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The tubulin code in mammalian sperm development and function

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Abstract

Microtubules are cytoskeletal elements that play key roles throughout the different steps of sperm development. As an integral part of the sperm flagellum, the molecular machine that generates sperm motility, microtubules are also essential for the progressive swimming of sperm to the oocyte, which is prerequisite for fertilisation. Given the central role of microtubules in all steps of spermatogenesis, their functions need to be tightly controlled. Recent work has showcased tubulin posttranslational modifications as key players in sperm development and function, with aberrations often leading to male infertility with a broad spectrum of sperm defects. Posttranslational modifications are part of the tubulin code, a mechanism that can control microtubule functions by modulating the properties of their molecular building blocks, the tubulin proteins. Here we review the current knowledge on the implications of the tubulin code in sperm development and functions and its importance for male fertility.

Keywords: Microtubules, tubulin code, tubulin isotypes, tubulin posttranslational modifications, spermatogenesis, sperm, fertility

1. Introduction - microtubules and the tubulin code

Microtubules (MTs) are the largest filaments of the eukaryotic cytoskeleton. They are essential for a wide variety of basic biological processes, such as regulation of cell shape, division, motility and differentiation. For many of these functions MTs form distinct structures like the mitotic spindle during cell division, or axonemes, the core structures of cilia and flagella. MTs adapt to distinct functions by interacting with a variety of different microtubule-associated proteins (MAPs) [1], as well as modulating their inherent dynamic properties [2]. Another key component that is emerging as a central regulator of MT functions is the ‘tubulin code’ (Fig. 1A), a concept that proposes that MT properties and functions are modulated by the incorporation of alternative tubulin gene products, known as isotypes, as well as by a variety of posttranslational modifications (PTMs) [3, 4].

The human genome encodes nine α - and nine β -tubulin isotypes. The current nomenclature of these genes can be found at: <https://www.genenames.org/data/genegroup/#!/group/778>. Reviewing the past literature on tubulin isotypes proves highly challenging, as several alternative nomenclatures have been used. In the current review, we translated the nomenclature used in previous papers to adapt it to the currently used names. Tubulin isotypes have highly similar amino acid sequences and can therefore freely intermingle to form mosaic MTs [5]. This early finding had initially mitigated the interest in tubulin diversity as a regulator of MT functions, however recent advances in recombinant tubulin production [6, 7] allowed to re-visit the functions of tubulin isotypes, showing that they can indeed affect intrinsic MT properties such as MT dynamics [8], as well as the interactions of MTs with molecular motors [9]. Moreover, advances in genomics provide a constantly growing number of mutations in tubulin isotypes that lead to specific pathologies [10, 11]. These findings underpin that tubulin isotypes fulfil unique roles in the MT cytoskeleton, which in most cases still remain to be uncovered.

The research on tubulin PTMs has seen strong advances in the recent years, especially following the discovery of the enzymes catalysing tubulin acetylation [12-15], tyrosination [16] and detyrosination [17, 18], generation of $\Delta 2$ -tubulin [19], (poly)glutamylation [19-21] and (poly)glycylation [22, 23] (Fig. 1B). Current advances show that tubulin acetylation at lysine (K) 40 of α -tubulin changes the flexural rigidity of MTs [24, 25], which makes MTs more resistant to mechanical stress by mechanical bending. Detyrosination, generation of $\Delta 2$ -tubulin, (poly)glutamylation and (poly)glycylation, by contrast, are PTMs that modulate the C-terminal tails of tubulins. These tails are exposed to the outer surface of MTs and thus predominantly regulate interactions between MTs and associated proteins [3].

Among all MT structures studied so far, ciliary and flagellar axonemes are highly enriched in all the above-listed PTMs. However, apart from forming this essential core structure of the sperm flagellum, MTs play other crucial roles throughout the entire process of sperm development in the testis. Here we review the current knowledge of the implications of the tubulin code in sperm development and function, which to date is best-studied in mouse models. Given that sperm are one of the most divergent cell types in mammals [26], it often remains to be determined whether these roles are unique to sperm cells, or are more broadly found in other mammalian cells. As the process of spermatogenesis can strongly vary between different mammalian species, we focus here mostly on rodent models.

2. Spermatogenesis – the first step in sperm development

Spermatogenesis is a tightly controlled cellular process that takes place in the seminiferous tubules of the testis (Fig. 2A). In mice, spermatogenesis comprises three main stages: (i) spermatogonial proliferation, which is the mitotic division of germ cells (spermatogonia); (ii) meiosis of spermatocytes and (iii) differentiation of the haploid spermatids into mature sperm

(Fig. 2B). The journey from spermatogonia to spermatids starts with multiple cascades of mitosis of so-called A-spermatogonia [27], followed by differentiation into intermediate, or B-spermatogonia, which lose contact with the basement membrane and undergo one more mitotic division to form the tetraploid primary spermatocytes. Spermatocytes then divide meiotically, first forming diploid secondary spermatocytes in meiosis I, which by the subsequent meiosis II form haploid spermatids [28]. All the differentiation steps from spermatogonia to spermatids are supported by epithelial cells called Sertoli cells, which not only provide the physical support, but also the necessary nutrients to sustain germ cell development (Fig. 2B).

The development of germ cells to spermatids requires the formation of bipolar MT spindles, as well as the midbody, two structures essential for cell division. While the centrosomes act as the MT-organising centres (MTOCs) in both mitotic and meiotic cell division, the spindle orientation in the two classes of dividing cells is different. Dividing spermatogonia undergo an ‘oriented cell division’ in which spindles are oriented mostly perpendicular to the basement membrane of the germinal epithelium [29]. This orientation is driven by forces from astral MTs, the motor protein dynein as well as components of the cell cortex. It ensures that one germ cell type (A-spermatogonia) is always in contact with the basement membrane to ensure self-renewal of the progenitor cells, while the other (B-spermatogonia) continues the cycle of division [28] (Fig. 2B). Cells undergoing meiotic division, by contrast, are arranged closer to the lumen of the seminiferous tubules and their centrosomes lack any particular direction of orientation [29]. This suggests that spermatogenesis requires signals from the basement membrane for proper orientation of their MTOCs to generate two different types of cells. By contrast, the formation of mature sperm cells, spermiogenesis, may not require such cues as the underlying cell divisions generate cells of the same type. During the entire process of spermatogenesis, MTs of the supporting Sertoli cells also play a crucial role. These MTs extend

along the entire radial length of the cell and are extremely dynamic, which is essential for the cells to respond to rapid changes in the developing germ cells (reviewed in [30]).

So far, little is known about the role of the tubulin code in early stages of sperm development. Most information found in the literature is restricted to analyses of the presence of specific tubulin isoforms and PTMs. For instance, early work has found that mammalian testicular cells express a specific subset of tubulin isoforms. Using antibodies raised against tubulin isoform-specific motifs [31, 32] it was shown that germ cells express mainly α -tubulins of the TUBA3 clade, as well as the TUBB4B β -tubulin isoform [33]. By contrast, Sertoli cells specifically express the β -tubulin isoform TUBB3 [34], which is otherwise known as a neuron-specific isoform. So far nothing is known about the functional importance of this cell-type specific isoform expression.

Tubulin glutamylation is a key PTM in sperm, however, it is mostly found at low levels during spermatogenesis. Germ-cell MTs are almost devoid of glutamylation, with only the mitotic midbody and centriolar MTs being glutamylated. In Sertoli cells, only apical MTs, i.e. MTs pointing with their minus ends towards the apical surface of the cells, are faintly glutamylated [35, 36]. By contrast, mice lacking the deglutamylase CCP5 (*Ccp5*-KO) are male sterile [37], and present a high degree of glutamylation even in the germ cells. *Ccp5*-KO mice develop defects during spermatogenesis, which further amplify as the spermatids mature [38]. It is thus likely that tight control of polyglutamylation in spermatogenesis is essential for correct MT functions.

