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Review

Direct and indirect effects of tubulin post-translational modifications on microtubule stability: Insights and regulations

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ABSTRACT

Microtubules (MTs) mediate various cellular functions such as structural support, chromosome segregation, and intracellular transport. To achieve this, the pivotal properties of MTs have to be changeable and tightly controlled. This is enabled by a high variety of tubulin posttranslational modifications, which influence MT properties directly, *via* altering the MT lattice structurally, or indirectly by changing MT interaction partners. Here, the distinction between these direct and indirect effects of MT PTMs are exemplified by acetylation of the luminal α-tubulin K40 resulting in decreased rigidity of MTs, and by MT detyrosination which decreases interaction with depolymerizing proteins, thus causing more stable MTs. We discuss how these PTMs are reversed and regulated, e.g. on the level of enzyme transcription, localization, and activity *via* various signalling pathways including the conventional calcium-dependent proteases calpains and how advances in microscopy techniques and development of live-sensors facilitate the understanding of MT PTM interaction and effects.

1. Introduction

Microtubules (MTs) are essential cytoskeletal elements in all eukaryotic cells. They participate in numerous cellular processes ranging from defining the cell shape, forming the mitotic spindle, and mediating intracellular trafficking, to cell migration and differentiation. Their structure, dynamics, and mechanics are modulated by the repertoire of different tubulin isoforms and posttranslational modifications, the so-called "tubulin-code" [1].

MTs are hollow tubes that are formed of mostly 13 protofilaments, strings of α- and β-tubulin dimers (Fig. 1A). The end with α-tubulin is called the minus (−) end and commonly is less dynamic than the end with β-tubulin, named plus (+) end. Both α- and β-tubulin bind guanosine triphosphate (GTP) and the polymerization to MTs is induced when a critical concentration of α-β-tubulin dimers (both monomers bound to GTP) is available. β-Tubulin catalyses the hydrolysis of GTP thereby acting as a GTPase. When the GTP hydrolysis is faster than the addition of new dimers, dimers containing guanosine diphosphate-bound β-tubulin reach the +end and the MT will depolymerize, a process called catastrophe.

MTs are highly dynamic and oscillate between periods of growth and depolymerization. This process is called dynamic instability and is highly regulated. Tubulins can undergo many chemically distinct post-translational modifications (PTMs): acetylation, methylation, phosphorylation, palmitoylation, polyamination, detyrosination, polyglycylation, polyglutamylation, ubiquitinylation and sumoylation (Fig. 1A) [2]. PTMs can label a subset of MTs as tracks for specific cargo-motor protein complexes [3], modulate interaction with certain microtubule-associated proteins (MAPs) that, for example, bundle MTs together, or change their stability by recruiting MT severing proteins or polymerization promoting factors, thereby acting as molecular switches of MT function [2].

Abbreviations: Acetyl-CoA, acetyl coenzyme A; aTAT1, α-tubulin-*N*-acetyltransferase; ATP, adenosine triphosphate; FRET, Förster resonance energy transfer; fry, furry; GTP, guanosine triphosphate; HDAC, histone deacetylase; MAPs, MT associated proteins; MTs, microtubules; NDST3, *N*-deacetylase and N- sulfotransferase 3; NLS, nuclear localization signal; PKA, protein kinase A; PKC, protein kinase C; PTM, posttranslational modification; ROS, reactive oxygen species; SIRT2, Sirtuin 2; SVBP, small vasohibin binding protein; TTL, tubulin tyrosine ligase; VASH, Vasohibin.

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Different tubulin modifications can occur on the same MT thereby adding further complexity to the "tubulin-code". Interestingly, C-terminal truncations of tubulin (detyrosination or possibly Δ2 and Δ3) and acetylation of α-tubulin frequently *co*-exist on the same polymer [4] and are both associated with increased stability of MTs. Tubulin detyrosination is an evolutionarily conserved process that affects almost all α-tubulin isoforms and occurs in a great variety of invertebrate and vertebrate species [2,5]. It is mediated by a group of enzymes termed tubulin tyrosine carboxypeptidases, a major one of these is the vasohibin (VASH)/small vasohibin binding protein (SVBP) complex [6,7]. Interestingly, tubulin detyrosination is a reversible process. After depolymerization of the MTs, the re-ligation of the tyrosine residue by tubulin tyrosine ligase (TTL) enables returning of the tubulin dimers to the polymerization-depolymerization cycle (Fig. 1C). In contrast to most other modifications labelling the C-terminus of tubulin, MT acetylation is occurring on the lysine 40 residue of α-tubulin oriented to the inside of MTs [8]. This enzymatic reaction is characterized by relatively slow kinetics compared to MT detyrosination thereby long-lived MTs are a prerequisite for efficient acetylation [8]. It is mediated by the α-tubulin-*N*-acetyltransferase 1 (aTAT1) [8] and is, similarly to detyrosination, reversible (Fig. 1D). Deacetylation preferably occurs on tubulin dimers and is mediated by several enzymes including histone deacetylase 6 (HDAC6) and sirtuin 2 (SIRT2) [9].

Due to their abundance and functional outcomes, both types of MT modifications attracted great attention from cell biologists and have been intensively studied in the past decades. The identification of aTAT1 and VASHs further helped to address the functional significance of these PTMs in different model organisms and cellular systems and are highlighted in several excellent reviews [2,10]. On the other hand, the molecular mechanisms regulating the activity of these enzymes are not well understood. In this review, we will focus on mechanistic and biochemical aspects in the regulation of MT detyrosination and acetylation which are determined by protein-protein interactions. We discuss the most recent findings describing the involvement of proteolytic enzymes in MT detyrosination, the role of motile cargo vesicles in MT acetylation, kinetics, and crosstalk between detyrosination and acetylation as well as novel tools which could further progress the field of MT research.

2. Primary and secondary effects of microtubule modifications on stability

2.1. The "tubulin-code"

MTs show diversity on several levels. First, there are different isotypes of both α- and β-tubulin. HGNC Database search reveals the existence of 9 α- and 10 β-tubulins in humans [11]. Most of these are differentially expressed throughout different tissues and can freely incorporate into MT lattices [12]. Therefore, the composition of MTs differs between each tubule and especially between tissues because of the varying incorporation of α- and β-tubulins. Besides α- and β- other tubulin proteins like γ-, δ and ε-tubulins exist, which have specialized functions such as MT nucleation (γ-tubulin) [13] or formation of centrioles (δ- and ε-tubulins) [14].

