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Václav Bočan

Microtubule inner proteins
Vnitřní mikrotubulární proteiny

Bachelor's thesis

Supervisor: RNDr. Lenka Libusová, Ph.D.

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Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

Declaration

I honestly declare that I wrote this thesis on my own and that I stated all used literature and other information sources. This work or its significant part was not used to previously acquire any other or same academic title.

V Praze / In Prague, 09.05.2019

Abstract

Microtubules are a prominent part of the cytoskeletal network in eukaryotic cells. They are involved in nearly all cellular processes, e.g. in vesicular trafficking, signal transduction, locomotion, or cell morphogenesis. To discharge that many functions, precise regulation of microtubule dynamics and architecture is essential. Such regulation is maintained by various microtubule-associated proteins, which usually bind from the outside. However, several proteins were found to bind in the lumen of microtubules. These microtubule inner proteins were shown to function either as post-translational modifiers of tubulin or stabilizers in time-persistent microtubular structures. A few inner proteins were identified, but our understanding of their attributes is still incomplete.

This thesis summarizes current knowledge of microtubule inner proteins. The scope is focused on their enzymatic and structural features. Tubulin acetyltransferase represents the enzymatic MIPs. Possible ways of lumen entry and impact on the tubulin lattice are described. Next, the structural roles of proteins inside microtubules, most prominent in the axoneme, are outlined. The relevance of microtubule inner proteins for cytoskeletal functions, flagellar motility, and future perspectives are discussed at the end.

Keywords

microtubules, microtubule-associated proteins, microtubule inner proteins, axoneme, microtubule doublets, α-tubulin acetyltransferase 1

Abstrakt

Mikrotubuly jsou jednou z hlavních složek cytoskeletu eukaryotických buněk. Podílí se na mnoha buněčných funkcích – řídí dopravu váčků, zapojují se do přenosu signálů, umožňují pohyb bičíků či spoluvytváří celkový tvar buňky. Při všech těchto dějích je nezbytná přesná regulace struktury a dynamiky mikrotubulární sítě. Za tu do značné míry zodpovídají proteiny asociované s mikrotubuly, které se váží na vnější stranu mikrotubulů. Jiné mohou ovšem vstupovat do mikrotubulů a vázat se k jejich vnitřnímu povrchu. Tyto vnitřní mikrotubulární proteiny posttranslačně modifikují tubulin, v jiných případech fungují jako vnitřní výztuha. Některé proteiny se již podařilo identifikovat, o jejich vlastnostech však mnoho nevíme.

Tato práce shrnuje dosavadní poznatky o proteinech uvnitř mikrotubulů – jejich enzymatické aktivity a význam jakožto strukturní opory. Jedním z mála známých enzymů vstupujících do mikrotubulů je tubulin acetyltrasferáza; práce shrnuje dopady jejího působení na vlastnosti mikrotubulů a popisuje cesty, kterými do nich může vstupovat. Dále jsou přiblíženy strukturní role vnitřních proteinů v axonemě. V závěru je diskutován význam vnitřních mikrotubulárních proteinů pro buněčnou fyziologii a pohyb. Nastíněno je také možné budoucí směřování výzkumu tohoto fenoménu.

Klíčová slova

mikrotubuly, proteiny asociované s mikrotubuly, vnitřní mikrotubulární proteiny, axonema, mikrotubulární dublety, α-tubulin acetyltransferáza 1

List of abbreviations

EM	electron microscopy
ET	electron tomography
FAP	flagellar associated protein
fMIP(s)	filamentous microtubule inner protein(s)
GTP	guanosine triphosphate
MAP(s)	microtubule associated protein(s)
MIP(s)	microtubule inner protein(s)
MT(s)	microtubule(s); microtubular
MTD(s)	microtubule doublet(s)
PF(s)	protofilament(s)
RIB	ribbon-associated protein

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1. Introduction

Microtubules (MTs) are key cytoskeletal structures organizing cytosolic content of eukaryotic cells. These complex polymers of tubulin serve as a scaffold for a plethora of cellular functions, e.g. vesicular trafficking (Rowning *et al.* 1997), organelles positioning (Fu *et al.* 2011), segregation of chromosomes during cell division (Esau and Gill 1965), or motility of cilia and flagella (Satir 1965). Thus, much attention has been paid to MTs since their discovery in half of the 20th century.

Microtubules are often referred to as hollow tubes or cylinders. They are described as such also in recent world-established textbooks, for instance, Molecular Biology of the Cell, 6th edition 2015, page 891, despite early electron microscopy (EM) micrographs clearly captured densities in their lumen (Bassot and Martoja 1966; Behnke 1967; Peters, Proskauer, and Kaiserman-Abramof 1968). Many other authors confirmed these observations, e.g. (Rodríguez Echandía, Piezzi, and Rodríguez 1968; Stanley *et al.* 1972; Burton 1984; Linck 1976). Since then, understanding of proteins inside MT inner space has been gradually improving. The spatial arrangement of microtubule inner proteins (MIPs) resolved with < 1 nm precision is available today and some MIPs are already identified. This was possible thanks to the recent development of structure-imaging techniques like cryo-EM (Maheshwari *et al.* 2015; Ichikawa *et al.* 2017).

In this bachelor thesis, I aim to delineate the current knowledge of structural and enzymatic features of MIPs. I divide the topic into three chapters: General description of MIPs, MIPs linked to post-translational modifications of microtubules (acetylation of tubulin), and MIPs serving as structural components of MTs (involved in lattice stabilization).

1.1 The microtubule

Microtubules are formed in the cytosol of all eukaryotic cells as prolonged tubes (Fig. 1). The outer diameter is 23–27 nm[i] (Gall 1966; Beese, Stubbs, and Cohen 1987; Ledbetter and Porter 1963), the inner diameter is 15 nm (Nogales *et al.* 1999). MTs are composed of two forms[ii] of the highly conserved protein tubulin, α-tubulin and β-tubulin (Bryan and Wilson 1971; Feit, Slusarek, and Shelanski 1971). More forms of tubulin exist, termed γ-, δ-, ε-tubulin, and so on (Oakley and Oakley 1989; Chang and Stearns 2000)). A particular form of tubulin consists of more isotypes – eight genes for α-tubulin and nine for β-tubulin were found in human so far, as reviewed in (Roll-Mecak 2019). α- and β-tubulin are proteins approximately 450 residues long, depending on the isoform. Both have the molecular weight of about 50 kilodaltons (kDa) (Ponstingl *et al.* 1981; Krauhs *et al.* 1981). They associate into heterodimers (Bryan and Wilson 1971). Upon MT assembly, these dimers form protofilaments (PFs) by "head-to-tail" polarized interactions, so that α- and β-tubulins alternate in the lattice (Erickson 1974b). PFs, in turn, assemble into the fully-formed MTs. Remarkably, the tubulin wall is not completely continuous – there are fenestrations 1 nm in diameter between tubulin subunits (Nogales *et al.* 1999).

Conserved number of 13 PFs are present in nearly all eukaryotic MT structures (Tilney *et al.* 1973), with several exceptions (e.g. in nematode *Caenorhabditis elegans*, general number of PFs is 11, except for a specialized subset of MTs in touch receptor neurons, which have 15 (Chalfie and Thomson 1982) and their diameter is larger (Chalfie and Thomson 1979)). MTs contain a discontinuity in lateral tubulin interactions between PFs number 1 and 13, termed the seam (Fig. 1).

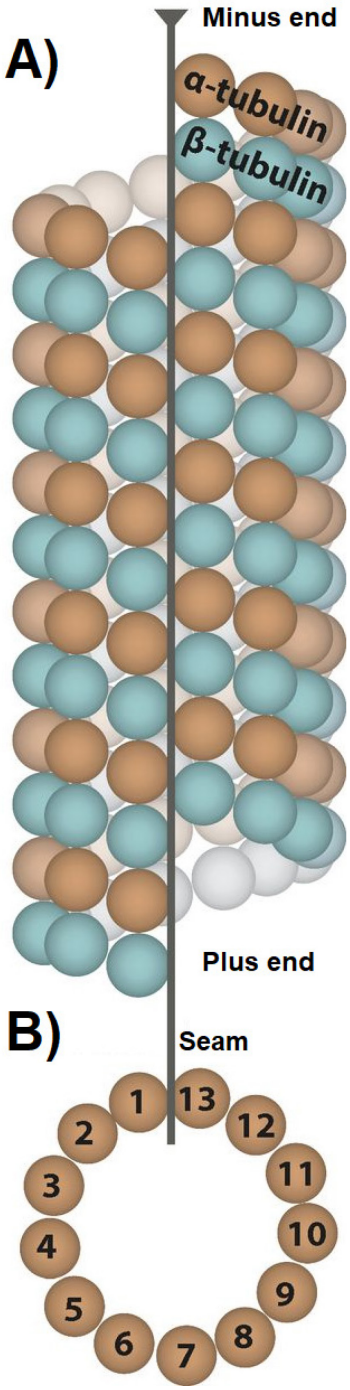

Fig. 1: **A)** Schematic representation of a microtubule. The position of the seam is indicated. **B)** Schematic cross-section of a microtubule. Individual protofilaments are numbered. (Deng *et al.* 2017), modified

[i] In recent literature, the diameter of microtubules is stated as a commonly known fact with no resources cited. The diameter might be obtained directly from various published 3D-structure models, though, as those in (Zhang, LaFrance, and Nogales 2018). In this case, the resulting value 26.3 nm for a 13-PFs microtubule (Eva Nogales, personal correspondence 2019) is of course in agreement with old EM data.