3. Spermiogenesis – shape-shifting of germ cells to form spermatozoa

Mature sperm are formed by a process called spermiogenesis. It starts with a haploid spermatid that undergoes progressive remodelling of the cellular and cytoskeletal architecture to form the

final motile sperm. An early step in spermiogenesis is the biogenesis of the acrosome [30], an apical nuclear cap containing enzymes necessary for the sperm to alter the structure of the zona pellucida of the oocyte during fertilisation [39]. Acrosome biogenesis happens in four phases in mammals (Fig. 2C; reviewed in [40]), and in each of them MTs play important roles. In the Golgi phase, MTs orient the *trans*-Golgi network in the direction of the nucleus [41] and aid in transporting vesicles from the Golgi complex to the nucleus. Disruption of MTs either by drug-mediated depolymerisation, or due to mutations in the MT-interacting protein, Hook1 in the *azh/azh* (abnormal spermatozoan head shape) mouse model can lead to acrosome biogenesis defects [42] as they affect the Golgi-nucleus vesicle transport.

The Golgi phase is followed by the cap phase towards the end of which the nucleus is polarised to one side of the spermatid. The morphological change involved alters the overall shape of the cell from a round to an elongating spermatid, thereby initiating the acrosome phase that is accompanied by nuclear compaction [43], which is coupled to the reshaping of the nucleus and facilitated by a MT-based structure, the manchette (Fig. 2C).

The manchette is a transitional structure formed upon nuclear elongation, and disassembling when nuclear compaction and acrosome biogenesis are complete. It is composed of up to 1000 single MTs that are linked by lateral interactions, thus forming a “skirt-like” structure around the nucleus (Fig. 3A,B). The manchette is nucleated acentrosomally and appears to emerge from a perinuclear ring that is formed adjacent to the elongating nucleus [44]. However, since the perinuclear ring lacks γ -tubulin, the “generic” MT nucleator [45], it is still unclear how manchette MTs are nucleated. Current hypotheses propose either the existence of alternate MT-nucleating proteins [46] in the perinuclear ring, or that MTs are nucleated elsewhere in the spermatid cytoplasm and the perinuclear ring captures it [47]. Irrespective of how manchette MTs are nucleated, it is established that this nucleation always takes place close to the nuclear membrane, and that it is critical for shaping the sperm head. This was established with the

azh/azh mutant mouse, which displays abnormalities in manchette development that result in sperm with head shape defects [48]. For the manchette to shape the head efficiently, MTs have to act in coordination, which is possibly achieved through the linker structures between manchette MTs [49]. These inter-MT linkers (Fig. 3B) connect MTs to the nuclear envelope and shape the nucleus, and thus the sperm head, by moving from the tip of the acrosome downwards in a zipper-like fashion [50]. This movement is driven by cytoplasmic dynein, a motor protein that can interact with both, the MTs and the nuclear envelope [51] (Fig. 3B).

Similar to the cytoplasmic MT of the germ cells, manchette MTs are mainly composed of the TUBA3 and TUBB4B isotypes [33, 52]. One study identified an additional, more divergent α -tubulin isotype, pRD α TT1 in manchette MTs [53], which surprisingly has never been further characterised.

Tubulin PTMs in round and elongating spermatids are similar to spermatocytes, with MT acetylation observed predominantly near the developing acrosome [42]. As the spermatids elongate, tubulin PTMs appear to be confined to manchette MTs. So far, functional data on the role of PTMs of manchette MTs are restricted to polyglutamylation. Lack of the deglutamylase CCP5 in *Ccp5*-KO mice led to an abnormal increase of polyglutamylation, which resulted in structural defects of the sperm manchette (Fig. 3C). This normally highly ordered MT array was disorganized, and acrosomes were detached from nuclei that were, by contrast, condensing normally [38]. While the underlying molecular mechanisms of the manchette defects are not yet uncovered, it is likely that MAPs and motor proteins that play essential roles in the formation of this highly ordered MT array [47] are controlled by polyglutamylation.

4. The sperm flagellum – the molecular machine that moves the sperm

4.1 Assembly of the sperm flagellum

The MT structure essential for sperm motility is the axoneme of the flagellum. Structurally, the sperm flagellum is divided into four distinct segments: the connecting piece (neck), middle piece, principal piece and the end piece (Fig. 4A). Assembly of the axoneme begins after meiosis, at the round-spermatid stage (Fig. 2C). First, the distal centriole docks to the nuclear membrane at the pole opposite to the acrosome with the help of the distal and sub-distal appendages, which initiates the formation of the connecting piece. Next, the formation of the core flagellar structure is initiated, and flagellar assembly continues throughout the elongation phase of spermiogenesis. During this period, other accessory features of sperm flagella like the outer dense fibres (ODFs), mitochondrial sheath and the fibrous sheath (FS) are added onto the axonemes (Fig. 4A; reviewed in [30, 54, 55]). This assembly of the sperm flagellum depends on two key transport pathways – intraflagellar transport (IFT) and intra-manchette transport (IMT) (Fig. 3B).

IFT is an evolutionarily conserved process that is not restricted to development of sperm flagella, but found in all types of cilia and flagella. The IFT transport complexes, also called IFT trains, transport the tubulin, structural proteins and the outer and inner dynein arm motor proteins as cargoes into the flagellum. IFT is powered by kinesins and dyneins that walk on the axonemal MTs [56-58] (Fig. 3B). IMT, on the other hand, is a transport process specific to the sperm, as it takes place on the manchette MTs, however it shares several molecular components with IFT. IFT is functional as soon as axoneme biogenesis begins in the round spermatids [30, 59]. IMT, by contrast, begins only once nuclear compaction in elongating spermatids begins. This timing coincides with the assembly of the flagellar components - the central pair complex and accessory axonemal structures like the ODFs and FS – suggesting that IMT cooperates with IFT to complete sperm tail formation [30, 47, 60-62]. While IFT is entirely MT-based, it

was suggested that the IMT involves both actin- and MT-based transport with actin motors specifically involved in short-distance transport [30].

4.2 Sperm maturation in the epididymis

Once flagella are assembled and nuclei compacted, spermatozoa are fully differentiated and thus structurally mature. However, they are not immediately functionally competent, as their flagella are still immotile. The activation of sperm flagella requires two extra-testicular maturation steps. The first step takes place while sperm migrate from the testis to the epididymis, an organ that stores mature sperm (Fig. 2A). The second maturation step takes place in the female reproductive tract.

While traveling through the epididymis, sperm undergoes several molecular changes in protein composition [63] and PTMs [64-66] that stabilise both nuclear and flagellar proteins. At the same time, sperm also undergoes activation of different signalling cascades, mainly regulated by cyclic AMP and several protein kinases and phosphatases. These biochemical and physiological changes are prerequisites for the acquisition of progressive motility and sperm capacitation, the last maturation steps that occur within the female reproductive tract to make sperm competent for fertilisation [67]. So far, no implication of the tubulin code in sperm maturation has been uncovered.

4.3 Control of flagellar motility

Motility of mammalian sperm is controlled by the axoneme that generates the sperm beat, as well as by accessory structures like the ODFs, mitochondrial sheath and the FS (Fig. 4A). These structures stabilise the overall flagellar beat [68] and in viscous fluids like within the female tract, they reduce the buckling instabilities of the flagella, thus preventing circular

swimming of sperm [69]. Irrespective of the supporting roles of accessory components, the three-dimensional flagellar beat is mainly generated by the coordinated activity of thousands of axonemal dynein motor proteins [70]. These dyneins work in coordination with their interactors the nexin-dynein regulatory complex (N-DRC), radial spokes, and the central-pair complex (Fig. 4A; reviewed in [71, 72]). While the dyneins provide the motor function, the radial spokes mechanically couple central-pair MTs and dyneins through the N-DRC. Moreover, the radial spokes also provide structural stability to the axonemes [73]. Axonemes contain two classes of dyneins. The outer dynein arms (ODAs) are thought to control the beat amplitude and frequency, while inner dynein arms (IDAs) the beat waveform [74]. Axonemal dyneins are bound to the A-tubules of the axonemal doublet MTs through their tails. To generate a flagellar bend, they “walk” towards the minus end of the neighbouring B-tubule [75] (Fig. 4A). This movement generates the force to slide doublets within the axoneme, resulting in the flagellar bend. To generate the flagellar beat, axonemal dyneins switch regularly and in a coordinated manner between active and inactive states [76, 77]. Indeed, structural analyses of dynein conformations during active flagellar beat in echinoderm sperm and *Chlamydomonas* flagella revealed that the dyneins exist either in a conformationally active (pre-powerstroke), or inactive (post-powerstroke) state [70] (Fig. 4A). The recent discovery of similar dynein conformations in mouse sperm flagella [78] underpinned the evolutionary conservation of this mechanism in generating flagellar beat. Apart from the sliding forces, axonemal dyneins also generate torque forces that induce an axonemal twist, which results in chiral flagellar beat patterns [79]. As a consequence of these different forces, sperm swim in a helical or a twisted ribbon path that allows progressive swimming [80, 81], which has been observed in many mammalian species. A comprehensive discussion of the biophysical aspects that underlie the regulated flagellar bending by axonemal components can be found in a recent review [81].

