Title: Insights of the tubulin code in gametes and embryos: from basic research to potential clinical applications in humans

Running title: tubulin in gametes and embryos

Summary sentence: The tubulin code is emerging as an important regulator of microtubule function that impacts gamete and embryonic development.

Keywords:
Tubulin, tubulin isotypes, tubulin post-translational modifications (PTMs), spermatozoa, oocytes, early embryo development.

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Grant support: This work was supported by the Doctoral Industrial program from Generalitat de Catalunya (2014 DI 065) and the CERCA Programme/Generalitat de Catalunya, the support of the Spanish Ministery of Economy and Competitiveness, "Centro de Excelecia Severo Ochoa 2013-2017".

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Abstract:

Microtubules are intracellular filaments that define in space and in time a large number of essential cellular functions such as cell division, morphology and motility, intracellular transport and flagella and cilia assembly. They are therefore essential for spermatozoon and oocyte maturation and function, and for embryo development. The dynamic and functional properties of the microtubules are in large part defined by various classes of interacting proteins including MAPs (Microtubule Associated Proteins), microtubule dependent motors, and severing and modifying enzymes. Multiple mechanisms regulate these interactions. One of them is defined by the high diversity of the microtubules themselves generated by the combination of different tubulin isotypes and by several tubulin post-translational modifications (PTMs). This generates a so-called tubulin code that finely regulates the specific set of proteins that associates with a given microtubule thereby defining the properties and functions of the network. Here we provide an in depth review of the current knowledge on the tubulin isotypes and PTMs in spermatozoa, oocytes, and preimplantation embryos in various model systems and in the human species. We focus on functional implications of the tubulin code for cytoskeletal function, particularly in the field of human reproduction and development, with special emphasis on gamete quality and infertility. Finally, we discuss some of the knowledge gaps and propose future research directions.

Introduction

Successful gamete and embryo development, including spermatozoon flagellar beating, meiotic and mitotic spindle assembly, and polarization of blastomeres, rely on the fine regulation of the microtubule network in space and in time.

Microtubules are intrinsically dynamic hollow filaments formed by lateral interactions of protofilaments. In mammalian cells, cytoplasmic microtubules have 13 protofilaments. Protofilaments result from the head to tail self-assembly association of alpha- and beta-tubulin heterodimers. Microtubules are polarized with an exposed alpha-tubulin subunit at the so-called minus-end and a beta-tubulin subunit exposed at the so-called plus-end **(Figure 1)**. In many animal cells, microtubule minus-ends are focused at the centrosome (the main microtubule organizing center, MTOC) and their more dynamic plus-ends extend out toward the cell periphery during interphase. However, microtubules can organize different types of networks highly related with the specific function of the different cell types. They can also form specialized cellular structures that can either be relatively stable like the axoneme of flagella and motile cilia, or highly dynamic like the bipolar spindle, the molecular machine that segregates the chromosomes during cell division.

Microtubules alternate between phases of growth and shrinkage, an intrinsic property known as “dynamic instability” [1]. Therefore the length of dynamic microtubules varies constantly. In the cell, microtubule dynamics is regulated by several classes of interacting and severing proteins that can promote their stabilization or, on the contrary, destabilize them. In addition, microtubule-dependent motors define their intracellular organization and/or use them as tracks to drive the directional transport of different cellular components to different cellular destinations. Despite their homogeneous alpha- beta-tubulin heterodimer core composition, two mechanisms generate diversity

at the microtubule surface thereby defining different binding affinities for the associated proteins resulting in major functional implications. These mechanisms rely on the presence of small sequence differences at the exposed C-terminus of the different alpha- and beta-tubulin isotypes, and on several tubulin PTMs, many occurring on specific residues in these C-terminal sequences [2] **(Figure 1)**.

The tubulin code is functionally important because it can either directly have an impact on the structural properties of the microtubules and/or define the specific binding affinities and properties of interacting proteins.

The first evidence for the role of tubulin PTMs in the regulation of the binding of MAPs to microtubules was obtained in 1994 for Tau and one specific class of tubulin PTMs: polyglutamylation [3]. Tau has a higher affinity for microtubules with short polyglutamylated chains (up to 3 residues). Instead, MAP1A associates preferentially with microtubules having long polyglutamylated chains. It was therefore proposed that the potential binding competition of different MAPs for the microtubules is solved through the control of tubulin polyglutamylation chain length [3, 4]. As a consequence, not only the combination of tubulin isotypes and different tubulin PTMs can regulate the binding of microtubule-associated proteins but the extent of some modifications (chain length) has also different functional consequences. Moreover, some tubulin PTMs have an impact on the functional properties of the associated motors such as their processivity, their velocity, and their directionality [5] **(Table 1)**. Overall, the tubulin code provides a complex and fine mechanism that defines microtubule function.

Several data indicate that the tubulin code has important functions for gamete and embryonic development in model organisms and human. Indeed specific tubulin isotypes are expressed, and enzymes that catalyze tubulin PTMs are present in their reproductive systems. Moreover, work in several animal models has provided evidence

for the essential roles of tubulin modifying enzymes with different knockouts or mutants showing reproductive problems. Although somatic cells such as sertoli and cumulus cells are essential for the proper development of the spermatozoa and oocytes, due to the lack of data for these cells, we review here the mechanisms that generate microtubule diversity and our current understanding of their role in gametes, fertilization, and early embryo development that come mostly from studies in animal models, with a special emphasis on the putative implications for fertility in humans.

The tubulin isotypes

The human genome contains 12 genes encoding alpha-tubulins (9 genes, 2 pseudogenes and 1 putative gene) and 10 genes encoding beta-tubulins (9 genes and 1 pseudogene) **(Table 2) (**HGNC Database, HUGO Gene Nomenclature Committee (HGNC), EMBL Outstation - Hinxton, European Bioinformatics Institute, Welcome Trust Genome Campus, Hinxton, Cambridgeshire, CB10 1SD, UK www.genenames.org. Data retrieved in June 2018**).** All alpha and beta tubulins have a highly conserved N-terminal globular domain and an unstructured C-terminal tail that extends outwards along the microtubule surface serving as docking site for a large number of associated proteins. These C-terminal tails show the highest sequence diversity amongst the different tubulin isotypes **(Table 2)**. For instance, TUBA4 A/B (tubulin alpha isotype 4 A/B), TUBA8 (tubulin alpha isotype 8) and TUBAL3 (tubulin alpha L3) do not contain the final tyrosine present in the other alpha tubulin isotypes [6].

Interestingly, while some tubulin isotypes are expressed in different cell types, others are restricted to certain cell types or to specific developmental stages [7-10]. For example, TUBB8 (tubulin beta isotype 8) is specifically expressed in human oocytes [11] and TUBB4 (tubulin beta isotype 4) is the main isotype expressed in bovine

ciliated tissues [12]. The functional and structural consequences of these specific expression patterns are still currently unclear [9].