[ii] A note on tubulin variants terms usage: "Tubulin forms" refers to gene families within tubulin gene superfamily (e.g. α-tubulin, γ-tubulin). "Tubulin isotypes" denotes distinct genes of one tubulin family (e.g. β2, β3-tubulin). "Tubulin isoforms" are alternatively spliced products of one particular tubulin isotype gene (not addressed in this thesis).

The 3D-structures of both α- and β-tubulin were resolved in 1998 with 3.7 Å spatial resolution (Nogales, Wolf, and Downing 1998). Both proteins share ~40 % amino acid sequence identity (Krauhs *et al.* 1981). They also have highly similar globular shape containing the Rossmann's fold, serving for guanosine triphosphate (GTP) binding. GTP might by spontaneously hydrolysed to guanosine diphosphate and exchanged back again for GTP from the cytoplasmic pool on β-tubulin only. In the α-tubulin structure, it is buried, and thus non-exchangeable for free GTP (Nogales, Wolf, and Downing 1998). Nucleotides bound to β-tubulin are locked in position when incorporated into the tubulin lattice and may dissociate only upon microtubule disassembly (Farrell, Kassis, and Wilson 1979; Margolis and Wilson 1978).

1.2 Dynamics of MTs

MTs are polarized structures with two distinct ends (Fig. 1). Polymerized dimers might be lost or new might be added at both ends in a dynamic and stochastic manner (Mitchison and Kirschner 1984a). Once in the lattice, however, tubulin has only a low chance of being lost or replaced (Margolis and Wilson 1978; Farrell, Kassis, and Wilson 1979; Soltys and Borisy 1985). The end terminated by β-tubulin is denoted as the plus end, the opposite end terminated by α-tubulin is being referred to as the minus end; usually, plus end grows more readily than minus end (Rodionov and Borisy 1997; Soltys and Borisy 1985). Simultaneous growing and shrinking may manifest as if the whole (unanchored) microtubule was moving, although individual tubulin monomers stay approximately at the same location (Rodionov and Borisy 1997). The unidirectional flow of tubulin throughout the tubule during such a situation is called treadmilling. A sudden massive disintegration of a MT by PFs peeling off and depolymerizing (Kirschner *et al.* 1974) is termed the catastrophe, which occurs when terminal β-tubulins have their GTP hydrolysed. The whole microtubule might eventually fall apart, or be rescued and switch back to growing phase if GTP molecules rebind terminal β-tubulins (Walker *et al.* 1988). Alternating phases of polymerization, catastrophes, and rescues are referred to as dynamic instability (Mitchison and Kirschner 1984a). This property enables the cell to rapidly rebuilt the whole microtubular network just by slight changes in interacting microtubule-associated proteins (MAPs) or tubulin post-translational modifications (Mitchison and Kirschner 1984a).

MTs are rarely constituted de novo in cytosol from free dimers, but rather origin from partially assembled curved or ring-shaped PFs seeds (Erickson 1974a). A nucleation primer, such as γ-tubulin rings on centrosomes, is required (Moritz *et al.* 1995). The γ-tubulin ring stabilizes the associated minus end of a tubule so that it does not depolymerize over time. By anchoring to microtubule organizing centres, e.g. centrosomes (Soltys and Borisy 1985), basal bodies, or other structures (Rodionov and Borisy 1997), the radial pattern of MTs in the cytoplasm is created (Mitchison and Kirschner 1984b).

The features of dynamic instability and treadmilling might be modified by various interactions with MAPs (Mitchison and Kirschner 1984a), including MIPs, and post-translational modifications of tubulin. α- and

β-tubulin isotypes composition also affects the dynamics of MTs (Vemu *et al.* 2017). The dynamic subset typically comprises MTs spread throughout the whole cytoplasm and plays the scaffolding role for vesicular transport and organelles positioning via attachment of motor proteins (dyneins and kinesins). Kinetochore MTs exhibit dynamic qualities as well. On the other hand, stable populations of MTs set up the axoneme, centrioles, and other structures (e.g. bundles of axonal MTs in neurons) (Orbach and Howard 2019).

1.3 The axoneme, centriole, and basal body

The term axoneme refers to the microtubular scaffold in eukaryotic cilia and flagella[iii]. A detailed model of this complex structure with ~40 Å resolution by electron tomography (ET) is available since 2006 (Nicastro *et al.* 2006), although first observations by EM were made 70 years ago (Grigg and Hodge 1949). The axoneme typically features a "9 + 2" arrangement: Peripheral microtubule doublets (MTDs) in 9-fold symmetry and two central single MTs (Fig. 2) (Nicastro *et al.* 2006; Afzelius 1959; Fawcett and Porter 1954). This layout is highly conserved among eukaryotes (Fawcett and Porter 1954), reviewed in (Mirvis, Stearns, and James Nelson 2018), although exceptions are reported even in mammals (e.g. 9 + 4 architecture in rabbit notochondral plane (Feistel and Blum 2006), 10 + 2 or 7 + 2 doublets observed in rabbit oviductal epithelia, and so on (Odor and Blandau 1985)). In the concentric peripheral MTDs, two microtubules are joined together. One, so-called the A-tubule, is fully assembled with the usual number of 13 PFs. The second one, termed the B-tubule, forms an arch attached to the A-tubule and most often consists of only 10 PFs of tubulin (11th PF is sometimes distinguished, but it is smaller in diameter and composed of MAPs instead of tubulin) (Nojima, Linck, and Egelman 1995).

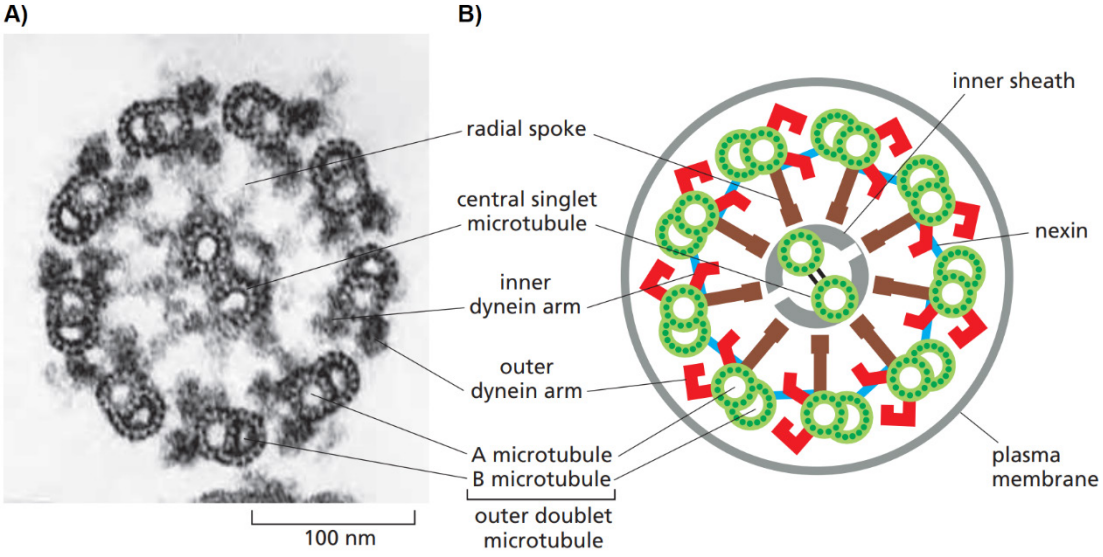

Fig. 2: The structure of the axoneme from alga *Chlamydomonas*. **A)** Cross section of the flagellar axoneme viewed by transmission EM. **B)** Schematic drawing of a cross-section of the axoneme. The distal tip (plus end of MTs) is facing the reader. (Molecular Biology of the Cell, 6th edition 2015, page 941)

[iii] The terms "cilia" and "flagella" are often used interchangeably (Orbach and Howard 2019). Both organelles possess the same axonemal architecture and differ only in the biomechanics of beating.

