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1 **Mechanisms and functions of the tubulin code**

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10

11 **Abstract:**

12 Microtubules are core components of the eukaryotic cytoskeleton with essential roles in cell  
13 division, shaping, intracellular transport, and motility. Despite their functional heterogeneity,  
14 microtubules have a highly conserved structure made from almost identical molecular  
15 building blocks; the tubulin proteins. Alternative tubulin isotypes and a variety of  
16 posttranslational modifications control the properties and functions of the microtubule  
17 cytoskeleton, a concept known as the ‘tubulin code’. This concept first emerged with the  
18 discovery that  $\alpha$ - and  $\beta$ -tubulin are each encoded by multiple genes, but it took decades  
19 before its functional importance begun to emerge. Here we review the current understanding  
20 of the molecular components of the tubulin code, and how they impact microtubule properties  
21 and functions. We discuss how tubulin isotypes and posttranslational modifications control  
22 microtubule behaviour at the molecular level, and how this translates into physiological  
23 functions at the cellular and organism levels. We further show how the fine-tuning of  
24 microtubule functions by some tubulin modifications affects homeostasis, and how its  
25 perturbation can lead to a large variety of dysfunctions, many of them linked to human  
26 disorders.

27

28

## 29 **Introduction**

30 Microtubules are cytoskeletal filaments with an outer diameter of 25 nm. Their hollow shape  
31 endows them with a unique mechanical rigidity<sup>1</sup> that allows for the assembly of large  
32 intracellular structures. Microtubules are intrinsically dynamic; they constantly alternate  
33 between phases of polymerization and spontaneous depolymerization, a process known as  
34 dynamic instability<sup>2</sup>. How to tame such a fluctuating system into highly ordered and  
35 controlled structures such as mitotic and meiotic spindles ensuring the correct division of  
36 cells<sup>3-5</sup>, axonemes that are the central molecular machines of cilia and flagella<sup>6-8</sup>, or the  
37 cytoskeleton of neurons that controls neuronal connectivity and function over an entire  
38 lifetime<sup>9-11</sup> is a fascinating problem that has caught the attention of a large scientific  
39 community for over half a century<sup>12</sup>.

40 Since the early ultrastructural analyses of microtubules by electron microscopy<sup>13,14</sup>, huge  
41 advances have been made in understanding the molecular structure of the microtubule lattice  
42 and the arrangement of the  $\alpha/\beta$ -tubulin heterodimers within<sup>15-19</sup>. The discovery of many  
43 microtubule-associated proteins (MAPs) as factors that influence microtubule assembly and  
44 dynamics revealed that microtubule assemblies could attain specific characteristics, and thus  
45 functions, by associating with selected subsets of MAPs<sup>20</sup>. Specific combinations of active  
46 molecular motors and structural MAPs can thus explain the mechanisms of self-organizing  
47 assemblies such as mitotic and meiotic spindles<sup>21,22</sup>.

48 The understanding of how microtubules form characteristic assemblies together with a  
49 plethora of MAPs and motors, and how these assemblies fulfil specific functions has strongly  
50 advanced in all areas of cell biology. Some of these MAPs are specific end-binding proteins  
51 that control microtubule dynamics and attachment to other cellular structures<sup>23</sup>. Other MAPs  
52 bind the entire microtubule lattice, and are thus considered to regulate microtubule dynamics  
53 and stability, but might also have more specific roles that remain to be explored<sup>20</sup>. By  
54 contrast, how microtubules themselves are functionally modulated by incorporation of  
55 specific tubulin gene products, called isotypes, or by tubulin posttranslational modifications  
56 (PTMs), has remained unclear until the beginning of the 21<sup>st</sup> century. Why these molecular  
57 processes, commonly conceptualised under the term 'tubulin code'<sup>24</sup> (Fig. 1), have for a long  
58 time resisted a thorough functional characterization became only recently apparent. Emerging  
59 molecular and functional studies reveal that in many cases, the tubulin code acts as a fine-  
60 regulator, and not as a binary switch of microtubule functions. In this review we will

61 summarize the current understanding of the tubulin code, its elements and their regulation,  
62 and discuss the functional implications of this code on the cell and organism levels.

63

#### 64 **The tubulin code elements**

65

66 Microtubules exist in every eukaryotic cell. The striking sequence conservation of tubulins  
67 throughout evolution is reflected in an almost identical fold of tubulin in virtually every  
68 species investigated so far<sup>25,26</sup>, with the consequence that tubulin of a variety of eukaryotic  
69 organisms assembles into highly similar microtubules: hollow tubes mostly, but not  
70 exclusively, built of 13 protofilaments in cells. As tempting as it appears to talk of  
71 evolutionary conservation in this case, in reality microtubules can be different between  
72 species, and even within single species functionally specialized microtubules have been  
73 observed.

74

#### 75 1) Tubulin isotypes

76 Tubulin isotypes arise from the expression of alternative tubulin genes, and their numbers  
77 vary largely between species and phyla. In yeast, for instance, there are two genes for  $\alpha$ -<sup>27</sup> and  
78 only one for  $\beta$ -tubulin<sup>28</sup>, whereas the human genome contains nine genes for each,  $\alpha$ - and  $\beta$ -  
79 tubulin<sup>29</sup>. There is no clear evolutionary trajectory of these tubulin genes, which is why  
80 orthologs can only be identified in evolutionary close species. This is reflected in the rather  
81 confusing nomenclature of the tubulin genes<sup>30</sup>. ‘Generic’  $\alpha$ - and  $\beta$ -tubulins are highly  
82 conserved between evolutionarily distant species, while more unique isotypes appear to have  
83 evolved when novel microtubule functions arose. A striking example is the co-evolution of  
84 blood platelets and  $\beta$ 1-tubulin (TubB1)<sup>31</sup>. Platelets are small cell fragments essential for blood  
85 coagulation that exist only in mammals. Platelets assemble a specialized microtubule array,  
86 the marginal band, which requires  $\beta$ 1-tubulin<sup>32</sup>, a highly divergent isotype in the vertebrate  
87 phylum. Another example is  $\beta$ 3-tubulin ( $\beta$ Tub60D) in *Drosophila melanogaster*, an isotype  
88 that is only expressed in subsets of cells during development. Genetic experiment  
89 demonstrated that this isotype cannot replace the generic  $\beta$ 2-tubulin ( $\beta$ Tub85D) in key  
90 microtubule functions such as axoneme assembly or spindle formation<sup>33</sup>, suggesting that it  
91 had evolved for a specific developmental processes in fly. While these particular cases clearly

92 underpin the notion that tubulin isotypes can be essential to form functionally specialized  
93 microtubules, it still remains an open question why so many other tubulin isotypes (Fig. 1) are  
94 almost identical in many species, including mammals. We will try to provide some answers to  
95 this question in this review.

96

## 97 2) Tubulin posttranslational modifications

98 Tubulin is subjected to a large number of PTMs (Fig. 1; Table 1). Some of them are found on  
99 a broad range of proteins such as phosphorylation<sup>34-50</sup>, acetylation<sup>51</sup>, methylation<sup>52</sup>,  
100 palmitoylation<sup>53</sup>, ubiquitination<sup>54,55</sup>, or polyamination<sup>56</sup>, while others were initially discovered  
101 on tubulin. Examples for such ‘tubulin-specific’ PTMs are the enzymatic, ribosome-  
102 independent incorporation of tyrosine (tyrosination)<sup>57,58</sup>, glutamate ([poly]glutamylolation)<sup>59-61</sup>,  
103 or glycine ([poly]glycylation)<sup>62</sup>, or the enzymatic removal of single amino acids from the C-  
104 terminus of tubulin, such as detyrosination<sup>63,64</sup>, or the generation of  $\Delta 2$ -<sup>65,66</sup> or  $\Delta 3$ -tubulin<sup>67</sup>  
105 (Fig. 1; Table 1; Box 1).

106 Most PTMs label distinct microtubule subpopulations in cells, and are expected to ‘encode’  
107 those microtubules for specific functions. Enzymes catalysing detyrosination<sup>68</sup>, acetylation<sup>69</sup>  
108 and polyglutamylolation<sup>70</sup> were shown to preferentially modify microtubules vs. the soluble  
109 tubulin dimers, underpinning that a targeted modification of selected microtubules in cells is  
110 mechanistically feasible.

111 In the past decade, great advances in the understanding of the biological roles of tubulin  
112 acetylation, [de]tyrosination, [poly]glutamylolation and [poly]glycylation have been made,  
113 which is why we will focus on those PTMs in this review. Most research has focussed on the  
114 role of those PTMs on tubulin, which appears to be the main substrate for glutamylolation and  
115 glycylation, however other, non-tubulin substrates have also been described (Box S1).

116

## 117 **Regulation of microtubule properties**

118 The concept that the incorporation of different tubulin variants can affect intrinsic properties  
119 of microtubules, such as flexibility, or assembly/disassembly dynamics, is as old as the  
120 discovery of tubulin isotypes<sup>71</sup>. However, mechanistic insights into how tubulin isotypes and  
121 PTMs control microtubule properties have mostly been obtained in the recent years.

122

### 123 1) Control of mechanic properties

#### 124 *1.1) The tubulin code can determine structural features of microtubules*

125 Recent advances in cryo-electron microscopy provided high-resolution structures of entire  
126 microtubules<sup>16,17,25,72,73</sup> that now directly visualise which amino acid residues of  $\alpha$ - and  $\beta$ -  
127 tubulins are critically involved in the formation of the microtubule lattice. The availability of  
128 these structures makes it now possible to model how different tubulin isotypes, which often  
129 differ in only a few amino acids, could alter the properties of microtubules, for instance due to  
130 their involvement in lattice contacts. Indeed, evolutionary distant mammalian and yeast  
131 tubulins both assemble into highly similar 13-protofilament microtubules, but show  
132 differences in microtubule structure and mechanics<sup>25,74</sup>. Novel approaches to generate  
133 recombinant mammalian tubulin<sup>75-77</sup> allowed to directly demonstrate a strong impact of  
134 mammalian  $\beta$ -tubulin isotypes on structural features of the microtubules: while  $\alpha$ 1B/ $\beta$ 2B-  
135 tubulin (TubA1B/TubB2B) assembled preferentially into 14-protofilament microtubules *in*  
136 *vitro*,  $\alpha$ 1B/ $\beta$ 3- (TubA1B/TubB3) microtubules mostly formed 13-protofilament  
137 microtubules<sup>77</sup>.