Tubulin PTMs: types and modifying enzymes

Several types of tubulin PTMs have been described including phosphorylation, polyamination, methylation, ubiquitination, palmitoylation, acetylation, (poly)glutamylation, (poly)glycylation and detyrosination. In this review, we will focus on the last four types of tubulin PTMs keeping a focus on their role in gamete maturation and early embryo development **(Figure 1).**

Tubulin acetylation. Tubulin acetylation was identified for the first time in 1985 [13]. It consists of the addition of an acetyl group to a lysine in a reversible reaction. Both alpha and beta tubulins can be acetylated on several sites: Lys40 (lysine 40), 60, 112, 311, 326, 370, 394, 401 among others on alpha-tubulin, and Lys58, 252 and 324 on beta-tubulin [14]. Although acetylated microtubules are usually reported as being more stable than non-acetylated ones, the mechanism by which acetylation confers microtubule stability is not clear and some authors have even questioned this correlation [15-22]. In fact, acetylation of beta-tubulin Lys252 negatively regulates tubulin polymerization. However, acetylation of Lys40 on alpha-tubulin is a marker for stable microtubules. Interestingly, Lys40 acetylation is the only modification that occurs inside the lumen of the microtubule [23]. It was recently proposed that this modification stabilizes the microtubule lattice by inducing the formation of a salt bridge, which in turn promotes structural changes in the microtubule angle [24]. However, no significant changes in the microtubule lattice were observed in Lys40 acetylated microtubules *versus* non-acetylated ones by cryo-electron microscopy [25]. Overall, tubulin

acetylation may modify the mechanical properties, structure and organization of the microtubules [24, 26].

Several enzymes have been shown to drive tubulin acetylation or deacetylation. Tubulin acetylation is catalyzed by several acetyltransferases including ARD1-NAT1 (arrest defective protein 1 – arylamide acetylase 1) [27], ELP3 (elongator complex protein 3) [28], San (separation anxiety) [29], GCN5 (general control nonrepressed protein 5) [30] and αTAT1 (alpha-tubulin N-acetyltransferase 1) [26, 31-33]. ARD1-NAT1 is a protein complex conserved from yeast to humans with ARD1 holding the enzymatic activity [27]. ELP3 was found to modulate the migration and differentiation of cortical neurons through the acetylation of alpha-tubulin [28]. San acetylates beta tubulin on Lys252 [29]. GCN5, on the other hand, promotes tubulin acetylation by interacting with localization factors such as Myc-nick [30]. Finally, αTAT1 is the major alpha-tubulin Lys40 acetyltransferase in mice. It is conserved in ciliated organisms and it was shown to be required for the acetylation of axonemal microtubules and for the normal kinetics of primary cilium assembly [31, 32]. The mechanism by which αTAT1 acetylates alpha-tubulin Lys40 inside the microtubule lumen was recently described. αTAT1 can enter the lumen at the microtubule ends and at bends or breaks in the microtubule lattice, and then spread Lys40 acetylation along the microtubule [34, 35].

Tubulin deacetylation is promoted by HDAC6 (histone deacetylase 6) [36, 37] and by SIRT2 (NAD-dependent protein deacetylase sirtuin-2) [38]. HDAC6 acts mainly on polymerized microtubules and is constitutively active whereas SIRT2 is a NAD dependent enzyme [36, 38]. SIRT2 acts specifically on alpha-tubulin Lys40. Although HDAC6 and SIRT2 interact and may function together [38, 39], altering HDAC6 levels is sufficient to increase tubulin acetylation in fibroblasts [40].

Tubulin tyrosination/detyrosination. Most alpha-tubulin isotypes have a glutamic acid followed by a tyrosine residue at their C-terminus. The removal of this tyrosine generates detyrosinated tubulin also named Glu-tubulin (because the newly exposed C-terminal amino acid is a glutamic acid, with the exception of TUBA3E, whose exposed amino acid is an alanine). Glu-tubulin in turn can be re-tyrosinated [41-43]. This generates a dynamic cycle of tubulin tyrosination/detyrosination [44, 45]. Although tyrosinated and detyrosinated microtubules can co-exist in the same cell, different cell types and specific microtubule-based structures have different ratio of tyrosinated/detyrosinated microtubules [43, 46, 47]. This ratio may in fact define specific biochemical properties of the microtubule network. Indeed, dynamic microtubules have been found to be mostly tyrosinated, whereas stable ones like for example those forming centrioles, are mostly detyrosinated [45, 48].

The further removal of the glutamic acid exposed at the C-terminus of detyrosinated alpha-tubulin generates the so-called Δ2-tubulin. This modification is irreversible [49]. Δ2-tubulin is particularly enriched in very stable microtubules like those of cilia (primary or motile) and neurons. Strikingly, it constitutes 35% of all brain tubulin [50]. The enzyme that catalyzes the removal of the last tyrosine residue on alpha-tubulin is the complex of vasohibin-1 with the small vasohibin binding protein [51]. The enzyme that catalyzes the addition of a tyrosine on Glu-tubulin is named tubulin tyrosine ligase (TTL) and requires ATP, magnesium ions and a terminal glutamic acid residue for activity [43, 52-54]. TTL is the founding member of the large TTLL family (see below).

Tubulin glutamylation and glycylation. Chains of different lengths of identical amino acids, either glutamic acids or glycines, can be added on glutamic residues present in the C-terminal tail domains of alpha- and beta-tubulins. These modifications generate

mono- / polyglutamylated or mono- / polyglycylated tubulin, respectively and can occur on any tubulin independently of any other tubulin PTM [55]. Glutamylation and glycylation compete for the same sites at the C-terminal tail domains of tubulins [56], although they can also co-exist in a single molecule of tubulin [57]. These tubulin PTMs are particularly versatile. Depending on the cell type and organism the branched aminoacid chains can range from as little as 1 to more than 20 residues [58].

Glutamylation was characterized for the first time in 1990 in brain tubulin where it represents about 40 to 50% of the total α-tubulin [58]. It is found in both stable and dynamic microtubules. On the other hand, stable microtubule structures, such as axonemes, are highly glycylated (glycine lateral chains of up to 34 residues branching from alpha- and/or beta-tubulin subunits were described in *Paramecium* [59-61], whereas dynamic microtubules, if they are modified, have shorter polyglycylated chains (in humans, see below) [60, 62]. This modification has been detected in one of the oldest eukaryotes, *Giardia lamblia*, suggesting an important role for glycylation throughout evolution [63].

The mechanism by which mono- and polyglutamylated / glycylated chains are assembled involves a 2-step specific reaction. The initial branching reaction involves the formation of a γ-amide bond between a glutamic acid residue in the main chain of tubulin and a free glutamate/glycine. Second, the addition of successive glutamic acid or glycine residues on the first branched one occurs through the formation of α-amide bonds (peptide-like bond) [64] **(Figure 1)**. The enzymes that catalyze these reactions were initially identified in mouse brain, *Tetrahymena thermophila* and zebrafish [65-67]. These enzymes are highly related to TTL (see above) and constitute the tubulin tyrosine ligase-like family (TTLL) [66, 68]. They all share a TTLL core domain and an "extended" TTL domain. The amino acids responsible for the function of the TTLL

enzymes are located in these conserved domains [68]. Mammalian organisms have 13 different TTLL enzymes (from TTLL1 to TTLL13) **(Table 3)**. In the human species, microtubules can be mono- or polyglutamylated. However, they can only be monoglycylated because the elongation enzyme (TTLL10) is not functional [69].