Bending and beating of cilia/flagella are carried out by attached dyneins – molecular motors exerting mechanical work by adenosine triphosphate hydrolysis (Gibbons and Rowe 1965; Vale and Toyoshima 1988). The arrangement of the flagellar cytoskeleton and the association patterns of dynein molecules and other neighbouring MAPs are conserved among large phylogenetic taxa (Nicastro *et al.* 2006). Nonmotile, or primary[iv], cilia lack the central pair of single MTs in their axoneme (Sorokin 1968). Nonetheless, motile cilia with the 9 + 0 arrangement were reported in mammalian embryonal node and oviductal epithelium (Nonaka *et al.* 1998; Odor and Blandau 1985). Nonmotile cilia play various roles as sensory organs and cell signalling centres (Teilmann *et al.* 2005, reviewed in Pazour and Witman 2003).

Mutations of axonemal components lead to improper cilia assembly and performance. Such conditions are called ciliopathies. To list some consequences of compromised cilia function: Defects in establishing lateral left-right body symmetry due to improper distribution of morphogens during development (manifesting as situs inversus in Kartagener syndrome) (Nonaka *et al.* 1998), polycystic kidney disease in patients with mutated genes essential for intraciliary transport (Pazour *et al.* 2000), or degeneration of sensory epithelia, e.g. retina (Pazour *et al.* 2002). These defects clearly illustrate the importance of cilia in human physiology.

Centrioles and basal bodies share many architectural features and are often described together. Both structures are highly stable and important organizers of MTs in cells (Byers, Shriver, and Goetsch 1978; Karsenti *et al.* 1984; Moritz *et al.* 1995). They typically comprise MT triplets with an additional C-tubule (10 PFs, similar to the B-tubule), arranged in a barrel-like manner with 9-fold symmetry (Harven and Bernhard 1956; Anderson 1972; Guichard *et al.* 2013; Li *et al.* 2019). Several exceptions are documented, for instance in *Caenorhabditis*, the centriole consists of 9 singlets (Wolf, Hirsh, and McIntosh 1978). A pair of centrioles constitutes a membraneless organelle called the centrosome, which seeds MTs from attached γ-tubulin rings (Moritz *et al.* 1995). Centrosomes play a pivotal role also in mitotic spindle establishment during cell division (Byers, Shriver, and Goetsch 1978). Basal bodies anchor eukaryotic cilia and flagella at their proximal end. The triplets of the basal body are continuous to doublets in the axoneme, except for the C-tubules which are terminated before entering the cilium (Geimer and Melkonian 2004). As well as the axoneme, centrioles and basal bodies contain MIPs (Li *et al.* 2012, 2019).

[iv] The literature is inconsistent in the usage of term "primary cilia": Its meaning could be restricted to a) immobile structures with 9 + 0 architecture (used in such way e.g. by (Nonaka *et al.* 1998)), or b) 9 + 0 cilia regardless of their actual ability to move. Here, the term is used in the former sense, same as the definition in review of (Satir and Christensen 2007).

2. Luminal content of MTs

Even though MTs might appear hollow, their lumen does not contain only equilibrated cytoplasm solution: Some proteins or other molecules localize there specifically. For instance, microtubule-stabilizing drug taxol has its binding site inside MTs (Nogales, Wolf, and Downing 1998). There were many EM observations of dense-core MTs from numerous structures and species, like spermatid axoneme of *Drosophila* (Stanley *et al.* 1972), microtubule singlets in the neurites of frog olfactory neurons (Burton 1984), touch sensory neurons in *Caenorhabditis* (Chalfie and Thomson 1979), or toad neurons and glial cells of adrenal gland and hypothalamus (Rodríguez Echandía, Piezzi, and Rodríguez 1968). These luminal patterns were proven not to be artefacts of heavy metal salts staining procedure (Stanley *et al.* 1972; Burton 1984). Burton showed that most of the dense cores disappear after depolymerizing the frog axonal microtubules and re-establishing them from free tubulin dimers in isolated nerves. Also, *in vitro* assembled MTs are devoid of luminal content (Garvalov *et al.* 2006). Initially, there were little data on what the spatial arrangement of the luminal material is. The authors just mentioned dots or dot-like density (Behnke 1967; Rodríguez Echandía, Piezzi, and Rodríguez 1968; Stanley *et al.* 1972); others proposed filamentous structure (Peters, Proskauer, and Kaiserman-Abramof 1968). Burton observed bead-like structures with a diameter of ~4.5 nm in oblique transections, but also elongated clusters, located either in the middle of the lumen, or attached to the inner MT surface (Burton 1984).

The collocation "microtubule inner proteins" was first used by Nicastro *et al.* in 2006 when studying the axonemes of *Chlamydomonas* and sea urchin by cryo-ET (Nicastro *et al.* 2006). Generally speaking, the occurrence of MIPs appears to be positively correlated with the stability of the microtubular structure – there are only rare reports from dynamic cytoplasmic tubules, e.g. from hepatoma cells (Garvalov *et al.* 2006) or fibroblasts (Koning *et al.* 2008). Such MTs probably do not require strong stabilization from the inner side (Stoddard *et al.* 2018). On the other hand, partially or completely stabilized microtubules, as are found in axons and dendrites (Baas *et al.* 1993), in the axoneme (Redeker *et al.* 1994), or in the centrioles and basal bodies (Bobinnec *et al.* 1998), are documented to routinely contain bound proteins in their lumen (Bouchet-Marquis *et al.* 2007; Li *et al.* 2012; Maheshwari *et al.* 2015).

MIPs are now thought to play a crucial structural role in axonemal MTDs and other persistent MT structures for assembling and enhancing the stability (Nicastro *et al.* 2006; Owa *et al.* 2019). Such MIPs could be conveniently explored by structural approaches – single particle cryo-EM and cryo-ET performed on *ex vivo* isolates of cilia/flagella from a ciliate *Tetrahymena thermophila*, biflagellate green alga *Chlamydomonas*, and sea urchin sperm (Nicastro *et al.* 2006; Ichikawa *et al.* 2017; Maheshwari *et al.* 2015; Stoddard *et al.* 2018). Moreover, MIPs can also be enzymatically active, like α-tubulin acetyltransferase 1 (αTAT1). Studies regarding this enzyme cannot exploit the techniques mentioned above since it is associating with the inner surface in a less affinitive and symmetrical manner, compared to structure-building MIPs in stable MTs.

3. MIPs with enzymatic activity

Tubulins are subject to many post-translational function-tuning modifications, e.g. polyglutamylation, detyrosination, or acetylation, summarized in (Y. Song and Brady 2015). Acetylation is of high interest since tubulin (de)acetylase can enter the lumen of polymerized MTs and perform there (Shida *et al.* 2010). α-tubulin acetyltransferase 1 (αTAT1) and its concomitant antagonist histone deacetylase 6 are the only two MIPs with enzymatic activity found so far.

The fact that α-tubulin might be post-translationally acetylated on lysine 40 is long-known (L'Hernault and Rosenbaum 1985; LeDizet and Piperno 1987). Lysine 40 is not conserved in β-tubulin (Krauhs *et al.* 1981), so β-tubulin cannot be modified at this position. Both tubulins can be acetylated by different enzymes than αTAT1 on other residues, too, but the relevance is mostly unknown (Choudhary *et al.* 2009). Flagellar or ciliary axonemes are heavily acetylated in both central singlets and MTDs (Orbach and Howard 2019). Conversely, α-tubulin in cytoplasmic MTs has its lysine 40 modified to a much lower extent (Piperno and Fuller 1985). Defined MT structures like axonal bundles (Lin *et al.* 2017), the mitotic spindle and midbody (a transient microtubular structure within daughter cell interface after cytokinesis) are acetylated as well (Piperno, LeDizet, and Chang 1987). Treatment by MT-stabilizing drug taxol induced reversible acetylation of whole MT net in mammalian cells (Piperno, LeDizet, and Chang 1987). From this observation, Piperno concluded that acetylation follows the stabilization and not the other way round, although more recent data support the stabilizing role of acetylation, not just being a passive mark of stability (Akella *et al.* 2010). (Xu *et al.* 2017) published convincing results indicating that acetylation confers local mechanical resistance to MTs. Interestingly, there is no difference in the tubulin lattice structure in both acetylated and non-acetylated MTs. Unknown MIP(s) were proposed to function as reporters of acetylation towards outer MAPs (Howes *et al.* 2014). Moreover, no MAPs binding specifically to acetylated regions were described (Howes *et al.* 2014), although kinesins show some preference for acetylated MTs (Reed *et al.* 2006). Overall, the relationship between acetylation, its readout, and mechanical/dynamic stability of MTs is still unclear, as reviewed by (Y. Song and Brady 2015).