138 *Caenorhabditis elegans*, a worm built of only 959 somatic cells, shows a large structural  
139 divergence between microtubules of different cell types. Most somatic cells contain 11-  
140 protofilament microtubules, however some neurons assemble hyper-stable 15-protofilament  
141 tubes, and cilia form their axonemal microtubule doublets with A-tubules of  
142 13 protofilaments<sup>78</sup> (Fig. 2a). This diversity of microtubule structures is mirrored by a  
143 relatively large sequence variability of *C. elegans* tubulin isotypes. Indeed, specific  $\alpha$ - and  $\beta$ -  
144 tubulin isotypes are required for the assembly of 15-protofilament<sup>79,80</sup>, or ciliary  
145 microtubules<sup>81,82</sup> in this organism. The concept that tubulin isotypes are determinants of  
146 protofilament numbers was further corroborated by a cross-species study. The formation of  
147 16-protofilament accessory microtubules, normally found in the sperm tails of the moth

148 *Heliothis virescens*, but not in the fly *Drosophila melanogaster*, could be induced by  
149 expressing the testis-specific *TUBB2* gene from *Heliothis virescens*<sup>83</sup> in *Drosophila*.  
150 Direct evidence for the intrinsic capacity of tubulin isotypes to determine microtubule  
151 structure was recently provided by assembling microtubules from purified *C. elegans* and  
152 bovine brain tubulin *in vitro*. Similar to previous observations in cells, *C. elegans* tubulin  
153 preferentially assembled into 11-protofilament microtubules, while bovine brain tubulin  
154 formed 13- and 14-protofilament microtubules<sup>26</sup>. While it cannot be excluded that PTMs  
155 present on these purified tubulins influence protofilament numbers, these *in vitro* experiments  
156 provide strong evidence for the concept that isotypes do directly determine the structure of  
157 microtubules. In cells, however, microtubules assemble in the presence of interacting proteins  
158 such as doublecortin<sup>84</sup>, or the yeast orthologue for EB1 - Bim1p<sup>74</sup>, which can further influence  
159 protofilament number.  
160 Finally, emerging evidence suggests that tubulin PTMs can also influence the structure of  
161 microtubules. The assembly of the characteristic 15-protofilament microtubules in *C. elegans*  
162 touch receptor neurons, for instance, is dependent on Mec-17 (aTAT1)-mediated tubulin  
163 acetylation, and absence of this enzyme leads to irregularities in protofilament numbers<sup>85</sup>.  
164 Mice lacking the polyglutamylase TLL9 show defects in the characteristic structure of  
165 ciliary axonemes, where some microtubule doublet are missing<sup>86</sup>.

166

### 167 1.2) Tubulin isotypes determine mechanical features of microtubules

168 Mechanical bending of microtubules requires sliding of adjacent protofilaments, which is  
169 controlled by non-covalent inter-protofilament interactions. Tubulin isotypes might affect  
170 those interactions, however so far, no direct evidence for the involvement of isotypes in  
171 microtubule flexibility has been reported. Indirect support comes from studies of blood  
172 platelets. Platelets attain their specific round shape and defined diameter by the assembly of a  
173 microtubule coil of precisely 12 turns; the marginal band<sup>87</sup>. The extreme bending of platelet  
174 microtubules depends on a specific  $\beta$ -tubulin isotype, TUBB1<sup>31</sup>, as mutation or absence of  
175 this gene lead to severe defects in the architecture of the marginal band<sup>32,88</sup> (Fig. 2b). TUBB1  
176 is the most divergent tubulin isotype in mammals and does not have close homologs in other  
177 phyla that do not have platelets. It thus appears that this particular  $\beta$ -tubulin isotype has  
178 specifically evolved to sustain the high degree of microtubule bending required for correct

179 platelet functions<sup>89,90</sup>, however, direct biophysical evidence for an increased flexibility of  $\beta$ 1-  
180 tubulin-containing microtubules is still missing.

181

### 182 *1.3) Can tubulin PTMs affect microtubule mechanics?*

183 Acetylation of lysine 40 of  $\alpha$ -tubulin<sup>51,91</sup> was for many years the most enigmatic PTM of  
184 tubulin, as it occurs in the lumen of microtubules (Fig. 1), thus causing a number of  
185 controversial discussions on its potential functions (reviewed in ref.<sup>92</sup>). The famous ambiguity  
186 was whether acetylation actually stabilises microtubules, or just labels stable microtubules.  
187 Recent work has now provided evidence that K40-acetylation protects microtubules from  
188 mechanical aging, a process in which microtubules lose their flexural rigidity following  
189 repetitive bending<sup>93</sup>. Consequently, acetylation avoids microtubule breakage inside cells, thus  
190 making them longer-lived<sup>94</sup> (Fig. 2c). A structural study showed that the modification of K40,  
191 located in an unstructured loop of  $\alpha$ -tubulin, reduces inter-protofilament interactions<sup>95</sup>  
192 (Fig. 2c), and might thus facilitate protofilament sliding and increase microtubule flexibility.  
193 Therefore, K40-acetylation of  $\alpha$ -tubulin is a tubulin PTM that directly regulates microtubule  
194 mechanics. Intriguingly, the loop containing K40 is one of the hotspots of sequence variation  
195 between tubulin isotypes, and might thus adapt different conformations as already shown for  
196 tubulin from budding yeast<sup>74</sup> and *C. elegans*<sup>26</sup>. This suggests that acetylation and expression  
197 of different  $\alpha$ -tubulin isotypes could cooperate to adjust mechanical features of microtubules  
198 in cells.

199 Little is so far known on how other tubulin PTMs affect microtubule mechanics. A potential  
200 role of detyrosination could be deduced by studying the role of a specific  $\alpha$ -tubulin isotype,  
201  $\alpha$ 4A-tubulin (TUBA4A). Loss of this isotype in blood platelets affects the architecture of the  
202 microtubule marginal band<sup>96</sup> (Fig. 2b), indicating that  $\alpha$ 4A-tubulin plays an essential role in  
203 the assembly of this coiled microtubule structure. However, as  $\alpha$ 4A-tubulin is a rather  
204 conserved, ‘generic’  $\alpha$ -tubulin, it is rather unlikely that it contains unique structural features  
205 that change microtubule mechanics. By contrast, a distinct feature of  $\alpha$ 4A-tubulin is the lack  
206 of the gene-encoded C-terminal tyrosine residue, which mimics detyrosination. Though it has  
207 not yet been tested whether detyrosination directly affects microtubule bending in platelets,  
208 the essential role of this PTM in microtubule flexing during heart and skeletal muscle  
209 contraction<sup>97,98</sup> suggests so. It remains to be determined if detyrosination directly renders

210 microtubules more flexible, or rather attracts proteins to the microtubules that then change  
211 their mechanical behaviour.

212

## 213 2. Control of microtubule dynamics

### 214 *2.1) Tubulin isotypes can control polymerization dynamics of microtubules*

215 Structural work demonstrates that the contacts between tubulin molecules within the  
216 microtubule lattice determine microtubule dynamics<sup>19</sup>. Microtubule dynamics is in fact a  
217 summary term for several of their properties: growth speed and persistence, as well as the  
218 propensity to spontaneously depolymerize, a.k.a catastrophe<sup>2</sup>. First experiments using  $\beta$ -  
219 isotype-specific monoclonal antibodies to fractionate brain tubulin<sup>99</sup> showed that different  $\beta$ -  
220 tubulin isotypes do indeed affect the dynamic properties of microtubules<sup>100-103</sup>. The use of  
221 recombinant tubulin with defined isotype composition confirmed these early experiments by  
222 demonstrating that microtubules assembled from pure  $\alpha$ 1B/ $\beta$ 2B-tubulin dimers were more  
223 resistant to spontaneous, or catalysed depolymerization as compared to  $\alpha$ 1B/ $\beta$ 3-  
224 microtubules<sup>77,104</sup> (Fig. 2d). Considering that  $\beta$ 3-tubulin is predominantly expressed in  
225 neuronal cells<sup>105</sup>, this suggests that neuronal microtubules are more dynamic, a concept that  
226 was suggested earlier based on the observation that brain tubulin which was biochemically  
227 depleted of  $\beta$ 3-tubulin shows an increased assembly speed<sup>100</sup>.

228 An even more striking impact of tubulin isotypes on microtubule dynamics was found with  
229 the more divergent *C. elegans* tubulin, which *in vitro* assembled more than three times faster  
230 as compared to mammalian brain tubulin<sup>26</sup>. Together, these observations have provided solid  
231 and direct evidence that isotypes control the dynamic instability of microtubules.

232

### 233 *2.2) Regulation of microtubule dynamics by tubulin PTMs*

234 So far there are a few examples of PTMs that can directly modulate microtubule dynamics.  
235 Phosphorylation of S172 of  $\beta$ -tubulin by the cyclin-dependent kinase Cdk1<sup>48</sup>, or by the dual-  
236 specificity tyrosine-regulated kinase (DYRK)<sup>50</sup>, as well as acetylation of K252 of  $\beta$ -tubulin  
237 by San acetyl transferase<sup>106</sup> preclude the tubulin dimer from incorporation into microtubules  
238 (Fig. 2d). On the other hand, polyamination of tubulin stabilises microtubules and prevents  
239 their depolymerisation<sup>56</sup> (Fig. 2d).

240 Other tubulin PTMs can control microtubule dynamics indirectly, by regulating the MAPs  
241 that affect microtubule stability. Detyrosination, for instance, can control binding of CLIP170  
242 or p150<sup>glued</sup>, which in turn affects microtubule growth speed and persistence<sup>107-109</sup> (Fig. 3a).  
243 At the same time, detyrosination also regulates the active disassembly of microtubules by the  
244 depolymerizing motors of the kinesin-13 family<sup>110</sup> (Fig. 3a). Polyglutamylation controls  
245 enzymatic severing of microtubules by spastin and katanin<sup>111-113</sup> (Fig. 3b), and could thus  
246 modulate the microtubule mass and dynamics in cells<sup>114</sup>. Additionally, polyglutamylation  
247 might control the binding of a variety of microtubule-associated proteins (MAPs)<sup>115,116</sup>, which  
248 could eventually stabilize microtubules (Fig. 3b)<sup>20</sup>.

249

## 250 **Control of microtubule-MAP interactions**

251 Microtubules are interaction platforms for a myriad of proteins, commonly referred to as  
252 MAPs<sup>20</sup>. While the term MAP is often associated with non-motile proteins that bind with high  
253 affinity to microtubules, in a larger sense, all proteins that interact with microtubules,  
254 including molecular motors, plus- and minus-end tracking proteins, and even microtubule  
255 depolymerizing proteins could be considered as MAPs. One of the central concepts of the  
256 tubulin code is that it could regulate interactions between MAPs and microtubules in a  
257 selective manner, thus introducing specificity and selectivity. Intuitively, the PTMs are  
258 perfectly situated as dynamic, rapidly adjustable regulators of such interactions, as they can  
259 take place on tubulin dimers within existing microtubules. Tubulin isotypes can also control  
260 MAP-microtubule interactions, though this type of regulation might be less dynamic, as  
261 newly synthesized isotypes need to be incorporated into microtubules via de-novo  
262 polymerization.

263

### 264 1) Tubulin isotypes and MAPs

265 In the past ten years many novel structures of MAPs bound to the microtubule lattice have  
266 been solved (for example ref.<sup>73,117-124</sup>). As these structures reveal the precise interaction sites,  
267 i.e. amino acid residues, between a MAP and tubulin, it can now be deduced how sequence  
268 differences between tubulin isotypes could affect these interactions.

269 A domain of the tubulin molecule that is involved in many, but not all microtubule-MAP  
270 interactions is the unfolded C-terminal tubulin tail. Notwithstanding the rather subtle  
271 differences in the primary sequences of tubulin tails in mammals, first direct experimental

272 evidence with chimeric yeast tubulins has demonstrated that single amino-acid differences,  
273 such as the presence of a lysine residue in the tail of  $\beta$ 3-tubulin, is sufficient to substantially  
274 reduce the run length of kinesin-1 on microtubules. Strikingly, this effect could be  
275 counteracted by adding additional glutamate residues in the form of a side chain similar to  
276 polyglutamylation on the  $\beta$ 3-tubulin tail, or by simply removing the lysine residue<sup>125</sup>. This  
277 example illustrates the potential cross-talk between isotypes and PTMs. Similarly,  $\alpha$ 4A-  
278 tubulin (TUBA4A), an isotype lacking the genetically encoded C-terminal tyrosine, thus  
279 mimicking tubulin detyrosination, can be enzymatically tyrosinated<sup>126,127</sup>. Finally, the  
280 distribution of glutamate residues within the C-terminal tails (i.e. the modification sites for  
281 glutamylation and glycylation) might affect the patterns of these two PTMs, as the modifying  
282 enzymes have some, yet not fully explored preferences for those sites (Box 1).