Second, both MTs and tubulins can be modified posttranslationally. These PTMs (e.g. detyrosination, tyrosination, acetylation) mediate properties of MTs like stability, rigidity, and binding of MAPs [2,15–17]. Furthermore, they enable the formation of MT subgroups which can be

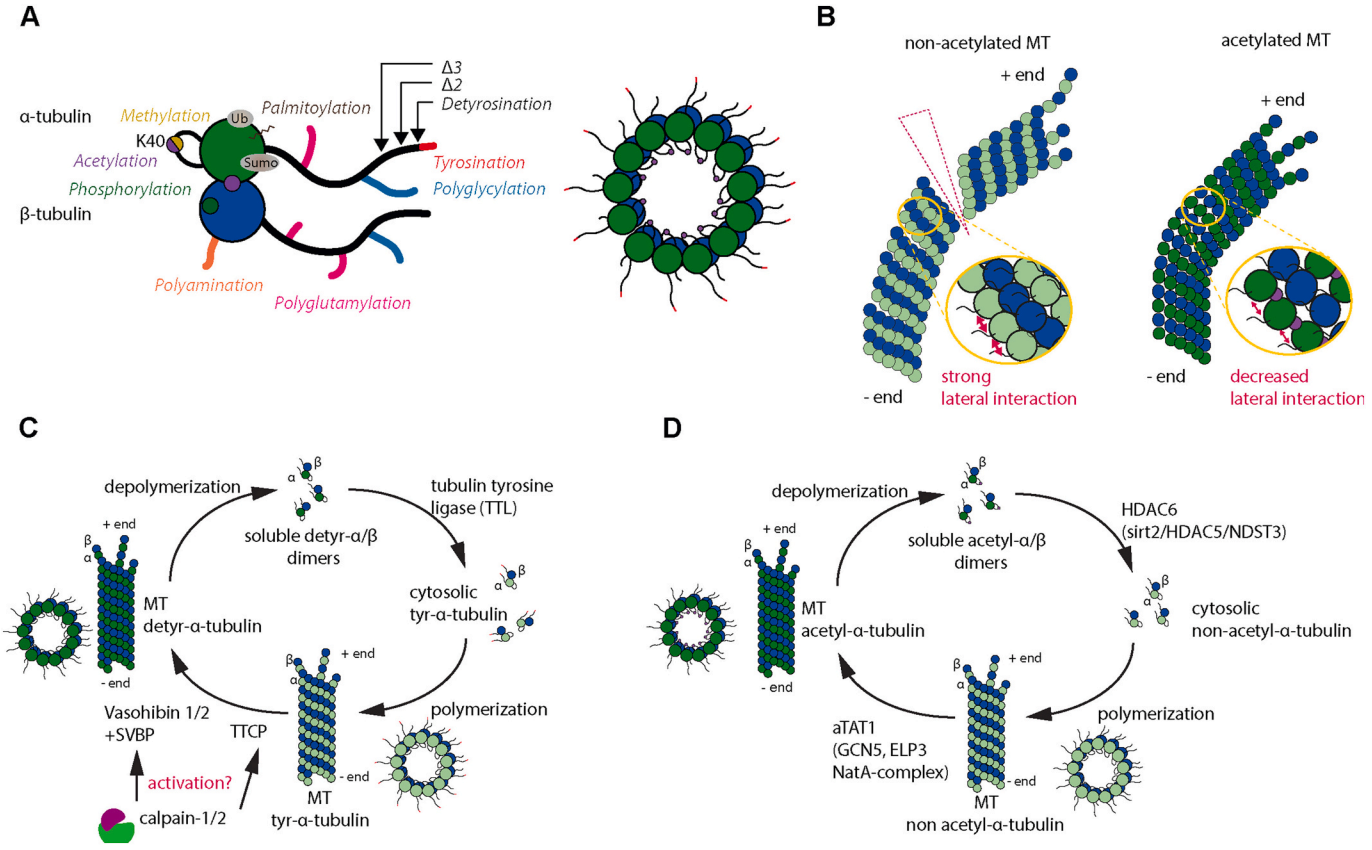

Fig. 1. Microtubule posttranslational modifications and enzymes involved in MT detyrosination and acetylation cycles.
A) Posttranslational modifications of α- and β-tubulin. Most PTMs occur at the C-terminal tails. K40 is the only modified residue within the lumen of MTs. Ub: Ubiquitinylation; Sumo: Sumoylation. Cross-section of an acetylated MT on the right. **B)** K40 acetylation decreases lateral interaction between protofilaments leading to increased flexibility of MTs. **C)** The MT detyrosination/tyrosination cycle including involved enzymes and possible ways of regulation. **D)** The acetylation/deacetylation cycle of MTs with enzymes catalyzing the modifications. Light green and dark green circles depict unmodified and modified α-tubulin.

marked by specific PTMs. The variety of tubulins and MT PTMs form spatial and temporal clues for interaction partners. They regulate cellular functions including transport mechanisms by molecular motors [2,3] and morphological characteristics which are important for cellular shape, cell migration, and adhesion [18]. Conversely, MT interactors play a central role in the regulation of PTMs by controlling the accessibility of the MT lattice or actively recruiting different enzymes. Therefore, the entirety of these features was named the "tubulin-code" [1].

Most PTMs occur on the C-terminal tails of α- or β-tubulin (Fig. 1A). The C-terminal tails are directed outwards from the MT lattice and therefore decorate the MT surface. The variety of PTMs ranges from addition or removal of single or multiple amino acids on the C-terminus of α-tubulin (detyrosination [6,7], tyrosination [19], Δ2- and Δ3-tubulin [20–22] to the addition of long and possibly branched side chains (polyglutamylation [23–25] and polyglycylation [26]) on the C-terminal tails of both α- and β-tubulins or even ligation of peptides (sumoylation [27]) and proteins (ubiquitinylation [28]). Other possible PTMs are polyamination of Q15 [29], phosphorylation of S172 [30], palmitoylation [31] and acetylation of K252 on β-tubulin [32] (Fig. 1A). The only known residue in the MT lumen that can be modified is K40 of α-tubulin which can be acetylated [33] and methylated [34,35]. This localization is remarkable because 1) acetylation occurs mainly on polymerized MTs and the enzyme mediating the acetylation, aTAT1, has to reach its substrate (the K40 methyltransferase SET domain containing 2 is preferring tubulin dimers) [36] and 2) the effects of K40 modifications have to be mediated from the lumen of MTs [8]. In the following chapters, we will focus on detyrosination and MT K40 acetylation as examples of different mechanisms to regulate MT stability.

