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Chadni Sanyal, Niels Pietsch, Sacnicte Ramirez Rios, Leticia Peris, Lucie Carrier, et al.. The detyrosination/re-tyrosination cycle of tubulin and its role and dysfunction in neurons and cardiomyocytes. *Seminars in Cell and Developmental Biology*, 2023, 137, pp.46-62. 10.1016/j.semcdb.2021.12.006 . hal-04282770

HAL Id: hal-04282770

<https://hal.science/hal-04282770v1>

Submitted on 13 Nov 2023

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The detyrosination/re-tyrosination cycle of tubulin and its role and dysfunction in neurons and cardiomyocytes

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Declarations of interest: none

Abstract

Among the variety of post-translational modifications to which microtubules are subjected, the detyrosination/re-tyrosination cycle is specific to tubulin. It is conserved by evolution and characterized by the enzymatic removal and re-addition of a gene-encoded tyrosine residue at the C-terminus of α -tubulin. Detyrosinated tubulin can be further converted to $\Delta 2$ -tubulin by the removal of an additional C-terminal glutamate residue. Detyrosinated and $\Delta 2$ -tubulin are carried by stable microtubules whereas tyrosinated microtubules are present on dynamic polymers. The cycle regulates trafficking of many cargo transporting molecular motors and is linked to the microtubule dynamics via regulation of microtubule interactions with specific cellular effectors such as kinesin-13. Here, we give an historical overview of the general features discovered for the cycle. We highlight the recent progress toward structure and functioning of the enzymes that keep the levels of tyrosinated and detyrosinated tubulin in cells, the long-known tubulin tyrosine ligase and the recently discovered vasohibin-SVBP complexes. We further describe how the cycle controls microtubule functions in healthy neurons and cardiomyocytes and how deregulations of the cycle are involved in dysfunctions of these highly differentiated cells, leading to neurodegeneration and heart failure in humans.

Keywords:

tubulin, tyrosination, neuron, cardiomyocyte, TTL, vasohibin

Footnotes:

Abbreviations:

A β , amyloid- β ; AD, Alzheimer's disease; CAP-Gly, cytoskeleton-associated protein glycine-rich; CCP, cytosolic carboxypeptidases; HSP, hereditary spastic paraplegia; KIF, kinesin superfamily protein; SMA, spinal muscular atrophy; HCM, hypertrophic cardiomyopathy; HPA, human protein atlas; L-dopa, L-3,4-dihydroxyphenylalanine; MAP, microtubule associated protein ; MARK4, microtubule-affinity regulating kinase 4; MCAK, mitotic centromere-associated kinesin; NMDA, N-methyl-D-aspartate; NX, normalized expression; SMN, survival motor neuron; SVBP, small vasohibin-binding protein; TCP, tubulin carboxypeptidase; +tips, +end tracking proteins; TTL, tubulin, tyrosine ligase; TrkB, tropomyosin-related kinase B; VASH, vasohibin.

1. General features

Tyrosination was first described in 1973 by an Argentinian team [1, 2]. They observed the incorporation of radiolabeled tyrosine (Tyr) on a peptide sequence, which was independent of the ribosome activity. They showed that tyrosination was abundant in brain and reversible. Two years later, they identified α -tubulin as the substrate for tyrosination [3-5]. After their initial proposition that the reactions of addition and cleavage of tyrosine were carried out by the same protein [6], they provided evidence that detyrosinating and tyrosinating activities were actually performed by distinct enzymes [7, 8]. A timeline of important discoveries about the cycle is presented in [Fig. 1](#).

1.1. Overview of the cycle, involved enzymes and generated pools of α -tubulin

The detyrosination/re-tyrosination cycle occurs on the C-terminus of α -tubulin ([Fig. 2](#)). The majority of α -tubulin genes encode a C-terminal tyrosine: they are 'tyrosinated'. Among the 9 human α -tubulins only $\alpha 4$ (TUBA4A) and $\alpha 8$ (TUBA8) are not tyrosinated: $\alpha 4$ -tubulin C-terminus bears no tyrosine and terminates with a glutamate (Glu) residue, and $\alpha 8$ tubulin bears a phenylalanine (Phe) instead of a Tyr [9]. The C-terminal Tyr (or Phe) can be cleaved by tubulin carboxypeptidases (TCPs). This cleavage produces 'detyrosinated' tubulin (previously called Glu-tubulin), which exposes a C-terminal Glu to which a Tyr can be re-added by the tubulin tyrosine ligase (TTL; [Fig. 2](#)). TCP and TTL enzymes are ubiquitous, and their activities are spatially and temporally regulated in tissues. Unlike the other post-translational modifications which can occur on proteins other than tubulins, the reactions of detyrosination and re-tyrosination are specific to α -tubulin.