283

## 284 2) Tubulin PTMs and MAPs

### 285 *2.1) The detyrosination/retyrosination cycle*

286 The idea that tubulin PTMs could dynamically regulate the interaction landscape of  
287 microtubules emerged together with the discovery of these modifications. Experiments in the  
288 1980ies already suggested differences in the interactions of MAPs with microtubules  
289 depending on their tyrosination state<sup>128</sup>, but were surprisingly not followed up. More recently,  
290 it was demonstrated that the C-terminal tyrosine of  $\alpha$ -tubulin plays an essential role for the  
291 localisation of CAP-Gly domain-containing proteins to the +TIP complex<sup>129,130</sup>. The  
292 underlying molecular mechanism was revealed by structural work showing that CAP-Gly  
293 domains specifically recognise C-terminal -EEY/F sequences, which are characteristic for the  
294 tyrosinated form of  $\alpha$ -tubulin<sup>131</sup>.

295 Another molecular mechanism that depends on the presence of tyrosinated tubulin in the  
296 microtubule lattice is the kinesin-13-mediated microtubule disassembly. Complete  
297 detyrosination can thus protect microtubules from active depolymerization with motor  
298 proteins of this family, such as mitotic centromere-associated kinesin (MCAK) and Kif2A<sup>110</sup>  
299 (Fig. 3a). This discovery provided a mechanistic rationale for the established notion that  
300 detyrosinated microtubules are more stable, which was mostly derived from observations in  
301 cells<sup>132-134</sup>, where depolymerizing kinesins might selectively spare detyrosinated  
302 microtubules, and consequently making them longer-lived.

303 Other microtubule interactors have greater affinity to detyrosinated microtubules. Studies  
304 using chimeric yeast tubulin revealed that kinesin-2, but not kinesin-1, has an increased  
305 motility and processivity on detyrosinated microtubules<sup>125</sup> (Fig. 3a). Similarly, CENP-E, a  
306 kinetochore-associated kinesin-7 motor, shows stronger interactions, and is thus more  
307 processive, on detyrosinated as compared to fully tyrosinated microtubules purified from HeLa  
308 cells<sup>135,136</sup> (Fig. 3a).

309 The minus-end directed motor dynein, in contrast, was not affected by the tyrosination status  
310 of microtubules<sup>125</sup>, whereas a complex of dynein, dynactin and the adaptor protein BicD2  
311 required tyrosination for its initial loading onto microtubules (Fig. 3a). This dependency on  
312 tyrosination is mediated by the p150<sup>glued</sup> subunit of dynactin – a CAP-Gly protein. Strikingly,  
313 once the complex is loaded on microtubules, it can walk through patches of detyrosinated  
314 microtubules without changes in motility<sup>137</sup>.

315

## 316 *2.2) The concept of fine-tuning microtubule-MAP interactions*

317 Two tubulin PTMs, polyglutamylation and polyglycylation, generate a variety of lateral  
318 glutamate or glycine peptide chains at different glutamate residues within the C-terminal tails  
319 of  $\alpha$ - and  $\beta$ -tubulins (Box 1). Using chimeras of yeast tubulin bodies with mammalian C-  
320 terminal tails, on which controlled patterns of polyglutamylation were generated by  
321 chemically adding glutamate chains of defined length allowed for the first time to show a  
322 differential sensitivity of kinesin motors to glutamylation patterns: kinesin-2 motility was  
323 already induced by glutamylation with chains of 3 glutamate residues, whereas for activating  
324 kinesin-1, glutamate chains of 10 residues length were required<sup>125</sup> (Fig. 3b). In contrast,  
325 neither the motility of dynein, nor the depolymerizing activity of kinesin-13 were affected by  
326 the presence of either of these glutamate chains<sup>125</sup>. These observations have far-reaching  
327 functional implications in the light of polyglutamylation levels found in cells. In brain,  
328  $\alpha$ -tubulins with 10 glutamate residues have not been detected, and the majority of  $\alpha$ -tubulin  
329 carries about 3 glutamate residues<sup>59</sup>. This implies that only kinesin-2, but not kinesin-1, might  
330 be directly regulated by tubulin polyglutamylation in neurons.

331 How a single biological process can be fine-tuned by different polyglutamylation levels has  
332 been first demonstrated for microtubule severing. Comparing virtually non-glutamylated and  
333 differentially glutamylated microtubules showed that spastin is activated by  
334 polyglutamylation of its substrate, the microtubule<sup>111</sup>. Using the polyglutamylase TLL7 to

335 generate microtubules with controlled polyglutamylation patterns further revealed an even  
336 more exciting aspect of spastin regulation: while the initial increase of tubulin  
337 polyglutamylation gradually induced the severing activity of spastin, further accumulation of  
338 the PTM reversed this effect<sup>112</sup> (Fig. 3b). This demonstrated that polyglutamylation can act as  
339 a rheostat, an exciting concept implying that the length of the glutamate chains, or/and the  
340 accumulation of glutamylation on different sites within a single tubulin molecule, could fine-  
341 tune the functional readout of this PTM. A similar concept had been proposed earlier for  
342 several other MAPs that showed binding differences to differentially glutamylated tubulin in  
343 blot-overlay assays<sup>115,116,138,139</sup>, however more direct evidence will be required to confirm  
344 those conclusions. Ultimately, the discovery that different polyglutamylases can specifically  
345 determine the length and distribution ( $\alpha$ - vs.  $\beta$ -tubulin) of glutamate chains<sup>140</sup> shows that the  
346 concept that many microtubule interactors are coordinated by different degrees and patterns of  
347 polyglutamylation is a realistic scenario in cells. Expressed in a cell- and tissue-specific  
348 manner, the large variety of modifying and demodifying enzymes could cooperate to generate  
349 defined glutamylation patterns (Box 1) to control intracellular distribution of microtubule-  
350 interacting proteins and organelles.

351

### 352 2.3) Regulatory mechanisms of tubulin PTMs in cells

353 The well-characterised roles of tubulin detyrosination in controlling CAP-Gly protein-  
354 microtubule interactions<sup>129,130</sup> and kinesin-13-mediated microtubule depolymerisation<sup>110</sup> are  
355 almost binary switches between two different functional states of microtubules. However,  
356 PTMs can also have more subtle effects on the interactions between MAPs and microtubules,  
357 and are consequently much harder to measure. Many observations were first made in cells,  
358 and were not always confirmed by *in-vitro* reconstitution assays with purified components.

359 In neurons, excessive detyrosination of tubulin abolished the preference of kinesin-1 motors  
360 to move into axons, suggesting that differential detyrosination between axons and dendrites  
361 could guide kinesin-1 into axons<sup>141</sup>. A preference of kinesin-1 to detyrosinated microtubules  
362 in cells has also been reported in non-differentiated cells<sup>142</sup>. Lysosomes accumulate on  
363 detyrosinated stretches of microtubules in a kinesin-1-dependent manner, and as a result their  
364 fusion with autophagosomes preferentially takes place at those microtubule sections<sup>143</sup>. These  
365 experiments suggested a preference of kinesin-1 motors for detyrosinated microtubule tracks,  
366 however *in vitro* experiments did not confirm this notion<sup>125</sup>.

367 Changes in acetylation also induced alterations of cargo transport in cultured cells<sup>144</sup>,  
368 particularly in neurons<sup>145-149</sup>. While the evidence for transport regulation in most of these  
369 studies is compelling, it is still an open question if acetylation alone leads to this effect.  
370 Indeed, neither mice lacking the tubulin deacetylase HDAC6<sup>150</sup>, nor the acetyl-transferase  
371 aTAT1<sup>151,152</sup>, show obvious defects in neuronal functions, which would be expected when  
372 neuronal transport is perturbed. Moreover, *in-vitro* assays with purified components have  
373 shown that the motility of kinesin-1 is not affected by the acetylation status of the tubulin  
374 tracks<sup>153,154</sup>, which makes it difficult to directly link the effects observed in cells with the  
375 molecular functions of this tubulin PTM.

376 Discrepancies between cell-based and *in-vitro* experiments might be explained by other  
377 factors that influence transport processes in cells, for instance a combined effect of multiple  
378 PTMs on microtubule tracks<sup>155</sup>. Indeed, a recent *in-vitro* study comparing microtubules  
379 assembled from brain (many PTMs) and HeLa (no PTMs) tubulin found a clear difference in  
380 the motility of the kinesin-3 KIF1A. So far it is not clear if this is caused by a single PTM or  
381 isotype, or the combination of them<sup>156</sup>. Moreover, motor proteins are not alone on transported  
382 vesicles and organelles<sup>157-159</sup>, and the additional adapter or helper proteins<sup>160</sup> could, in  
383 combination with the motor proteins, sense the PTM status of the microtubules. Another  
384 possibility is that MAPs that bind the microtubule tracks affect the use of these tracks by  
385 specific motors<sup>161</sup>, and that the preferential binding of certain MAPs is regulated by tubulin  
386 PTMs. It thus appears that while some of the published data are contradictory and confusing,  
387 they in fact open a large window of novel options of how the interplay between the tubulin  
388 code and a hypothetical MAP code<sup>162</sup> could control microtubule-based functions, which is an  
389 exciting field to be explored in the near future.

390

### 391 **Cellular and physiological roles**

392 Microtubules adapt to an amazing variety of structures and behaviours in different cell types  
393 of multicellular organisms, and even within single cells. Tubulin isotypes and PTMs  
394 contribute to the assembly of those microtubule structures by modulating their intrinsic  
395 properties, as well as their interactions with a multitude of interacting proteins. On the  
396 organism scale, the tubulin code can help microtubules adapt to changing physiological  
397 requirements in long-lived cells, ensuring homeostasis. Indeed, a growing number of studies  
398 shows that perturbations of the tubulin isotypes and PTMs can have devastating consequences  
399 at the organism level.

400

401 1) The tubulin code in cilia and flagella

402 Eukaryotic cilia and flagella are based on an evolutionarily conserved microtubule structure,  
403 the axoneme, which consists of nine circularly arranged doublet microtubules, plus two  
404 central singlet microtubules for motile cilia and flagella<sup>163</sup>. In motile cilia, the microtubule  
405 doublets are interconnected with ciliary dynein motors, thus forming the machinery to  
406 generate the characteristic ciliary beating. Motile cilia and flagella are important for cell  
407 movement, for example for spermatozooids or ciliated microorganisms such as *Tetrahymena* or  
408 *Paramecium*<sup>164</sup>, or for the generation of liquid flow, as the multiciliated ependymal cells in  
409 the brain ventricles, or in the trachea of the respiratory system<sup>165</sup>. Many PTMs of tubulin are  
410 strongly enriched on axonemal microtubules, and even appear to be evolutionarily linked to  
411 this organelle (Box 2).

412

413 *1.1) Tubulin PTMs play key roles in cilia and flagella*

414 Whenever glutamylation is perturbed in different cellular or organism models, motile cilia  
415 and flagella are among the most obvious structures showing functional aberrations. Deletion  
416 of polyglutamylating enzymes directly affected ciliary beating in the unicellular organisms  
417 *Chlamydomonas reinhardtii*<sup>166</sup> and *Tetrahymena thermophila*<sup>167</sup>, or in the multiciliated  
418 ependymal cells in mice<sup>168</sup>. On the ultrastructural levels, glutamylation is predominantly  
419 found on the B-tubules<sup>169,170</sup>, which are the interaction sites of the axonemal dynein heads that  
420 generate the ciliary beating. It was thus intuitive to assume that polyglutamylating levels of  
421 axonemal microtubules directly control dynein activity, and thus the beating of the cilia,  
422 which indeed is the case<sup>166,167</sup> (Fig. 4a).