3.1 α-tubulin acetyltransferase 1

αTAT1 is the only known enzyme catalysing the acetylation of α-tubulin and was discovered relatively recently – after identification of both lysine 40 tubulin deacetylases (Hubbert *et al.* 2002; Shida *et al.* 2010; Akella *et al.* 2010). Additional paralog named αTAT2 with different spatial expression was found in *Caenorhabditis*; this paralog is absent from the human genome. Aside from the acetylation of lysine 40 in α-tubulin using acetyl-coenzyme A as an acetyl donor, αTAT1 displays no other activity towards α- or β-tubulin or any other substrate (including histones). Both polymerized and free tubulin are acetylated, assembled MTs are

however highly preferred (Shida *et al.* 2010; Coombes *et al.* 2016). Disruption of α-tubulin acetylation either by mutating lysine 40 to arginine or by altering the active site of αTAT1 leads to lowered MT stability in *Tetrahymena* and touch insensitivity in *Caenorhabditis* (Akella *et al.* 2010).

Three-dimensional structure of αTAT1 is known (Kormendi *et al.* 2012; Taschner, Vetter, and Lorentzen 2012). αTAT1 possesses a conserved catalytic domain common for histone acetyltransferases, yet αTAT1 is specific for tubulin instead of histones and utilizes different catalytic mechanism (Shida *et al.* 2010). Tubulin variable loop containing lysine 40 is rather acidic and fits a positively charged groove on the αTAT1 surface; the amino acid charges are opposite for histone acetylases (Taschner, Vetter, and Lorentzen 2012). Approximately first half at the N terminus of the 421-residue-long protein accounts for the catalytic domain. The C-terminal half, which is partially missing in some isoforms, is probably unstructured. One can speculate whether it plays a role in the inner surface binding. A short sequence at the N-terminus also lacks a stable fold, yet it is important for the catalytic function. This intrinsically disordered protein might require MT lattice as a "chaperone" for adopting its active conformation (Kormendi *et al.* 2012). αTAT1 occurs as a monomer in physiological concentrations. Importantly, dimensions of αTAT1 ($30 \times 62 \times 30$ Å^3) can account for observed densities in cytosolic MTs with a diameter 4–7 nm (Burton 1984; Garvalov *et al.* 2006; Kormendi *et al.* 2012).

3.2 αTAT1 lumen entry

Lysine 40 was surprisingly found facing the lumen of MTs, as revealed from the tubulin structure (Nogales *et al.* 1995). A possible explanation that this residue is acetylated when the tubulin is depolymerized was disproved – αTAT1 acetylates MTs much faster than free tubulin (Piperno, LeDizet, and Chang 1987). It was concluded that αTAT1 must enter the lumen of MTs. Aside from the luminal surface, αTAT1 interacts with the outer side as well. The probability of entering the lumen is thus increased (Ly *et al.* 2016; Howes *et al.* 2014). Considering the size of αTAT1, it is highly unlikely that the enzyme enters MT lumen via lattice fenestrations. Rather, the terminal openings or large lattice defects must be utilized (Taschner, Vetter, and Lorentzen 2012; Coombes *et al.* 2016). The patches of acetylation should be then located nearby entry points, which was confirmed (Akella *et al.* 2010; Ly *et al.* 2016). Additionally, transient lattice openings (so-called "breathing" of MTs) were also suggested to enable lumen entry (Shida *et al.* 2010), this seems to be just a minor way of entry. When a MT grows, previously terminally-located patches are buried under newly polymerized tubulin (Ly *et al.* 2016). In other words, αTAT1 diffuses in lumen extremely inefficiently because of perpetual binding to the walls. Non-terminal patches are thus either remnant of previous terminal acetylations, results of activity of αTAT1 molecules buried by treadmilling, or sites with lattice defects serving as entry points. This model was challenged by Szyk who observed rapid αTAT1 diffusion and continuous acetylation in MTs (Szyk *et al.* 2014). Notwithstanding, their data were based on *in vitro* assembled MTs, which possess numerous lattice defects (e.g. changed PFs number by taxol stabilization (Howes *et al.* 2014)) and

lack MAPs, which could limit the access to the lattice. This may explain the discrepancy between *in vitro* and *in vivo* experiments. Apparently, *in vitro* experiments should be interpreted cautiously (Ly *et al.* 2016).

In luminal space, the concentration of binding sites for αTAT1 is high (17 mM), so the enzyme rebinds rapidly after dissociation with estimated rebind time of 6×10^{-5} s^{-1}. This hinders free spreading alongside the MT and accounts for observed slow diffusion (under 1 μm^2 s^{-1}) and acetylation in patches. Length of acetylated spots increased when binding affinity was lowered by higher concentrations of salts. That means that not the steric hindrance, but the high binding rate is the cause of low αTAT1 mobility (Coombes *et al.* 2016). The authors further hypothesize that short-lived MTs probably do not survive long enough for αTAT1 to enter them whereas long-lived stabilized MTs have higher acetylation probability because of accumulated lattice defects (serving as secondary entry points) over time. Indeed, older microtubules tend to curve (Xu *et al.* 2017), which leads to lattice defects. As acetylated MTs gain breakage resistance, αTAT1 is thought to prevent defect spreading by local acetylation in mechanically stressed MTs (Xu *et al.* 2017). αTAT1 itself does not prefer acetylated patches for binding, though (Howes *et al.* 2014). In conclusion, various findings suggest there is positive feedback between stabilization and acetylation of MTs and acetylation could be used as a marker for MT age, as concisely reviewed in (Janke and Montagnac 2017).

3.3 Tubulin deacetylation

The predominant α-tubulin deacetylase (at lysine 40 residue) is histone deacetylase 6 (HDAC6) (Hubbert *et al.* 2002). HDAC6 can deacetylate both free dimers and MTs from the inner side. However, it strongly prefers dimers (Skultetyova *et al.* 2017), although the first report claimed the opposite (Hubbert *et al.* 2002). This is probably due to the structural context nearby the lysine 40 loop, not the inaccessibility of this loop when hidden inside MTs. The enzyme binds to the external surface of MTs with no preference for ends (Skultetyova *et al.* 2017). HDAC6 is enriched at the leading edge of moving cells, where acetylated MTs are scarce (Hubbert *et al.* 2002). Second known deacetylase acting on α-tubulin lysine 40 position is sirtuin 2 (North *et al.* 2003). It was proven to interact with HDAC6. Sirtuin 2 can handle both free dimers and assembled MTs *in vitro*, although no work confirmed that it really accesses the lumen. HDAC5 is a third identified enzyme deacetylating α-tubulin at lysine 40 (Cho and Cavalli 2012). It can perform on axonal MTs (Lin *et al.* 2017), evidence for lumen entry is again missing.

4. Structural MIPs

MIPs with structural functions are predominantly found in stabilized MTs, as reviewed in (Ichikawa and Bui 2018). Oppositely to the enzymes involved in tubulin (de)acetylation, none of the structural MIPs has been studied to such a broad extent. Thus, the precise mechanisms of action of structural MIPs are mostly still to be revealed (Kirima and Oiwa 2018). The lack of structures resolved at the atomic level hinders the research of MIPs-tubulin interactions, even though some progress has been already made.

4.1 MIPs in the axonemal doublets

More is known about the axonemal MIPs in comparison to the centrioles and basal bodies. However, because of broad architectural similarity, some findings might be applicable for all these structures. An early approach to study the axoneme was to reveal the organization inside cilia and flagella by EM. Among these pioneering works, several authors noticed densities located in the MTDs lumen. Witman described constant "beak-like structure" in MTDs of *Chlamydomonas* (Witman *et al.* 1972), also dealt with in (Hoops and Witman 1983). These densities appear constantly inside B-tubules of MTDs number 1, 5, and 6, suggesting that MTDs of the axoneme are not all identical. Similarly, individual PFs in MTDs differ in their mechanical properties (Witman *et al.* 1972). Stanley and colleagues (Stanley *et al.* 1972) precisely followed the developmental stages of spermatids maturation in *Drosophila*; they referred to an accumulation of dense material in the A-tubule near the part of the wall which is shared with the B-tubule. Central microtubule density was clearly visible in many EM micrographs in this paper. The appearance of such dense cores in axonemal MTDs was even used as a diagnostic trait for one of the spermatid developmental stages. The authors also speculated that since early axonemal structures were more often deformed, the axoneme may gain rigidity during its maturation. This conclusion is in concordance with recent data showing the role of MIPs for microtubule stability (Stanley *et al.* 1972; Owa *et al.* 2019). Axonemal MTs still undergo dynamic instability but to a much lower extent than cytoplasmic MTs (Orbach and Howard 2019).