4.4 Functional roles of tubulin PTMs in sperm flagella

Given that sperm flagellum is highly enriched with a variety of tubulin PTMs, it is not surprising that many of the modifying enzymes (Fig. 1B) play important roles, and their mutation or loss leads to a panoply of sperm defects that affect both assembly and function of sperm flagella.

The broadest spectrum of functional implications has been found for polyglutamylation. Knockout mouse models for glutamylating enzymes from the TTL family [20, 21], or deglutamylases from the CCP family [19] show a variety of characteristic and unique phenotypes. TTL1, TTL5 and TTL9 are important for axoneme architecture. Knockout of either TTL1 or the polyglutamylase subunit 1 (PGS1), which are both part of the same multi-protein complex [20, 82] leads to massive defects in axoneme assembly reaching from missing MT doublets, absence of central pair MTs, or complete axoneme disorganization (Fig. 4B). Even if spermatids had formed an axoneme, flagella were much shorter with a thickened midpiece [83-85]. As a consequence, *Ttll1*-KO and *PGS1*-KO mice are male sterile. Similar defects have been observed in a syndrome known as Multiple Morphological Abnormalities of the Flagellum (MMAF), which is caused by mutations in structural proteins of the axonemes [86]. The discovery of these defects in *Ttll1*-KO and *PGS1*-KO suggest that MMAF can also be caused by perturbations of tubulin PTMs. Strikingly, the massive architectural defects appear to be specific to sperm axonemes, as the ultrastructure of other motile cilia in *Ttll1*-KO mice showed the normal 9+2 organisation. However, motile cilia in the trachea and the nasal cavity of *Ttll1*-KO mice are not fully functional as mice frequently cough and emit sneezing-like sounds indicating defective mucus elimination [85].

Mice lacking the glutamylase TTL5 (*Ttll5*-KO) are subfertile with sperm displaying structural defects such as detached heads and coiled flagella. *Ttll5*-KO sperm shows reduced progressive motility, which is likely to be the cause of subfertility of these mice [87]. Human patients with

mutations in *TLL5* are either male infertile, or subfertile [88]. Axonemes of sperm flagella from *Tll5*-KO mice show a specific structural defect: the absence of doublet n°4 (Fig. 4B). Strikingly, a distinct, but similarly specific defect was found for mice lacking the polyglutamylase TLL9. *Tll9*-KO mice are sterile with reduced sperm count and flagella that are frequently stalled. Although the gross morphology of the flagella was normal, ultrastructural analysis revealed a shortened MT doublet n°7, with the consequence that this doublet is absent towards the distal part of the principal piece (Fig. 4B) [89]. So far, no reports are available on the role of other glutamylases from the TLL family.

Out of the six deglutamylases from the CCP family [19, 90], two knockout mouse models show male infertility, *Ccp1*-KO [91] and *Ccp5*-KO [37]. While loss of CCP5 causes massive perturbations in sperm development that mostly cause total absence of flagella [38] (Fig. 3C), *Ccp1*-KO mice develop sperm with flagella, which are, however, structurally abnormal and often immotile [91, 92]. Sperm in these mice have a bulbous head, coiled flagella and disorganized ODFs (Fig. 4B). Though the sperm acquire most of these defects within the testes, the coiling of the tail was observed during maturation within the epididymis [91].

In contrast to polyglutamylation, which despite its enrichment on axonemal MTs is also found on a variety of other MTs, polyglycylation is so far almost exclusively observed in cilia and flagella [3, 93]. In mammals, glycylation is catalysed by two initiating enzymes, TLL3 and TLL8, and one enzyme that elongates glycine chains, TLL10. Because polyglycylation of tubulin by TLL10 requires prior glycylation by either TLL3 or TLL8 [22], it can be entirely abolished by inactivating those two enzymes, as recently confirmed in the *Tll3/Tll8*-double-KO mouse. *Tll3/Tll8*-double-KO mice are subfertile but displayed no defects in overall sperm development or maturation. Nevertheless, *Tll3/Tll8*-double-KO sperm showed asymmetric flagellar beat with increased flagellar curvature, which resulted in reduced progressive motility. Most strikingly, sperm lacking glycylation switched from straight to

circular swimming patterns, which is not observed for wild-type sperm [78] (Fig. 4B). The increased flagellar curvature results in flagella bending into one preferred direction, thus altering the overall axonemal twist that is suggested to control the flagellar beat, and could consequently lead to the observed abnormal swim paths [79]. Ultrastructural analyses of *Ttll3/Ttll8*-double-KO sperm revealed no gross architectural defects of the axoneme, but conformational aberrations of the flagellar dyneins (ODAs and IDAs) that were more frequently in their inactive post-powerstroke states, or in mixed conformations that are rarely found in the wild type (Fig. 4B) [78]. Glycylation thus appears to specifically control, or fine-tune, the conformational states of axonemal dyneins, thus coordinating their power strokes and thereby the flagellar beat.

An open question is the role of polyglycylation catalysed by TTLL10. In humans, TTLL10 is expressed but enzymatically inactive, and sperm flagella are, in contrast to all other mammalian species studied so far, not polyglycylation [22, 94]. Why an evolutionary conserved PTM could be abolished without major physiological consequences has remained a conundrum. A possible answer might lay in the observation that glycylation and glutamylation compete for the same modification sites on tubulin [23, 95], a function that does not require the elongation of glycine chains by TTLL10.

Another PTM, acetylation, is abolished by knockout of the unique acetyltransferase ATAT1. While the axonemal structure of *Atat1*-KO sperm flagella appears normal, the overall flagellar length and the numbers of sperm with progressive motility are both reduced, and mice are subfertile [96]. So far, there are no insights into the role of tubulin detyrosination in sperm flagella. This is most likely related to the fact that mice lacking the unique enzyme catalysing tubulin tyrosination, TTL [16], die postnatally [97]. On the other hand, mouse models for the recently discovered detyrosinating enzymes from the vasohibin (VASH) family [17, 18] are not yet available. VASH proteins interact with a short protein called SVBP that is important

for their enzymatic activity [18]. A first analysis of a *Svbp*-KO mouse revealed that mice are fertile, however, no detailed analyses of sperm flagella or functions have so far been performed [98].

4.5 Roles of tubulin isotypes in sperm flagella

Owing to the high degree of sequence identity among α - and β -tubulins, it is difficult to attribute specific roles to isotypes found in axonemal MTs. Studies in *Drosophila* established that a testis-specific β -tubulin (*TubB85D*) is essential for spermatogenesis, as recessive mutations in this gene led to defects in all MT-dependent processes starting from meiosis of the spermatogonia [99]. Mutations in *TubB85D* also resulted in a range of axonemal defects, including loss of central-pair MTs, or reduced numbers of doublet MTs [100]. Studies in *Drosophila* further established that the presence of a glycine residue at position 56 in β -tubulins is essential for the attachment of ODAs to the A-tubule, and thus for flagellar motility [101]. While the evolutionary divergence between flies and mammals prevents the identification of orthologs for tubulin isotypes, work in *Drosophila* revealed the presence of the C-terminal motif -EGEFEEE- that is characteristic for axonemal β -tubulin isotypes [102]. This motif is also found in mammalian TUBB4B, which has previously been identified as the main axonemal β -tubulin isotype expressed in the germ cell lineage [33]. Subsequent studies in bovine respiratory multiciliated cells showed that this motif is pivotal for the localization of the β -tubulin into cilia, as well as for ciliary beating [103]. Moreover, glycine 56 is present in all mammalian β -tubulin isotypes, except for TUBB3 and TUBB6, which are not expressed in the germ cell lineage [33].