2.2. *Effects of microtubule posttranslational modifications on stability*

PTMs of MTs can have a direct effect on MT structure and thus parameters such as stability and rigidity, or influence MT characteristics indirectly *via* MAPs. Although α-tubulin K40 acetylation was correlated with long MT lifetime [37] and is important for kinesin-1-mediated transport [3], the effects of acetylation on MTs, especially on the structure and stability, were unknown until recently. A study by Eshun-Wilson et al. from 2019 showed differences in the structure of the MT lattice between acetylated and deacetylated MTs using cryo-electron microscopy and molecular dynamics simulations [15] (Fig. 1B). They demonstrated that acetylation of K40 changes the conformation of the flexible loop containing K40 inside the MT lumen and reduces the degrees of freedom of this loop. Their results indicate that acetylation reduces the lateral interaction between MT protofilaments and the interaction surface of two α-tubulins (Fig. 1B). The acetylated K40 is more directed towards the hydrophobic inner core and has a bigger distance to the M-loop of the second α-tubulin and therefore cannot support the important interaction between K60 of the first α-tubulin and H283 of the second α-tubulin. These results could explain the findings of Portran et al. from 2017 that acetylated MTs are less rigid and therefore making them more resilient against mechanical stress [38]. This direct effect of a PTM on the MT structure is unique among MT PTMs.

In contrast to this primary effect, secondary effects on stability are mediated by interaction with MAPs which are recruited on modified MTs and are able to (directly or indirectly) alter the mechanic properties of MTs. The unstructured C-terminal tails of tubulins are located on the outer surface of polymerized MTs. This exposed localization predestines their modifications to influence the interaction with MAPs [39,40]. Structural MAPs like MAP1 and MAP2 conduce to the stability of MTs by binding along the length of the tubule. This interaction is influenced by the PTMs decorating the MTs. Intermediate levels of polyglutamylation result in the highest binding affinity of MAP1B and MAP2 to MT lattice while MAP1A prefers highly polyglutamylated MTs [41]. Also binding of tau is dependent on modifications of the C-terminus of tubulins. Its interaction with unmodified α- and β-tubulins is negligible but increases with addition of glutamates on the C-termini. Again, the affinity decreased on highly modified MTs [42]. Tau was thought to stabilize MTs but recently this idea was challenged as tau was shown to be present at the dynamic ends of MTs which can be mainly found at growth cones of neurons [43].

Besides static MAPs also the interaction of end binding proteins and motor proteins with MTs is strongly influenced by PTMs. Tyrosinated MTs, for instance, recruit depolymerizing motors like kinesin-13 or mitotic centromere-associated kinesin (MCAK) [44] and proteins that enhance polymerization like cytoplasmic linker protein (CLIP170) [45–47], thereby increasing MT dynamics, which in turn reduces the stability and lifetime of MTs. Contrary, detyrosination results in decreased binding of these proteins, thus detyrosinated MTs are protected from active depolymerization by e.g. MCAK and KIF2A [44,48]. This makes detyrosinated MTs more stable and they are preferred tracks for active transport *via* the processive motors kinesins-1 and -2, that show increased binding, velocity and processivity on detyrosinated MTs [48,49]. This notion is supported by a recent paper from Chen et al. that shows that detyrosinated, tyrosinated and Δ2 tubulin have no effect on the dynamics of MTs *in vitro* [50]. However, once interaction partners EB1 and H2 (truncated form of CLIP-170) were added, an increase in dynamics was observed with increasing tyrosination levels.

However, detyrosination is not the only PTM influencing MT-MAP interactions causing changes in MT dynamics. Initial polyglutamylation is recruiting spastin which leads to severing of the MT [51,52]. This results in breakage and appearance of shorter MTs, but it can increase MT polymerization due to the increase in available +ends. Further polyglutamylation inhibits spastin action [52] illustrating the complexity, specificity and interactivity of PTM effects on MTs. Additionally, detyrosination and polyglutamylation also modulate the motility of motor proteins on MTs. Kinesin-2 motor proteins for example are preferentially binding to detyrosinated and slightly polyglutamylated MTs, which leads to overall higher motility because of greater processivity [48]. Kinesin-1 motility is increased on MTs with high levels of polyglutamylation [48] in contrast to axonemal dynein which shows higher motility on MTs lacking long polyglutamate side chains [53]. Recently it was shown that the moving action of motor proteins (dynein, kinesin-1 and Klp2) itself causes damages to the MT lattice. This can cause MT catastrophes or mechanical failure such as breakage [54]. Counter-intuitively, this motor-induced damage can also lead to more stable microtubules. The damages in the MT lattice can be repaired by incorporation of freely available GTP bound tubulin dimers [54] - a process called "self-repair" [55]. This incorporated GTP-bound tubulin dimers form so-called "GTP islands" in the lattice of MTs [56]. They are considered to promote rescue events counteracting MT catastrophes by increasing the concentration of GTP-bound tubulin dimers at the +end once the depolymerization reaches the GTP island [57–59]. Thus, PTMs recruiting different motor proteins can indirectly change MT stability [50].

Overall, tubulin PTMs exert a plethora of different effects on MT structure, stability and function thereby corroborating the crucial regulatory potential of these PTMs.

3. Microtubule detyrosination and tyrosination

3.1. *Microtubule detyrosination via vasohibins and its regulation*

The removal of the C-terminal tyrosine of most α-tubulin isoforms, named detyrosination, is one of the most prominent PTMs. This PTM is reversible *via* the re-ligation of tyrosine *via* TTL (Fig. 1C). The irreversible removal of the penultimate and 3rd last glutamate is further PTMs, leading to Δ2 and Δ3 α-tubulin.

Detyrosination of α-tubulin in MTs is generally associated with stable MTs and as such serve in intracellular transport. Tyrosinated MTs on the other hand are more dynamic, are preferentially bound by +TIP binding proteins like CLIP170 and p150/glued [2,45,47] and serve in general a

more structural purpose. Thus, detyrosination/tyrosination of MTs can act as a binary on/off switch for MT function [60].