As a complex structure, MTDs (in contrast to cytoplasmic microtubules) are hard to assemble *in vitro*. MTDs reconstitution from free tubulin was achieved recently for the first time (Schmidt-Cernohorska *et al.* 2019). This might render highly useful for future biomechanical and compositional studies. The preceding approach was to isolate MTDs *ex vivo* from ciliated organisms (Maheshwari *et al.* 2015; Ichikawa *et al.* 2017; Stoddard *et al.* 2018). A first thorough report on MIPs was the structure of the axoneme from *Chlamydomonas* and sea urchin sperm by cryo-ET (Nicastro *et al.* 2006). The achieved resolution was mere 40 Å so that only three luminal structures (denoted MIP1–3) were discerned, with no subunits. These MIPs were later proven to be multi-subunit complexes rather than individual peptides (Maheshwari *et al.* 2015; Ichikawa *et al.* 2017). Anyway, by recognizing MIPs as a consistent and periodical feature of MTDs, this experiment established

a framework for a novel research topic. The authors even stated: "Many proteins associate with the outer surfaces of doublet MTs, but we did not expect to find periodic densities on the inner surfaces of A- and B-tubules in both sea urchin and *Chlamydomonas* axonemes." Reaching resolution of ~19 Å, an article by (Maheshwari *et al.* 2015) described the spatial distribution of MIPs in MTDs in relation to assigned α- and β-tubulin subunits in the lattice by utilizing β-tubulin-specific kinesin decorations in *Tetrahymena*.

Today, even better-resolved structure (~5.7 Å) based on *Chlamydomonas* cilia is available, again from single particle cryo-EM analysis (Ichikawa *et al.* 2017). This latest model coined many additional subunits of MIPs. In this high-resolution model, 29 putative MIPs (including subunits) were detected. Their spatial distribution and the naming convention are depicted in Fig. 3. A more detailed model is still needed, though, since the overall resolution of 5.7 Å does not permit full discerning which densities are independent polypeptides. Henceforth, new MIPs have been added, e.g. by (Stoddard *et al.* 2018), indicating these numbers not to be final. It is now unequivocal that nearly the whole luminal surface of axonemal MTDs is covered in MIPs (evident in Fig. 4). Their identity (summarized in Table 1) is mostly unknown, as well as their function (Stoddard *et al.* 2018; Kirima and Oiwa 2018). Loops in the tubulin structure which are thought to interact with MIPs (practically whole luminal surface of tubulin) are more conserved in ciliated organisms than in cilia-lacking species. This observation suggests that correct interactions between MIPs and tubulin lattice are necessary for proper flagella motility (Ichikawa *et al.* 2017). On top of that, a similar pattern of MIPs arrangement was found in *Tetrahymena*, *Chlamydomonas* and sea urchin sperm, further validating this hypothesis (Maheshwari *et al.* 2015).

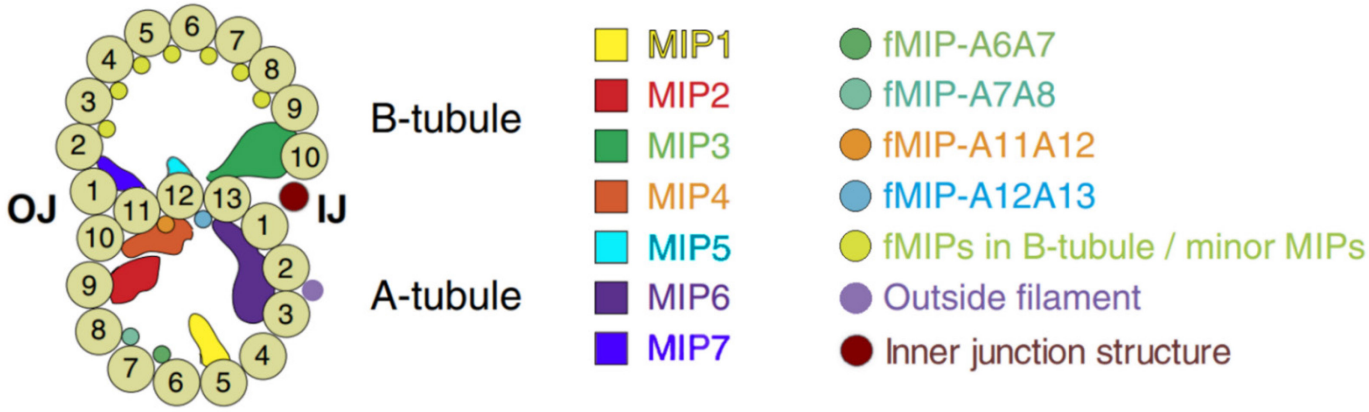

Fig. 3: Naming convention and schematic localization of globular MIPs (various shapes) and fMIPs (small circles) in a flagellar MTD. Tubulin PFs of A- and B-tubules are numbered; OJ, outer junction (formed by closed tubulin lattice), IJ, inner junction (formed by MIPs). Reprinted from (Ichikawa *et al.* 2017), modified.

Aside from MIPs with common globular protein shapes, filamentous MIPs (fMIPs) were observed in axonemal MTDs (Ichikawa *et al.* 2017). MIPs of both morphologies show various interactions with surrounding proteins; some fMIPs in B-tubule were not detected to have other contacts besides those with tubulin, though. The observed shapes and thickness of these fMIPs lead to the conclusion that they are mostly made of

extended α-helices or coiled-coil motifs. Even though the term fMIPs was coined in recently (Ichikawa *et al.* 2017), outer filamentous MAPs in the axoneme were observed before (Nicastro *et al.* 2006). Surprisingly, some of the MIPs partially penetrate the holes between tubulins of adjacent PFs. Cryo-EM revealed contacts spanning through the tubulin lattice: MIP2a and MIP4c, located in A-tubule, interact with MIP7, located in the B-tubule (Ichikawa *et al.* 2017) (Fig. 4). Stoddard then suggested that MIPs can regulate the binding of dynein and other structural components of cilia to the external surface of MTDs. Thus, MIPs might control ciliary motility, frequency of beating, and shape changes during the stroke cycle (Stoddard *et al.* 2018).

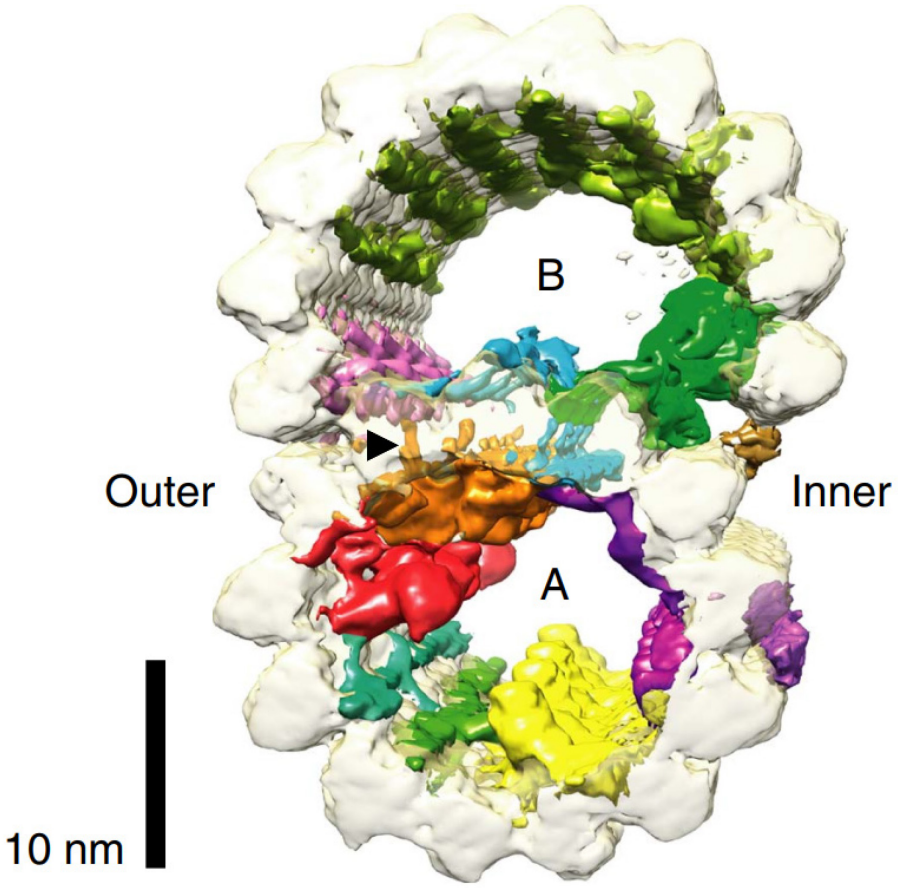

Fig. 4: Structure of an axonemal MTD from cryo-EM. MIPs are in colour, tubulin in white. The black arrowhead marks the tubulin trans-lattice MIPs interaction. A, B – A-tubule and B-tubule, respectively. Doublet outer part and inner part (facing to the centre of the axoneme) is denoted. Reprinted from (Ichikawa *et al.* 2017), modified.