5. Mechanisms controlled by the tubulin code

So-far very little is known about molecular mechanisms underlying the observed physiological roles of the tubulin code, and in particular tubulin PTMs, in sperm formation and function. It is conceivable that most of these defects can be linked to molecular mechanisms that have been discovered in other experimental systems, such as the regulation of MT interactions with motor proteins, severing enzymes and several other MAPs by tubulin PTMs (reviewed in [3]).

In vitro studies have shown that two PTMs, detyrosination and polyglutamylation, control the motility and processivity of the kinesin motors [9]. Given that kinesins play a key role in IMT and IFT, this might explain at least in part the manchette defects and the massive perturbation of axonemal assembly observed in the *Ccp5*-KO mice in which polyglutamylation is strongly increased in early stages of sperm development [38]. In other mouse models with perturbed polyglutamylation, axonemes are assembled but show structural and functional defects. The structural defects could be related to dysfunctions of the IFT. Indeed, IFT is a highly controlled process, which in *Chlamydomonas* has been shown to use the B-tubule for anterograde and the A-tubulin for retrograde transport [104]. Moreover, previous work in *Chlamydomonas* has shown that B-tubules are enriched in polyglutamylation and detyrosination, while A-tubules lack these PTMs, and are thus tyrosinated [105, 106]. Together with *in-vitro* work showing that some kinesins show a preference for polyglutamylated MTs [9], while dynein is insensitive to this PTM, but requires tyrosinated MTs for efficient initiation of its motility [107], this suggest that the observed path preferences of IFT trains [104] could be controlled by these two PTMs. While the validity of these findings in *Chlamydomonas* flagella remain to be confirmed in other organisms, and in particular in sperm flagella, a possible result of the deregulated axonemal PTMs is likely to cause a perturbation of IFT, with consequences on flagellar assembly and integrity.

The enrichment of polyglutamylated tubulin at the B-tubules of axonemal MT doublets has also implications on axonemal motility. B-tubules are the interaction sites of the axonemal dynein heads, and their PTMs could thus directly influence the activity of these motors, thereby regulating the flagellar beat. Indeed, while polyglutamylated tubulin appears to have no impact on cytoplasmic dynein [9, 107], it was shown to control flagellar beating in unicellular organisms [108, 109]. It is thus likely that defects in flagellar beat of mouse models lacking enzymes that catalyse tubulin glutamylation are caused by a perturbed interaction of axonemal dyneins with the B-tubules. Strikingly, in a mouse model lacking tubulin glycylation, axonemal dyneins are disordered and at the same time, polyglutamylated tubulin of axonemal MTs is increased [78]. It is conceivable that additionally to the lack of glycylation, accumulation of polyglutamylated tubulin could cause the observed uncoordinated interactions of the dyneins with the B-tubule of the opposing MT doublets.

The impact of acetylation on the sperm flagellum could be explained by the role of this PTM in making MTs resistant to repeated mechanical stress by bending [24, 25], which is very prevalent in the beating flagellum. Moreover, given that the site of acetylation, K40, is located at the inside of MTs, it is possible that acetylation also affects the arrangement of microtubule inner proteins (MIPs), which are present in the lumen of axonemal doublet MTs [26].

Overall, mutations or knockout of single enzymes catalysing tubulin PTMs lead to distinct flagellar phenotypes. This could either be related to unique, yet to be determined, roles these enzymes play in the assembly and functioning of sperm axonemes. Additionally, it is likely that glutamylases and glycylation enzymes from the TLL family achieve control of specific MT functions by generating distinct PTM patterns on the axonemal MTs [21, 22], which could, as recently demonstrated in neurons [110], control distinct molecular functions, such as IFT, stability of axonemal MTs, or the function of axonemal dyneins and thus flagellar motility.

Conclusions

Sperm flagella are perhaps the most complex MT-based molecular machines in mammals. Their essential role in sexual reproduction requires a reliable, highly controlled flagellar beat to assure efficient swimming, which is essential to reach the oocyte in the female reproductive tract. Additionally, all steps of sperm development depend on MT functions. It is thus not surprising that regulatory mechanisms of MT functions are particularly essential for sperm. Here we reviewed the current knowledge of the implications of the tubulin code in sperm functions. So far, little is known about the functions of single tubulin isotypes, while the generation of knockout mouse models for different tubulin-modifying enzymes has revealed the essential functions of many of them – and consequently of the PTMs they catalyse – in sperm development and in particular in flagellar assembly and function.

Emerging studies linked not just male fertility disorder, but also other ciliary and non-ciliary human pathologies to defects in tubulin PTMs (reviewed in [111]), however in many cases it is unclear how these pathologies develop. To uncover how changes in tubulin PTMs translate into the observed defects, sperm cells are a great model system as almost every mouse model has so far shown defects either in spermatogenesis or sperm function. The tight link between the tubulin code, and in particular tubulin PTMs, and male fertility that has been discovered in mouse models qualifies tubulin-modifying enzymes as potential risk factors for male sterility in humans that should be exploited for novel diagnostic and treatment options in the near future.

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Author contributions

SG and CJ wrote the manuscript. TH prepared illustrations in discussion with SG and CJ.

Competing interests

The authors declare no competing interests

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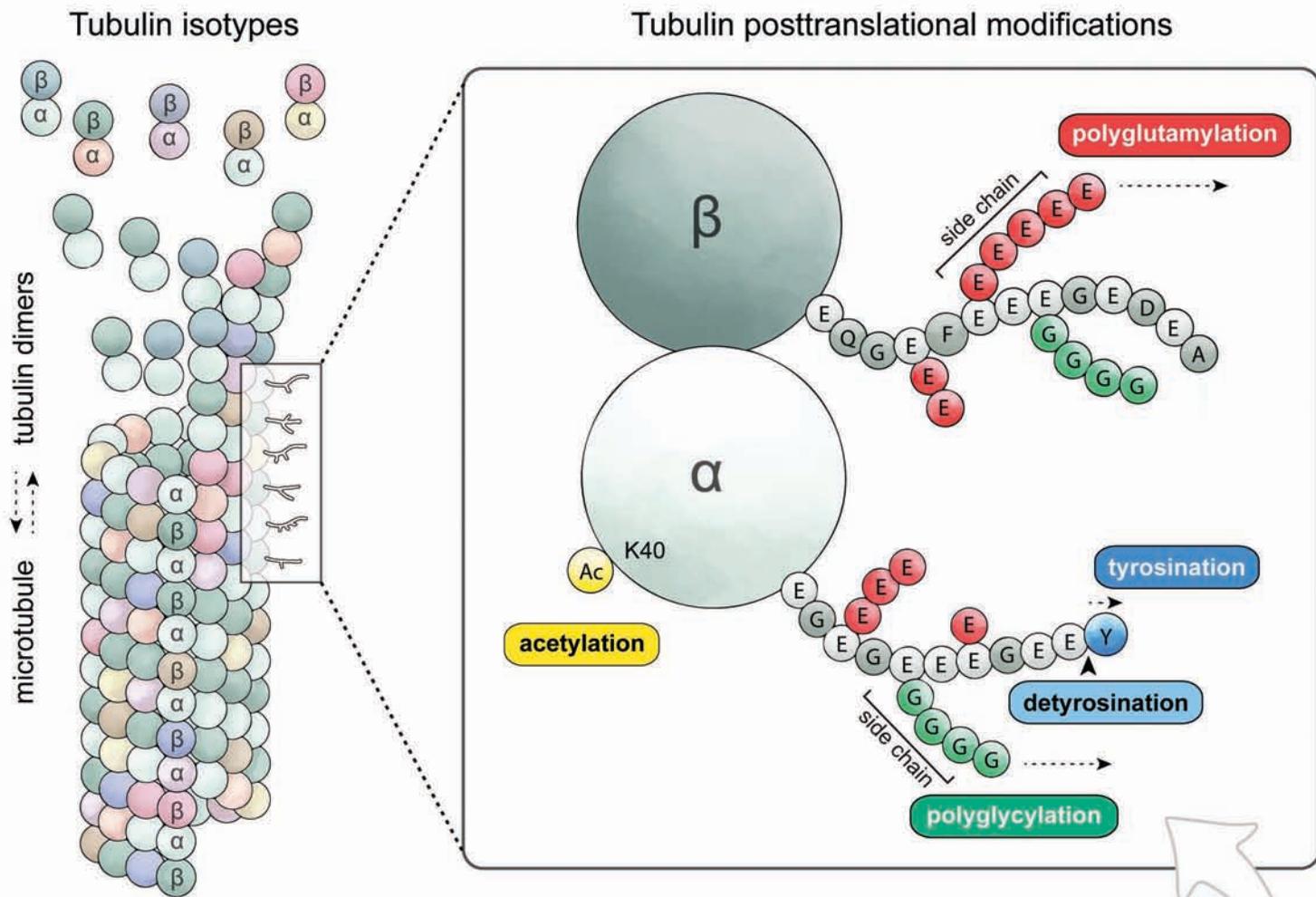
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A The tubulin code