Although detyrosination of α-tubulin in MTs is the longest known PTM of MTs (reviewed in [2]), the enzymes responsible for the removal of the C-terminal tyrosine were discovered just recently. VASH 1/2 in complex with SVBP were independently identified by two research groups using a chemical proteomics approach with a unique irreversible tubulin tyrosine carboxypeptidase inhibitor (Alkyne-epoY) [6] and by a haploid genetic screen in human cells [7]. They were originally discovered as secreted proteins important for the regulation of angiogenesis and therefore tumour growth and metastasis [61–63]. The transglutaminase-like cysteine proteases possess an uncommon catalytic triad made of cysteine, histidine and the backbone of a leucine residue and are abundant in eukaryotes [64]. However, no substrates were known until the identification of their cytosolic function. Several studies showed the enzymatic activity of the VASH1/2:SVBP complex in MT detyrosination in the context of cell division [64] and human pathology [65,66]. Furthermore, the structures of the complexes have been solved by x-ray crystallography [67–73] and cryo-electron microscopy [74]. However, it becomes increasingly clear that VASH:SVBP cannot account for all MT detyrosination, and another, yet unknown tubulin tyrosine carboxypeptidase exists.

The detyrosination activity of the VASH:SVBP complex is mainly regulated by the formation of the complex itself. This was shown by *in vitro* assays using bovine brain tubulin and in HEK293 cells [6,64]. Vasohibins are almost insoluble in the absence of SVBP and need SVBP for their stability and proper folding [6,7,75]. While VASH1 alone does not detyrosinate MTs, VASH2 has some basal activity. SVBP strongly increases the detyrosination activity of both homologues and thus acts as a *bona fide* activator of VASHs [64]. While tubulin is the only known intracellular substrate of Vash1:SVBP, Vash2:SVBP can additionally partially detyrosinate EB1 [6], a modification described in migrating human epithelial cells and in glioblastoma cells [76]. If and under which condition this occurs in other cell types still needs to be determined.

A recent bioinformatical approach suggests a calcium-dependent activation of VASHs themselves, based on the regulation principle of other transglutaminase-like cysteine protease and the conservation of an essential calcium-binding amino acid of the calcium-dependent periplasmic cysteine protease LapG [77]. However, no indication of calcium-binding can be found in the later solved structures of VASH1 and VASH2 [70], even when purified in the presence of 200 mM calcium [67]. Recently, we could show the possible involvement of calpains on MT detyrosination, which would allow a calcium dependency of this PTM in a more general term and will be discussed in Section 3.2.

3.2. Role of conventional calpains in the regulation of MT detyrosination

Calpains are a family of calcium-dependent non-lysosomal cysteine proteases which includes classical penta-EF domain containing calpains and non-classical calpains. Among classical calpains, only the conventional calpains 1, 2 and 11 consist of a unique big catalytic subunit (CAPN) and an identical regulatory small subunit CAPNS1. The most studied, ubiquitously expressed and catalytically active conventional calpains are calpain 1 and 2, also known as μ- and m-calpain based on their *in vitro* calcium-affinity. Calpain 11 on the other hand is only expressed in testis and has not been studied in regard of the cytoskeleton [78].

Calpains have a wide variety of substrates including membrane receptors, transporters, steroid receptors as well as kinases and phosphatases, and thus are implicated in major cellular functions [79]. The involvement of calpains 1 and 2 in physiological remodelling and pathophysiological degradation of the actin and microtubule cytoskeleton in neurons and other cell types is well studied [80]. This includes the cleavage of substrates such as spectrin [81,82], MAPs (tau, MAP1B and 2) [83–86], and as shown in cell free assays maybe even degradation of soluble tubulin itself [85]. Other calpains, except calpain 6 which is mentioned in Section 4.2, have not been linked to cytoskeletal functions. An early study showed calpain 1 localization to the cytoskeleton in several human cell lines and pointed to a distinct subcellular localization of calpain 1, 2 and their endogenous regulator calpastatin [87]. However, such localization seems to be cell line dependent. Calpains have been clearly identified as having a role in the reorganization of the cytoskeleton in primary hippocampal neurons. This appears to be especially important during axonal growth and plasticity. Furthermore, they are involved in cell death [88,89] and pathology (e.g. paraplegia [90]) which was shown in *Caenorhabditis elegans, Drosophila melanogaster* and *Danio rerio*. Surprisingly, the conditional neuronal knockout of CAPNS1 under the nestin promoter has no effect on gross brain development in mice but leads to changes in axonal and dendritic morphology and spine density [91], processes in which the detyrosination/tyrosination state of MTs and therefore their dynamics and stability are of major importance. Calpain 1 and 2 are mainly calcium-activated, i.e. triggered by elevated intracellular calcium levels following extracellular signalling (Fig. 2). However, both brain-derived neurotrophic factor and epidermal growth factor rapidly activate calpain 2 in different cells (HEK293 cells, neurons) *via* a mitogen-activated protein kinase-dependent phosphorylation, a process independent of calcium, as shown by experiments sequestering intracellular calcium by BAPTA-AM [92].

Although the involvement of conventional calpains in MT stability and function has been shown excessively, its regulation and direct influence on PTM or MAPs are largely unknown. A few studies showed the effect of calpains on microtubule acetylation, which is discussed in Section 4 in detail [90,93,94], but not on other PTMs.

In recent work, we highlighted the importance of calpain 1 and 2 for efficient MT detyrosination in VASH1:SVBP overexpressing HEK293T cells, as knockdown of both calpains lead to a strong reduction of detyrosination [95]. New calpain cleavage sites of VASH1 were

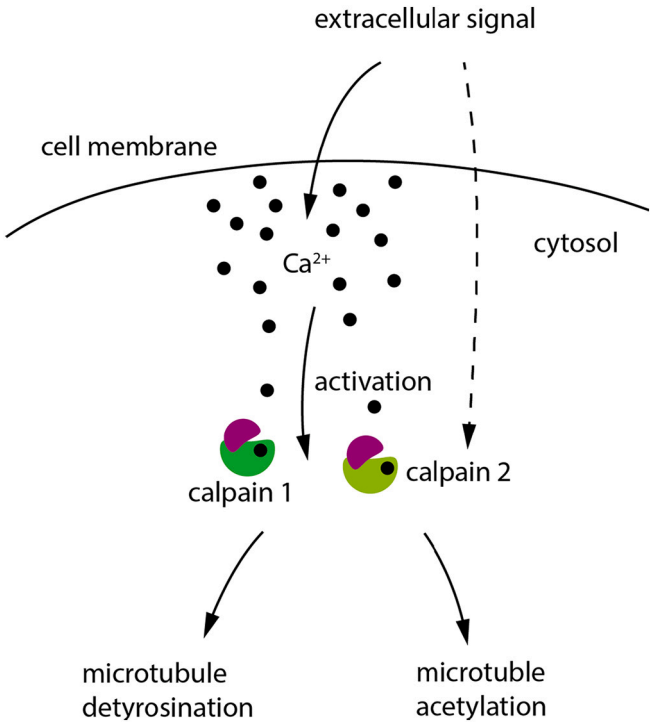

Fig. 2. Calpains play an important role in the regulation of microtubule post-translational modifications.
Modification of the cytoskeleton *via* calpains. Extracellular signalling leads to increased intracellular calcium concentration and in turn activation of calpains 1 and 2 or to a calcium-independent activation of calpain 2. Calpain activity leads to increased MT detyrosination and acetylation.