TCPs prefer tubulins incorporated in microtubules, whereas TTL acts only on the newly depolymerized tubulin dimers (see chapter 2). This differential regulation of enzymes leads to the complete absence of free detyrosinated tubulin in cells. Detyrosinated tubulin is only found on persistent microtubules and is therefore a marker of microtubule stability. The two populations of tyrosinated and detyrosinated α -tubulin present in cells have been extensively studied using highly specific antibodies. A rat monoclonal antibody called YL1/2 developed against yeast tubulin specifically recognizes tyrosinated α -tubulin [10, 11], whereas polyclonal antibodies developed against C-terminal peptides of α -tubulin without the tyrosine allow analysis of detyrosinated tubulin [12-14]. The first immunofluorescence studies revealed distinct but overlapping populations of tyrosinated and detyrosinated microtubules in kidney cell lines (CV1 and PtK2 cells) in culture [12, 15]. However, ultrastructural studies performed by electron microscopy on these cells revealed that microtubules are made up of the two forms of tubulins in a more or less enriched manner [15, 16]. The so-called 'tyrosinated microtubules' consist mainly, but not exclusively, of tyrosinated tubulin. The same is true

for 'detyrosinated microtubules' which contain mainly, but not exclusively, detyrosinated tubulin. In cycling cells, majority of the microtubules are strongly tyrosinated, whereas only a few are heavily detyrosinated. In contrast, the bulk of tubulin is detyrosinated in highly differentiated cells such as neurons and cardiomyocytes [16-19].

1.2. Link to microtubule dynamics

Detyrosination has been long recognized as a marker of microtubule stability. Detyrosinated microtubules are indeed resistant to disassembly by the microtubule depolymerizing drug nocodazole. In contrast, tyrosination is a marker of dynamic microtubules as tyrosinated microtubules are highly sensitive to nocodazole [20-22]. Such observations led to the question of what the intrinsic dynamics of the two species of microtubules are, i.e., their intrinsic ability to polymerize, depolymerize and form stable filaments. It was shown that detyrosination *per se* does not modify the dynamics of the microtubules. Indeed, treating permeabilized cells with carboxypeptidase A (which detyrosinates microtubules) or with an antibody that blocks TTL activity did not significantly change microtubule resistance to drug-induced depolymerization [23, 24]. Moreover, purified preparation of α -tubulin species (tyrosinated and non-tyrosinated) from bovine brain showed the same dynamics parameters [25], as do the recombinant, engineered human variants (tyrosinated, detyrosinated and $\Delta 2$) [26].

A mechanistic explanation for the long-recognized correlation between microtubule stability and tubulin detyrosination remained elusive until tyrosination status was shown to have an important effect on microtubule depolymerizing activity of kinesin-13 family members in cells [27]. These motors, and specifically MCAK (mitotic centromere-associated kinesin) and its homologue KIF2A (kinesin superfamily protein 2A), are directly inhibited by tubulin detyrosination. Microtubules that are transiently stabilized in cells can be detyrosinated by TCPs and thus become resistant to depolymerizing motors of the kinesin-13 family, a property which further strengthens their stability. Such mechanism gives rise to subpopulations of polymers with increased stability, which were shown to be important for neuronal development and error correction in mitosis [27, 28]. The kinesin-2 family member KIF3C, which is majorly expressed in the axons of nervous system, also acts as a microtubule destabilizing factor that preferentially binds to tyrosinated microtubules and therefore regulates microtubule dynamics in the axonal growth cone after axonal injury [29]. Another member of the kinesin-2 family, KIF17, preferentially binds to detyrosinated tubulin and induces microtubule stabilization by relocalizing adenomatous polyposis coli (APC) to the plus ends [30]. A recent *in vitro* study also demonstrated synergy between EB1 and Clip-170 (cytoplasmic linker protein of 170 kDa) to selectively increase tyrosinated microtubules dynamics [26]. Thus, in cells, tyrosination can act through a combination of effectors to increase microtubule turnover.

1.3. Other truncated variants of α tubulin, $\Delta 2$ and $\Delta 3$ tubulins

The earliest studies aiming at quantifying the levels of detyrosinated and tyrosinated tubulin pools in tissues led to the discovery of α -tubulin species, which could not be re-tyrosinated *in vitro* [31-33]. This non-tyrosinable tubulin fraction, which accounts for about one-third of brain tubulin [14, 34], was isolated after tyrosination of brain tubulin by TTL and elimination of the tyrosinable fraction by affinity chromatography on a column of YL1/2 antibody [13, 25]. Its molecular nature differs from tyrosinated α -subunit by the truncation of the two C-terminal glutamate residues and has thus been named " $\Delta 2$ tubulin" [13, 35]. $\Delta 2$ tubulin was found in differentiated structures such as neuronal microtubules, cilia and flagella (for review see [36]), and also in kinetochore microtubules [37]. In contrast, almost all α -tubulin molecules were tyrosinable in skeletal muscle [38]. An additional form of tubulin, " $\Delta 3$ tubulin", corresponding to α -tubulin lacking the last three amino acids, has been also described in neural tissue [14]. This α -tubulin is more abundant in the embryonic brain than in the adult brain, suggesting that it is important during development.