B Enzymes catalysing tubulin posttranslational modifications

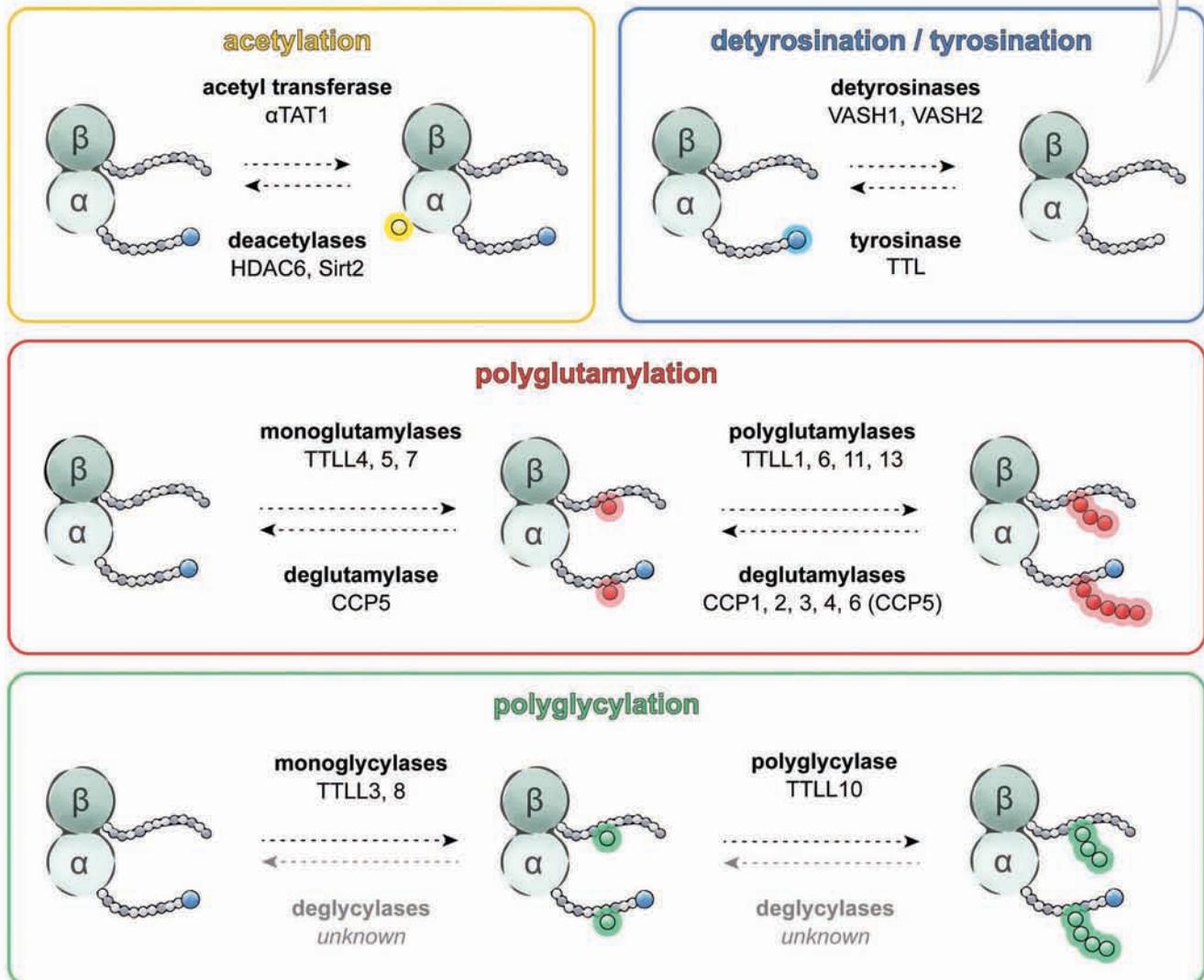


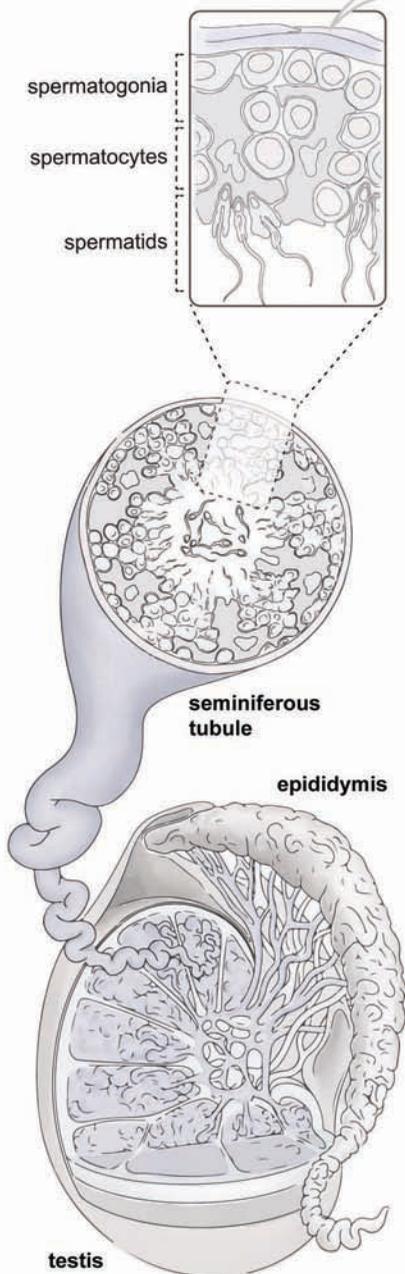
Figure legends

Figure 1: The tubulin code

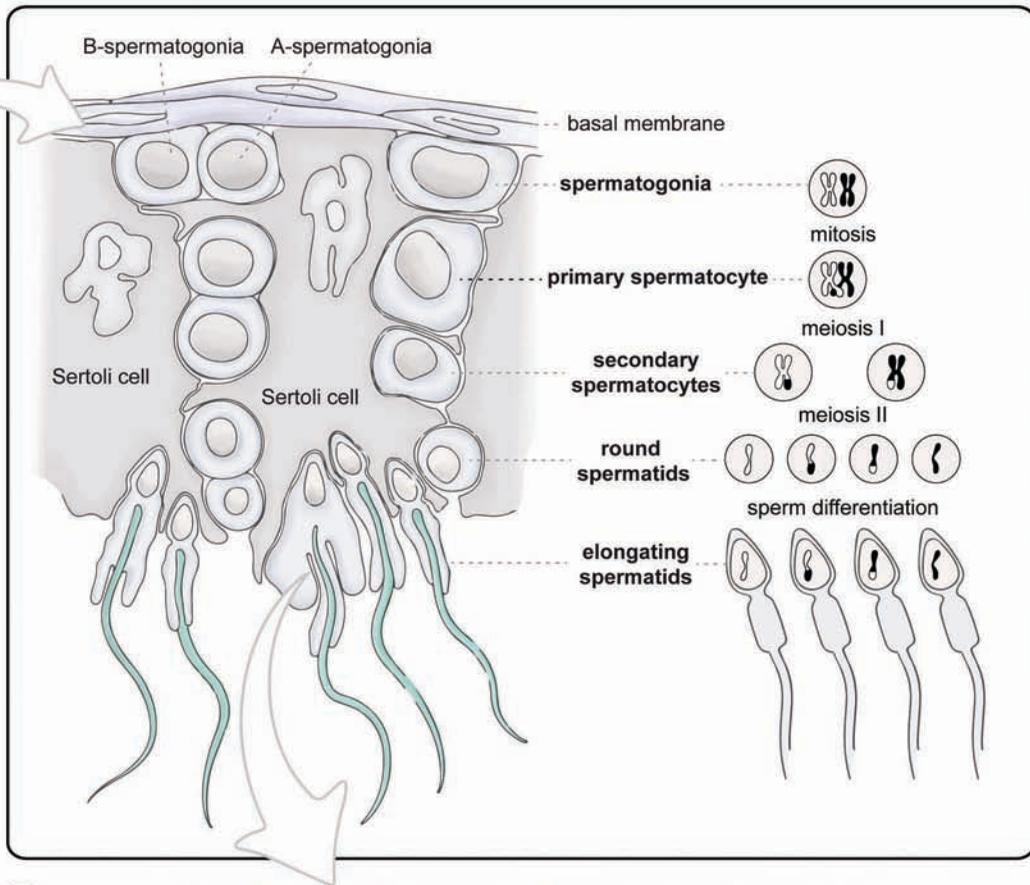
A) In mammals, both α - and β -tubulin are expressed from 9 different genes each, giving rise to isotypes with subtle differences in their amino acid sequences. Isotypes intermingle when assembling into MTs. Tubulin and assembled MTs undergo a series of posttranslational modifications (PTMs), many of which are enriched within the C-terminal tails of α - and β -tubulins.