The enzymes responsible for the removal of the mono- or polyglycylated chains have not been identified yet. The enzymes that remove the mono- or polyglutamylated chains form a family of enzymes called cytosolic carboxypeptidases (or CCPs), also known as Nna-like proteins or AGTBPs. These enzymes were first identified in studies of axonemal regeneration and Purkinje cell degeneration in animal models [70, 71]. The CCP family consists of six members (CCP1 to CCP6) **(Table 4)**. They share a conserved carboxypeptidase domain (300 residues approximately), which has catalytic activity and a shorter N-terminal domain (150 residues approximately) that has been proposed to act as a folding, regulatory or binding domain [72-75]. The functional redundancy of the TTLL and CCP enzymes may provide a failsafe mechanism to support essential microtubule functions.

The tubulin code and its functional implication in spermatozoa

Mechanism of spermatozoa movement generation. Spermatozoa have a very specific microtubule-based axoneme that is the main constituent of the flagella that drives spermatozoa motility, which is essential for natural fertilization. The axoneme is a distinctive structure that consists of a central pair of microtubules surrounded by a circular sheaf of nine doublets of microtubules (called 9+2). Each doublet consists of a complete 13 protofilaments microtubule (named A-tubule) associated with an incomplete 10 protofilaments microtubule (named B-tubule). The axoneme is

surrounded by the outer dense fibers (ODFs) and the fibrous sheaths [76] that provide elasticity and support to the beating flagellum and are therefore important for spermatozoa motility and stability [77] **(Figure 2)**.

Tail movement is powered by axonemal dynein. Axonemal dynein was first identified in *Tetrahymena pyriformis* cilia [78] and later found to be a central components of motile cilia and flagella in all species. It is a microtubule minus-end directed multiprotein ATP-dependent motor [79]. Dyneins power the reciprocal sliding of adjacent microtubule doublets by interactions of inner and outer dynein arm complexes with the microtubules generating the beating of the flagella [78, 80-84]. Inner and outer arm dyneins have more stable interactions with the A-tubule within a microtubule doublet **(Figure 2)**. These dynein complexes then interact transiently in an ATP dependent manner with the B-tubule of an adjacent microtubule doublet, producing a sliding movement of the microtubule doublets and the bending of the flagellum [85].

Tubulin PTMs in the spermatozoa flagella. Axonemal microtubules are highly modified by acetylation, polyglutamylation and polyglycylation [86-88]. The distribution of some of these PTMs along the spermatozoon axoneme and in each of the microtubule doublets is not homogeneous [89], generating a complex biochemical code that may promote transient and specific interactions with individual proteins.

Tubulin acetylation occurs uniformly along the microtubule doublets of the flagella and in the centrioles in all the species analyzed [90].

Polyglutamylation can occur on both the axoneme and the centrioles of mammalian spermatozoa at any stage during their formation and maturation [91, 92]. The length of the polyglutamate chains is not homogeneous along the spermatozoon axoneme or in the outer and inner microtubule doublets in several organisms [90, 92-94]. For instance,

in mice and humans, the presence of branching glutamates decreases along the tail length and they are enriched on doublets 1, 5 and 6 as well as in the outer microtubule doublets in comparison to the inner ones. The linear decrease of tubulin polyglutamylation along the spermatozoon axoneme correlates with a decrease of dynein immunostaining [90, 92, 94], in agreement with the proposed role of tubulin polyglutamylation in the regulation of the interaction of axonemal dynein with the microtubule doublets. However, data from other species suggest that this is not a general rule. Indeed, the distribution of this modification in the cilia of *Paramecium* is the opposite of the one found in human spermatozoa axonemes [95], reinforcing the observation that each tubulin PTMs pattern may be species specific.

Mono- / polyglycylated tubulin is also strongly enriched in the axonemes of cilia and flagella [59, 96]. This tubulin PTM is preferentially found on the outer doublets 3 and 8 of the axonemes of mammalian spermatozoa, and like glutamylation it decreases towards the flagellar tip [95]. In mammalian spermatozoa, monoglycylation is enriched in doublets 3 and 8 of the axoneme, whereas as described above, mono- / polyglutamylation is enriched in doublets 1, 5 and 6. Since the two types of modifications compete for the same site on the tubulin sequence, they are mutually exclusive. Interestingly, in *Sea urchin* spermatozoa flagella, tyrosinated tubulin is mainly found at doublets 1, 5 and 6, that are polyglutamylated. Instead detyrosinated tubulin is enriched in doublets 3 and 8 [97, 98], that are polyglycylated. Therefore, it seems that polyglutamylation and tyrosinated tubulin on one hand and detyrosination and polyglycylation on the other show some interdependence.

The PTMs in spermatozoa tubulin can be established before or during their differentiation and maturation depending on species. In *Drosophila melanogaster*, most tubulin PTMs are already present in young and mature cysts, a germline stage that

corresponds to spermatogonia and spermatocytes/spermatids, respectively. Tubulin (poly)glycylation however, is the only modification that occurs towards the end of spermatozoa maturation, specifically during *Drosophila melanogaster* spermatozoa individualization, which is the physical separation of the 64 spermatids of the cyst into individual spermatozoa. [69, 99, 100]. Similar results were observed in *Paramecium* [101], suggesting that (poly)glycylation is the last modification occurring during the maturation of the spermatozoon. In mammals, there is also a differential expression of the tubulin family members (alpha, beta and gamma) and distribution of tubulin PTMs during the process of spermatozoa differentiation and maturation [102, 103]. In rats, the distribution of tyrosinated, detyrosinated and acetylated tubulins is specific for the different structures and cells of the seminiferous epithelium (spermatids, spermatocytes and Sertoli cells) [104].

Tubulin modifying enzymes and isotypes in spermatozoa. In agreement with the high level of PTMs found in the spermatozoon flagellum, most of the modifying enzymes are present in testis and in spermatozoa of animal models and in humans. For example, the mRNAs for all the CCPs are present in mice and human testis [105, 106]. In addition, transcriptomic analysis showed that mRNAs for 5 different alpha (TUBA1A, TUBA1C, TUBA3C, TUBA4A and TUBA4B) and beta (TUBB, TUBB2A, TUBB3, TUBB4B and TUBB6) tubulin genes are present in human spermatozoa [106].