$\Delta 2$ tubulin was generally found on stable and persistent microtubules [34, 36], whereas $\Delta 3$ tubulin appeared to be related to dynamic microtubules in neurons, resembling tyrosinated-tubulin rather than the other truncated variants [14]. Like detyrosination, the cleavage of Glu and formation of $\Delta 2$ microtubule does not modify *per se* the intrinsic dynamics of the microtubule [25, 26]. The C-terminal $\Delta 2$ and $\Delta 3$ truncated forms of α -tubulin are generated by cytosolic carboxypeptidases (CCP), enzymes of the deglutamylase family, which modify both lateral polyglutamate chains (involved in tubulin polyglutamylation) and primary tubulin chain [14, 39-41]. Very little is known about the physiological role of these truncated α -tubulin variants, apart from the fact that $\Delta 2$ tubulin accumulates during neurodegeneration processes or heart failure [42, 43] (see 5.1 & 5.2). These removals of C-terminal glutamates allow tubulin to break out from the detyrosination/re-tyrosination cycle, and possibly to be locked in a non-tyrosinable state. However, we cannot rule out the possibility of a re-glutamylation of $\Delta 3$ tubulin and $\Delta 2$ tubulin in cells, leading to the re-formation of $\Delta 2$ and detyrosinated tubulin, respectively. Such reactions on the primary tubulin chain by polyglutamylases of the TTL-like family known to generate lateral chains of polyglutamates on α and β tubulins might indeed be considered.

1.4. Molecular impacts

The known readers of the tubulin tyrosine signal emerging from the tubulin detyrosination/re-tyrosination cycle are kinesin motors and CAP-Gly proteins. The tyrosination status of microtubules is

long known to modulate interaction with kinesins [44], and apart from the effect on kinesins inducing changes in microtubule dynamics (see above), it can regulate the trafficking of these cargo-transporting motors. In neurons, kinesin-1 (KIF5A) and kinesin-2 (KIF17) molecular motors prefer to travel on detyrosinated microtubules [45-47], whereas kinesin-3 (KIF1A) and kinesin-5 (KIF11) preferentially associate with microtubules enriched in tyrosinated tubulin [48, 49]. These motors are essential to the polarized transport within axons or dendrites (see 3.3). The preference of kinesin-3 for tyrosinated microtubules was also observed in HeLa cells where it was involved in the lysosome positioning [50]. Nevertheless, in HL-1 cardiomyocyte cell line, kinesin-2 transport of Kv1.5 vesicles was not restricted to specific microtubules [51]. In vitro studies showed some discrepancies. By using single molecule imaging, kinesin-1 showed preferential moving on stable, detyrosinated or acetylated microtubules in live COS cells, whereas kinesin-2 and kinesin-3 appear non-selective [51]. The preference of kinesin-1 (KIF5B) for detyrosinated microtubule was not observed in vitro using engineered α/β tubulin dimers, and kinesin-2 motility required microtubule detyrosination in these experiments [52]. Kinesin-7 (CENP-E) prefer detyrosinated microtubule tracks in reconstituted systems in cells and/or in vitro [53]. Although, specificities are still not well established, and might depend on methods used and tissues analyzed, it is clear that kinesins can preferentially use or avoid detyrosinated or tyrosinated tracks.

The tyrosination state of microtubules can also affect transport by dynein. The C-terminal tyrosine of tubulin indeed controls transport initiation by the dynein-dynactin complex via the protein CLIP-170 in neurons [54], whereas it did not impact dynein motility [54, 55]. Clip170, such as the dynactin subunit P150Glued, is a CAP-Gly (cytoskeleton-associated protein glycine-rich) protein whose location at the microtubule +ends is controlled by tyrosination [56, 57]. These proteins specifically bind to the C-terminal EEY/F sequence of α -tubulin as well as on +tips (+end tracking proteins) such as EB1 via their CAP-Gly domain [58]. They do not interact with detyrosinated microtubules [57]. Apart from their role in dynein trafficking, interaction of CAP-Gly proteins with microtubule +ends are crucial for microtubule-cell cortex attachment. The latter is thus modulated by the tyrosination state of tubulin [57].

Thus, through the diverse molecular effectors/mechanisms cited above, the tubulin detyrosination/re-tyrosination cycle plays a role in important cellular and tissues functions, as will be detailed in the following parts of this review for cardiomyocyte/heart and neuron/brain physiology.

