia fasciculata*. There are interesting similarities and differences between these preparations. The mouse enzyme shows a sedimentation constant of 10S, which corresponds to a molecular weight of 300 kDa, and is probably organized into a multimeric structure[44]. Although the preparation has been purified ~1,000-fold, this has not been sufficient to identify the polypeptides that are responsible for polyglutamylation. With the generation of monoclonal antibodies that can immunoprecipitate the glutamylation activity from this enriched fraction, it has recently been possible to identify the mouse protein Pgs1 as a subunit of brain tubulin polyglutamylase. This 32-kDa protein does not show catalytic activity, but could be implicated in localizing the enzyme to sites of glutamylation[45].

Work in HELA CELLS has shown that polyglutamylation is cell-cycle regulated. Although the polyglutamylase activity peaks in G2, the level of glutamylated

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Box 2 | Analysis of tubulin modifications

Several techniques have been used to investigate post-translational modifications. First, high-resolution ISOELECTRIC FOCUSING can separate polypeptides according to their net charge. This technique resolves brain tubulin into more than 20 spots, far more than is expected from the number of isotypes that are expressed. Second, monoclonal antibodies raised against peptides that specifically mimic the modified, or unmodified, tubulin carboxyl termini have been used to study the localization of modified microtubules. They have also been microinjected into cultured cells to obtain information about possible functions of these modifications. Third, mass spectrometric analysis of isolated carboxy-terminal tubulin peptides has been essential in detecting polyglutamylation and polyglycylation. Digestion of tubulin with specific proteinases such as endoproteinase LysC, or chemical cleavage at methionine residues with cyanogen bromide, releases the carboxyl terminus of α- and β-tubulin, respectively. The peptides can be purified by ion-exchange chromatography and high-performance liquid chromatography and analysed by mass spectrometry. Modified peptides typically give rise to a series of regularly spaced peaks that are separated by mass increments of 57 (glycine) or 129 (glutamate) mass units. The exact position of the modified residue can be determined by sequencing these peptides by EDMAN DEGRADATION, which typically produces a 'gap' in the sequence. Modern mass-spectrometric techniques such as TANDEM-MS can be used to directly sequence branched peptides[101,102]. In the future, a wider use of 'tubulin proteomics' to investigate modifications under different cellular conditions could be helpful to gain insights into the function of these modifications.

ISOELECTRIC FOCUSING
A method to separate proteins according to their isoelectric point; it is carried out by electrophoresis in a pH gradient.

EDMAN DEGRADATION
A classical method of peptide sequencing by stepwise degradation and identification of the amino-terminal amino acid.

TANDEM-MS
(or MS/MS). A variant of mass spectrometry that is used to sequence peptides and determine their structure.

TRYPANOSOMES
Flagellate protozoans, ubiquitous parasites of insects, birds and mammals; some species are important human pathogens.

CENTROSOME
The main microtubule-organizing centre of animal cells. It functions as a spindle pole during mitosis.

POLYCYSTIC KIDNEY DISEASE (PKD)
A genetic disease that is characterized by the formation of multiple cysts in the kidney, which ultimately leads to loss of renal function and the need for dialysis or transplantation.

KINESIN
A microtubular motor protein that generally moves towards the plus end of microtubules.

tubulin is maximal during mitosis. Differences in the extractability of the glutamylation activity with detergents indicates the existence of at least two isozymic variants, which glutamylate either HeLa β-tubulin, or HeLa α-tubulin and brain tubulin[46].

Polyglutamylation activity has also been purified from the trypanosomatid *C. fasciculata*. Isolated cytoskeletons from TRYPANOSOMES contain an activity that incorporates glutamic acid into tubulin in an ATP-dependent manner[47]. This activity also accepts mammalian brain tubulin as well as synthetic peptides that mimic the carboxy-terminal residues of α- and β-tubulin as substrates[48]. Like the mouse enzyme, the trypanosomal glutamylase is strongly inhibited by salt, but it seems to behave as a single polypeptide with a sedimentation constant of 3S, which corresponds to a molecular weight of 40–50 kDa. The main protein that copurifies with glutamylation activity in this system has been identified as *C. fasciculata* Nek[49], a NIMA (never in mitosis gene A)-related kinase (Nek) that localizes to the basal body and is found in a decreasing gradient along the flagellum — this matches the glutamylation profile of the axoneme. Interestingly, kinases of this family influence CENTROSOME organization and have also been implicated in POLYCYSTIC KIDNEY DISEASE (PKD)[50]. Recently, it has been shown that the underlying defect in this disease is a failure to assemble a functional primary cilium[51], which is a main site of tubulin polyglutamylation. These results provide a potential link between a tubulin modification and an important human disease. The human genome encodes at least 11 Neks[52]. Disruption of the *Nek1* gene in mice causes pleiotropic effects, including male sterility and PKD[53], which is potentially consistent with a role for Nek1 in tubulin modification. As *C. fasciculata* Nek was not functional when expressed in a heterologous system, it remains to be established whether it catalyses tubulin glutamylation directly or whether it is a regulatory enzyme. The identification of further polypeptides that constitute the mouse enzyme will shed more light on how glutamylase preparations from evolutionarily distant organisms relate to each other. Finally, polyglutamylation is a reversible modification, and a deglutamylase activity has been detected in neurons[54].