Relevance of the tubulin code in spermatozoa. As explained above, the activity of the inner and outer dynein arms promote the spermatozoa movement. This process is regulated by tubulin PTMs. Inner dynein arms are positive charged. Since axonemal B-

tubules are highly polyglutamylated, this repulsion may promote their transient interaction providing a basic mechanism for driving spermatozoa motility [107-109]. On the other hand, the activity of the outer dynein arm is regulated by acetylation of α-tubulin Lys40 in the B-tubule that favors the unbound dissociation state [110] **(Figure 2)**. Therefore, the main expected phenotype from interfering with the tubulin code is defective spermatozoon motility.

Information on the functional consequences of interfering with tubulin PTMs has been obtained in mutants for specific tubulin modifying enzymes. Interfering with tubulin acetylation induces defects of spermatozoa motility and tail morphology, which in turn results in the diminished probability of successful fertilization and a reduction of litter size in mice. Mice mutants for the acetylase αTAT1 have been extensively characterized. These mice develop testis and the epididymis with a reduced weight and as well a reduced number of spermatozoa. Mature spermatozoa isolated from cauda epididymis showed morphological aberrations most of the spermatozoa still had attached the cytoplasmic droplet, indicating problems in achieving a complete maturation. The length of the spermatozoa flagella was also diminished and showed a decrease in the amplitude of the lateral beat, therefore, reducing progressive motility. However, the flagella microtubule structure was correct, suggesting that acetylation is affecting spermatozoa maturation, motility and morphology through another mechanism [33]. Consistently, asthenozoospermic human sperm samples show a reduction of acetylated tubulin levels compared with normozoospermic cones [111].
The functional relevance of tubulin polyglutamylation in the spermatozoa has been addressed with different approaches and in different model systems. In *Sea Urchin* spermatozoa, blocking polyglutamylation with specific antibodies was found to

decrease the amplitude of the flagellar beating, especially at its tip [112, 113] and induce a complete loss of spermatozoa motility. Additional evidence was obtained by interfering with the modifying enzymes TTLLs and/or CCPs. Mutations in mouse TTLL1 or TTLL5 (polyglutamylation), result in different levels of infertility, due to immotile spermatozoa or defects in progressive motility characterized by abnormal axonemal structures with shortened or absent flagella [114, 115]. Furthermore, mutations in one the de-glutamylating enzyme CCP1 in mice, result in male sterility associated with low concentrations of spermatozoa with abnormal shape and motility [71]. In *Chlamydomonas*, the tubulin polyglutamylase TTLL9-FAP234 protein complex was shown to determine the flagellar length [116] by controlling tubulin turnover and thereby the axonemal stability [117]. Therefore, it seems that the tight regulation of tubulin polyglutamylation is essential for spermatozoa functionality.

(Poly)glycylation seems to affect, specially, the flagella stability because the polyglycyne chains would help to reduce the negative charge of the C-terminal tails of alpha- and beta-tubulin heterodimers [99]. In *Drosophila melanogaster*, TTLL3 mutants show a male sterility phenotype characterized by only 2.4% of the males being able to generate some offsprings. Although the flagellum can assemble in these mutants, it is rapidly destabilized, either disassembling or becoming highly disorganized [69]. In *Zebrafish*, the depletion of the same enzyme resulted in axonemes lacking the central microtubule doublets [56] and thereby showing elongation and stability defects. In *Sea urchin* spermatozoa, blocking tubulin glycylation not only indues morphology defects but also motility ones characterized by spermatozoa showing an erratic swimming movement with a reduction of the beating amplitude and frequency as well as an incorrect positioning of the spermatozoon head during swimming [99].

Interestingly, differences in the tubulin isotype and tubulin modifying enzymes transcription profiles have been reported for human teratozoospermic sperm samples when compared to control normozoospermic ones. For instance, the expression of TUBA3C, TUBA4A and TUBB4B is down-regulated in teratozoospermic samples, whereas that of TUBB2B is up-regulated. On the other hand, most of the modifying enzymes including TTLL5, TTLL6, TTLL7, TTLL9, TTLL13 pseudogene, CCP2 and CCP5 are down-regulated whereas SIRT2 is up-regulated [106]. These data indicate that teratozoospermic samples have lower levels of polyglutamylated and polyglycylated tubulin and higher levels of acetylated tubulin than normozoospermic ones. The functional implications of these differences are currently unclear. Interestingly, the truncation of the C-terminal tail domains of B2-tubulin in *Drosophila melanogaster* resulted in the absence of spermatozoa axoneme and infertility [118, 119]. Moreover, recent data correlate the presence of acetylated alpha-tubulin isoforms (TUBA3C and TUBA4A) with sperm samples with poor motility [111].

In summary, specific tubulin isotypes and PTMs are present in spermatozoa. All the data support the idea that the tubulin code plays an essential role in the differentiation, maturation and functionality of the spermatozoa in humans and other organisms.

The tubulin code and its functional implications in oocytes

The mechanism of MI and MII spindle assembly. One essential function of the microtubules in the oocyte is the assembly of the meiotic spindle. The morphology and functionality of the human oocyte spindle is associated with the oocyte quality and the ART (Assisted Reproduction Technologies) success because poor oocytes quality is a common cause of female infertility. The spindle is generally described as a barrel shape

structure, with 47.7% of oocytes with both spindle poles flattened. Compared with the human oocyte size (approximately 120 μm in diameter) the spindle is very small, 11.8±2.6 μm in length and 8.9±1.7 μm in width, with a slightly shifted metaphase plate to the pole closer to the oocyte surface [120]. There is relatively few information about how the human oocyte spindle is assembled and organized. Until recently, it was generally accepted, but not demonstrated, that oocytes eliminate or degenerate their own centrosome and any other acentriolar microtubule organizing centers (aMTOCs)– no spindle pole markers could be found in human oocytes (pericentrin, γ-tubulin) [121] – during oogenesis. Hence, spindle assembly completely relies on the RanGTP or chromosome dependent pathway [122, 123]. The fact that aMTOCs do not participate in spindle assembly seems to be specific for humans. In other species such as mice, aMTOCs largely contribute to the spindle assembly and bipolarization by a three-step aMTOC fragmentation and clustering mechanism [124-128]. It is especially curious that PLK4, which is a master regulator of centriole formation, is essential for spindle formation in mice oocytes that possess a ring of aMTOCs at spindles poles and are free of centrioles [129, 130].