423 In mice, many of the enzymes involved in glutamylation appear to be important for sperm  
424 development and function, as a recurrent phenotype of knockout models is male infertility.  
425 The morphological defects range from impaired flagellar motility to erroneous axoneme  
426 assembly, which could be related to dysfunctions of either centrioles serving as basal bodies  
427 for axoneme assembly, or the axonemes themselves<sup>86,171-173</sup>. Even early steps of  
428 spermatogenesis can be perturbed. In mice lacking the deglutamylase CCP5, the sperm  
429 manchette, a transient microtubule structure essential for the formation of sperm heads, is  
430 dysfunctional. Spermatozooids fail to evacuate their cytoplasm, show supernumerary basal  
431 bodies, and are unable to assemble functional flagella<sup>174</sup>. Perturbed polyglutamylating in mice

432 also induces defects in other motile cilia such as airway cilia<sup>86,175</sup>, which could lead to  
433 respiratory disorders as pathogens cannot be efficiently cleaned out of the trachea.

434 Tubulin glycylation was considered a PTM highly specific to axonemes of motile cilia and  
435 flagella<sup>176</sup> until the recent demonstration of its presence in some primary cilia<sup>177</sup>. Depletion of  
436 glycylation led to loss of motile cilia from ependymal cells in mice<sup>168</sup>, and to a significant  
437 shortening of primary cilia in cultured cells<sup>177</sup>. Photoreceptors of the mammalian retina  
438 contain the highly specialised connecting cilia, which progressively shortened in the absence  
439 of glycylation. The late-onset retina degeneration observed in mice lacking the glycyrase  
440 TTLL3<sup>178</sup> is likely related to a suboptimal cargo transport through the connecting cilium, a  
441 process that is highly solicited in photoreceptors<sup>179</sup> (Fig. 4a).

442 Intriguingly, loss of glycylation in murine photoreceptor cells is accompanied by an increase  
443 of glutamylation<sup>178</sup>, indicating that, as shown earlier in *Tetrahymena thermophila*<sup>180,181</sup>, both  
444 PTMs compete for the same modification sites on tubulin, and are therefore functionally  
445 interconnected. Indeed, patients with mutations in the deglutamylase CCP5 also develop  
446 retina degeneration<sup>182-185</sup>. Loss of CCP5 is likely to lead to an accumulation of  
447 polyglutamylation, similar what has been demonstrated for mice lacking the deglutamylase  
448 CCP1<sup>178,186</sup>. The concept emerging from these observations is that mutations in a range of  
449 different tubulin-modifying enzymes can not only functionally, but biochemically lead to  
450 similar defects, and thus, be linked to similar diseases. Along these lines, mutations in the  
451 glutamylase TTLL5 have also lead to retina degeneration in humans<sup>187,188</sup>, however it appears  
452 that in this case it is not the perturbation of tubulin glutamylation, but of another substrate  
453 (Box S1), which causes the loss of photoreceptors in the corresponding mouse model<sup>189</sup>.

454 Other tubulin PTMs such as detyrosination,  $\Delta$ 2-tubulin<sup>190</sup> and acetylation<sup>176</sup> are also enriched  
455 on axonemes, but little is so far known on their functional roles. Mice lacking  $\alpha$ TAT1 are  
456 subfertile<sup>152</sup>, suggesting that this PTM is needed for proper axoneme function, perhaps due to  
457 its capacity of rendering microtubules more resistant to mechanical fatigue<sup>93,94</sup>.

458 Finally, primary cilia are also modified with a range of tubulin PTMs. Those non-specialised  
459 types of cilia are present on many cells in the vertebrate organism, and serve as sensory  
460 organelles and signalling hubs. Defective primary cilia can lead to a variety of diseases  
461 commonly referred to as ciliopathies<sup>191</sup>. Tubulin PTMs might play similar roles in primary  
462 cilia as in their motile counterparts, however much less is so far known about direct regulation  
463 of their functions by tubulin PTMs. First examples show that acetylation<sup>69</sup>,

464 glutamylation<sup>192,193</sup> and glycylation<sup>177</sup> are required for correct assembly and function of  
465 primary cilia (Fig. 4a).

466

### 467 1.2) Cilia-specific roles of tubulin isotypes

468 Early studies demonstrated the presence of distinct tubulin isotypes in cilia of different  
469 species<sup>194</sup>, however it was at the time not clear if this heterogeneity was related to tubulin  
470 isotypes or PTMs. The development of antibodies specific to mammalian isotypes<sup>99,101</sup>  
471 revealed  $\beta$ 4-tubulin (TUBB4) as a major  $\beta$ -tubulin isotype in two functionally different types  
472 of cilia: the connecting cilia of photoreceptor cells, as well as in the motile airway cilia in  
473 trachea<sup>195</sup>. It is therefore likely that  $\beta$ 4-tubulin possesses properties that are essential for the  
474 formation of the axoneme.

475 The idea that specific tubulin isotypes convey unique properties to axonemal microtubules  
476 was recently experimentally supported by the observation that purified axonemal tubulin from  
477 *Chlamydomonas* displayed a distinct assembly/disassembly behaviour as compared to  
478 mammalian brain tubulin<sup>170</sup>. Strikingly, *Chlamydomonas*  $\beta$ -tubulin shares specific sequence  
479 motifs with mammalian TUBB4A, which are absent in other mammalian tubulin isotypes.  
480 This strongly suggests that the primary peptide sequence of ciliary  $\beta$ -tubulin isotypes  
481 determines some of the characteristic features of axonemal microtubules, such as particularly  
482 low growth and shrinkage rates<sup>170</sup>. Work in *Drosophila* further demonstrated that a specific  
483 amino acid residue encoded in all axonemal  $\beta$ -tubulins, glycine 56, is essential for the  
484 attachment of the outer dynein arms, and thus, for the motility of the axonemes<sup>196</sup> (Fig. 4a).

485 In *Caenorhabditis elegans*, an organism without motile cilia, cells with primary cilia express  
486 characteristic tubulin genes<sup>81</sup>. Deleting one of them, the  $\alpha$ -tubulin gene TBA-6, led to a loss  
487 of the microtubule doublet structure in the sensory cilia, which instead contained 18 singlet  
488 microtubules, and displayed defects in intra-flagellar transport and vesicle-sorting<sup>82</sup>. A unique  
489 feature of TBA-6 is its C-terminal tail, which, in contrast to all other  $\alpha$ -tubulin isotypes, is  
490 longer, contains positively charged amino acid residues, and, most strikingly, no glutamate  
491 residues that could serve as sites for posttranslational glutamylation. In the light of *in vitro*  
492 reconstitution experiments demonstrating that the C-terminal tails of brain tubulin hinder the  
493 formation of B-tubules<sup>197</sup>, it is appealing to hypothesise that the particular tail of TBA-6  
494 permits doublet formation due to its different biophysical features, and perhaps because it  
495 cannot be polyglutamylated.

496 Thus, while still little is known about the underlying mechanisms, solid evidence for essential  
497 roles of particular tubulin isotypes in axonemal structure and function exist. So far, these data  
498 stem mostly from two model organisms in which the primary sequences of tubulin isotypes  
499 are more divergent than in mammals. While this makes it difficult to draw direct parallels to  
500 other organisms, these examples show that single amino-acid substitutions in the highly  
501 structured tubulin body, as well as variations in the peptide sequence of the C-terminal tails  
502 can be essential to build and maintain axonemes. Most excitingly, these sequence variations  
503 can influence the posttranslational modification of a given isotype, thus directly linking the  
504 two core elements of the tubulin code in one single biological function.

505

## 506 2) The tubulin code in neurons

### 507 *2.1) A differential distribution of tubulin PTMs in neurons?*

508 In contrast to most other cell types of multicellular organisms, neurons are particular as their  
509 entire microtubule cytoskeleton is highly posttranslationally modified. Neuronal  $\alpha$ -tubulin is  
510 acetylated at K40<sup>198,199</sup>, detyrosinated<sup>199,200</sup>, and further converted into  $\Delta 2$ -tubulin<sup>66</sup>.

511 Moreover, neuronal microtubules are abundantly polyglutamylated on  $\alpha$ -<sup>59</sup> and  $\beta$ -tubulin<sup>60,61</sup>.  
512 All these PTMs accumulate as neurons differentiate and mature<sup>199,201,202</sup>, underpinning the  
513 concept of tubulin PTMs as neuronal differentiation markers. Biochemical analyses of  
514 purified brain tubulin so far provided approximate measures of the levels of individual  
515 PTMs<sup>59,65,93,203-205</sup>.

516 A first careful mapping of tubulin tyrosination and acetylation by immunofluorescence and  
517 immune-electron microscopy revealed that acetylation is present all-along the axon, but much  
518 less so at the growing end of the axon, where reversely, tyrosinated (i.e., non-detyrosinated)  
519 tubulin is predominant<sup>206</sup>. This fits the expectation of axonal microtubules being long-lived,  
520 and thus more acetylated and detyrosinated, while the growing end of the axon, including the  
521 growth cone, contains freshly assembled, non-modified microtubules. An elegant approach  
522 used fraying of microtubules of cultured neurons to show that single, continuous microtubules  
523 change their PTM status towards distal end of the axon<sup>207</sup>, which might have important  
524 implications for their functions in growth cones (Fig. 4b).

525 It took two decades and the advent of superresolution microscopy until another study  
526 described the presence of two different microtubule populations, one acetylated and barely  
527 tyrosinated (i.e. detyrosinated or  $\Delta 2$ -tubulin) in the centre of neuronal dendrites, and the other

528 tyrosinated and barely acetylated at the dendrite periphery<sup>208</sup>. Amazingly, these two different  
529 microtubule species show opposite polarity, thus supporting two different types of transport:  
530 retrograde, kinesin-1 driven transport on the central, highly modified microtubules, and  
531 anterograde transport by kinesin-3 on the peripheral, less modified microtubules (Fig. 4b). At  
532 this point it is not clear if the different PTMs are merely markers of different microtubule  
533 subtypes, or if they directly control the motors that walk on them. In axons, this polarity and  
534 PTM segregation of microtubules does not exist, and consequently all kinesin motors walk  
535 towards the axon distal ends.

536

### 537 2.2) Functions of tubulin PTMs in neurons

538 Different PTMs in neurons might play distinct roles in neuronal development and  
539 homeostasis. The balance between detyrosination and tyrosination appears to be important in  
540 early neuronal development, as massive accumulation of detyrosination in TTL-knockout  
541 mice leads to perinatal death due to neurodevelopmental defects<sup>209</sup>. Cultured hippocampal  
542 neurons from these mice lack tyrosinated microtubules in axonal growth cones and show  
543 massive abnormalities in neuronal pathfinding<sup>210</sup>. Perturbations of the tubulin detyrosination  
544 cycle can also lead to human disease. While mutations of TTL might be rare to find in adult  
545 patients due to the expected massive developmental defects induced by the nearly-complete  
546 absence of tyrosinated tubulin<sup>209</sup>, they are more likely to be found in genes encoding enzymes  
547 of the detyrosinase family<sup>211,212</sup>. Indeed, mutations in SVBP were recently linked to  
548 microcephaly and intellectual disability<sup>213,214</sup>, thus confirming the importance of this PTM in  
549 neurodevelopment.

550 Phosphorylation of  $\beta$ -tubulin S172 by the DYRK kinase controls microtubule dynamics in  
551 differentiating neurons in *Drosophila melanogaster*<sup>50</sup>. Alterations in this PTM lead to defects  
552 in dendrite branching and excitability of these neurons, which results in neurological defects  
553 similar to defects found in Down syndrome and autism spectrum disorders.