B) Enzymes catalysing tubulin PTMs shown in (A). Acetylation of α -tubulin at lysine (K) 40 is catalysed by the α -tubulin N-acetyltransferase (α TAT1) [14, 15], and removed by the histone deacetylase 6 (HDAC6 [12]), or Sirtuin 2 (Sirt2 [13]). Most α -tubulin isotypes contain a gene-encoded C-terminal tyrosine, which is enzymatically removed by detyrosinases of the vasohibin family (VASH [17, 18]). Detyrosinated tubulin is enzymatically re-tyrosinated by tubulin tyrosine ligase (TTL [16]). Polyglutamylation and polyglycylation are catalysed by members of the tubulin-tyrosine ligase like (TTLL) enzyme family, which add glutamate or glycine residues to specific glutamate residues within the C-terminal tails of both, α - and β -tubulin [20-23]. Deglutamylation is catalysed by enzymes from the cytosolic carboxypeptidase (CCP) family [19, 90], while deglycylation has so far not been identified.

A The male reproductive system



B The process of spermatogenesis



C Morphological changes during spermiogenesis

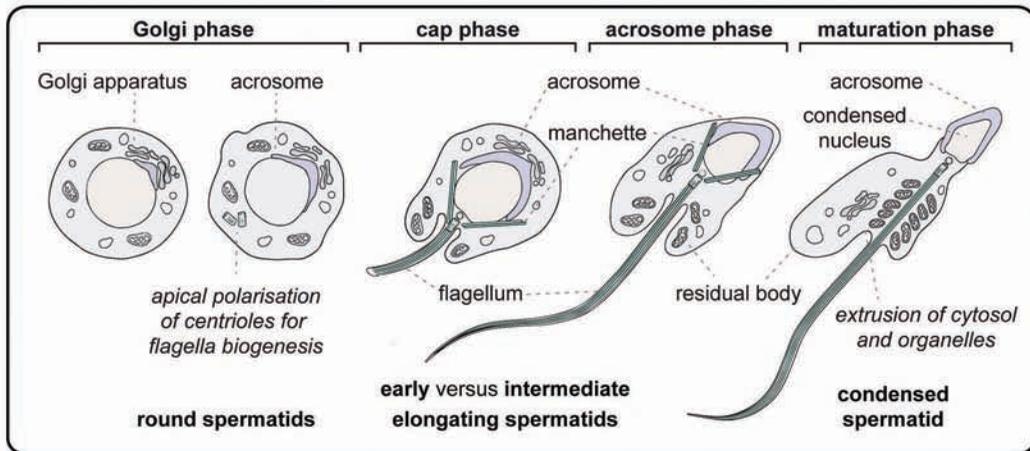


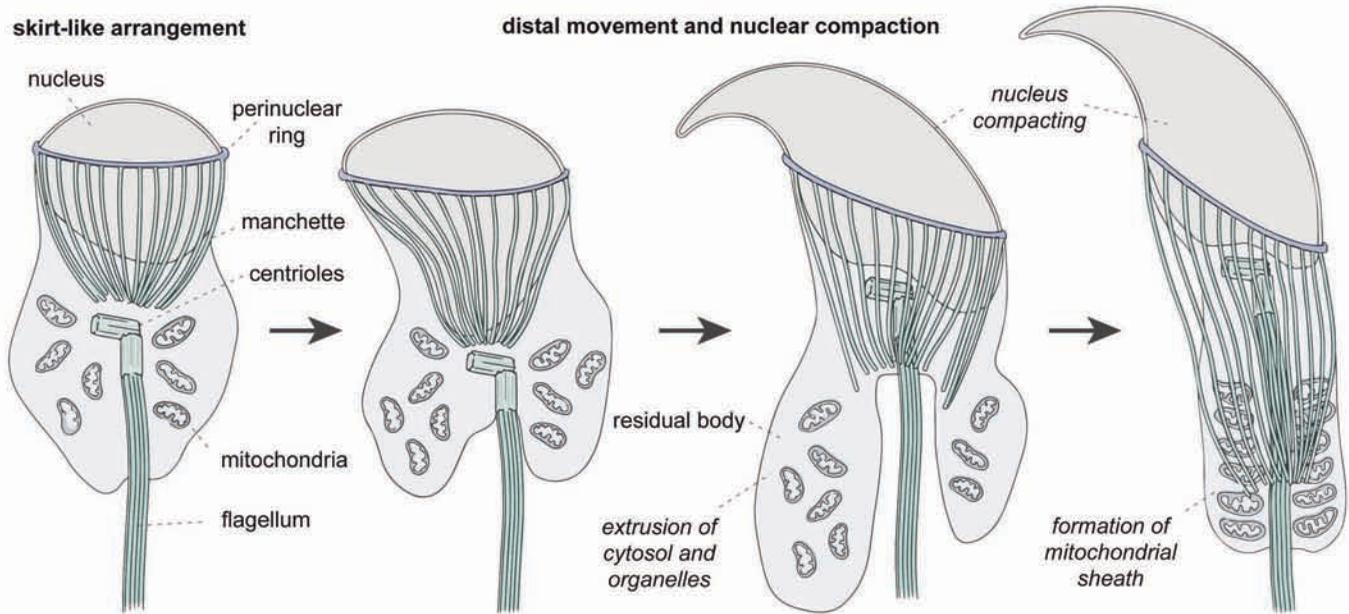
Figure 2: Mammalian spermatogenesis

A) Spermatogenesis in mammals takes place in the seminiferous tubules of the testes in a multi-step process. The maturing sperm cells are arranged in an ordered manner within the tubules, and are surrounded by the supporting Sertoli cells. Fully assembled, but still immature sperm cells are transported to the epididymis, where the final maturation steps take place.