2. Enzymes of the cycle

2.1. TTL

As a part of the evolutionary conserved tubulin tyrosination cycle, re-tyrosination, which is the process of the re-addition of a Tyr residue to the C terminus of de-tyrosinated α -tubulin, is achieved by TTL through a peptide bond to the α -carboxyl of a glutamate residue [1, 3, 59, 60]. TTL requires ATP, Mg^{2+} , and K^+ for its activity. Purish *and colleagues* examined the steady-state kinetics of bovine brain TTL [61]. Their data were consistent with a sequential addition of tubulin and tyrosine, a K_m (Michaelis constant) of 1.9 μM for tubulin, and a maximum velocity of 0.2 $\mu M/min$.

Raybin and Flavin were the first to partially purify TTL using standard biochemical procedures from brain in 1977, followed by Murofushi in 1980, who described a preparation close to homogeneity [32, 59]. Then, an improved and efficient affinity purification procedure was established using monoclonal antibodies directed against TTL [62]. The porcine TTL enzyme was identified by protein sequencing and cDNA cloning in 1993 [60]. TTL is a globular, monomeric protein made up of a single polypeptide with a molecular weight of about 43 kDa, which is highly conserved in the phylogeny. TTL was the first discovered member of a large family of proteins with a TTL homology domain. Members of this family, the TTL-like (TLL) enzymes, catalyse ligations of amino acids to tubulins or other substrates and include polyglutamylases and polyglycyclases [63-66].

It was shown very early that TTL is highly specific for tubulin dimers [15, 16, 32]. Therefore, tyrosination is seen in the newly assembled dynamic microtubules. Recent structural studies showed that TTL forms a complex with the tubulin dimer, which prevents tubulin incorporation into microtubules, and thereby could influence partition between free dimer and microtubules in cells [67, 68]. It was also known for a long time that TTL tyrosinates only the α -subunit of the α/β tubulin heterodimer [60]. Crystallisation studies showed how TTL discriminates between α and β tubulins, and how the tubulin-TTL interaction is regulated [67, 68]. TTL is comprised of an N-terminal domain, a central domain, and a C-terminal domain [67], all shared with TLL enzymes. These three domains and an adenosine nucleotide moiety contribute to TTL activity. TTL has a distinctive bipartite substrate identification (α - β interface) and binding strategy. Binding of TTL to this interface positions the active site of TTL in such a manner that it is only able to recognise the C-terminal tail of α -tubulin (which contributes to almost $\sim 80\%$ of the interaction surface), but not of β -tubulin, which is too short to reach the active site [68]. TTL binding requires the two consecutive C-terminal acidic residues, which are specific of α -tubulin, explaining why $\Delta 2$ -tubulin, which lacks the C-terminal Glu residue, cannot be tyrosinated by TTL. These models also explain the insensitivity of TTL to C-terminal polyglutamylation of α -tubulin [69] since the major site of polyglutamylation is oriented away from the TTL-interacting site on tubulin. To date, no other physiological substrate of TTL has been identified, defining the de-tyrosination/re-tyrosination cycle exclusive to α tubulin.

Various Tyr analogues, including Phe and L-3,4-dihydroxyphenylalanine (L-Dopa), can be incorporated into tubulin in place of Tyr both *in vitro* and *in vivo* [4, 70-72], which could have

implications in disease treatment (see paragraph 5.1; [73]). More recently, it was shown that TTL has an even wider and unusual substrate tolerance, and this property was used for chemoenzymatic protein functionalization [74, 75]. TTL was repurposed to attach unnatural Tyr derivatives to proteins containing a short C-terminal tubulin-derived recognition sequence. This strategy, called Tub-tag labeling, enables a subsequent bioorthogonal modification (by click chemistry) of proteins *in vitro* and in cells with a great variety of probes.

It is predicted that TTL has several phosphorylation sites, which could contribute to its regulation [60, 76]. Interestingly, mutations at the protein kinase C-sensitive Ser152 of TTL displays more than 90% reduction in enzymatic activity, suggesting that phosphorylation at this site regulates TTL activity [68]. In addition, two highly conserved regulators of microtubule dynamics might control TTL activity in cells. The microtubule-associated neuronal protein MAP1B interacts with TTL in neurons, suggesting its potential regulation in α -tubulin tyrosination [77]. Stathmin competes with TTL for tubulin binding *in vitro* [78]. The interaction of stathmin with free tubulin, which depends on stathmin phosphorylation status [79], could therefore regulate tubulin tyrosination by TTL in cells.