There is a tight structural relation between the tubulin lattice and bound MAPs. The axoneme has 96-nm- repeating architecture (Oda *et al.* 2014), which is transferred to all MTDs associated complexes, like MIPs (Table 1), dynein or nexin arms. The elemental repeating unit is the αβ-tubulin dimer with a repeat length of 8.0 nm (Linck 1976; Nogales *et al.* 1999). MIPs bind either to a single PF, two adjacent PFs, or two non-neighbouring PFs (Maheshwari *et al.* 2015). Also, all possibilities of tubulin units binding (α-tubulin only, β-tubulin only, both subunits of the same dimer, two subunits of adjacent dimers of the same PF) were detected (Maheshwari *et al.* 2015; Ichikawa *et al.* 2017).

Table 1: Summary of known MIP structures, their identity (together with UniProt database entry identifier code for *Chlamydomonas* homolog), binding site, and periodicity. Only one fMIP is listed for lack of data. * denotes binding from the outside of given protofilament (in the ribbon region). (Ichikawa *et al.* 2017) is a reference for all entries except for MIP3c, MIP4e, MIP6c, MIP6d, and other ribbon associated MIPs.

Complex	**Subcomplex**	**Identity (UniProt entry identifier)**	**Binding site**	**Periodicity, nm**	**Reference**
MIP1	MIP1a	FAP85 (A8J250)	A4, A5, A6	48	(Kirima and Oiwa 2018; Stoddard *et al.* 2018)
	MIP1b	?	A4, A5, A6	16	(Stoddard *et al.* 2018)
MIP2	MIP2a	?	A9, A10, A11	48	
	MIP2b	?	A8, A9, A10	48	
	MIP2c	?	A8, A9, A10	48	
MIP3	MIP3a	FAP52 (A8ILK1)	A12*, A13*, A1*, B9, B10	16	(Owa *et al.* 2019)
	MIP3b	?	A12*, A13*, A1*, B9, B10	16	
	MIP3c	FAP45 (A8I9E8)	B7, B8, B9	48	(Owa *et al.* 2019)
MIP4	MIP4a	?	A10, A11, A12	48	(Stoddard *et al.* 2018)
	MIP4b	?	A10, A11, A12	48	(Stoddard *et al.* 2018)
	MIP4c	?	?A10, A11, A12	48	(Stoddard *et al.* 2018)
	MIP4d	?	A10, A11, A12	48	(Stoddard *et al.* 2018)
	MIP4e	RIB72B (Q8LKK4)	A11, A12	48	(Stoddard *et al.* 2018)
MIP5	MIP5a	?	A11*, A12*	48	
	MIP5b	?	A11*, A12*	48	
MIP6	MIP6a	?	A13, A1, A2, A3	16	(Stoddard *et al.* 2018)
	MIP6b	?	A13, A1, A2, A3	16	(Stoddard *et al.* 2018)
	MIP6c	?	A2, A3	8	(Stoddard *et al.* 2018)
	MIP6d	?	A2, A3	8	(Stoddard *et al.* 2018)
MIP7	—	?	A11*, B1, B2	16	
Inner junction structure	—	FAP20 (A8IU92)	A1*, B10	4/8?	(Yanagisawa *et al.* 2014)
Other ribbon associated MIPs	—	RIB43a (Q9M6B0)	?	?	(Norrander *et al.* 2000)
	fMIP A11A12?	tektin (A8J8F6)	A11?, A12?	various	(Norrander *et al.* 1996; Sui and Downing 2006)
	—	PACRG (B1B601)	?	?	(Ikeda *et al.* 2007)

Regarding stability, the PFs in a MTD are heterogeneous. This was illustratively documented by solubilization of axonemal components by increasing concentrations of detergent sarkosyl (*N*-lauroylsarcosine) (Witman *et al.* 1972); same order of fractions was obtained with $NaNO_3$ extraction (Orbach and Howard 2019). Central singlets are dissolved first (singlet of higher and lower stability can be distinguished). Next, B-tubule is partially disassembled together with the second central singlet, but PF B1 remains attached to A-tubule. Eventually, the A-tubule disaggregates into individual PFs and so-called ribbon region (Witman *et al.* 1972). Ribbon region is the most stable MT structure in the axoneme; it is composed of PFs A11, A12, and A13, but sometimes, additional PFs were present (Linck and Langevin 1982). Such endurance is caused by several MIPs binding to that region (Linck 1976; Hinchcliffe and Linck 1998). Low stability of central singlets, on the other hand, could be linked to the lack of MIPs in them.

There are almost no data on lumen entry for structural MIPs. Findings which are valid for αTAT1 do not necessarily apply to stably bound MIPs. MT structure, dynamicity, and lattice defects determine which ways of lumen entry are possible and what will be the turnover of MIPs. Stabilized axonemal MTs differ markedly from cytoplasmic in these parameters (Orbach and Howard 2019), although even axonemes do treadmill (Stephens 1999; L. Song and Dentler 2001). Some proteins of the axoneme appear to have negligible turnover. For instance, flagellar associated protein (FAP) 20 is not transported via intraflagellar transport. It enters the cilium during formation period probably just by diffusion and incorporates into the MTDs from proximal to the distal part of the axoneme. Newly synthesized FAP20 would not incorporate into previously assembled axoneme (Yanagisawa *et al.* 2014). On the other hand, more than 80 polypeptides of the axoneme are being exchanged with cytoplasmic pool. Among these, ribbon-associated protein (RIB) 43a and other ribbon components were replaced to a low extent, but tektin, a component of the ribbon region, turns over rapidly (Stephens 2000). Importantly, tubulin turnover occurs independently of exchange of other axonemal components (L. Song and Dentler 2001). This claim argues against MIPs being incorporated into MTDs by associating to treadmilling tubulin.

4.2 Identified MIPs

4.2.1 Flagellar associated protein FAP20

FAP20 constitutes the inner junction structure between PFs A1 and B10 in the axoneme and basal body (Yanagisawa *et al.* 2014). Other proteins must colocalize to this structure since FAP20 alone is not large enough to account for the whole density. FAP20 is a very basic protein – positively charged residues could plausibly interact with the acidic tails of tubulin facing outwards. This interaction is possible since the inner junction structure is partially accessible from the outside. The protein contains a domain of unknown function 667 (DUF667), which was found in other proteins possibly linked to cilia (Yanagisawa *et al.* 2014).

No turnover of FAP20 was observed, suggesting immensely slow dynamics in axonemal MIPs. The human homolog of FAP20 localizes to primary cilia (Mendes Maia *et al.* 2014) and has 89 % amino acids sequence identity with *Chlamydomonas*. Zebrafish FAP20 knock-down showed phenotypes linked to ciliopathies, such as defects in heart-looping (Yanagisawa *et al.* 2014). *Drosophila* FAP20 mutant males produced immotile spermatozoa. Mutant strains of *Chlamydomonas* were unable to swim and their axonemes were less stable. Alongside with inner junction structure, "beak-like structures" were also lost, although FAP20 is not their direct component. FAP20 likely connects to these structures via other MIPs of the B-tubule. Other roles of FAP20 outside MT lumen are also possible because the protein is enriched in the nucleus and nucleolus in *Drosophila*, too (Mendes Maia *et al.* 2014).

4.2.2 Flagellar associated proteins FAP45 and FAP52

FAP45 was linked to MIP3c density in B-tubule (Owa *et al.* 2019). FAP52 was identified as large MIP3a, which bridges the inner junction and, together with FAP20, connects to the outside of the A-tubule (Fig. 3). MIP3a interacts with MIP3c, too. Both FAP45 and 52 are conserved proteins among eukaryotes with motile cilia. Predicted tertiary structures of both proteins fit into observed densities – FAP45 possesses coiled-coil structure; the human ortholog is accordingly called coiled-coil domain-containing protein 19 (CCDC19) (Owa *et al.* 2019). CCDC19 is expressed preferentially in the human nasopharyngeal epithelium and was linked to inhibition of nasopharyngeal carcinoma growth (Liu *et al.* 2012). FAP52 human ortholog is WD40 repeat domain 16 (WDR16) (Owa *et al.* 2019), which was shown to be associated with laterality disorders in human, caused erroneous nodal cilia beating (Ta-Shma *et al.* 2015). *Tetrahymena* FAP45 and 52 single knock-out strains exhibited only mild or insignificant perturbations in swimming speed. The double mutant, however, swam significantly more slowly with decreased beating frequency (Owa *et al.* 2019). Expectedly, B-tubules were more labile in mutants compared to wild-type.