Functions of polyglutamylation. The genetic experiments in *Tetrahymena* point to an essential function for polyglycylation in axonemal organization, but evolutionarily ancient trypanosomes have functional axonemes in spite of the fact that their tubulins lack polyglycylation and are instead highly polyglutamylated[17]. Although this might argue that there is some degree of functional redundancy between the two polymodifications, it is noteworthy that other primitive organisms maintain both modifications, which indicates that they might have distinct functions. Moreover, the introduction of a highly anionic glutamate side chain versus a neutral glycine side chain is likely to have very different effects on the tubulin molecule.

Accumulating evidence indicates that polyglutamylation can differentially influence the interaction between microtubules and their associated proteins. *In vitro* assays have shown that microtubule-associated proteins (MAPs) such as KINESIN and tau show the strongest affinity for microtubules that have been modified by the addition of glutamic-acid side chains with a length of three residues, whereas their affinity for longer side chains is decreased[55,56]. By contrast, the binding of MAP1B to microtubules remained strong even for microtubules with longer side chains. Importantly, both conventional and monomeric kinesins[57] have conserved basic residues that interact with the negatively charged carboxyl terminus of tubulin. Site-directed mutagenesis of these basic residues has shown that the processivity of kinesins depends on electrostatic interactions between the kinesin neck region and the extreme carboxyl terminus of tubulin[58]. Is is conceivable that glutamylation could be used by the cell to increase the processivity of kinesins — for example, during mitosis when the spindle microtubules become highly glutamylated.

Box 3 | Evolutionary considerations

Tubulin modifications developed early during eukaryotic evolution, possibly together with the appearance of motile cilia and flagella. They are present in many eukaryotes, but the three ancient groups — trichomonads (*Tritrichomonas mobilensis*), DIPLOMONADS (*Giardia lamblia*) and trypanosomatids (*Trypansoma brucei*) — show differing sets of tubulin modifications. Whereas tubulin acetylation and polyglutamylation are found in all of these species, detyrosination only occurs in trypanosomatids and therefore seems to have developed later during evolution. Polyglycylation is present in the diplomonad *Giardia lamblia*, the most primitive of these species, but it is lost in the branches leading to the trypanosomatids and trichomonads[94].

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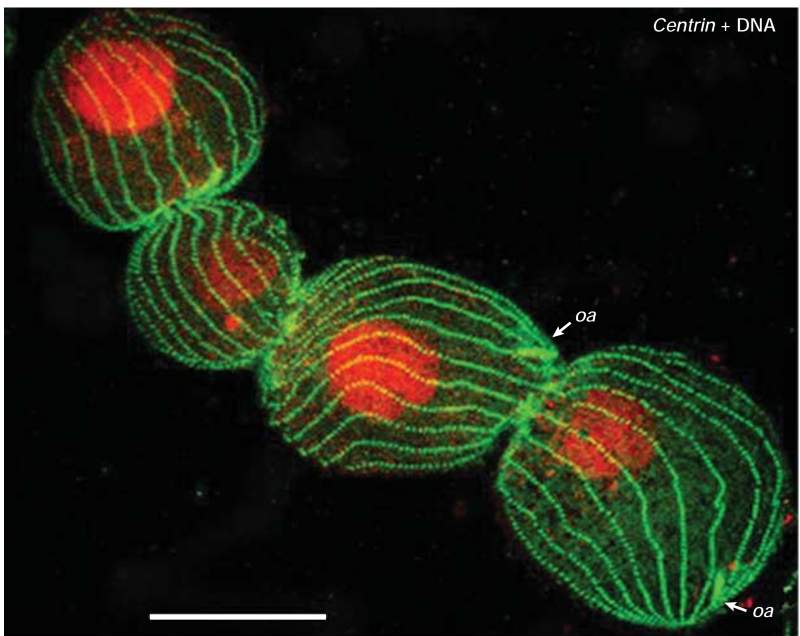