2.2. TCPs

Even though the process of detyrosination was observed more than 40 years ago [8], the identity of TCPs has remained elusive until recently. Many procedures of brain TCP purification were proposed [7, 80, 81], but none of them resulted in the identification of the enzyme. However, these initial purification attempts gave an idea about the distinct biochemical characteristics and specificities of TCPs, which were helpful for their recent identification, such as its sensitivity to sulfhydryl reactive compounds and cations [82]. All the data accumulated since 1977 favor preferential detyrosination by TCPs on polymerized tubulin [8, 31, 80, 83-86]. Moreover, association of TCP activity with microtubules was demonstrated in living cells, like COS-7, CHO, NIH3T3, and PC12 cells [87]. As a result of TCP preferential activity, tubulin detyrosination mainly occurs on long-lived, stable microtubules [15, 16].

The identity of key brain TCPs was discovered in 2017 by two teams, which showed simultaneously and with two different approaches, that protein complexes formed by vasohibins (VASH) and small vasohibin-binding protein (SVBP) have detyrosinating activity [88, 89]. While Moutin *and colleagues* used chemical proteomics with an irreversible TCP inhibitor (epoY; [88]), Brummelkamp *and colleagues* incorporated a haploid genetic screening strategy [89]. The first team developed a purification procedure to enrich TCPs in brain homogenate including an affinity step with the designed inhibitor and followed the TCP activity with radiolabelled tyrosinated microtubules. After mass spectrometric analysis, they identified only one candidate of high interest, namely VASH1 [88]. The other team searched for regulators of tubulin detyrosination by treating the mutant library with

paclitaxel (known to favour tubulin detyrosination in cells [90]), and found SVBP as a positive regulator of detyrosination. VASH proteins (VASH1 and VASH2) are members of the transglutaminase-like cysteine protease superfamily [91] and bind with nanomolar affinity to SVBP, a chaperone protein necessary for their stability [92, 93]. Both teams showed that VASH1-SVBP and VASH2-SVBP complexes have tubulin detyrosinating activity in cells and *in vitro* [88, 89]. The complexes can cleave a Tyr or a Phe at the end of α -tubulin to produce detyrosinated tubulin, and the predicted catalytic cysteine (Cys) is essential. As expected, the enzymatic complexes had a clear preference for microtubules than for free α/β tubulin dimers [88]. VASH1 and VASH2, which exhibit 52.5% homology in the human genome [94], were originally known to regulate angiogenesis, where they play opposite roles [94, 95]. These proteins are eventually ubiquitous, and their precise role in many tissues remain to be clarified. However, as remaining detyrosinating activity is detected after VASH1/2 knockdown in neurons [88] and knock-out (KO) in cell lines [89], other TCP enzymes(s) remain(s) to be identified.

Recent success in obtaining the crystal structures of both VASH1-SVBP and VASH2-SVBP complexes at high resolution from groups all over the world [93, 96-100] allowed to determine the overall organisation of the protein (Fig. 3). The central protease core domain of VASHs can be divided into three major subdomains namely, an N-terminal domain (ND), which encloses the C-terminal region of SVBP forming a three-helix bundle, a small three-helical lobe, and a C-terminal domain (CD) comprising of antiparallel β -sheets flanked by two α helices. SVBP is interposed between these two domains via specific molecular interactions. The active site of VASH1/2 is part of a hydrophobic groove comprising of a non-canonical, catalytic Cys-His-Leu residues triad [93, 96-99]. These three residues, which are positioned at the intersection of the N- and C-terminal domains, form an integral part of VASH-SVBP and are conserved across species. Mutation of these residues followed by *in vitro* and cellular tubulin detyrosination assays resulted in the loss of catalytic activity, which confirmed the importance of the triad in vasohibins' function. To gain a further understanding on the active site framework, VASH-SVBP was crystallised in presence of inhibitors like epoY [88, 93, 98], TPCK [88, 93], and parthenolide [90, 98]. As observed, the tyrosine group of epoY, phenyl and tosyl groups of TPCK, and the α -methylene of parthenolide all bind to the same hydrophobic groove of VASH (specifically the Cys) and this region has been named as the tyrosine-binding pocket, which plays a role in tubulin detyrosination. Crystallization of the complex with α -tubulin C-terminal peptides [96, 100] or derived peptides [93] were also obtained, allowing to define the specific determinants on the enzyme that recognize the negatively charged tail of tubulin.

Interestingly, through surface electrostatic potential analysis of the VASH2-SVBP complex structure, Wang *et al.* found an additional patch of basic residues (conserved in VASH1), situated farther away from the tyrosine-binding groove [93] (Fig. 3). Mutational studies of these residues *in vitro* and in cells demonstrated the role of these VASH amino acids in microtubule binding. Li *et al.* [101]

recently used cryo-electron microscopy (cryo-EM) to dwell deep into the structure of VASH1(core domain)-SVBP bound to microtubules. They proposed that VASH interacts with microtubules via three major interfaces (Fig. 3). Interfaces 1 and 2 (that are related to certain residues mutated in [93], see Fig. 3) are located between VASH and a first α -tubulin, which is detyrosinated by VASH. Additionally, VASH interacts with a second α -tubulin molecule from the adjacent protofilament via interface 3. This inter-protofilament mode of interaction, which is only possible within the microtubule lattice and not with free $\alpha\beta$ tubulin heterodimers, further re-establishes the substrate preference of VASH towards microtubules. Core domains of VASH do not have any contact with β -tubulin, and SVBP does not seem to contribute to the substrate recognition [101].