554 Acetylation is a prominent tubulin PTM in neurons, however its absence in aTAT-knockout  
555 mice induced surprisingly mild neurological defects<sup>151</sup>, the most remarkable being the loss of  
556 touch sensation<sup>215</sup>. This mirrors touch-sensation defects in acetylation-defective  
557 *Drosophila*<sup>216</sup>, as well as in *C. elegans*, where aTAT1 (Mec-17)-mediated acetylation<sup>217</sup> is  
558 important for the formation of the characteristic 15-protofilament microtubules<sup>85</sup> that are  
559 essential for touch sensation<sup>218</sup>. Mutation of K40 in the major neuronal  $\alpha$ -tubulin isotype in

560 *Drosophila* ( $\alpha$ Tub84B) further highlighted the importance of acetylation in dendritic  
561 refinement of sensory neurons<sup>219</sup>. A number of reports have linked tubulin acetylation to  
562 neurodegeneration, mostly via the deacetylase HDAC6<sup>149,220-224</sup>. The interpretation of these  
563 experiments is however not straight-forward, as HDAC6 deacetylates not only  $\alpha$ -tubulin  
564 K40<sup>225</sup>, but also the mitochondria transport adaptor protein Miro1<sup>226</sup> and the actin regulator  
565 cortactin<sup>227</sup> (Box S1).

566 Polyglutamylation, on the other hand, has been demonstrated to directly and cell-  
567 autonomously cause neurodegeneration using genetic approaches in mice. The well-  
568 established mouse model for Purkinje cell degeneration, the *pcd* mouse<sup>173</sup>, carries a mutation  
569 in the gene *Nna1*<sup>228</sup>, later shown to be the deglutamylase CCP1 (also known as  
570 *Nna1/AGTPBP1*)<sup>229</sup>. CCP1 deficiency causes accumulation of hyperglutamylated tubulin in  
571 the cerebellum, the main brain region undergoing degeneration in *pcd* mice<sup>229</sup>. The rapid  
572 degeneration of Purkinje cells can be avoided for the entire lifetime if TLL1, the major  
573  $\alpha$ -tubulin polyglutamylase in neurons<sup>230</sup>, is deleted selectively in Purkinje cells of *pcd* mice.  
574 This demonstrates the causality of TLL1-catalysed hyperglutamylation for the degeneration  
575 of these neurons<sup>231</sup> (Fig. 4b).

576 But why then do not all brain regions in *pcd* mice degenerate sooner or later? Another  
577 member of the CCP family<sup>232,233</sup>, CCP6, was found to be expressed specifically in brain  
578 regions that do not degenerate in *pcd* mice<sup>229</sup>. Indeed, deletion of CCP6 additional to CCP1  
579 induced a massive hyperglutamylation in the entire mouse brain, resulting in the degeneration  
580 of neurons that were unaffected in *pcd* mice<sup>231</sup>.

581 The discovery of a novel infant-onset human condition linked to inactivating mutations in  
582 CCP1 with remarkable similarity to the *pcd* mouse model<sup>234-236</sup> established deregulated  
583 polyglutamylation as a novel cause of human neurodegeneration. It is conceivable that more  
584 subtle alterations of this PTM could be linked, or even causative, for other, late-onset human  
585 pathologies.

586 Exploring the molecular mechanisms by which abnormal polyglutamylation leads to  
587 neurodegeneration provide a handle to decipher the physiological role of this PTM in the  
588 nervous system. So far, defects in axonal transport have been reported in different types of  
589 neurons<sup>231,237</sup>, while a causative role of the microtubule-severing enzyme spastin was  
590 excluded<sup>231</sup>. However, it is likely that other microtubule-based processes, such as the binding  
591 and distribution of neuronal MAPs, could also be affected if polyglutamylation is perturbed.

592

593

594 *2.3) Tubulin isotypes in the nervous system*

595 Two  $\beta$ -tubulin isotypes,  $\beta$ 2- (TUBB2) and  $\beta$ 3- (TUBB3) tubulin are strongly enriched in  
596 neuronal microtubules<sup>238</sup>. While  $\beta$ 2-tubulin is also expressed in other cell types,  $\beta$ 3-tubulin is  
597 almost exclusively found in neurons<sup>239,240</sup>. In the light of a recent study showing that  
598  $\beta$ 3-tubulin-containing microtubules depolymerise faster<sup>77</sup>, previous observations of  
599 differential expression of this TUBB3 in different types of neurons<sup>241</sup>, or its upregulation  
600 during regeneration of sensory nerves<sup>242</sup> now suggest that TUBB3 expression directly  
601 regulates microtubule dynamics in a cell-type and function-dependent context. This concept  
602 was confirmed in a TUBB3-knockout mouse, which displays defects in axonal regeneration<sup>243</sup>  
603 (Fig. 4b). Those defects are reminiscent of phenotypes found in aTAT1-knockout mice<sup>215</sup>,  
604 indicating once again that different elements of the tubulin code concur in optimising  
605 microtubule functions. Indeed, an increase of tubulin acetylation and polyglutamylation was  
606 detected in TUBB3-knockout mice<sup>243</sup>, suggesting that neurons attempted to compensate for  
607 the loss of  $\beta$ 3-tubulin by adjusting microtubule dynamics, or interactions with MAPs and  
608 molecular motors.

609

610 3) Microtubule functions in muscles

611 The observation that enzymatic activity of TTL in muscles is about two times higher as  
612 compared to the brain<sup>244</sup>, and reaches a temporal maximum during myofiber development in  
613 skeletal muscles<sup>245</sup> indicated very early that the detyrosination/tyrosination cycle could play a  
614 particularly important role for muscle microtubules. The first functional insight, however,  
615 came only recently from the observation that mechanotransduction in skeletal and heart  
616 muscle is affected by the detyrosination status of muscle microtubules<sup>97</sup> (Fig. 4c). High-speed  
617 imaging revealed that microtubules in the heart muscle buckle with every beat – an  
618 impressive example of the mechanical resistance of microtubules. The buckling of  
619 microtubule provides a viscous resistance to the actin-myosin force, thus controlling the  
620 viscoelasticity of the muscle.

621 The viscoelasticity of muscles is directly dependent on tubulin detyrosination, which controls  
622 the anchorage of microtubules to the desmin structures of muscle fibres. Absence of

623 detyrosination leads to disruption of microtubule-desmin contacts, and consequently perturbs  
624 cardiac muscle function<sup>98</sup> (Fig. 4c). Abnormally high detyrosination levels of microtubules  
625 lead to overly stiff cardiac muscles, and related to human heart failure<sup>246</sup>. Strikingly,  
626 myocardiocytes from heart-failure patients recovered elasticity when treated with the drug  
627 parthenolide to reduce tubulin detyrosination<sup>247</sup>, or with the microtubule-destabilizing drug  
628 colchicine<sup>246</sup>. Genetically, increased detyrosination levels could originate from two  
629 mechanisms, upregulation of the detyrosinating enzymes Vash1 or Vash2, or overexpression  
630 of  $\alpha$ 4A-tubulin (TUBA4A), which lacks C-terminal tyrosine and mimics detyrosination.  
631 Indeed, TUBA4A was found to be overexpressed in failing hearts<sup>246</sup>.

632 The discovery of the role of microtubules and their posttranslational detyrosination in  
633 controlling muscle functions provides a striking example for an unexpected role of  
634 microtubules and the tubulin code. Other tubulin PTMs and isotypes<sup>248,249</sup>, yet to be explored,  
635 might also play important roles in the regulation of muscle functions.

636

#### 637 4) The tubulin code in cell division

##### 638 *4.1) Regulation of the mitotic and meiotic spindles*

639 The division of eukaryotic cells essentially depends on microtubules as components of mitotic  
640 and meiotic spindles. Spindles are amazingly complex<sup>250</sup>, and yet highly dynamic assemblies  
641 of microtubules<sup>251</sup> that ensure the correct separation of the genetic material into two daughter  
642 cells. A huge amount of work has so far gone into explaining how the self-assembly of  
643 different molecular compounds can give rise to such complex, highly controlled microtubule  
644 structure<sup>252,253</sup>, but the potential impact of the tubulin code has so far rarely been explored.

645 A first direct evidence for a role of tubulin isotypes in controlling spindle behaviour was  
646 found in *C. elegans*, where two  $\alpha$ - and two  $\beta$ -tubulins are expressed in the embryo. Despite  
647 the high similarity of those two  $\alpha$ - and two  $\beta$ -tubulin isotypes, each isotype confers distinct  
648 dynamic properties to mitotic spindle microtubules, and thus each of them was essential for  
649 proper spindle function<sup>254</sup>.

650 Several tubulin PTMs have been found on spindle microtubules. In mammalian cells,  
651 detyrosination is enriched on inner spindle microtubules, but virtually absent from astral  
652 microtubules<sup>255</sup>. A similar distribution has been shown for polyglutamylation<sup>111</sup>, which

653 together with an increased polyglutamylase activity in mitosis<sup>256</sup> suggested a role of this PTM  
654 in cell division (Fig. 4d).

655 A recent study has now uncovered a mechanistic role for detyrosination in cell division. In  
656 mitosis, unaligned chromosomes are transported to the metaphase plate by the kinetochore-  
657 associated kinesin-7 motor CENP-E<sup>257</sup>. This mechanism was perturbed by a complete  
658 inhibition of detyrosination in dividing cells, suggesting that CENP-E can ‘read’ the  
659 detyrosination of spindle microtubules. Indeed, *in vitro* reconstitution experiments revealed a  
660 preference of CENP-E for detyrosinated microtubules<sup>135</sup> (Fig. 4d).

661 Finally, phosphorylation of serine 172 of  $\beta$ -tubulin by the cyclin-dependent kinase Cdk1  
662 prevents the incorporation of the tubulin dimer into the microtubule lattice, which might  
663 control microtubule dynamics in mitosis<sup>48</sup>. Indeed, mimicking S172 phosphorylation in  
664 budding yeast perturbed cell division, thus confirming the importance of this PTM for correct  
665 spindle function<sup>258</sup>.

666

#### 667 4.2) *Generating asymmetries in dividing cells*

668 In mammals, TTL is the sole enzyme to catalyse re-tyrosination of tubulin, thus its absence  
669 leads to a massive accumulation of detyrosinated tubulin, and TTL-knockout mice die  
670 perinatally<sup>209</sup>. Among the dysfunctions observed in these mice, one striking phenotype was a  
671 severe disorganisation of the brain. This defect could be explained by the failure of spindle  
672 alignment in TTL-knockout cells<sup>129</sup>: Spindle position depends on the interactions of astral  
673 microtubules with the cell cortex<sup>259</sup>, which are mediated by CAP-Gly proteins<sup>260</sup>. The +TIP  
674 localisation of CAP-Gly proteins depends on tyrosination<sup>130</sup>, which is normally enriched on  
675 astral microtubules<sup>255</sup>. Therefore, the nearly-complete absence of tyrosinated tubulin in TTL-  
676 knockout cells leads to dysfunctional +TIP complexes, and consequently to impaired spindle  
677 orientation, which in turn determines the fate of daughter cells after neuronal progenitor  
678 division<sup>261,262</sup>.

679 During meiosis in mouse oocytes, detyrosination is asymmetrically distributed between the  
680 two meiotic half-spindles, and thus involved in non-Mendelian segregation of chromosomes,  
681 known as meiotic drive<sup>263</sup>. Strikingly, the half-spindle that migrates toward the oocyte cortex  
682 progressively accumulates tyrosination, which implies an active role of TTL rather than of a  
683 detyrosinating enzyme in the generation of this asymmetry<sup>264</sup>.