4.2.3 Flagellar associated protein FAP85

FAP85 constitutes part of the MIP1a density (Kirima and Oiwa 2018). In several identified MIPs (FAP85 is among them), the EF-hand motif was found (Kirima and Oiwa 2018; Stoddard *et al.* 2018). This motif serves for Ca^{2+} binding (Kretsinger and Nockolds 1973) and probably regulates the beating of whole flagella (DiPetrillo and Smith 2010). No apparent homolog of FAP85 was found in human. There are no data on FAP85 mutant phenotypes.

4.2.4 Tektins

Several proteins associated with the ribbon region of axonemal A-tubules were detected using polyacrylamide gel electrophoresis and thoroughly studied by R. Linck (Linck 1976; Linck, Amos, and Amos 1985). These proteins, present in axonemal doublets, centrioles, and basal bodies but missing from cytoplasmic MTs

(Amos, Amos, and Linck 1985), reviewed in (Linck 1990), were named tektins (Linck *et al.* 1982). They constitute filaments 3-nm-wide, more resistant to solubilization than tubulin PFs of ribbon region, and with remarkably high helical content. They closely resemble intermediary filaments by their α-helical segments forming coiled-coil structure, interrupted by a short linker (Linck and Langevin 1982; Amos, Amos, and Linck 1986; Norrander *et al.* 1996). The tektin filament is composed of tektin-A and B heterodimers and tektin-C homodimers in equimolar ratios (Linck and Stephens 1987; Pirner and Linck 1994; Norrander *et al.* 1996). Tektins were even believed by some (Nojima, Linck, and Egelman 1995) to make up one of the 13 PFs of the A-tubule instead of tubulin.

Tektins contain specific 9-residue-long signature motif (Norrander *et al.* 1996) and are used in phylogenetic studies (Whinnett *et al.* 2006). A recent article assessed the evolution of tektin genes among eukaryotes – they are surprisingly missing in many lineages with flagella (e.g. the ciliates or placozoans) (Bastin and Schneider 2019). At least five tektin genes are present in human, some of them are expressed exclusively in cilia/flagella-bearing cells, e.g. sperm cells (Bastin and Schneider 2019; reviewed by Amos 2008). Despite great effort, the detailed structure of tektin polymers was not revealed. Linck suggested that tektin might play a role as a "ruler" (mentioned in a review Linck and Norrander 2003), but this was ruled out by Oda and collaborators, who identified FAP59/129 to be the 96-repeat imposing component in MTDs (Oda *et al.* 2014). Mutation in tektin leads to impaired tracheal epithelium and sperm cells motility in mice, rendering the males infertile and lowering tracheal debris clearance capacity (Tanaka *et al.* 2004).

4.2.5 Ribbon-associated protein RIB43a

A component of stable ribbon region of basal body and axoneme with molecular weight 43 kDa was isolated in *Chlamydomonas* and named as RIB43a (Norrander *et al.* 2000). Although predicted to possess coiled-coil structure and forming thin fibrils, RIB43a homology to tektins is low. Expression of a murine homolog is restricted to testes (Arango *et al.* 2004). No research has been conducted on human homolog and no ciliopathies have been linked to RIB43a mutation so far.

4.2.6 Ribbon-associated protein RIB72

When RIB72A and B (72 kDa) are missing in mutant strains of *Chlamydomonas*, MIP1, 4 and, 6 are partially or completely depleted from the A-tubule lumen. Specifically, RIB72B constitutes the MIP4e density in the A-tubule (Stoddard *et al.* 2018). The attempt to assign RIB72A to a density in MTDs lumen was unsuccessful. In *Chlamydomonas*, association of RIB72 to the ribbon region was shown by (Ikeda *et al.* 2003). RIB72A/B probably interact with other ribbon region MIPs, tektin, and RIB43a. RIB72 presumable homologs from sea urchin (termed Sp77 and Sp83) were found to localize to basal bodies and ribbon regions of axonemal MTDs. Sp83 antibody also stained centrioles and partially astral tubules, even in human cells (Hinchcliffe and Linck 1998). Intriguingly, Sp83 antibody seemed to additionally stain central pair in the axoneme, too. RIB72A and B both

localize to basal bodies and cilia in *Tetrahymena* (Stoddard *et al.* 2018), but only in cilia and not basal bodies in *Chlamydomonas* (Ikeda *et al.* 2003). RIB72 conserved homologs in other species typically contain three DM10 domains, which are needed for proper ciliary localization. Human RIB72 homolog hsEFHC1 is however expressed widely in soma and dendrites of neurons (Suzuki *et al.* 2004), suggesting that RIB72 might have gained new roles and cellular localization in vertebrates. Another common but not universal feature of the RIB72 protein family is the EF-hand motif – it is present in RIB72A, but not in RIB72B (Ikeda *et al.* 2003; Stoddard *et al.* 2018).

RIB72A knock-out *Tetrahymena* strain manifests no change in the localization pattern of RIB72B, and vice versa (Stoddard *et al.* 2018). Double knock-out strain for both these proteins reached only half of the maximum swimming speed compared to the wild-type strain, also the beating frequency of cilia was lowered. Furthermore, cilia of mutant *Tetrahymena* strain were abnormally curved and desynchronized. Mutation of human homolog hsEFHC1 was shown to result in juvenile myoclonic epilepsy by increasing calcium current in neurons (Suzuki *et al.* 2004).Parkin-coregulated gene product protein (PACRG) is a potential interacting partner of RIB72 (Ikeda *et al.* 2007). It localizes alongside the whole axoneme and basal body. It could be extracted only in higher sarkosyl concentrations, therefore it is probably buried inside MTDs lattice, presumably nearby the ribbon region. More robust evidence is missing, though.

4.2.7 Tau

Neuronal axon-specific (Binder, Frankfurter, and Rebhun 1985) protein tau was identified as a MAP long time ago (Witman *et al.* 1976). Tau stabilizes MTs and promotes their nucleation (Drechsel *et al.* 1992; Brandt *et al.* 1994). Tau is intrinsically disordered (von Bergen *et al.* 2005) and retains such properties even when bound to MTs (Kadavath *et al.* 2015). In contrast, insoluble plaque-forming misfolded tau triggering Alzheimer's disease contains highly structured β-sheets (von Bergen *et al.* 2005), reviewed in (Ittner and Götz 2011).

Tau can bind either MTs or free tubulin dimers (Kadavath *et al.* 2015). Binding is facilitated, aside from other parts, by three or four sequence repeats, but they render nonefficient when isolated from the rest of the protein. Rigid stoichiometry of one tau molecule per two tubulin dimers was observed, confirming that it incorporates in the MT lattice (Witman *et al.* 1976; Gustke *et al.* 1994). Tau protein binds from the exterior of MTs but possibly protrudes through the lattice fenestrations. Inside, tau reaches taxol-binding pocket on β-tubulin, as shown by immunogold cryo-EM assay. The loop on tau which binds to the taxol-pocket on tubulin is sequentially similar to a loop on α-tubulin which interacts with the pocket when neither tau or taxol is present (Kar *et al.* 2003). Inaba and colleagues designed a tau-derived peptide which interfered with binding kinetics of taxol (Inaba *et al.* 2018). Contradict data were published, though, reporting tau to bind at the interface of tubulin heterodimers and not interfering with the taxol pocket (Kadavath *et al.* 2015). Tau knock-out mice

are viable and without severe pathologies; neuronal aberrations arise in older animals, summarized in (Ke *et al.* 2012).

4.3 MIPs in basal bodies and centrioles

Cryo-ET structures from *Chlamydomonas* basal bodies showed that the arrangement of basal body MIPs is similar to that of axonemes (Fig. 5) (Li *et al.* 2012). Some differences in reported repeat patterns might be attributed to overall low resolution – a more detailed structure is needed to carefully evaluate repeat distances. The density corresponding to MIP3 which connects A- and B-tubule had a 4-nm repeat: That means two molecules of this MIP are bound to one tubulin dimer PFs A13 and B8. Nearby, a complex filling the inner junction of A/B-tubule interface is visible. This density called the inner junction complex and identified as FAP20 in the axoneme (Yanagisawa *et al.* 2014) lead to misinterpretations of old EM micrographs and claims that B-tubule is composed of 11 PFs (Tilney *et al.* 1973; Linck 1976). Besides the aforementioned similarities, some MIPs and MAPs are unique for the basal body. Intriguingly, there was a sudden change of elemental tubulin monomer repeat length alongside the PF C1 from 4 nm (proximally) to 8 nm (distally). New densities emerged behind this transition, one of them was a crescent-shaped MIP in the C-tubule, connecting PF C3 to C6 and C7 (Li *et al.* 2012). The authors attribute this phenomenon to the presence of δ-tubulin in C-tubule. Apparently, there are zones with distinct architecture alongside the basal body – axoneme continuum.