identified after *in vitro* digestion followed by mass spectrometry using N-terminomics [95]. They differ from previously described calpain 1-mediated truncation on the N- and C-terminus (ΔN, ΔC VASH) [96] of released VASHs, which could not be found in this screen. Those new cleavage sites lie in the unstructured N-terminus of VASH1 and are more terminally than ΔN-cleavage [96]. They seem to not affect VASH1 catalytic activity on α-tubulin, as shown in both cell-free assays followed by Western Blots and in HEK293T cells as visualized by immunocytochemistry: A result explainable by the fact, that the cleavage does not affect the described SVBP binding regions [6,61]. Additionally, this N-terminal cleavage does not seem to affect the number of growing microtubules or the polymerization rate visualized by overexpression of the +end protein EB3 in cellular assays, as no changes could be observed upon overexpression of truncation constructs [95]. However, possible effects of calpain 1 cleavage on VASH1:SVBP and MT binding kinetics and stability were not measured and therefore cannot be excluded. The newly identified cleavage is substantially different from the previously described ΔN- and ΔC- truncations of VASH1 by calpain [96], which would render the protein inactive regarding the detyrosination activity [95] and it is likely, that the mechanisms regulating cytosolic and extracellular functions of VASHs are different from each other. The mechanisms underlying the activating effects of calpain on MT detyrosination are still unknown. Furthermore, conventional calpains could act on a different, yet unknown, carboxypeptidase or influence e.g. binding of MAPs to MT and thus influences MT detyrosination.

3.3. Tubulin re-tyrosination and regulation

Tubulin detyrosination is a reversible process, as TTL can add the C-terminal tyrosine (Fig. 1B). Re-ligation of tyrosine is a common and well-characterized PTM, as its activity of ligating tyrosine to α-tubulin and the enzyme itself are long identified [19,97]. TTL is ubiquitously expressed, specific for tyrosination of α-tubulin and like its detyrosinating counterpart, conserved in eukaryotes. Knockout of TTL leads to perinatal death in mice [98]. Structural studies showed that TTL affects mainly soluble tubulin-dimers, rather than assembled MTs [99,100] and thus allows the re-use of dimers during polymerization and therefore is essential for maintaining the detyrosination/tyrosination cycle. TTL requires adenosine triphosphate (ATP), Mg^{2+} and K^{+} [97]. Recently, the structures of frog and chicken TTL were solved and show why tyrosine ligation is ATP-dependent [99,100]. Based on amino acid sequence analysis, it was suggested that TTL could be regulated by protein kinases A and C (PKA and PKC) as several phosphorylation sites could be identified [101]. It was suggested that phosphorylation, especially by PKC, could regulate TTL activity: Especially PKC-mediated phosphorylation of the conserved S152 could inactivate TTL as it is located in close proximity to ATP/Mg^{2+} binding residues, which might be affected by the addition of a negative charge [101]. This is in line with the finding that PKC activation by phorbol esters leads to a loss of tyrosinated tubulin [102]. Although S152 itself is, judged by the protein structure, not involved in ATP binding, a phosphomimetic mutation of this residue leads to an almost complete loss of TTL activity [100]. The mechanism behind this strong effect is currently unknown. Besides, there is no further experimental evidence of TTL phosphorylation and resulting TTL regulation. Potential phosphorylations could be individually regulated and could have diverse effects on TTL activity. Despite the importance of tyrosinated tubulin and kinases in cellular functions such as axonal regeneration, it is currently unclear if kinases influence tyrosinated tubulin levels *via* TTL [103]. Thus, the regulation of TTL activity by phosphorylation deserves further investigation. TTL activity seems to be regulated by its binding partner MAP1B, as mouse MAP1B knockout neurons do not recover tyrosinated MTs after depolymerization and knockout mice show lower tyrosinated MT levels in brain sections [104]. Thus, although TTL is well established as the enzyme that re-ligates tyrosine to tubulin, surprisingly little is known about the regulation of TTL activity.

4. Microtubule acetylation and deacetylation

4.1. Microtubule K40 acetylation is mainly pursued by aTAT1

Acetylation of tubulins was first described almost 40 years ago [105]. Since then, two distinct acetylation sites have been identified: lysine 40 (K40) of α-tubulin and lysine 252 (K252) of β-tubulin [8,32] (Fig. 1A).

Interestingly K40 is the only residue which can be modified that is inside the lumen of MTs [35,106]. It is located in a short and flexible loop and was found to be predominantly acetylated by aTAT1. Besides aTAT1 other α-tubulin acetyltransferases were proposed, namely elongator protein 3 (ELP3) [107,108], homolog of yeast general control nonderepressible 5 (GCN5) [94] and N^{α}-acetyltransferase complex [109], which are supposed to have minor α-tubulin acetylation activity and might be responsible for the observed residual acetylation activity after depletion of aTAT1 [110]. For aTAT1 no other substrates than α-tubulin K40 acetylation are known. The minimum catalytic domain of aTAT1 is formed by residues 2–193 [8] and aTAT1 was shown to have a clear preference for polymerized MTs (K_M: 1.6 ± 0.36 μM) *versus* free tubulin dimers (K_M: 2.0 ± 0.16 μM) (Fig. 1D). However, its enzymatic activity was found to be rather slow with a k_{cat} of $615 \pm 34 \times 10^{-6}$ s^{-1} at polymerized MTs and 98 ± 1.8 × 10–6 s^{-1} at free tubulin dimers [8] and in 2014 it was confirmed that aTAT1 is indeed active inside of the MT lumen [106].

Regarding the exact dynamics of aTAT1 binding to MTs, contradictory claims have been published. First, Szyk et al. suggested that aTAT1 randomly binds along the MT lattice *in vitro* with no preference for the MT ends [106]. They propose that aTAT1 diffuses fast in the lumen and acetylates K40 residues stochastically. Contrary, using MTs from HeLa cells whose plasma membranes were extracted, Ly et al. claimed that mainly the ends of new grown MTs were acetylated by aTAT1 [111]. However, on *in vitro* MTs, they did not recognize a clear preference of aTAT1 for MT ends. Besides, Coombes et al. proposed that aTAT1 mainly binds to ends of *in vitro* MTs but also at sites of damage or openings in the MT lattice [112]. Furthermore, they suggest that the high concentration of substrates limits the diffusion of aTAT1 in the lumen, thereby causing slow intraluminal diffusion. Thus, the dynamics of aTAT1-MT-interactions are not fully clear yet and remain to be determined.