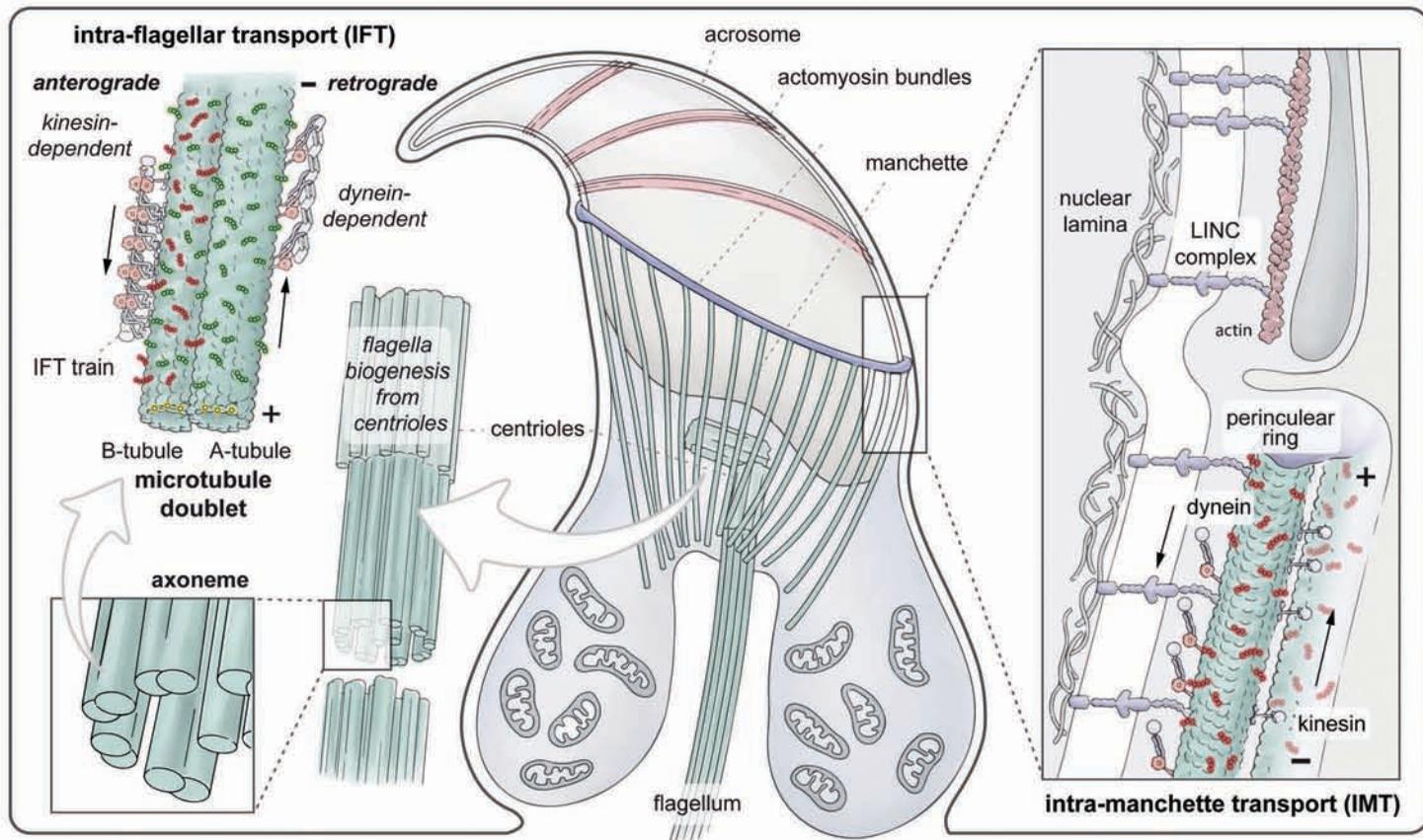
B) Spermatogenesis begins when the male germ cells (spermatogonia) differentiate into spermatocytes through multiple mitotic divisions. Spermatocytes then undergo meiotic divisions to form haploid round spermatids. Spermatogenesis requires a close nutritional and structural support from Sertoli cells that embed the developing sperm cells.

(C) Once the round spermatids are generated by meiotic divisions (B), the process shifts to spermiogenesis, during which spermatids undergo specific morphological changes resulting in elongated and condensed spermatids. Elongated spermatids have compacted nuclei and fully assembled flagella. The MT manchette begins to develop at the end of the cap phase, plays a key role in nuclear compaction, and is disassembled at the end spermatozoa maturation (see Fig. 3A).

A Development of the sperm manchette and sperm head compaction



B Microtubule structures in the condensing spermatid



C Perturbed tubulin polyglutamylation causes spermatogenesis defects

Ccp5-KO (increased polyglutamylation)

defects observed: perturbation of spermatogenesis with disorganised manchette and absence or mis-assembled axonemes

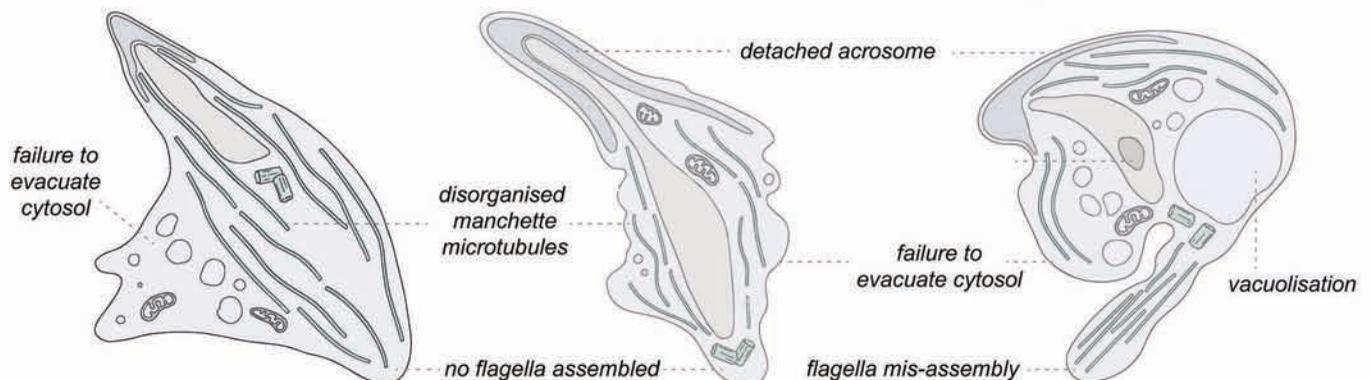


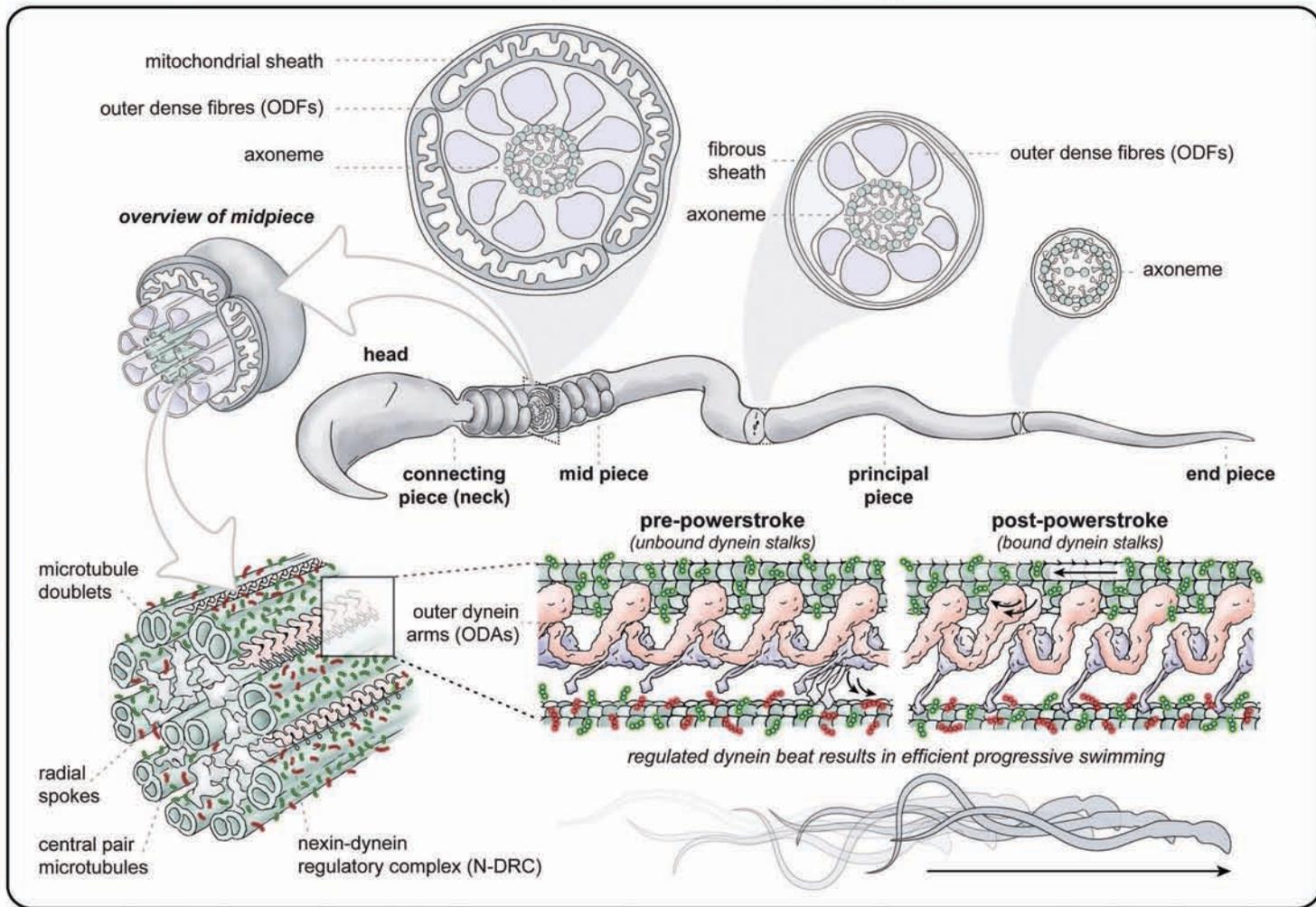
Figure 3: Microtubule structures and functions in condensed spermatids

(A) The manchette is a transient MT structure that is arranged in a skirt-like fashion around the nuclei of round spermatids. As spermiogenesis proceeds, the manchette moves distally towards the developing flagella, thereby shaping the acrosome and compacting the nucleus. In this process cytosol and organelles are extruded into residual bodies, and mitochondria move to the developing flagellum where they form the mitochondrial sheath (Fig. 4A).