Figure 2 | **Polyglycylation mutants affect cytokinesis in *Tetrahymena*.** A *Tetrahymena* cell with a mutation that prevents β-tubulin polyglycylation fails to complete cytokinesis, whereas nuclear division (the DNA is labelled in red) occurs normally. The underlying defect might be a failure to sever longitudinal microtubules. Centrin (green) denotes the position of the cortical array of basal bodies. *oa*, oral apparatus. Scale bar represents 25 μm. Reproduced with permission from REF. 34 © (2002) Macmillan Magazines.

Antibodies against glutamylated tubulin (GT335 and B3) specifically inhibit the beating of cilia and flagella[59]. In human ciliated epithelial cells, anti-glutamylation antibodies were more potent than anti-polyglycylation antibodies at reducing the ciliary-beat frequency[60]. The antibodies are likely to inhibit ciliary beating by preventing the interaction between ciliary dynein and the B-tubules of the outer axoneme doublets.

Centrioles are the main sites of glutamylation in non-neuronal cells. Interestingly, microinjection of the GT335 antibody into HeLa cells leads to a disappearance of centrioles and a loss of the centrosome as a defined organelle[61]. This effect is very specific for the GT335 antibody and it highlights a potential role for polyglutamylation in the maturation and long-term stability of centrioles. Polyglutamylation has been found to occur early during the genesis of centrioles[60] and it is thought that polyglutamylation of the outer centriole walls could recruit specific proteins that help to constitute the centriolar matrix and anchor the centriole microtubules[62].

DIPLOMONAD
A primitive single-celled organism with two nuclei and no mitochondria; includes the human intestinal parasite *Giardia lamblia*.

B-TUBULES
Incomplete, 10-protofilament microtubules that comprise a part of the outer axoneme doublets.

The tyrosination cycle of α-tubulin

α-tubulin is tyrosinated. The addition of a carboxy-terminal tyrosine to most α-tubulins is an intensively studied post-translational modification (FIG. 3). As a carboxy-terminal tyrosine is encoded for in most α-tubulins by the messenger RNA, the primary modification is its removal by a tubulin tyrosine carboxypeptidase (TTCP)[63]. The resulting detyrosinated tubulin exposes a carboxy-terminal glutamic acid and is therefore often referred to as Glu-tubulin (not to be confused with polyglutamylated tubulin). The transfer-RNA-independent incorporation of tyrosine into tubulin was demonstrated in brain homogenates some 30 years ago[64–66]. This ATP-dependent reaction restores the carboxy-terminal tyrosine to yield Tyr-tubulin and is catalysed by tubulin tyrosine ligase (TTL). Interestingly, removal of the penultimate glutamic acid leads to the formation of Δ2-tubulin[67], which is no longer a substrate for TTL[68] and thereby escapes the tyrosination cycle. Coordination between different post-translational modifications has been indicated by the recent finding that, whereas most brain tubulin is polyglutamylated, brain Δ2-tubulin seems to be specifically polyglycylated[69]. This, however, challenges the previous finding that Δ2-tubulin can carry 1–4 glutamyl units[67], so more work is needed to clarify this point.

A differential distribution of post-translational modifications within a supramolecular microtubule structure has been reported for the axoneme of sea urchin sperm. The A- and B-TUBULES of the outer axoneme doublets strongly differ in the extent to which their tubulin is post-translationally modified: whereas the A-tubules are mostly unmodified and contain Tyr-tubulin, the B-tubules contain detyrosinated tubulin and are extensively modified by polyglycylation[70]. This differential distribution has also been reported for the axonemes of *Chlamydomonas* flagella[71] (FIG. 4). As the tyrosinated A- and central-pair tubules reach past the detyrosinated B-tubules at the tip of the axoneme, they produce a distal zone that is enriched in tyrosinated tubulin. Moreover, a decreasing gradient of polyglutamylation and polyglycylation along the flagellum has been described[72,73], with tubulin in the proximal part of the flagellum being more strongly modified than that in the distal part.