Recently, it was shown that aTAT1 associates with vesicles purified from murine brains, facing the cytosolic site, and is being actively transported to the proximity of its substrate. Even and colleagues proposed a model where aTAT1 is transported together with vesicles along axons and that the residues 242–333 are needed for the vesicular association. Loss of aTAT1 led to impaired axonal transport in mouse slice cultures, primary neurons and *in vivo* [113].

As MT K40 acetylation increases stability directly and changes the interaction with other proteins (e.g. motor proteins), this modification has to be tightly controlled.

4.2. Regulation of microtubule K40 acetylation

K40 acetylation on MTs increases the flexibility of the MTs which results in more resilience against mechanical stresses. This has several implications for the structural roles of MTs and MT-dependent processes like intracellular transport. Therefore, K40 acetylation is no ubiquitous modification of α-tubulin. An example is the distinct acetylation of MTs in dendrites of primary rat hippocampal neurons which are oriented with the +end towards the soma [3]. To achieve this distinct acetylation pattern, the activity of aTAT1 and the other (minor) acetylating enzymes has to be controlled. Although MT acetylation is intertwined with many cellular functions like touch sensation in *C. elegans* and mice [8,114], cell migration [115,116] and autophagy [117] in HeLa and MDA-MB-231 cells, little is known about the regulation of MT acetylation. Here we will focus on the regulation of the major acetylase aTAT1.

Regulation of MT acetylation by aTAT1 is possible on several levels: 1) regulation of aTAT1 expression, 2) regulation of aTAT1 activity (by e.

g. phosphorylation or availability of its cofactor and 3) localization of aTAT1. Additionally, involved signalling pathways with still unknown mechanisms (like calpains) regulate MT acetylation.

ATAT1 expression is negatively inhibited by the histone acetyltransferase p300 [117]. It could be shown in HeLa cells that upon cell stress (e.g. amino acid starvation, high NaCl concentration) p300 is exported from the nucleus and recruited to acetylated MTs. This nuclear exclusion of p300 relieves the aTAT1 expression inhibition and subsequently causes MT hyperacetylation [117].

A study by Shah et al., using mouse brains, HeLa and COS7 cells, revealed the role of TGF-β-activated kinase 1 which can phosphorylate S237 of aTAT1, thereby enhancing its enzymatic activity [118]. Furthermore, MT hyperacetylation was shown as a response to reactive oxygen species (ROS) and it was suggested that aTAT1 might be phosphorylated by 5′ adenosine monophosphate-activated protein kinase as a response to ROS production [117]. Besides, a recent paper shows that in mouse and *D. melanogaster* neurons the availability of the acetyl-group donor acetyl-coenzyme-A (acetyl-CoA) is limited by the acetyl-CoA producing enzyme ATP-citrate lyase whose expression is under control of the activity of the protein elongator [119].

The special localization of the K40 residue in the lumen of MTs allows for certain degrees of modulation because aTAT1 has to enter the lumen to bind its substrate efficiently. However, both the mode of entry into the lumen of MTs and the MT-binding kinetics of aTAT1 remain under debate and are not yet fully understood. Further studies, clarifying the entry mode, could show the regulatory potential of aTAT1 luminal entry.

Additionally, the subcellular localization of aTAT1 in HeLa and MEF cells could be governed by its phosphorylation status [120]. It harbours both, a nuclear export signal and a putative nuclear localization signal (NLS) on its C-terminus, which are recognized by exportin and importin-α, respectively. Unphosphorylated aTAT1 is localized in both the nucleus and the cytosol where it can perform MT acetylation. The subcellular distribution changes when aTAT1 is phosphorylated on T322 and S315 by serine-threonine kinases, including cyclin-dependent kinases 1 and 2 and PKA. This phosphorylation is masking the NLS of aTAT1. Additionally, phosphorylated aTAT1 binds to 14-3-3 proteins which further increases masking of the NLS and results in cytosolic accumulation of aTAT1 and increased MT acetylation. Moreover, since aTAT1 is able to acetylate MTs in both its unphosphorylated and phosphorylated state and has no known targets in the nucleus, the nuclear localization of unphosphorylated aTAT1 could be solely due to regulatory reasons and nuclear exclusion of aTAT1 by these site-specific phosphorylation events could represent a possibility to fine-tune the amount of MT acetylation.

The other K40 acetylating enzymes are also involved in regulatory signalling pathways, however, the mechanisms remain unclear. The calcium-dependent proteases calpains were shown to affect not only detyrosination but also acetylation of MTs. In *D. rerio,* knockdown of calpain 1 was reported to result in hyperacetylated MTs *in vivo* [90]. However, this effect seems to be specific to certain brain areas because other areas showed decreased MT acetylation after calpain 1 knockdown [90]. A study by Tonami and colleagues showed a role of the enzymatically inactive calpain 6 in the stabilization of MTs in HeLa cells [93]. They showed that calpain 6 interacts with MTs and increases acetylation. However, it is not clear if this interaction is direct and which enzyme causes the increase in α-tubulin acetylation. Calpains were shown to cleave the transcription factor c-Myc which results in a fragment called Myc-nick [94]. This cleavage product seems to have no transcription regulatory function due to the lack of its dimerization and DNA-binding domain but rather has cytoplasmic effects [94]. It could be shown that Myc-nick recruits the α-tubulin acetylase GCN5 to MTs in rat fibroblasts and HEK293 cells [94]. Therefore, calcium influx in stimulated cells could increase calpain-dependent cleavage of c-Myc and subsequently lead to increased MT acetylation. The authors tested that knockdown of only calpain 1 and 2 did not block the formation of Myc-nick, so other calpains could be involved as well.