Numerous microtubule-associated proteins are also involved in spindle assembly and for the proper centromere -microtubule attachment, such as Aurora A and B, katanins, dyneins and kinesin-2A [129, 131-136]. For instance, kinesin-2A (a kinesin-13 member motorprotein whose activity is regulated by tubulin tyrosination – see **Table 1**) is described to be essential for meiotic spindle assembly and migration, chromosome alignment and polar body extrusion. In *C.elegans*, katanin also mediates a correct meiotic spindle assembly. In oocytes of this species, the tubulin isotype TUBB2B is essential of the katanin severing microtubule function (katanin interaction with tubulin is mediated by polyglutamylation – see **Table 1**). The tubulin code may provide a

451 regulatory mechanism to determine the binding affinities as well as activity of all these
452 proteins.
453
454 **Tubulin PTMs in the oocytes.** However, the identification and characterization of
455 tubulin PTMs in the oocytes of model systems and in humans is still relatively poor due
456 to the difficulties in sample collection in most cases, the limited availability of specific
457 reagents and the transient state of tubulin PTMs. More descriptive and functional
458 analysis has been obtained for the tubulin PTMs present in mitotic spindles of somatic
459 cells [46, 47, 137-139]. Since meiotic spindles diverge form the mitotic ones in a
460 molecular and morphological way, this information is difficult to extrapolate. So far,
461 only data regarding acetylated and tyrosinated tubulin can be found in the literature. In
462 mice oocytes, the pattern of tubulin acetylation varies with the different phases of cell
463 division. It is high at the spindle poles in metaphase, distributed on the whole spindle
464 in anaphase and restricted to the midbody in telophase [140, 141]. During mouse oocyte
465 activation, tubulin acetylation increases, due to a reduction of HDAC activity [141]. In
466 sheep and lamb oocytes, MII spindles are also acetylated but Δ2-tubulin,
467 polyglutamylated and detyrosinated tubulin are not present [142]. Oocyte mice meiotic
468 spindles are enriched in tyrosinated [143] and detyrosinated tubulin in an asymmetric
469 fashion. The meiotic spindle cortical side is enriched in tyrosinated tubulin whereas the
470 egg side is enriched in detyrosinated tubulin. This asymmetry within the spindle is
471 induced by the cortex-localized protein CDC42 and, therefore, only acquired in the late
472 MI when the spindle is positioned close to the cortex, ready to extrude the first polar
473 body [144].
474

Tubulin modifying enzymes and isotypes in oocytes. Like in spermatozoa, most of the TTLLs and CCPs are also expressed in the human oocyte. Interestingly the human oocyte contains mRNAs encoding several alpha tubulin isotypes (TUBAL3, TUBA1B, TUBA1C, TUBA3C, TUBA3D, TUBA3E and TUBA4B), but only one for beta tubulin: TUBB8. TUBB8 should provide an important advantage to the oocyte microtubule functionality because any mutation on this tubulin isotype would have a vast effect on the oocyte viability [11, 106].

Relevance of the tubulin code in oocytes. Oocytes are prone to be genetically unstable, and this instability increases in aged oocytes due to spindle assembly and kinetochore-microtubule attachment defects as well as an inefficient spindle assembly checkpoint control, the loss of sister chromatid cohesion and spindle-associated proteins (PLK1 and gamma-tubulin) mislocalization [145-149]. All the data on tubulin acetylation in mice oocytes suggest that alteration in the levels of tubulin acetylation could contribute to these defects.

Indeed, more than 35% of the mice oocytes depleted of the tubulin deacetylase Sirt2, show abnormal spindle assembly, chromosome organization and congression defects and aneuploidy. Interestingly, aged mice (45 – 58 weeks) have reduced expression levels for Sirt2 and therefore, the levels of tubulin acetylation in the MII meiotic spindle is higher than in young mice (6 – 8 weeks) [150]. Oocytes from mice at a post-ovulatory age also have spindles with an increase level of tubulin acetylation [151]. However, this may not be a general rule since no change in tubulin acetylation was reported for young and aged human oocytes [149]. The inhibition of HDAC6 with Tubastatin A in mice induced several defects in oocytes including maturation arrest, abnomal spindle morphology, chromosome alignment and kinetochore-microtubule attachment defects

as well as defects in spindle migration [152, 153]. Recently, the chromosome cohesion factor Esco1 was shown to act as a tubulin acetylase in mice [154]. Interestingly, interfering with Esco1 activity also induced meiotic arrest associated with meiotic spindle defects and erroneous kinetochore-microtubule attachments [154].

The tubulin deglytamylase CCP1 is expressed in oocytes and cumulus cells in mice. Other members of the family with redundant functions such as CCP4 and CCP6 are not expressed in ovaries. Female mice with mutations in CCP1 and thus a high ratio of tubulin polyglutamylation are subfertile, producing a reduced number of oocytes after ovarian stimulation [155]. However unexpectedly, no defects in the assembly of the meiotic spindle or in chromosome congression were observed. One possibility is that other CCPs (such as CCP2 and/or CCP3) can compensate the loss of CCP1 activity in these mice. Another phenotype is the reduction of maturation of secondary follicles to antral follicles, suggesting that tubulin polyglutamylation is involved in the growth of the oocyte growing and its maturation [155]. The correct proportion of tubulin polyglutamylation in the oocyte and the cumulus cells may therefore provide a fine cross-talk important for oocyte development and maturation.

The genetic analysis of ART patients with primary female infertility problems revealed the existence of at least 26 independent mutations in the TUBB8 gene. These mutations are associated with oocyte maturation defects characterized by an abnormal or absent first meiotic spindle. Most of these mutations affect the folding, assembly and stability of the microtubules and the binding of motor proteins [11, 156]. Consistently, expression of the mutant forms of TUBB8 by microinjection of the mRNAs in mouse oocytes decreased significantly the extrusion of the polar body and this correlated with the presence of highly disorganized spindles. These phenotypes were further confirmed in somatic cells [11, 157]. Strikingly, for at least 4 specific TUBB8 mutations and/or

deletions, the oocytes extrude the first polar body but the 2nd meiotic spindle does not assemble. Once fertilized, the resulting embryos arrest at the cleavage stage [157]. Altogether these data show that mutations in TUBB8 promote defects that result in oocyte and/or embryo development arrest. In some cases these mutations are transmitted paternally because TUBB8 mutations do not have a major effect on spermatozoa [11, 156, 158].

The tubulin code and its functional implication in embryos

Tubulin PTMs in embryo self-assembly and lineage specification. Only a handful of articles have reported the tubulin isotypes and PTMs in embryos. The main phenotypes upon interfering with tubulin isotypes and PTMs are related to cilia dysfunctions during development.

In humans, from the 8-cells to blastocyst stage, cells reorganize within the embryo and establish cell polarity by forming two differentiated cell layers, the extra-embryonic trophectoderm, that will give rise to the embryonic contribution to the placenta and extra-embryonic tissues, and the inner cell mass (ICM) that will develop as the embryo proper. In mice (day E2.5 – E4.5), the mechanism driving cell internalization is mediated by differences in their surface contractility [159] generated by the cortical tension [160] and the asymmetrical distribution of macromolecules such as PAR proteins between cell-contact domains (baso-lateral) versus non cell-contact domains (apical) [161, 162] achieved through the positioning of the bipolar spindle close to the apical domain. When the division plane is parallel to the apical domain, the daughter cell that receives the apical domain of the polarized mother cell specializes as a trophectodermal cell, whereas the other daughter become part of the inner cell mass [163]. The rapid and complex mechanism of cellular polarization also involves

remodeling of the microtubule network that is very dynamic. The only information on tubulin PTMs currently available is on tubulin acetylation during pre-implantation development in mouse embryos. The first mitotic spindle in the zygote is acetylated, particularly at the spindle poles during metaphase [140, 141]. Acetylated microtubules are also present at later stages of mouse early development. At the 16- and 32-cell stages, the cortex of the cells is enriched in acetylated microtubules, a pattern that is more pronounced in the inner cells [164, 165]. However the functional relevance of this differential pattern is not known.