Recently published work dealing with procentrioles – precursors of centrioles – from *Chlamydomonas* achieved resolution of 2.1 nm and discerned 11 MIPs total, distributed in all three tubules (Fig. 6) (Li *et al.* 2019). They all had a periodicity of 8 nm except for MIP2, which had 4 nm. These data are in striking contrast with what is published from the axoneme; again, missing details in low resolution might be the cause. 7 of 11 identified MIPs are located nearby the inner junctions; MIP9 closely resembles 11th tubulin PF with its 8-nm periodicity. In mammalian and *Drosophila* mature centrioles, 4 MIPs were distinguished. MIP1, otherwise constantly present in structures, was not among them (Greenan *et al.* 2018).

In conclusion, some MIPs are common in all structures observed, others are missing from several, some seem to be unique to a certain structure. For instance, MIP2, associated with PF A9, is constantly seen in the axoneme, basal body and procentriole of several species including mammals (Greenan *et al.* 2018; Ichikawa *et al.* 2017; Li *et al.* 2012, 2019). Precise naming convention should be developed to prevent confusion among centriolar, basal body and axonemal MIPs. Interspecific differences could further complicate the situation. A recent observation of MIPs arranged into an interrupted helix in human spermatozoa distal tip illustrates this problem (Zabeo *et al.* 2018) – such architecture of MIPs was not previously observed in any model organism.

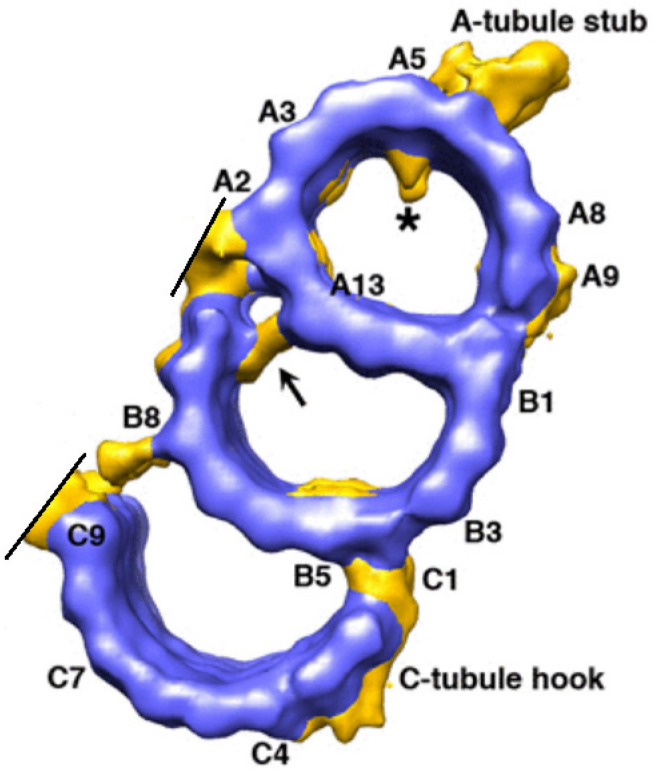

Fig. 5: MIPs associated with basal body microtubule triplet of Chlamydomonas, cryo-ET data. Microtubule lattice in purple, MIPs densities in yellow. Several PFs are labelled. * marks density similar to axonemal MIP1, arrow points to density bridging PFs B8 and A13 in inner junction, similar to MIP3. Reprinted from (Li *et al.* 2012), modified.

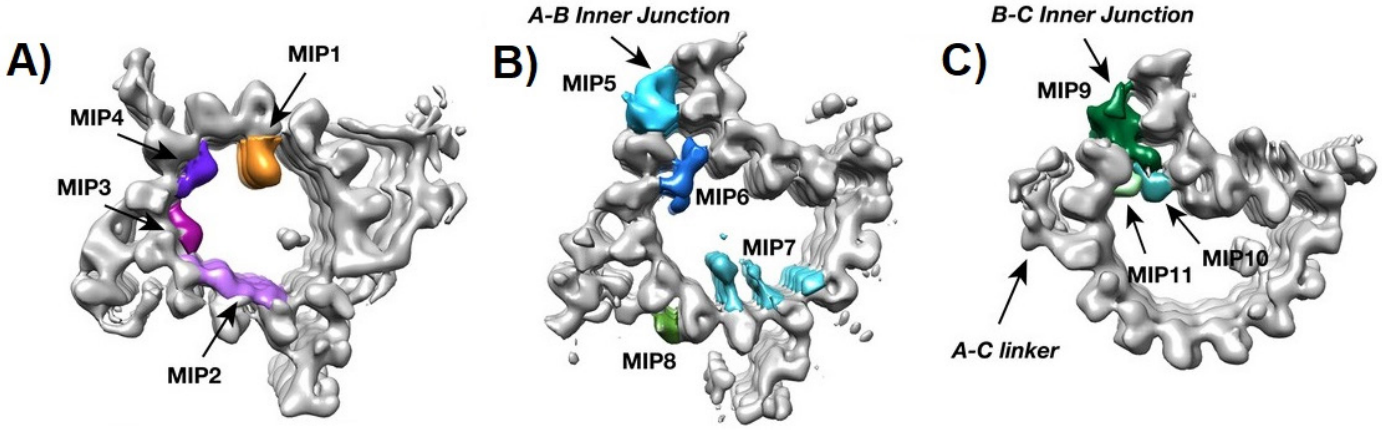

Fig. 6: MIPs associated with procentriole triplet of *Chlamydomonas*, cryo-ET data. **A)** A-tubule. **B)** B-tubule. **C)** C-tubule. Reprinted from (Li *et al.* 2019), modified.

5. Conclusions

First direct observation of MIPs in the microtubule lumen is dated more than 50 years back (Bassot and Martoja 1966). MIPs have attracted the attention of scientists since then, but only with recent tools, we are able to truly appreciate the structural and functional importance. There were considerable breakthroughs in the last two decades – the revelation of αTAT1 residence inside MTs and three-dimensional reconstruction of MIPs in axonemal doublets. These findings triggered many following discoveries on MIPs.

Some light was already shed on the roles which MIPs play in the inner microtubular space. Two main purposes emerged so far – involvement in tubulin post-translational (de)acetylation and enhancing the resilience of axonemal MTDs. Future work will likely restate these two categories and add more functions we are not aware of yet. Interestingly, dense luminal material was initially proposed to be transported via microtubules like in "pipes" (Rodríguez Echandía, Piezzi, and Rodríguez 1968). Burton, too, supported such a notion (Burton 1984). (Garvalov *et al.* 2006) concluded the same. This hypothesis has never had many proponents and no rigorous proof has been ever presented.

There is an obvious dichotomy between the two classes of MIPs. Structural regularly repeating MIPs have not been reported in unstable cytoplasmic microtubules, with an alleged exception of tau. One can simply justify this lack of structural components in dynamic MTs by the predominant stabilizing effect of these MIPs which is undesirable in general cytoplasmic microtubular net. On the other hand, αTAT1, an active enzyme, targets all kinds of MTs (although linked mostly to stable ones) with stochastic probability and without regular repeating. No models on how αTAT1 makes its way through the densely packed lumen of axonemal MTDs to access the acetylation site have been presented. Also, the acetylation pattern (e.g. PFs preference) is not known.

One may speculate whether the lumen of MTs could be considered as an independent cellular compartment. The relevance of MT compartment for cell physiology is elusive. Observed protrusions through MT lattice support MIPs–MAPs theoretical interactions and signal transduction from lumen to the outside. MTD wall arrangement may reflect changes in MIPs bound inside, thus enabling interactions with different MAPs from the outside. On the contrary, MTs inner binding capacity, volume, and potential to transport material are probably negligible compared to the bulk mass of cytoplasm. Either way, the inner binding capacity of microtubules might be utilized for drug delivery, slowly releasing the luminal content (e.g. bound taxol) via the two relatively small terminal opening (Odde 1998). This idea was recently elaborated further using a tau-derived peptide (Inaba *et al.* 2018).

Future research of inner MT components is of high importance. Not only it could elucidate more about the nature of the microtubular scaffold itself, it might also help to diagnose and cure some of the numerous

ciliopathies in human. Some of the newly discovered MIPs could account e.g. for α-tubulin acetylation readout since this modification does not induce any measurable change in the lattice distinguishable from the outside (Howes *et al.* 2014). That way, MIPs would be a crucial factor for interpreting the code of tubulin structural and signal properties (Cross 2019). Additionally, MIPs are likely indispensable elements in cilia assembly steps which are now poorly understood. Hopefully, an atomic-level structure of the axoneme and its MIPs will be obtained soon – it will immensely boost the pace of microtubule inner proteins research.

I plan to use the insight gained during writing this thesis in my master's project. I will focus on unstable cytoplasmic subset of MTs and try to identify candidates for MIPs residing in them. Conveniently, I can take advantage of αTAT1 being identified as a MIP and use it as a positive control during the proteomic analysis of MAPs in microtubular fraction.

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