4.3. *Tubulin deacetylation*

Acetylation of α-tubulin is a reversible modification. The acetyl-residue on K40 can be removed by the enzymes histone deacetylase 6 (HDAC6), SIRT2 [9,121], HDAC5 and *N*-deacetylase and N-sulfotransferase 3 (NDST3). α-tubulin is the major substrate of HDAC6 [122] but it also deacetylates other proteins such as tripartite motif-containing protein 50 [123], cortactin [124] and the MAP heat shock protein 90 kDa [125] as it was shown in multiple cell lines, *in vitro* and in mice. As the effects of HDAC6 spread over such a variety of signalling pathways it is challenging to address specific effects on MT acetylation by altering HDAC6 activity. First findings suggested that HDAC6 deacetylates polymerized MTs [121,126], but this was refuted later, resulting in a MT acetylation and tubulin dimer deacetylation cycle [127,128], similar to the detyrosination-tyrosination cycle by VASH1/2:SVBP and TTL (Fig. 1D). Besides its own interaction with tubulins, HDAC6 is also able to bind other MAPs like end binding protein 1 [129] and tau [130]. This enables other modulations of MT properties by the interplay of these MAPs. Tau, for instance, was shown to inhibit HDAC6 [131], which could lead to elevated MT acetylation levels.

Contrary to HDAC6, the second tubulin deacetylase SIRT2 can act on both polymerized MTs and free tubulin dimers and depends on NAD^+ [9]. However, the deletion of murine SIRT2 did not impact MT acetylation levels *in vivo* [132,133]. The lack of effect of SIRT2 deletion could indicate that it is not a major tubulin deacetylase or could potentially have a role in specific cellular processes. Again, multiple other substrates of SIRT2 are known (*e.g.* TP53, FOXO1, FOXO3a) and these are parts of diverse pathways, namely regulation of oxidative stress response, mitosis, cell death and differentiation [134–136] in human cancer cell lines and mice making it hard to isolate specific effects on MTs in cellular assays. A negative regulatory element of SIRT2 is furry (fry) which was first discovered in *D. melanogaster* [137]. As the MT deacetylase SIRT2 is inhibited by fry, the depletion of fry in HeLa and HEK293 cells results in reduced acetylation of spindle MTs. This lack of SIRT2 inhibition indicates that fry preserves spindle MT acetylation *via* SIRT2 inhibition during mitosis [137]. Interestingly, SIRT2 deacetylation regulates the autoacetylation activity of p300 [138] and is itself acetylated by p300, which in return inhibits SIRT2 [139], which is a commonality with aTAT1. Further investigations on p300 would clarify the role of this enzyme in the MT acetylation-deacetylation cycle.

Other pathways which play a role in MT deacetylation may represent specific cellular states. Specifically, HDAC5 was shown to be phosphorylated and activated by PKC upon axonal injury leading to increased MT deacetylation in living mice and in mouse primary neurons [140]. Therefore, HDAC5 mediated tubulin deacetylation could result in more dynamic MTs that are needed in case of injury. Very recently, NDST3 was proposed as a new tubulin deacetylase in human retina pigmented epithelial cells [141]. Its role was suggested in the regulation of lysosomal acidification.

This pluralism of regulatory mechanisms and the ability to change the acetylation level of MTs as a reaction to cellular conditions highlights the importance of MT acetylation for both the mechanical properties and the functional roles of MTs.

5. Interplay of microtubule detyrosination and acetylation

5.1. *Causalities and kinetics*

While individual PTMs alone may already profoundly alter the structure, function and stability of MTs, it has been additionally suggested that a substantial interplay between distinct PTMs such as α-tubulin detyrosination and acetylation adds another level of regulatory complexity. Consequently, evidence has been provided that the precise tubulin PTM pattern may allow distinguishing individual

functionally different MT subpopulations [142]. Here, a change in the tubulin modification pattern has been described with gradually decreasing tubulin detyrosination as MDCK epithelial cells acquire apical-basal three-dimensional polarity, while MT acetylation remained largely unchanged [142]. In line with this, the interplay of MT detyrosination and acetylation has also been demonstrated in neurons for the kinesin-1 motor, which preferentially associates with K40 acetylated and/or detyrosinated MTs and selectively moves into the axon, thereby acting as the earliest known marker of axonal identity [49,143]. Independently it was shown that neither α-tubulin K40 acetylation nor detyrosination alone could recapitulate the observed kinesin-1 track selectivity in a purified system [49]. While the primary effect of detyrosination was found to be the regulation of the kinesin-1 binding affinity for MTs, K40 acetylation was mostly thought to affect α-tubulin conformation or the recruitment of specific MAPs. Strikingly, however, the effect of detyrosination on the kinesin-1 landing rate *in vitro* was shown to be dampened by using MEC-17 (a homolog of aTAT1 in *D. melanogaster*) acetylated MTs suggesting that the combination or interplay of both PTMs might be functionally relevant [49]. Furthermore, this mechanism could potentially provide a negative feedback loop to avoid excessive landing of aTAT1-containing kinesin-1 driven vesicles on already acetylated MTs (Fig. 3).

Despite potential functional interplay, MT acetylation and detyrosination are currently thought to be independently regulated, which was investigated by quantifying focal adhesion dynamics in primary astrocytes [144]. While decreased detyrosination induced by overexpression of human TTL in rat embryonic astrocytes showed no effect on cell migration speed, direction or persistence, aTAT1-depleted astrocytes with decreased MT acetylation showed a reduced cell migration speed [144]. Nonetheless, in the same study frequent co-appearance of both α-tubulin modifications on distinct regions of the same MT was reported. Future studies may provide additional insight into the precise mechanisms regulating the spatiotemporal distribution of combined MT detyrosination and acetylation.

It has been discussed previously that *in vivo* the levels of both K40 acetylation and α-tubulin detyrosination increase over time [145]. Although both modifications are generally associated with more stable MTs, detyrosination has been described to be a hallmark of long-lived and thus less dynamic MTs rather than a direct cause of MT stability [146]. On the other hand, MT acetylation seems to confer higher mechanical stress tolerance to MTs likely by altering lateral contacts of protofilaments [15,38]. However, the mechanism by which both PTMs influence each other temporally remains elusive. Based on previous studies, a potential three-step scenario might be constructed (Fig. 3). First, long-lived MTs become increasingly detyrosinated over time either *via* the VASH1/2:SVBP complex and/or *via* a calpain-dependent pathway. Second, detyrosinated MTs show an increased affinity to kinesin-1, which in turn exhibits a higher landing rate on detyrosinated MTs and may transport aTAT1-containing vesicles. In a third step, aTAT1 might be released from these vesicles to enter the MT lumen thereby introducing K40 acetylation. Finally, future studies and novel tools, as outlined in the following chapter, are required to address this mechanism and to elaborate on the details of these PTM interplay kinetics.