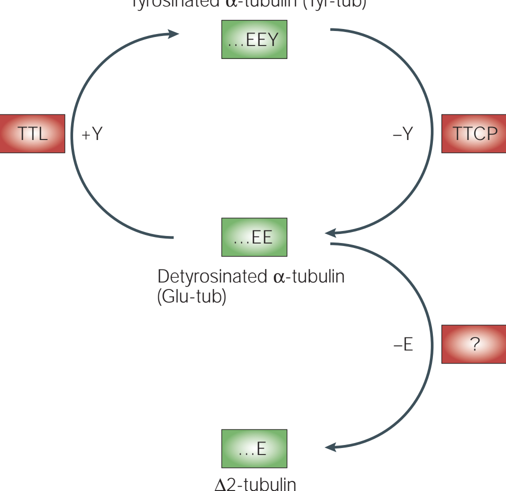

Figure 3 | **The tyrosination cycle of α-tubulin.** The carboxy-terminal tyrosine of α-tubulin can be removed by the tubulin tyrosine carboxypeptidase (TTCP) to generate Glu-tubulin (Glu-tub). In an ATP-dependent reaction, the carboxy-terminal tyrosine (Tyr-tub) can be restored through the enzymatic activity of tubulin tyrosine ligase (TTL). Glu-tubulin can lose the penultimate glutamate residue through the activity of an unknown peptidase to generate Δ2-tubulin, which cannot function as a substrate for TTL and is therefore removed from the cycle.

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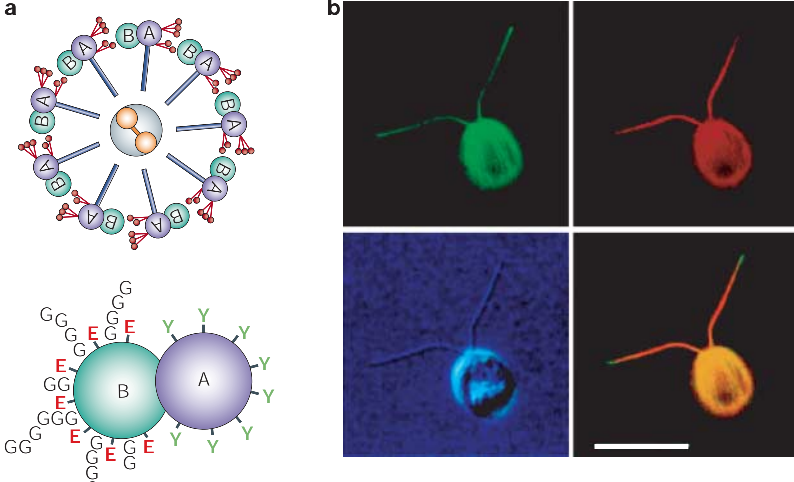

Figure 4 | **Differential distribution of modified microtubules within the flagellar axoneme.** **a** | The upper figure shows a cross-section through a typical flagellum, with the 9 + 2 arrangement of microtubules, dynein arms reaching towards the B tubules and radial spokes that are directed towards the centre. The A and B tubules of the outer axoneme are differentially modified (lower figure). A tubules contain tyrosinated (Y) tubulin. The B tubules contain detyrosinated (E) and polyglycylated (G) microtubules. **b** | Immunofluorescence of *Chlamydomonas*, labelling tyrosinated tubulin (green, upper left panel), and detyrosinated tubulin (red, upper right panel). The merged image (lower panel, right) shows a distal zone that is rich in tyrosinated tubulin (green) at the tips of *Chlamydomonas* flagella. Lower panel (left), phase contrast image. Scale bar represents 10 µm. Part **b** is reproduced with permission from REF. 70 © (1998) The Company of Biologists Ltd.

Tyrosine ligases and carboxypeptidases. Purification of TTL was first described by Murofushi *et al.*[74], but only when monoclonal antibodies against TTL were generated could sufficient amounts of the mammalian enzyme be immunoaffinity purified for peptide sequencing and cDNA cloning[75]. Sequence analysis showed that TTL is a member of the group of glutathione-synthetase ADP-forming enzymes[76], which have an ATP-dependent ligase activity. Although there are candidate homologues of TTL in the sequenced genomes of *Drosophila melanogaster*, *Caenorhabditis elegans* or *Leishmania major*, no functional characterization of these enzymes has been carried out so far.