684

685 4.3) *Controlling centrosome functions*

686 Centrosomes are microtubule organising centres, which in many different cell types serve as  
687 facilitators of mitotic spindle bipolarity, and converted into basal bodies become the  
688 organising centres of cilia and flagella<sup>265</sup>. Polyglutamylation is particularly enriched on the  
689 centrioles<sup>266</sup>; complex microtubule structures at the core of the centrosome<sup>267</sup> (Fig. 4d).  
690 Different patterns of polyglutamylation have recently been mapped to distinct domains of  
691 centrioles, suggesting that the modification could serve as a guidance signal for centriole-  
692 associated proteins that localise to highly defined positions within these complex  
693 structures<sup>268,269</sup>.

694 So far, no experiments selectively abolishing polyglutamylation of centrioles were reported.  
695 Injection of anti-glutamylation antibodies into dividing cells<sup>270</sup> led to centriole disassembly  
696 and cell-cycle defects<sup>266,271</sup>, thus providing a first glimpse onto a potential importance of  
697 polyglutamylation in centriole maintenance and functions. In the light of the central role  
698 centrosomes play in cell division<sup>5</sup>, this could indicate that polyglutamylation, by tightly  
699 controlling centriole assembly, and perhaps also centriole maturation and duplication, could  
700 control cell cycle timing and fidelity. Indeed, spermatozooids of *CCP5*-knockout mice show  
701 supernumerary centrioles, suggestive of a centriole duplication defect due to increased  
702 polyglutamylation<sup>174</sup>. Considering the large number of implications of centrosome duplication  
703 defects in human diseases<sup>272</sup>, it is possible that aberrant centriole polyglutamylation could be  
704 one of the causes of such disorders.

705

706 4.4) *Controlling cell proliferation via the primary cilium*

707 As mentioned above, many tubulin PTMs are enriched in ciliary axonemes and basal bodies,  
708 and might thus affect cell proliferation by controlling the functions of primary cilia. Primary  
709 cilia are signalling centres that control cell division and proliferation, and their dysfunction  
710 might lead to cancer<sup>273,274</sup>. The first direct link between a tubulin PTM, cell proliferation and  
711 cancer was found for the glycyclase *TLL3*. Mice lacking *TLL3* show a reduced number of  
712 primary cilia in the colon epithelium, which hyper-proliferates and shows faster tumour  
713 growth<sup>275</sup>. To which extent perturbed primary cilia are the unique reason for this phenotype  
714 remains to be established. A remarkable observation in this study was that hyperproliferation  
715 of the colon tissue led to no visible morphological changes in non-cancerous colon tissue, and  
716 the defect only became apparent after tumour induction. This illustrates how subtle effects of

717 defective tubulin PTMs can be overlooked despite their role in a key physiological process  
718 and tumorigenesis.

719

## 720 **Conclusions and perspectives**

721 Here we have reviewed current advances in the functional understanding of the tubulin code.  
722 So far, exciting new links between the tubulin code and a range of cellular functions have  
723 been discovered, however many questions still remain open. Considering that the elements of  
724 the tubulin code, i.e. tubulin PTMs and multiple tubulin genes, were discovered in the  
725 1970ies, it is surprising that so little advance had been made. Why is this so?

726 In 1976, Fulton and Simpson formulated the first ‘multi-tubulin hypothesis’: *“The surfaces of*  
727 *a tubulin molecule must interact with many other tubulin surfaces ... as well as with*  
728 *associated molecules .... Many of these structural interactions appear to have been conserved*  
729 *throughout evolution, and this probably imposes severe restraints on variations in the amino*  
730 *acid sequence. ... On the other hand, subtle changes may have occurred that do not alter the*  
731 *basic topology of tubulin but do provide specialized associative properties or binding sites for*  
732 *particular functions.”*<sup>71</sup>.

733 The discovery of tubulin isotypes that are often highly similar, or tubulin PTMs that label  
734 specific microtubule species in cells without being ‘essential’ in the classical cell-biological  
735 sense has beautifully confirmed this early hypothesis, but also somewhat dampened the  
736 interest in the tubulin code. At the end of the 1980ies, it became clear that most tubulin  
737 isotypes are interchangeable without obvious functional consequences in cells<sup>276</sup>, which led to  
738 the questioning of their functional importance (vs. evolutionary redundancy)<sup>277,278</sup>. At the  
739 same time, research on tubulin PTMs was impeded by the absence of appropriate means of  
740 manipulation, which was overcome mostly in the 21<sup>st</sup> century by the discovery of a number of  
741 modifying enzymes, and some were discovered only recently<sup>211,212</sup>. Surprisingly however,  
742 many of the tubulin-modifying enzymes showed only mild phenotypic defects when deleted,  
743 even though in some cases the levels of tubulin PTMs changed significantly when only one  
744 modifying enzyme was mutated. In most cases, only some specific cell types or organs show  
745 signs of dysfunction, or degeneration, and only in rare cases, such as TTL<sup>209</sup>, deletion of a  
746 single enzyme has severe consequences for development and survival.

747 It thus appears that tubulin isotypes and PTMs might have in many cases rather subtle effects  
748 on gross microtubule functions, but could be important to control complex, long-lasting

749 cellular functions, in some cases by regulating only selected microtubule populations in a cell.  
750 While this confirms the initial predictions of the multi-tubulin hypothesis<sup>71</sup>, it made and  
751 makes the functional analyses of the tubulin code challenging: Detecting subtle alterations of  
752 microtubule functions requires more sensitive methods to measure microtubule behaviour in  
753 cells or in purified systems, or long-term observations of organism, including detailed  
754 histological and behavioural analyses. However, it also bears a great opportunity for a  
755 conceptual leap in cell biology. Evolution has shown that both, tubulin isotypes and PTMs  
756 would be eradicated if they were not needed for cell survival (Box 2), which strongly suggests  
757 that tubulin isotypes and PTMs are bound to be functionally important in organisms that have  
758 retained them.

759 The fact that so far both elements of the tubulin code have almost systematically slipped  
760 through the meshes of various analytical approaches indicates the urgent need of more  
761 adapted methodology, and, more importantly, the need to broaden our concept of biological  
762 functions in space and time. Indeed, cellular processes can last over a lifetime, and cells such  
763 as neurons span lengths of over 1 m in our body. Regulatory processes that can easily be  
764 neglected in the cell culture dish might have a key role in controlling such complex systems  
765 over a longer time. The role of the tubulin code in these processes has recently been proven  
766 by the discovery that deregulation of tubulin PTMs can lead to the degeneration of  
767 neurons<sup>231,234</sup> or photoreceptors<sup>178</sup>, and there is a whole spectrum of neurological disorders  
768 linked to mutations of tubulin isotypes (Box S2). Exploring the role of the tubulin code is a  
769 great challenge for the coming years, and will certainly contribute to uncover novel, so far  
770 unexplored principles of the regulation of cellular functions.

771

772 **Display items**

773 **Box 1: Complex PTMs on the C-terminal tubulin tails**

774 The complexity of tubulin PTMs is particularly high on the C-terminal tails of these proteins,  
775 where detyrosination, polyglutamylation and polyglycylation take place (Fig. 1). The  
776 complexity that arises from these PTMs, and their interplay with tubulin isotypes, will be  
777 briefly discussed in this box.

778 The majority of  $\alpha$ -tubulin genes in most organisms encode a C-terminal tyrosine or  
779 phenylalanine, which can be enzymatically removed<sup>64</sup> and re-added without the requirement  
780 of ribosomes<sup>58</sup>. These discoveries were surprising in two ways: first, the initial PTM is  
781 actually the removal, and not the addition of a functional group, and second, it was the first  
782 time an enzymatic incorporation of an amino acid into a peptide chain without mRNA and  
783 ribosome was observed. Thus, while the tubulin PTM became known as tubulin tyrosination,  
784 it is more appropriate to consider the detyrosination as the actual modification – with one  
785 exception: in cells expressing tubulin isotypes missing the C-terminal tyrosine, such as the  
786 mammalian  $\alpha$ -tubulin TubA4A<sup>279</sup>.

787 The enzymatic removal of C-terminal tyrosine can be followed by further amino acid  
788 cleavages, which on mammalian  $\alpha$ -tubulin give rise to  $\Delta 2$ - and  $\Delta 3$ -tubulins (lacking the first  
789 and second glutamates before the C-terminal tyrosine; Fig. 1)<sup>66,67</sup>. It thus appears that tubulin  
790 C-terminal tails might be subjected to extensive amino acid editing, most likely beyond what  
791 is currently known. A first glimpse of this possibility was found with the discovery that an  
792 antibody specific to  $\Delta 3$ -tubulin also labelled  $\beta$ -tubulin, which implied that four C-terminal  
793 amino acids of  $\beta$ -tubulin have been removed to generate the specific epitope for this  
794 antibody<sup>67</sup>. Structural data of the enzyme adding tyrosine to tubulin, the tubulin-tyrosine  
795 ligase (TTL) show that the mode of binding between enzyme and the tubulin is so specific  
796 that even  $\Delta 2$ -tubulin cannot be retyrosinated<sup>122</sup>.

797 Polyglutamylation and polyglycylation were initially discovered on tubulin. Both PTMs  
798 consist of the generation of secondary peptide chains as branches from the main chain, using  
799 the ( $\gamma$ ) carboxy-group of a glutamate as modification site (Fig. 1). As C-terminal tails of  
800 tubulin are rich in glutamates, there are many potential sites on which these two PTMs could  
801 be added. Theoretically, this could give rise to a large variety of combinatory signals on both,  
802  $\alpha$ - and  $\beta$ -tubulin, however so far, only little insight has been gained in the complexity of these  
803 PTMs in living cells. Both PTMs were discovered by mass spectrometry approaches that were

804 particularly designed to analyse the C-terminal tubulin tails, as otherwise these highly acidic  
805 tails are mostly lost in proteomic analyses. Analysing purified brain tubulin – the gold  
806 standard in tubulin biochemistry – the main modification sites found were E445 on  $\alpha$ 1-tubulin  
807 (TubA1)<sup>59</sup>, E435 on  $\beta$ 2-tubulin (TubB2)<sup>61</sup>, and E438 on  $\beta$ 3-tubulin (TubB3)<sup>60</sup>. Using a  
808 similar approach, polyglycylation was discovered on ciliary tubulin isolated from  
809 *Paramecium tetraurelia*, and accumulations of up to 34 glycine residues per tubulin molecule  
810 were observed<sup>62</sup>.

811 The enzymes catalysing the glutamylation and glycylation reactions, both members of the  
812 tubulin tyrosine ligase like (TTL) family (Table 1) show enzymatic preferences for either  $\alpha$ -  
813 or  $\beta$ -tubulin, or for the generation of short vs. long glutamate or glycine chains<sup>140,280</sup>. To  
814 which extent these enzymes also modify specific positions out of the many possible  
815 modification sites within the tubulin tails has so far remained an open question. Nevertheless,  
816 the existing selectivity of the modifying enzymes already provides an indication that these  
817 two PTMs generate highly controlled patterns on cellular microtubules. To do so, TTL  
818 enzymes need to be selectively activated, or localized. Little is so far known about regulatory  
819 circuits involved in such control mechanisms, however first insights indicate that such control  
820 mechanisms exist: the protein CSAP was shown to directly activate TTL enzymes<sup>281</sup>, and  
821 some other proteins were shown to interact with TTLs thus localising them to specific  
822 organelles such as cilia<sup>193</sup> or centrosomes<sup>282</sup>.