5.2. Novel tools to study acetylation and detyrosination processes

Recently, live sensors for MT PTMs have been developed. Tyrosinated MTs can be visualized by the fluorescently labelled nanobody A1aY1 [147], which is highly specific for tyrosinated MTs and can be used *in vitro* and *in vivo* without altering (cellular) MT functions such as dynamic instability, growth rates, plus-end binding proteins and motor protein binding. This allows following tyrosinated MTs over time and during diverse cellular functions/stages. A live sensor for K40 acetylated α-tubulin with similar properties but with a different construction was proposed this year in a preprint [148]. This sensor is based on the rigor mutant of kinesin-1 which has a strong preference for acetylated MTs. It can be used for visualization of acetylated MTs intracellularly over space and time.

Live sensors enable studies on the spatiotemporal distribution and

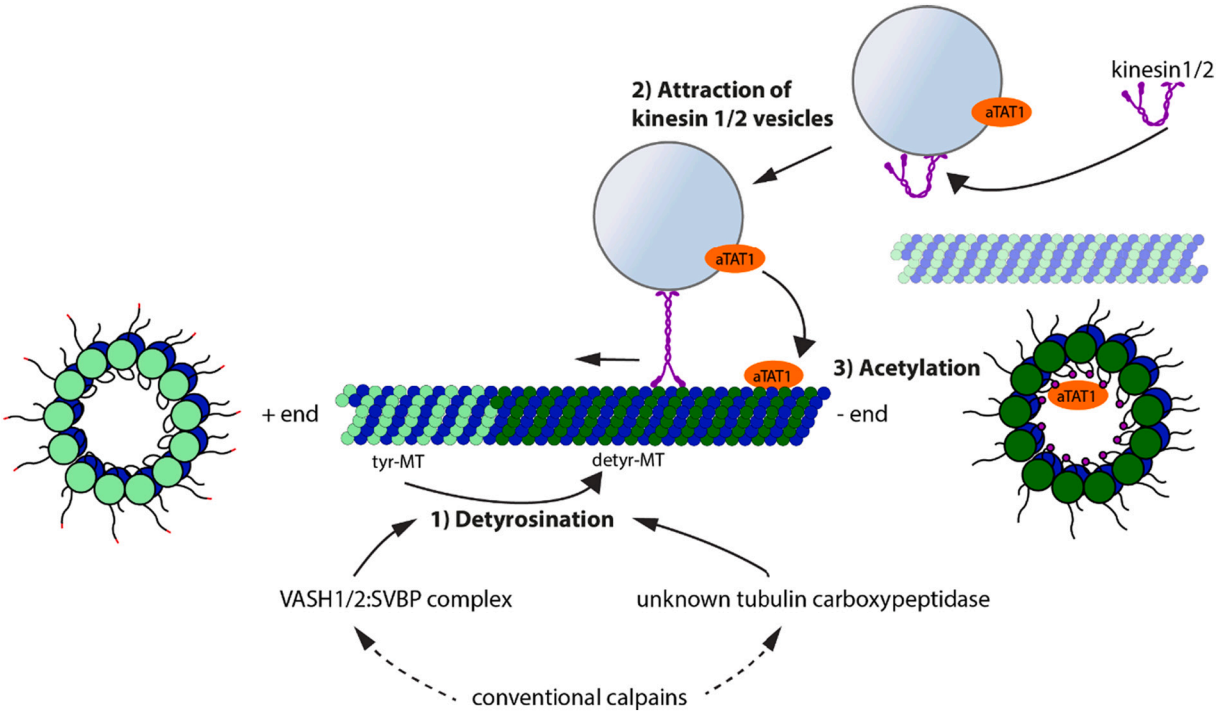

Fig. 3. Interplay of diverse MT PTMs: A hypothetical model of how MT detyrosination could lead to increased MT acetylation.
1) MT detyrosination occurs on long-living MTs. 2) Increased recruitment and processivity of aTAT1-containing kinesin-1/2 propelled vesicles, 3) aTAT1 is released and acetylates MTs.

regulation of specific PTMs throughout cells. They can be very helpful for gaining more insights into the mechanisms by which MT PTMs change cellular properties and functions. Moreover, they would allow for a live readout for alterations of the balance between detyrosinating/tyrosinating and acetylating/deacetylating enzymes or studies targeting the regulation of these enzymes.

The development of super-resolution microscopy methods enabled more detailed studies on the distribution of MT PTMs. First, the organization of MT subpopulations can be differentiated as presented recently [4]. Using expansion microscopy and stimulated emission depletion microscopy, it was shown that tyrosinated MTs are located close to the plasma membrane around a core of acetylated MTs in dendrites of primary rat hippocampal neurons [4]. Further studies using these advanced imaging techniques would be able to unveil the patterns of PTMs on MTs which could give more insight into the interplay of PTMs and their effects.

Another very useful tool to study the interconnectivity of MT PTMs would be Förster resonance energy transfer (FRET) pairs of sensors for certain PTMs. Using these FRET sensors would enable to study the kinetics of the modifications and how they influence each other. Together these tools could allow uncovering the details of the "tubulin-code".

6. Conclusions

PTMs of MTs provide a wide range of possible adaptation of MT properties. Although they are well characterized, and many specific physiological and pathophysiological cellular states are associated with specific MT modifications, their causalities and regulation are still less well understood. Not only can individual PTMs, such as acetylation and detyrosination, exert direct or indirect effects on MT stability, but also their interaction with a variety of MT-binding proteins and molecular motors, as well as their interplay, may further diversify individual MTs in terms of their properties. These modifications could be specific for (parts of) individual MTs or bundles and can be spatially and temporally regulated depending on a diversity of extra- and intracellular signals. This enables several levels of regulation of MT properties far beyond the modification of an individual tubulin. Together with the reversibility of most modifications, these allow MTs to fulfill their diverse functions in cells. The rapid progress made in the development of super-resolution imaging techniques and cryo-electron tomography combined with new high-throughput data analysis methods (e.g. deep learning) can advance our knowledge about MT structure and distribution of PTMs in different cell types, physiological states of the cell and compare them between different model organisms. Recent developments of sensors for specific PTMs together with advances in live microscopy facilitate the study of the temporal and causal relations between individual modifications and provide means to further understand their regulation not only *in vitro* but also in cellular assays. MTs are the target and the mediator of cellular changes occurring in pathophysiological conditions and understanding the precise regulation of PTMs and effects on MT function will aid future drug design. Therefore, comprehending their regulation is essential and the scientific community is putting further efforts in elaborating on the details of MT PTM regulation.

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JB, YP, MB and MM contributed to conceptual framework and structure of this review

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JB and YP prepared figures

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Declaration of competing interest

The authors declare no conflict of interest associated with this manuscript.

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