To note, in all these structural studies, only the central (core) domains of VASHs and the C-terminal part of SVBP could be examined. The importance of the disordered N- and C-terminal flanking regions of VASH and N-terminal region of SVBP is yet to be examined. Detyrosinating activities were observed in their absence, but the efficiency of activity was not thoroughly examined [88, 93].

3. Physiological impact of the cycle on neurons and brain

Microtubules are key players in the development and functioning of brain, and tubulin is its main protein component representing 20% of the soluble proteins [102]. Mass spectrometry analysis showed that the major α -tubulin isotypes are α 1A/B in the neonatal rat brain, while α 4A (whose gene does not encode a C-terminal Tyr, resulting in a detyrosinated form) is expressed later during development [102]. Adult brains were estimated to be composed of 85% of α 1A/B and 15% of α 4A proteins, both proteins being majorly non-tyrosinated [103]. A recent study also gives an overview on the spatio-temporal expression patterns of tubulin in the whole mouse brain, its hippocampus, and cultured hippocampal neurons [104]. This study, which was limited by the high degree of identity between tubulin isotypes, provides useful information about α - and β -tubulin antibodies suitability and cross-reactivity. It was shown that, like brain, the hippocampus, and cultured neurons exhibit a similar tendency, with prominent α 1 levels in adult and increased α 4A with age [104].

The cycle was discovered in brain, where it is tremendously active. Brain tissue contains high levels of detyrosinated tubulin when compared to most other tissues. The cycle-derived tubulin variant Δ 2 is also abundant in this tissue (representing 35-50% of total tubulin in adult brain, [34, 105]) in contrast to most other organs, and rather in neurons (representing one-third of α -tubulin) than in glial cells [34]. In mature hippocampal neurons (21 days of *in vitro* culture), tyrosinated, detyrosinated and Δ 2 tubulins were estimated to represent 36, 39, and 25 % of total, respectively (personal communication obtained as in [14] using mCherry- α tubulins as standards).

Alterations to the detyrosination/re-tyrosination cycle following the manipulation of TTL and TCPs have extensive consequences as described below (and in [Table](#)).

3.1. TTL-deficient mouse: the cycle is vital

The importance of the cycle in the whole animal was first discovered when TTL-deficient mouse was generated: these mice died just after birth [106]. The large majority of α -tubulin in the TTL-null tissues was present in the detyrosinated and $\Delta 2$ -tubulin forms. In post-mitotic cells such as neurons, tyrosinated tubulin was absent, whereas a small pool of tyrosinated tubulin coming from tubulin neosynthesis persisted in dividing cells. The TTL-deficient mice did not present any external morphological malformation and no obvious malformation of organs but showed notable anomalies in cerebral connectivity and organization of the cerebral cortex layers. During the later stages of development, proper cortical formation depends on the correct establishment of the cortico-thalamic loop. In the developing brains, the cortico-thalamic loop is characterised by the formation of the internal capsule, whereas in adult brains, it is identified by the axonal projections from the neocortex to the thalamus and vice-versa. This loop is critical in the relay and integration of sensory afferents from the thalamus to the cortex and motor efferents from the cortex to the thalamus. Death of TTL-deficient mice is most probably due to the impaired cortico-thalamic loop formation, meaning that they neither show the formation of the internal capsule nor the cortico-thalamic axonal projections. Establishing these connections is necessary to generate the cytoarchitectural differentiation of the cortex as the embryonic hypoplasia is lethal [107, 108].

At the cellular level, cultured TTL-deficient neurons exhibited morphogenetic abnormalities, such as erratic neurite outgrowth and premature axonal differentiation as observed in hippocampal neurons [106], decreased axon outgrowth, supernumerary axonal branches, and enlarged growth cone as observed in precerebellar nuclei neurons [109]. Interestingly, the growth cone's cytoskeletal organization is disrupted with detyrosinated microtubule entering the peripheral actin-rich region [109]. At the molecular level, the TTL-deficient neuron defects were linked to abnormal CLIP170 distribution [57], reduction of Kinesin-13's microtubule-depolymerizing activity [27, 110], defective recruitment of myosin IIB linked to a rise of Rac1 small GTPase activity [109], and possibly in the alterations of polarized trafficking of kinesin and dynein motors, which are sensitive to the tyrosination status of tubulin ([see 1.4](#)) and crucial to neuronal integrity [111].