Much less is known about TTCP. A 250-fold purification of this activity has been described[77], but no polypeptides have been identified. Purification has proven difficult, as radioactively tyrosinated microtubules must be used as substrates, and the specific release of the carboxy-terminal tyrosine has to be distinguished from non-specific proteolysis.

Functions of the tyrosination cycle. Detyrosination occurs after microtubule assembly, as the TTCP prefers polymers, whereas the TTL prefers tubulin dimers, as substrates[78,79]. Although detyrosination itself does not stabilize microtubules[80], it can be used as a marker for how long a microtubule has been assembled. Signal-transduction pathways leading to the formation of Glu-microtubules — microtubules that have been detyrosinated — have been investigated, and they involve the GTPase Rho[81], and Dia[82], a homologue of the yeast FORMINS. These Glu-microtubules seem to be stabilized by suppression of dynamics at the PLUS END through a capping complex of unknown composition[83].

Detyrosination might be important for the coordination of different cytoskeletal elements, as vimentin INTERMEDIATE FILAMENTS preferentially co-align with detyrosinated microtubules[84]. This interaction seems to be dependent on kinesin and can be blocked with antibodies against detyrosinated tubulin or kinesin, or by injecting chemically modified, non-polymerizable detyrosinated α-tubulin[85,86].

In an attempt to analyse the function of the tyrosination cycle directly, the TTL gene has been knocked out in mice. Unexpectedly, homozygous TTL-knockout mice are born without any obvious malformations, but die within the first day after birth. TTL is necessary at a certain developmental stage in some neuronal cell types. The phenotype is currently under analysis (D. Job and J. Wehland, personal communication).

Δ2-tubulin (which, as mentioned above, is not a substrate for TTL) constitutes a considerable proportion of brain tubulin. In cultured mammalian cells, its appearance can be used to screen for cell lines that have a low TTL activity. In this way, it has been possible to obtain NIH-3T3-derived clonal cell lines that are deficient for TTL activity[87]. Although these cells have a normal microtubular cytoskeleton, they frequently give rise to tumours when injected into nude mice. There is evidence that, during tumorigenesis, TTL activity is specifically lost. In fact, in the majority of breast cancer cells that have been investigated, TTL activity was reduced and the detyrosination level was elevated as a consequence[88]. An antibody-based assay for elevated levels of detyrosinated and Δ2-tubulin might therefore be of use in tumour diagnosis.

It has been shown that α-tubulin can also incorporate 3-nitrotyrosine[89], a modified amino acid that is generated by the reaction of nitric oxide species with tyrosine. Nitrotyrosination is catalysed by TTL *in vitro* and *in vivo*[90]. It has been suggested that this incorporation is irreversible and that the accumulation of nitrotyrosine leads to microtubule dysfunction and cellular injury in epithelial lung carcinoma cells[89]. Nitrotyrosine has also been used to suppress the detyrosination of microtubules during myogenic differentiation in L6 MYOBLASTS. This treatment blocked early myogenic events and differentiation into myotubes, indicating a potential role for Glu-microtubules in the initiation of a correct differentiation program[91].

Additional modifications

Phosphorylation. Phosphorylation of a serine residue (Ser441/444) within the carboxy-terminal tail of β-tubulin has been reported[92], but it is not a widely observed modification and its precise function is unknown. Phosphorylation of class III β2-tubulin accompanies neurite outgrowth and is dependent on the polymer level, which raises the possibility that it is part of a differentiation program[93]. Phosphorylation has an important role in the regulation of some MAPs, but it interesting to note that it only seems to be a minor modification for microtubules. The reason might be

RHO-FAMILY GTPases
Ras-related small GTPases that mediate signal transduction to cause rearrangements of the actin- and microtubule-filament network.

FORMINS
Rho-GTPase effector proteins that link signal-transduction pathways to actin assembly proteins.

PLUS END
The end of a microtubule at which addition of tubulin dimers occurs most rapidly.

INTERMEDIATE FILAMENTS
10-nm protein filaments that constitute one of the three main cytoskeletal filaments of eukaryotic cells.

MYOBLAST
A specialized cell type that, by fusion with other myoblasts, forms myotubes that eventually differentiate into skeletal-muscle fibres.