Tubulin isotypes in embryos. Although mRNAs encoding several tubulin isotypes were identified in embryos: four for alpha-tubulins (TUBA1A, TUBA1B, TUBA1C and TUBA3C) and six for beta-tubulins (TUBB2A, TUBB2B, TUBB3, TUBB4A, TUBB4B and TUBB6), their functional implications have not been yet elucidated [106].

Relevance of the tubulin code in embryos. The most common consequence of depleting tubulin modifying enzymes in any animal system is the formation of dysfunctional cilia with altered motility and/or structure. Unlike motile cilia and flagella, primary cilia are immotile and their axoneme lacks the central pair of microtubules. Cilia play essential roles in development as transmitters of external osmotic and mechanical signals, and as key coordinators of signaling pathways, like Hedgehog, essential during development [166]. Ciliary defects can promote alterations in organ laterality, hydrocephaly, cystic kidney disease and retinal degeneration, among others [167]. In mouse embryos, the primary cilia appear first at day 6 of development, in epiblast cells and subsequently in all the cells derived from them [168]. Microtubules

forming the axoneme of primary cilia are also highly modified [169]. Several studies in *Zebrafish* embryos have shown that interfering with PTMs produces developmental defects. Altering the levels of tubulin acetylation produces hydrocephaly, curved body shape, short body axis [32] and reduced rates of cilia assembly [31], among other phenotypes. CCP5 as well as TTLL3 or TTLL6 depletion also induce developmental defects such as body axis curvatures and hydrocephalus [67, 160, 170]. A similar TTLL3 mutation in *Drosophila* reduce the survival of embryos to only 50% [69]. In mice, mutations in CCP1 cause the *Purkinje cell degeneration* phenotype that exhibits numerous defects in adults such as the degeneration of cerebellar Purkinje neurons and ataxia [71].

Not only the tubulin code ensures the proper development of the embryo, but also MAPs and other centrosome-associated proteins play an important part. Recently, a genome-wide association study of human early embryos generated from IVF cycles identified maternal PLK4 genetic variants that influence the rate of aneuploidy arising from mitotic origin [171]. Later, another study demonstrated that infertile women carrying a specific PLK4 genetic variant form fewer good quality blastocysts and have an increased risk of early recurrent miscarriages [172]. All these studies show that, in general, the cytoskeleton configuration, dynamics and the tubulin code are essential for the development of a viable embryo and a healthy organism, and they also have a big influence in the IVF success rate.

Future perspectives and applications

Most of the research done so far on the expression of tubulin isotypes and on their PTMs has been done in somatic cells and model organisms such as *Sea urchin*, *Tetrahymena thermophila* and mice. The importance of the tubulin isotypes and their

PTMs in human reproduction is far from being understood. However, recent data from animal models and in humans suggest that they have an important and direct functional role in reproduction. There is a need for additional research on human gametes and embryos, which could include single oocyte proteomics and single cell embryos qPCR. These data may be particularly relevant for the ART clinics. The detailed analysis of tubulin PTMs in human spermatozoa could provide novel molecular markers for spermatozoa selection beyond those currently in use. It could be particularly relevant to study the tubulin PTMs and isotypes expression and distribution in normozoospermic samples with fertility problems, because although these spermatozoa are classified as "normal" in terms of morphology, motility and concentration, they could be aberrant at the molecular level.

Beyond the analysis of the TUBB8 gene in oocytes, other alpha-tubulin isotypes and their expression pattern could be determined in patients with oocyte maturation problems. The tubulin isotype expression pattern and the tubulin PTMs distribution of an *in vivo* matured MII oocyte could be compared to the oocytes matured *in vitro* to examine whether external factors, associated with the maturation media may induce changes. Another interesting question is whether the tubulin isotypes and PTMs may change in aged oocytes with respect to younger ones. In aged oocytes, aneuploidy is a common alteration that can cause embryonic arrests at the pre- and post-implantation level, and spontaneous abortions. Many factors can contribute to the appearance of aneuploid oocytes, such as a higher concentration of reactive oxygen species (ROS), altered gene expression or a permissive spindle assembly checkpoint. It will be particularly relevant to address whether aging can also affect the expression and/or functionality of tubulin PTMs and isotypes apart from acetylation. The abnormal expression of tubulin PTMs and isotypes would affect the binding and processivity of

motor proteins necessary for the congression and/or separation of the chromosomes to the metaphase plate, as well as the correct assembly of the meiotic spindle. We posit that the analysis of tubulin isotypes and PTMs in human oocytes might provide answers to some of these questions.

Concluding remarks

The cytoskeleton is a key element for the proper organization of the cell, axoneme formation, intraflagellar transport, cell shape determination and cell division, among others. All these functions are in part regulated by the expression of various tubulin isotypes and by the combination of tubulin PTMs that altogether determine the tubulin code. Defects in this code in spermatozoa, oocytes and embryos in animal models and humans showed severe phenotypes**,** leading to gamete maturation problems and embryo pre- and post-implantation defects **(Table 5)**. This emphasizes the importance of the tubulin code in development. Understanding the tubulin code in the human reproductive system may provide novel tools in ART.

Authors' role

F.A. collected the information, designed the figures/tables and performed the analysis and writing of the manuscript. M.B. critically revised the manuscript and provided expert knowledge. R.V. and I.V. designed the concept and the figures/tables, provided expert knowledge and critically revised the manuscript. All authors have seen and approved the final version.

Acknowledgments

We want to thank all the members of Clínica Eugin and Vernos lab for critical discussion on the various aspects of the tubulin isotypes and PTMs in reproduction and their support in writing this review. We want to thank Désirée Garcia for her advice during the manuscript preparation.