823

## 824 **Box 2: An evolutionary link between tubulin PTMs and cilia and flagella**

825 Tubulin PTMs are strongly enriched on axonemal microtubules, and most of them have  
826 essential ciliary functions<sup>176</sup> (Fig. 4a). Strikingly, most of the known tubulin PTMs appear to  
827 be evolutionarily linked to cilia and flagella. TTL enzymes, which catalyse tubulin  
828 glutamylation<sup>140</sup> and glycylation<sup>181,280</sup>, for instance, can be easily identified in different  
829 organisms based on their highly conserved TTL domain<sup>140,181,230,280</sup>. Homologs of *TTL* genes  
830 are absent from eukaryotes without cilia, such as the yeasts *Saccharomyces cerevisiae* or  
831 *Schizosaccharomyces pombe*, as well as many plants. However, whenever an organism has  
832 ciliated cells, *TTL* genes can be identified in its genome, given that a well-annotated genome  
833 sequence is available. For example, *Batrachochytrium dendrobatidis* is a fungus that can  
834 grow cilia, and consequently assembles basal bodies and axonemes<sup>283</sup>. Performing a BLAST  
835 search with murine TTL1, a polyglutamylase, reveals the presence of highly homologous

836 proteins. While so far, no systematic evolutionary study has been published, the presence of  
837 TLL homologs could be considered a strong indication for the presence of glutamylation  
838 and/or glycylation, and could be used as a starting point for a subsequent functional  
839 characterisation.

840

841 **Figure 1. The elements of the tubulin code.**

842 Microtubules dynamically assemble from dimers of  $\alpha$ - and  $\beta$ -tubulins. Tubulins are highly  
843 structured, forming the ‘tubulin bodies’, while their C-terminal amino acids form unstructured  
844 tails that protrude from the microtubule surface. The tubulin code stands for the concept that  
845 different tubulin gene products together with a variety of posttranslational modifications  
846 (PTMs) modulate the composition of individual microtubules. Tubulin isotypes (depicted in  
847 different colours: dark grey and brown for  $\alpha$ -tubulins, light grey and pink for  $\beta$ -tubulins) are  
848 encoded by different tubulin genes, and can intermingle during microtubule polymerisation.  
849 Tubulin PTMs are catalysed by a range of enzymes (Table 1), and are located either at the  
850 globular, highly structured tubulin bodies (e.g. acetylation, phosphorylation), or at the  
851 unstructured C-terminal tails of tubulin (e.g. detyrosination,  $\Delta$ 2- and  $\Delta$ 3-tubulin,  
852 (poly)glutamylation, (poly)glycylation). Tubulin PTMs can generate binary switches (on/off  
853 signals) by adding/removing single functional residues (acetylation, phosphorylation,  
854 detyrosination,  $\Delta$ 2- and  $\Delta$ 3-tubulin), or can gradually modulate the strength of their signals by  
855 adding different numbers of residues (polyamination, (poly)glutamylation, (poly)glycylation).  
856

857 **Figure 2. The impact of the tubulin code on microtubule properties.**

858 **a.** Tubulin isotypes can determine protofilament numbers. In *C. elegans*, two isotypes specific  
859 to touch-receptor neurons (*mec-7* and *mec-12*) determine the 15-protofilament microtubule  
860 architecture in these cells<sup>78-80</sup>. **b.** Tubulin isotypes can be essential for the formation of a  
861 geometrically defined microtubule array, the marginal band. This band assembles from  
862 microtubules along the outer rim of blood platelets, and is essential for the shape and correct  
863 function of the platelets. Two tubulin isotypes,  $\alpha$ 4A- (TubA4A)<sup>96</sup> and  $\beta$ 1-tubulin (TubB1)<sup>32</sup>  
864 are essential for the correct assembly of the marginal band, and lack of either of these isotypes  
865 leads to dysfunctions of blood platelets. **c.** Tubulin PTMs can change mechanical properties  
866 of microtubules. Acetylation of  $\alpha$ -tubulin at K40 structures the loop of  $\alpha$ -tubulin in a way that  
867 weakens the interactions between neighbouring protofilaments (red arrowheads)<sup>95</sup>. At the  
868 same time, acetylation reduces the flexural rigidity of microtubules, making them more

869 resistant to mechanical bending, thus avoiding microtubule breakage and disassembly<sup>93,94</sup>.  
870 (upper panels are adapted from ref.<sup>95</sup>) **d.** Tubulin isotypes can control microtubule dynamics.  
871 Microtubules containing  $\beta$ 3-tubulin are more dynamic than the ones assembled from  $\beta$ 2B-  
872 tubulin<sup>77,104</sup>. Phosphorylation of  $\beta$ -tubulin S172 by Cdk1<sup>48</sup> or DYRK<sup>50</sup>, or acetylation of K252  
873 by San<sup>106</sup> impede the incorporation of tubulin dimers into microtubules. Tubulin  
874 polyamination, in contrast, renders microtubules particularly resistant to depolymerisation<sup>56</sup>.  
875  
876

877 **Figure 3. The impact of the tubulin code on MAP-microtubule interactions.**

878 **a.** Both, tyrosinated and detyrosinated microtubules can attract specific subsets of MAPs.  
879 MCAK<sup>110</sup>, CLIP-170<sup>129,130</sup> and dynein in complex with BicD2<sup>137</sup> are attracted to tyrosinated  
880 microtubules, while the kinesin motors CENP-E<sup>135</sup> and kinesin-2<sup>125</sup> preferentially associate  
881 with detyrosinated microtubules. **b.** Different levels of tubulin polyglutamylation can fine-  
882 tune functions of microtubule-interacting proteins. The activity of the microtubule-severing  
883 enzyme spastin is upregulated by initial polyglutamylation of this substrate  
884 microtubules<sup>111,112</sup>, however, further accumulation of this PTM inhibit spastin activity<sup>112</sup>.  
885 Molecular motors, such as kinesin-1 and kinesin-2<sup>125</sup>, or flagellar dynein<sup>166</sup> can be also  
886 differentially regulated by varying degrees of polyglutamylation. While kinesin-2 is induced  
887 by moderate levels of polyglutamylation, kinesin-1 requires higher levels of this PTM to  
888 stimulate its performance<sup>125</sup>.  
889

890 **Figure 4. Cellular and physiological role of the tubulin code.**

891 Functions for specific tubulin PTMs and isotypes are summarised. Note that only the known  
892 functions are highlighted, which does not exclude that other PTMs or isotypes are present,  
893 and/or have additional functions on those microtubules. Overview representations of cells  
894 show all known tubulin PTMs using colour coding, while in zoom representations only  
895 specific PTMs and isotypes are shown for clarity.

896 **a. Cilia and flagella.** Axonemal microtubules are highly modified with a range of tubulin  
897 PTMs. Glycylation has so far only been found on axonemes. Both, polyglutamylation and  
898 glycylation accumulate toward the proximal part of the cilia, while acetylation appears to be  
899 equally distributed all-along axonemes<sup>168,177,284</sup>. Basal bodies are highly polyglutamylated. In  
900 axonemes polyglutamylation specifically decorates the B-tubules of the microtubule  
901 doublets<sup>169,170</sup> and controls dynein activity and ciliary beating<sup>166,167</sup>. In *D. melanogaster*, the

902  $\beta$ 2-tubulin isotype is essential for the binding of outer dynein arms<sup>196</sup>. In all types of cilia,  
903 glycylation controls cilia length and stability<sup>168,177,178</sup>, and its absence is linked to  
904 photoreceptor degeneration<sup>178</sup>, or cell-cycle defects due to loss of primary cilia<sup>275</sup>. **b.**  
905 **Neurons.** Acetylation and detyrosination decorate distinct microtubules of opposite polarities  
906 in dendrites. These two microtubule subpopulations control transport directionality in  
907 dendrites, but it is not known if the PTMs directly control the motor proteins involved<sup>208</sup> (note  
908 that polyglutamylation is also present, but not shown). Polyamination stabilises yet  
909 unidentified microtubule populations in neurons<sup>56</sup>, while the presence of  $\beta$ 3-tubulin (TubB3)  
910 in neurons enhances microtubule dynamics<sup>77</sup>, which is essential for axon regeneration<sup>243</sup>.  
911 Polyglutamylation regulates bidirectional axonal transport driven by kinesins and dynein<sup>231</sup>,  
912 and abnormal accumulation of this PTM leads to neurodegeneration<sup>231,234</sup>. Most neuronal  
913 microtubules are highly posttranslational modified, except for the highly dynamic ones in the  
914 growth cone<sup>207</sup>. Tyrosinated microtubules are essential for growth cone guidance<sup>210</sup>. **c.**  
915 **Muscles.** Detyrosinated  $\alpha$ -tubulin isotype TubA4A and posttranslational detyrosination of  
916 microtubules in muscle cells are essential for their buckling, which in turn defines their  
917 capacity to bear load and influences the viscoelastic behaviour of muscle cells during  
918 contraction<sup>98,285</sup>. Aberrant detyrosination is linked to heart failure<sup>246</sup>. **d. Cell cycle and**  
919 **centrosome.** Tubulin acetylation, polyglutamylation<sup>111</sup> and detyrosination<sup>255</sup> are enriched in  
920 central mitotic spindles and on midbody microtubules. Tyrosinated microtubules are essential  
921 for spindle orientation<sup>261,262</sup> due to the requirement of this PTM for dynein loading onto astral  
922 microtubules<sup>137</sup>. The enrichment of detyrosinated microtubules on central spindle  
923 microtubules<sup>255</sup> guides the kinetochore-associated CENP-E motor towards the metaphase  
924 plate, thus assuring correct chromosome congression and separation<sup>135</sup>. Centriolar  
925 microtubules are highly polyglutamylated<sup>266</sup>, with a specific localisation of this PTM at the C-  
926 tubules<sup>268</sup>. The high levels of polyglutamylation on centrioles is essential for centrosome  
927 integrity throughout mitosis<sup>266,271</sup>.  
928

929 **Table 1:** Known tubulin posttranslational modifications (PTMs) and enzymes

Tubulin PTM	Chemistry	Modification sites	Forward enzymes	Reverse enzymes
Acetylation	Enzymatic addition of acetyl-moiety to lysine residue	$\alpha$ -tubulin K40 <sup>91</sup>	$\alpha$ -tubulin acetyltransferase 1 (aTAT1) <sup>69,217</sup>	histone deacetylase 6 (HDAC6) <sup>225</sup> ; sirtuin 2 (Sirt2) <sup>286</sup>
		$\beta$ -tubulin K252 <sup>106</sup>	San acetyl transferase <sup>106</sup>	<i>Not known</i>
Methylation	Enzymatic addition of methyl-moiety to lysine residue	$\alpha$ -tubulin K40 <sup>52</sup>	SET-domain-containing 2 methyltransferase (SETD2) <sup>52</sup>	<i>Not known</i>
Detyrosination; retyrosination	Enzymatic removal of C-terminal tyrosine residue from $\alpha$ -tubulin <sup>64,287</sup> ; ribosome-independent incorporation of tyrosine <sup>58,288</sup>	$\alpha$ -tubulin C-terminal Y	Detyrosinases are vasohibin proteins <sup>211,212</sup> in complex with the small vasohibin-binding protein (SVBP) <sup>289-293</sup>	Tubulin tyrosine ligase (TTL) <sup>294</sup>
Generation of $\Delta 2$ -tubulin; $\Delta 3$ -tubulin	Enzymatic removal of C-terminal glutamates from $\alpha$ -tubulin after detyrosination <sup>66,67</sup>	$\alpha$ -tubulin penultimate C-terminal E	cytosolic carboxypeptidases (CCP) <sup>229,295,296</sup>	<i>No reverse reaction known to date, tyrosination of <math>\Delta 2</math>-tubulin with TTL is not possible<sup>66,122</sup></i>
[poly]glutamylation	Enzymatic addition of glutamate to $\gamma$ -carboxy-group of glutamate side chains, followed by elongation of the nascent chain with further glutamates	$\alpha$ - and $\beta$ -tubulin tubulin C-terminal tails (multiple residues are modified) <sup>59-61</sup>	tubulin tyrosine ligase like (TTLL) protein, multiple members in most organisms (9 glutamylases in mammals) <sup>140,230,297</sup>	cytosolic carboxypeptidases (CCP), multiple members in most organisms (6 deglutamylases in mammals) <sup>229,295,296</sup>
[poly]glycylation	Enzymatic addition of glycine to $\gamma$ -carboxy-group of glutamate side chains, followed by elongation of the nascent chain with further glycines	$\alpha$ - and $\beta$ -tubulin tubulin C-terminal tails (multiple residues are modified) <sup>62,284</sup>	tubulin tyrosine ligase like (TTLL) protein, multiple members in most organisms (3 glycyllases in mammals) <sup>181,280,298</sup>	<i>No reverse reaction or enzymes known</i>
Polyamination	Enzymatic addition of polyamines to the $\gamma$ -carboxamide group of a glutamine residue side chains	$\alpha$ - and $\beta$ -tubulin, major modification: $\alpha$ -tubulin Q15 <sup>56</sup>	Transglutaminases (TG) <sup>56</sup>	<i>No reverse reaction or enzymes known</i>
Phosphorylation	Enzymatic addition of phosphate group to serine/threonine/tyrosine residue	$\beta$ -tubulin S172 <sup>48</sup>	Cyclin-dependent kinase 1 <sup>48</sup>	<i>Not known</i>
		$\beta$ -tubulin S172 <sup>50</sup>	dual-specificity tyrosine-regulated kinase (DYRK) <sup>50</sup>	<i>Not known</i>
		$\beta 3$ -tubulin S444 <sup>42</sup>	<i>Not known</i>	<i>Not known</i>
		$\alpha$ -tubulin Y432 (determined on C-terminal $\alpha$ -tubulin peptide, insensitive to carboxypeptidase A	Spleen tyrosine kinase (Syk) <sup>46</sup>	<i>Not known</i>