(B) Acrosome biogenesis and nuclear compaction involves both actin and MTs, linked to the nuclear lamina through the LINC complexes. The actomyosin bundles surrounding the compacting nucleus support the short-distance vesicle transport from Golgi to the developing acrosome [30]. Manchette MTs are involved in long-distance transport of vesicles via intra-manchette transport that uses the molecular motors kinesin and dynein. Sperm flagella are assembled from the centrioles, the MTOCs. Flagellar assembly involves intraflagellar transport (IFT), which transports cargo by IFT trains along the MTs using kinesins and dynein. The flagellar axoneme is composed of 9 doublet MTs, an A-tubule of 13 protofilaments and a B-tubule of 10 protofilaments (see Fig. 4A).

(C) Loss of the deglutamyase CCP5 causes multiple defects in spermiogenesis. *Ccp5*-KO mice accumulate hyperglutamylated MTs in developing sperm cells, which display defects like incomplete acrosomes and defective manchettes (left), detached acrosomes along with disorganised manchette (centre), or entirely disorganised manchettes, disconnected acrosomes, deformed nuclei with inclusions, highly vacuolated spermatids with disorganised flagella (right). Developing spermatids cannot extrude the cytosol and mostly fail to form flagella. Consequently, *Ccp5*-KO mice do not produce mature sperm cells and are male sterile [38].

Structure and composition of the sperm flagellum



B Perturbed tubulin posttranslational modifications cause flagellar defects

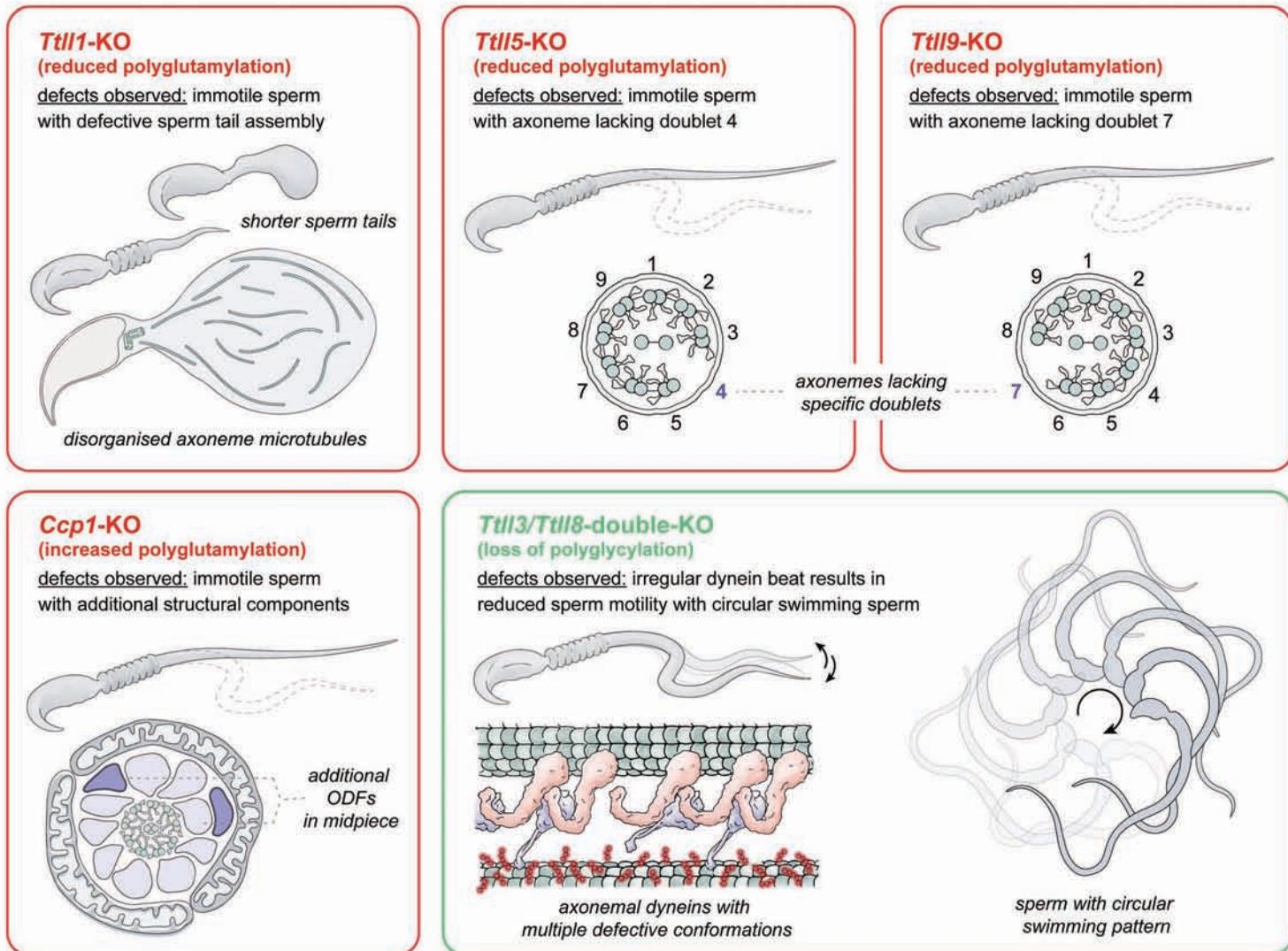


Figure 4: The sperm flagellum

A) Mature murine spermatozoa have a compacted hook-shaped nucleus enclosed by the acrosome, and connected to the flagella through the connecting piece, or neck. The proximal part of the flagellum is the midpiece, which contains the mitochondrial sheath and 9 outer dense fibres (ODFs). In the subsequent principal piece, the mitochondrial sheath is replaced by the fibrous sheath. ODFs gradually reduce towards the distal part of the flagellum, and are completely absent in the endpiece, which contains only the axoneme. The 9 doublet MTs of the axoneme are lined by axonemal dyneins bound to the A-tubules of MT doublets. To generate axonemal motility, dyneins move with their stalk regions on the opposing B-tubules in two main conformations, a pre-powerstroke with unbound, and a post-powerstroke with dynein stalks bound to the B-tubules. Coordinated dynein activity is essential for efficient progressive swimming of spermatozooids.

B) Loss of the glutamylase *TLL1* or its subunit *PGs1* [83-85] leads to either absence of flagella, or to assembly of short flagella with disorganised MTs. Sperm flagella lacking the glutamylases *TLL5* [87] or *TLL9* [89] assemble flagella lacking specific MT doublets in the axoneme, which perturbs flagellar motility. Loss of the deglutamylase *CCP1* causes hyperglutamylation, the presence of additional ODFs in the flagellar midpiece, and perturbed beating of sperm flagella [91, 92]. Male mice are either sterile (*Tll1*-KO, *PGs1*-KO, *Tll9*-KO, *Ccp1*-KO) or subfertile (*Tll5*-KO). Complete absence of glycylation after deletion of the glycylation enzymes *TLL3* and *TLL8* leads to a perturbed flagellar beat resulting in circular swim patterns of sperm, which causes subfertility. The perturbed swim patterns are most likely related to altered axonemal dynein arm configurations [78].