Conflict of interest

We declare no conflict of interest

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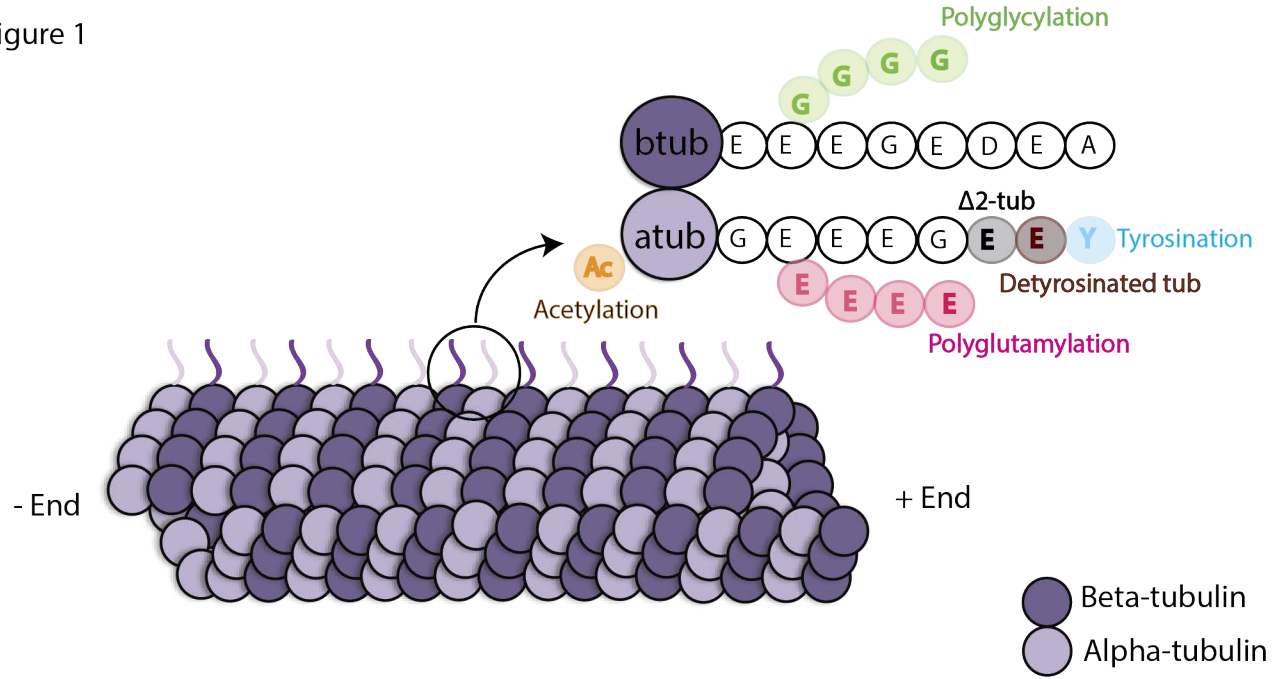
Figure 1: Microtubules and tubulin PTMs. All the described tubulin PTMs, with the exception of acetylation, occur at the C-terminal of alpha- and beta-tubulins exposed at the microtubule surface. Detyrosination and Δ2 tubulin are specific modifications of tyrosinated isotypes of alpha-tubulin. Glutamylation and glycylation occur on the C-terminal tail of both alpha and beta-tubulin. The best characterized acetylation occurs

on Lys40 of alpha-tubulin inside the lumen of the microtubule. In this figure, the C-terminal of alpha-tubulin corresponds to the TUBA1A or TUBA1B isotype, and beta-tubulin to TUBB2A or TUBB2B isotype. A: Alanine, Ac: Acetylation, D: Aspartic Acid, E: Glutamic Acid, G: Glycine, Y: Tyrosine.

Figure 2: Molecular mechanism of flagellar beating. The axoneme of the spermatozoon is formed by 9 outer doublets of microtubules (A and B) and an inner microtubule doublet. The movement of the flagella is powered through the interaction of the inner and outer dynein arms extending from the A tube of the outer doublets with the B tube of the adjacent doublet. These interactions are regulated by tubulin PTMs: polyglutamylation for the inner dynein arm and acetylation for the outer dynein arm. Ac: Acetylation, E: Glutamic Acid.

Figure 1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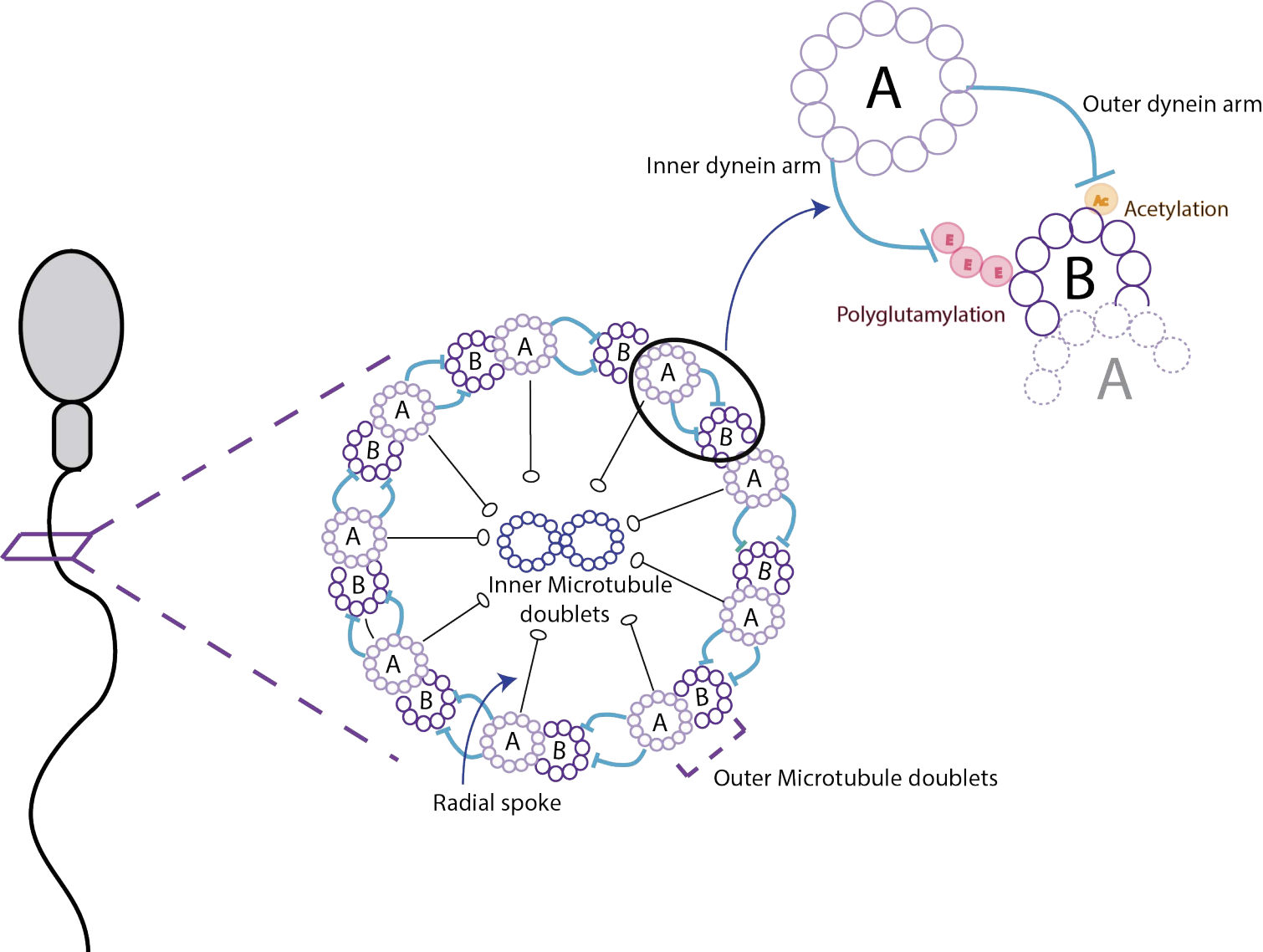
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Table 1: Microtubule associated proteins regulated by the tubulin code Tubulin PTMs and isotypes that alter the binding affinities and/or the activity of the indicated MAPs and motor proteins.