		treatment, which excludes Y451) <sup>46</sup>		
		$\alpha$ - and $\beta$ -tubulin Y residues (not identified) <sup>37,44</sup>	Src kinase <sup>37,44</sup>	<i>Not known</i>
Ubiquitinylation	Enzymatic addition of the small protein ubiquitin to lysine residues of tubulin <sup>54,299</sup>	$\alpha$ -tubulin, major modification: site K304 <sup>55</sup>	<i>Not known</i>	<i>No reverse reaction or enzymes known</i>
Sumoylation	Enzymatic addition of the small protein sumo to lysine residues of tubulin <sup>300</sup>	$\alpha$ -tubulin (modification site unknown) <sup>300</sup>	<i>Not known</i>	<i>No reverse reaction or enzymes known</i>
Palmitoylation	Enzymatic addition of long-chain fatty acid palmitate to tubulin <sup>53,301</sup>	$\alpha$ -tubulin, major modification site: K376 <sup>53</sup>	<i>Not known</i>	<i>No reverse reaction or enzymes known</i>

930

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944

### 945 **Author contributions**

946 Both authors researched data for the article, contributed to discussion of the content, wrote the  
947 article and reviewed and edited the manuscript.

948

### 949 **Competing interests**

950 The authors declare no competing interests.

951 **Glossary**

952 **Axonemes**

953 A structure built from microtubules and associated proteins at the core of all eukaryotic cilia  
954 and flagella. In motile cilia, axonemes consist of nine microtubule doublets arranged around a  
955 central microtubule pair, accessory proteins and flagellar dynein motors that assure the  
956 beating of cilia. Primary cilia lack the motor protein and central-pair microtubules.

957

958 **A-tubule, B-tubule**

959 Components of the microtubule doublets of axonemes. The A-tubules are generic  
960 microtubules made of 13 protofilaments, while B-tubules are partial microtubules made of 10  
961 protofilaments that partially share the wall of the A-tubules (Fig. 4a).

962

963 **CAP-Gly domain-containing proteins**

964 Cytoskeleton-Associated-Proteins (CAP) containing a glycine (Gly)-rich domain. CAP-Gly  
965 proteins contain a well-conserved GKNDG sequence motive that specifically  
966 recognizes -EEY/F sequences<sup>131</sup>, which targets them to the plus ends of tyrosinated  
967 microtubules.

968

969 **+TIP complex**

970 A group of microtubule-interacting proteins localized to the plus ends of microtubules<sup>302</sup>. For  
971 most of these proteins, plus-end localisation is mediated by a group of the end-cinding (EB)  
972 protein, such as mammalian EB1, EB2 and EB3, or yeast Bim1p.

973

974 **Meiotic drive**

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

976

977 **Ependymal cells**

978 Glial cells lining the ventricles of the mammalian brain, as well as the central canal of the  
979 spinal cord. Ependymal cells have multiple motile cilia, whose coordinated beating

980 determines the direction of flow of cerebrospinal fluid<sup>165</sup>. They are also called  
981 ependymocytes.

982

### 983 Basal body

984 A microtubule-based multiprotein structure at the base of cilia and flagella<sup>303</sup>. The core  
985 microtubule structure, the centriole (Fig. 4d), is the same that constitutes the centrosomes of  
986 dividing cells<sup>304</sup>.

987

### 988 Primary cilia

989 A solitary microtubule-based organelle emanating from the cell surface of most mammalian  
990 cells. Primary cilia are thought to be environmental sensors and signalling hubs of the cell<sup>305</sup>,  
991 and their dysfunction was linked to a variety of ciliopathies and cancers<sup>274</sup>. Primary cilia  
992 contain axonemes without dynein motors and are thus non-motile.

993

### 994 Connecting cilia

995 A highly modified primary cilium connecting the cell body to the outer segment of  
996 photoreceptor cells in the retina<sup>306</sup>.

997

### 998 Growth cone

999 Dynamic structure at the tip of a growing neurites, able to sense the environment and guide  
1000 neurite outgrowth and connection<sup>307</sup>. Growth cones are temporal structure in developing  
1001 neurons.

1002

### 1003 Purkinje cell

1004 GABAergic neurons located in the cerebellar cortex. Purkinje cell are among the largest  
1005 neurons in the brain with highly ramified dendritic tree.

1006

### 1007 Microcephaly

1008 A medical condition in which the brain and head of patients is smaller than expected<sup>308</sup>.  
1009  
1010 Viscoelasticity  
1011 The property of materials that exhibit both viscous and elastic characteristics when  
1012 undergoing deformation.  
1013  
1014 Desmin  
1015 Muscle-specific intermediate filament assembly essential for the structural integrity and  
1016 function of muscle fibres<sup>309</sup>.  
1017  
1018 Kinetochores  
1019 A multiprotein structure associated with the centromeres of duplicated chromosomes in  
1020 eukaryotic cells. Kinetochores are the docking sites for spindle microtubules to pull sister  
1021 chromatids apart<sup>310</sup>. Kinetochores further control correct sister chromatid attachment via  
1022 checkpoints<sup>311</sup>.  
1023  
1024 Astral microtubules  
1025 A microtubule population that exists only during mitosis. Astral microtubules connect the  
1026 centrosome to the cell cortex and serve to orient the mitotic spindle in the cell<sup>312</sup>.  
1027

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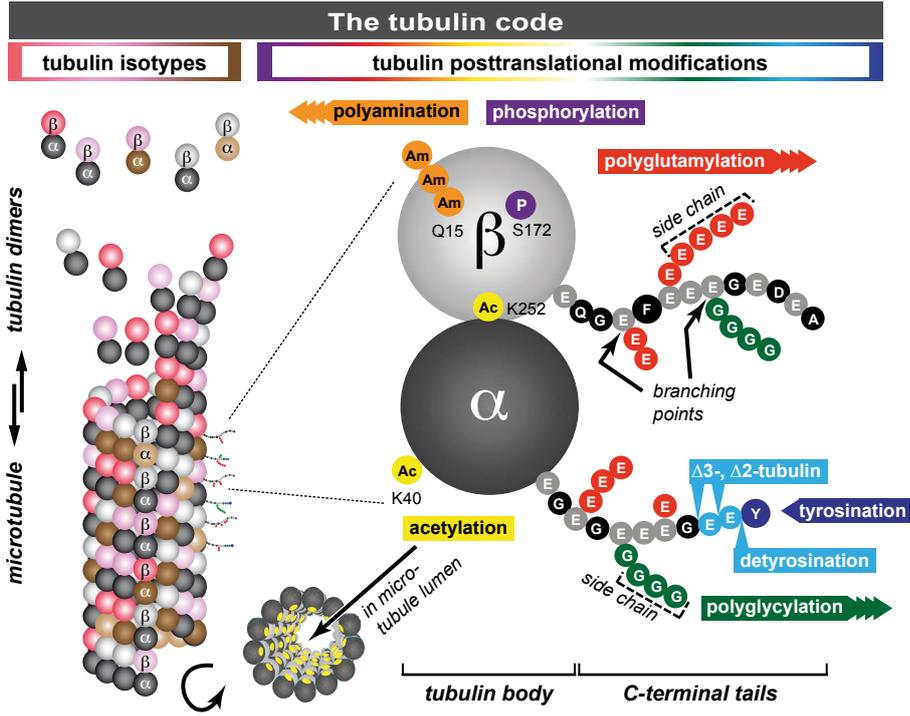
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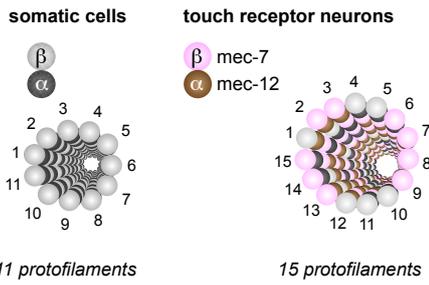
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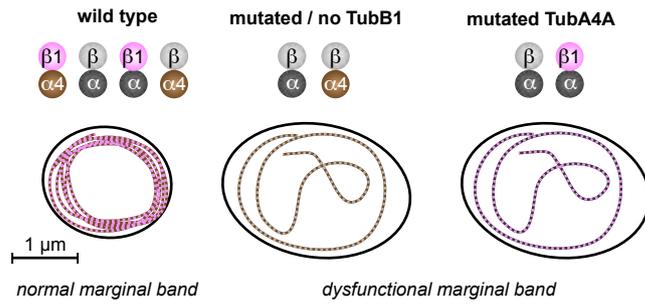
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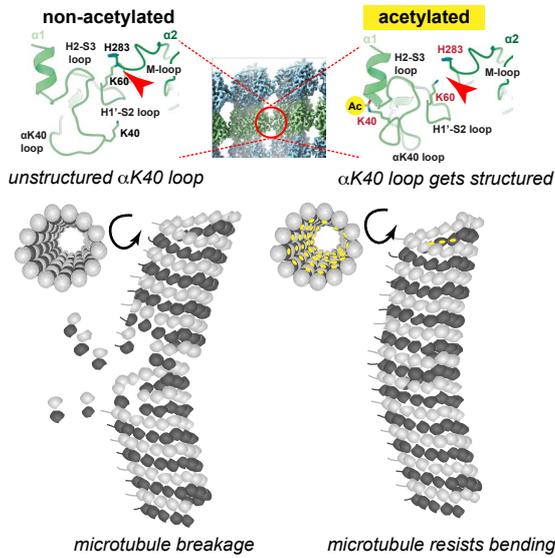
**a** specific microtubules in touch-receptor neurons in *C. elegans*



**b** marginal band microtubule bending in blood platelets



**c** K40 acetylation of  $\alpha$ -tubulin decreases flexural rigidity of microtubules



**d** microtubule dynamics is regulated by tubulin isotypes and posttranslational modifications