3.2. SVBP-deficient mouse and human mutant

The loss of VASH1 and VASH2 enzymes, as obtained in the absence of their chaperone SVBP, have less impact than the loss of TTL in mice [112]. SVBP KO mice were viable, fertile, and exhibited

normal general development. However, the level of detyrosinated tubulin was markedly lower and the tyrosinated tubulin level was much higher in the brain and neurons of SVBP-deficient mice than in wild-type mice. Remaining detyrosinated tubulin is likely due to the presence of α 4A tubulin and other unknown detyrosinating enzymes [88, 89]. SVBP deletion resulted in a 7% reduction of mouse brain volume (microcephaly) with the white matter region being majorly affected, suggesting deficiencies of axonal tracts. With respect to the behavioral patterns, SVBP-KO mice were more active and less anxious than their wild-type littermates. There were no major manifestations of memory defects but a markedly reduced social behavior. Importantly, a biallelic inactivating *SVBP* variant causes a neuropathology in humans [112, 113]. The syndrome involves brain microcephaly (affecting more severely the white matter), intellectual disability and delayed gross motor and speech development. Thus, the mouse model clearly mimics several features of human patients. SVBP-deficient hippocampal neurons in culture displayed a net delay in axonal differentiation and abnormal morphology with excessive number of primary neurites, axonal and dendritic branches.

Overall, it is evident that the inactivation of TTL and VASH-SVBP enzymes has a drastic impact on the detyrosination/re-tyrosination cycle in neuron, leading to undesired modifications of microtubule interactions and dynamics, and hence neuron development. Growth cone pathfinding and polarization of developing neurons are specifically affected, leading to neurodevelopmental defects. (for review, see [114]).

3.3. Importance of the detyrosination/re-tyrosination cycle in synapse of mature neuron

In contrast to its role in neuron development, less attention has been made to the significance of the detyrosination/re-tyrosination cycle in mature neurons. Mature neurons are highly polarized cells, with one thin, long axon transmitting information and several shorter processes, the dendrites, to receive information (Fig. 4). In these cells, microtubules serve as rails for motor protein-based intracellular transport and are critical for the maintenance of morphology and intercellular communication via synapses. In mature hippocampal neurons in culture, dynamic tyrosinated microtubules are abundant in the cell body and localize in the outer part of the dendrites, near the plasma membrane [49, 115]. More stable microtubules, bearing acetylated, detyrosinated and Δ 2 tubulin, co-exist with tyrosinated microtubules in the cell body but show a segregated localization in dendrites, where they form microtubule bundles at the inner part of the dendrites [49, 114, 115]. In the axon, microtubules are predominantly stable and enriched in all post-translational modifications including detyrosinated and Δ 2 tubulin, except at their distal part where tyrosinated microtubules are specially enriched (Fig. 4).

Microtubule dynamics, which are closely linked to the de-tyrosination/re-tyrosination cycle, were recently shown to be crucial for the synapse both in the pre- and postsynaptic compartments (reviewed in [116, 117]). Change in the balance between tyrosinated and de-tyrosinated microtubules was even observed in synaptosomal fractions of the dentate gyrus during contextual fear conditioning [118]. Interestingly, a recent study showed the crucial role of TTL, and thus of tubulin re-tyrosination in regulating structural plasticity [119]. In heterozygous TTL (KO) mice, reduction of expression of the tyrosinating enzyme resulted in early memory defects and age-dependent hippocampal synaptic dysfunction. The tubulin re-tyrosination impairment affected dendritic spine density both in cultured neurons and mice brains.

Axonal microtubules play important roles in receptors trafficking, presynaptic bouton organization, synaptic vesicle recycling and mitochondrial anchoring, while postsynaptic microtubules grow from dendritic shaft and target dendritic spines in an activity-dependent manner to strengthen the synaptic connection [120-122] (and reviewed in [116, 117]). Stimulation of postsynaptic N-methyl-D-aspartate (NMDA) receptors led to a higher frequency and longer duration of spine invasions by microtubules. Conversely, chemical induction of long-term depression decreased microtubules invasions, indicating that their targeting into spines is sensitive to synaptic activity [120, 121, 123-125].

Nothing is known about the post-translational modification status of presynaptic microtubules, but for the postsynaptic compartment, dynamic tyrosinated microtubules were shown to transiently invade dendritic spines and play an important role in synapse function and plasticity [120]. And, interestingly, controlled TTL overexpression promoted invasions of spines by dynamic microtubule and protected neurons from spine pruning induced by amyloid peptides [119] (see 5.2).