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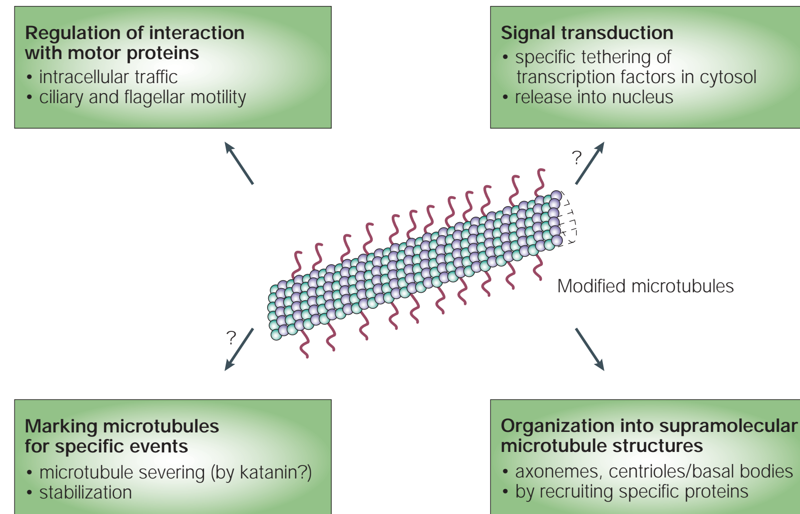

Figure 5 | **Proposed roles for post-translationally modified microtubules.** A schematic overview of possible functions for modified microtubules. Glutamate or glycine side chains are depicted in red and these modifications could have various functions. There is accumulating experimental evidence that implicates tubulin modifications in the generation of higher-order microtubule structures, such as axonemes, and in the regulation of interactions with associated proteins. The modifications could also have a role in signal transduction and in marking microtubules for specific events, but this is still largely speculative and awaits experimental verification.

that tubulin itself is a highly anionic protein and the introduction of a negatively charged phosphate group might not be sufficient to change the structure or charge of the protein. It is therefore possible that 'new' modification variants such as polyglutamylation or polyglycylation arose during evolution to introduce bulky side chains and multiple charged residues and thereby provide sufficient structural change to signal from microtubules.

Palmitoylation. Tubulin has been shown to incorporate radioactively labelled [^{3}H]palmitate, predominantly on the α-subunit at cysteine 376 (REFS 92,94,95). Mutation of the corresponding cysteine residue to serine in the budding yeast *Saccharomyces cerevisiae* reduced the *in vivo* palmitoylation level by 60% (REF. 96). Mitosis proceeded normally in these cells, but some defects in the positioning of ASTRAL MICROTUBULES and in the translocation of the spindle through the BUD NECK were observed. This indicates that palmitoylation of α-tubulin could contribute to the establishment of correct microtubule interactions with the cell cortex.

ASTRAL MICROTUBULES
A subset of microtubules within the mitotic spindle that are not attached to the kinetochore but extend from the centrosome to the cell cortex.

BUD NECK
A constriction between the mother and daughter cell in the budding yeast *Saccharomyces cerevisiae*.

Conclusions and perspectives

Summarizing the recent functional studies on tubulin modifications, the following picture emerges: polyglycylation and, most probably, polyglutamylation, have essential functions in the generation of higher-order microtubular structures such as axonemes and basal bodies. They influence ciliary and flagellar motility and can regulate microtubule-associated events such as severing. The mechanism of their action is very probably the selective recruitment of proteins and the regulation of interactions between microtubules and structural, as well as motor, MAPs (FIG. 5). Tubulin acetylation is not generally essential but seems to have a function in the regulation of cell motility. The tubulin tyrosination cycle still remains enigmatic — it is not required in cultured cells, but the lethal TTL knockout points to an important, but as yet unidentified, function.

By analogy with the histone amino-terminal modifications, combinations of the described tubulin modifications could create an elaborate 'code' that can be read by associated proteins. In addition to the generation of higher-order microtubular structures, this could also be important for signal-transduction events, as microtubules can tether certain transcription factors in the cytosol[97] and specifically release them into the nucleus after receiving upstream signals. It will be interesting to see whether the introduction or alteration of microtubule modifications has a role in this process.

Clearly, the field is still at the very beginning of understanding the functions of tubulin modifications. Further identification of the enzymes that are involved and their functional analysis in genetically tractable organisms such as *Tetrahymena* will help us gain further insights. A closer look at the role of tubulin modifications in certain cilia-based diseases, such as PKD, could also prove to be very fruitful. In the future, these studies will provide a better understanding of the modifications of tubulin — one of the main cytoskeletal elements of eukaryotic cells.

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Competing interests statement

The authors declare that they have no competing financial interests.

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