Tubulin PTM	Proteins and the type of regulation	Bibliography
Acetylation	Kinesin-1- increased microtubule binding affinity and behavior Axonemal dynein- increased microtubule binding affinity	[110, 173, 174, 175]
Polyglutamylation	Increased Kinesin-2 velocity and processivity, independently of the polyglutamylation chain length. Both alpha and beta-tubulin. Increased Kinesin-1 processivity. Only with long polyglutamylation chains. Both alpha and beta-tubulin. Axonemal dynein - increased microtubule binding affinity Tau - increased microtubule binding affinity MAP2 - increased microtubule binding affinity MAP1A/B - increased microtubule binding affinity Spastin – stimulates Spastin activity	[3, 107, 108, 109, 176]
Polyglycylation	Not characterized	
Tyrosination	kinesin-2- Inhibitory effects on processivity and velocity	[5,176,178,179]

	Kinesin-13 (MCAK)- Increased microtubule depolymerization rate Dynein-dynactin complex- Initiation Kinesin-1 – axon movement	
Detyrosinated tubulin	Kinesin-2 (anterograde intraflagellar transport)- increased processivity and velocity. MCAK and KIF2A- inhibition of microtubule depolymerization activity CENP-E/Kinesin-7- enhancement of chromosome dependent microtubule transport Kinesin-1- Increased landing rate	[139, 173, 174, 180]
Δ2-tubulin	Not characterized	
Alpha-tubulin isotypes	Not characterized	
Beta-tubulin isotypes	Kinesin-1- motor processivity reduced by TUBB1 and TUBB3	[176]

Table 2: Alpha and beta tubulin isotypes in humans. Only genes and putative genes are represented. TUBA: Alpha-tubulin, TUBB: Beta-tubulin. A: Alanine, D: Aspartic Acid, E: Glutamic Acid, F: Phenylalanine, G: Glycine, L: Leucine, K: Lysine, M: Methionine, N: Asparagine, P: Proline, Q: Glutamine, R: Arginine, S: Serine, T: Threonine, V: Valine, W: Tryptophan, Y: Tyrosine.

Genes	C-terminal aminoacid chain	Tissue expression
ALPHA-TUBULIN ISOTYPES		
TUBAL3	RDYEEVAQSF	Intestine, colon, oocyte, mucosa
TUBA1A	GEGEEEGEEY	Fetal brain, embryo, bone marrow
TUBA1B	GEGEEEGEEY	Brain, respiratory system, embryo
TUB1C	ADGEDEGEEY	Respiratory system, oocyte, embryo, spinal cord
TUBA3C	EAEAEEGEEY	Testis, sperm, oocyte, brain, respiratory system
TUBA3D	EAEAEEGEEY	Testis, platelet, oocyte, brain, liver, uterus
TUBA3E	EAEAEEGEAY	Testis, heart, placenta, oocyte
TUBA4A	SYEDEDEGEE	Brain, skeletal muscle, platelets
TUBA4B	MPALSLPTRW	Oocyte, respiratory system, oviduct
TUBA8	FEEENEGEEF	Platelet, heart, bone marrow
BETA-TUBULIN ISOTYPES		
TUBB	DFGEEAEEEA	Respiratory system, brain, embryo, placenta
TUBB1	AEMEPEDKGH	Blood, leukocytes, muscle, liver
TUBB2A	FEEEEGEDEA	Brain, liver, hair follicle
TUBB2B	FEEEEGEDEA	Brain, embryo, testis

TUBB3	EEESEAQGPK	Respiratory system, nervous system
TUBB4A	FEEEAEEEVA	Brain, testis, ovary, heart, colon
TUBB4B	FEEEAEEEVA	Respiratory system, testis, brain, oocyte
TUBB6	FEDEEEEIDG	Breast, respiratory system, muscle, placenta
TUBB8	DEEYAEEEVA	Oocyte, blood system

Table 3: The TTLL family in mammals.

TTLLs classified according to their activity and substrate specificity (alpha and/or beta tubulin, or other proteins)

Protein	Activity	Substrate	Function	Bibliography
TTL	Tyrosinase	Alpha-tubulin Depolymerized tubulin	Tyrosination	[43,54]
TTLL1	Polyglutamilase	Alpha-tubulin	Initiation and Elongation	[66]
TTLL2	Polyglutamylase	Not characterized	Not characterized	
TTLL3	Polyglycylase	Alpha- and Beta-tubulin	Initiation	[56,69]
TTLL4	Polyglutamylase	Alpha- and Beta-tubulin	Initiation	[68]
TTLL5	Polyglutamylase	Alpha-tubulin	Initiation	[68]
TTLL6	Polyglutamylase	Alpha-tubulin	Elongation	[68]
TTLL7	Polyglutamylase	Beta-tubulin Polymerized tubulin	Initiation and Elongation	[68,181,182]
TTLL8	Polyglycylase	Alpha- and Beta-tubulin Other proteins	Initiation	[69]
TTLL9	Polyglutamylase	Alpha-tubulin	Elongation	[183]
TTLL10	Polyglycylase	Alpha- and Beta-tubulin	Elongation	[69,184,185]

		MAPs		
TTLL11	Polyglutamylase	Alpha-tubulin	Elongation	[68]
TTLL12	Not characterized	Not characterized	Not characterized	
TTLL13	Not characterized	Not characterized	Not characterized	

Table 4: The CCP family in mammals. CCPs classified according to their activity and substrate specificity (alpha and/or beta-tubulin, or other proteins).

Protein	Activity	Substrate	Function	Bibliography
CCP1	Deglutamylase	Alpha- and Beta-tubulin Myosin light chain kinase 1	Removal of glutamates from lateral chains and the branching one (TTLL6 dependent)	[74,186,187,188]
CCP2	Deglutamylase	Alpha and Beta tubulin	Removal of glutamates from lateral chains	[105]
CCP3	Deglutamylase Deaspartylase	Alpha and Beta tubulin	Removal of glutamates from lateral chains. Removal of aspartates.	[105]
CCP4	Deglutamylase	Alpha and Beta tubulin. Myosin light chain kinase 1	Removal of glutamates from lateral chains	[74,187]
CCP5	Deglutamylase	Alpha and Beta tubulin.	Removal of the branching glutamates.	[188,189]
CCP6	Deglutamylase	Alpha and Beta tubulin. Myosin light chain kinase 1	Removal of glutamates from lateral chains and the branching one.	[74,187]