The differences in microtubule subtypes control the spatial regulation of specific molecular partners and intracellular transport in neurons (see 1.4). The gradual change of highly de-tyrosinated to tyrosinated microtubule distribution from the proximal to the distal part of the axon allow the sequentially regulated transport of the tropomyosin-related kinase B (TrkB) by two kinesin motors: the de-tyrosinated tubulin-sensitive kinesin-1 first, and the tyrosinated tubulin-sensitive kinesin-3 later [126]. Presynaptic microtubules also provide tracks for inter-bouton synaptic vesicle transport. Localized enrichment of dynamic microtubules at the bouton specifies an unloading zone to ensure the delivery of synaptic vesicle precursors by a tyrosinated tubulin-sensitive kinesin-3 (KIF1A) molecular motor [127]. At the same place, there is a hotspot for the nucleation of dynamic microtubules depending on γ -tubulin and the augmin complex [128]. As TTL rapidly tyrosinates soluble tubulin, newly formed microtubules at these sites of high remodeling are likely tyrosinated. As neurons contain high amounts of de-tyrosinated or $\Delta 2$ tubulin pools, we can however not exclude the polymerization of non-tyrosinated microtubules at the bouton. Thus, presynaptic dynamic, most probably tyrosinated, microtubules are needed for efficient delivery of synaptic vesicles.

Stable detyrosinated postsynaptic microtubules regulate the transport by kinesin-1 of synaptic α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and gamma aminobutyric acid (GABA) receptors to excitatory and inhibitory synapses, respectively [129 , 130 , 131, 132]. Kinesin-1 is also involved in mitochondrial trafficking and localization close to synapses [133, 134]. Another detyrosinated tubulin-sensitive motor, kinesin-2 (KIF17), is implicated in the transport of the NMDA receptors [135]. On the other hand, the tyrosine-sensitive kinesin-3 delivers synaptotagmin IV vesicles to the synapses via dynamic microtubule invasion into dendritic spines [136]. Finally, we can also speculate that tyrosinated microtubule invasion into active spines could enhance synaptic activity by locally recruiting CLIP-170-mDia1 complexes that accelerate actin polymerization and spine enlargement [57, 137].

All these studies point to a modulatory role of the detyrosination/re-tyrosination cycle in synapse functioning and maintenance. Stable detyrosinated microtubules emerge as essential for the transport and localization of mitochondria and synaptic receptors, whereas dynamic tyrosinated microtubules which are recruited to spines upon neuronal activity are likely to deliver specific cargos and regulate actin remodelling. Thus, these mechanisms allow specific spines to be targeted by pathways that impact their morphology and plasticity.

Another important mechanism involved in synaptic activity regulation is the autophagy-lysosomal pathway, that was recently linked to the tyrosination status of microtubules. Initiation of autophagy, autophagosome-lysosome interaction and fusion were shown to happen on detyrosinated microtubules through a mechanism involving Kinesin-1 [138]. In neurons, autophagy is essential for homeostasis since misfolded proteins or damaged organelles cannot be diluted through cell division. These recent results are undoubtedly of high interest for neurons in which axonal autophagy is tuned by synaptic activity and has high vulnerability to stress.

4. Physiological impact of the cycle in cardiomyocytes and heart

4.1. Microtubules in cardiomyocytes, essential tracks for transport and cellular homeostasis

Cardiomyocytes, such as neurons, are terminally-differentiated cells that have very low, if any, proliferative capability (reviewed in [139]). They are densely packed with myofibrils, mitochondria, nuclei, sarcoplasmic reticulum and invagination of the sarcolemma, namely the transverse tubules, leaving only a small portion of the cell volume (10%) for cytoskeletal proteins [140]. The non-sarcomeric cardiomyocyte cytoskeleton is mainly composed of actin filament, microtubules, and the intermediate filament desmin. These structures provide mechanical support to the cardiomyocytes,

and, because of their longitudinal and transversal distributions (See Fig. 5), they can sense and transmit mechanical signals to the nucleus to regulate gene expression [141-143].

Microtubules are critical for contractile function, tension-sensing and signal transduction in cardiomyocytes. The first evidence of the regulatory role of microtubules in cardiomyocyte contraction was provided by Klein in 1983, who showed that the microtubule-depolymerizing agent colchicine increased the spontaneous beating rate of neonatal rat cardiac muscle cells in a concentration-dependent manner [144]. In cardiomyocytes, microtubules can be divided into three subpopulations, namely perinuclear, cortical and interfibrillar microtubules [145]. Perinuclear microtubules are around the nuclei and provide connection between the cytoskeleton and nuclear lamina by interaction with the linker of nucleoskeleton and cytoskeleton complex (LINC; [146]). An elegant study has shown that knockdown of desmin, LINC or its cytoskeleton-binding partner nesprin in rat ventricular myocytes results in rapid nucleus shrinkage and chromatin compaction in a microtubule-dependent manner, suggesting that the cardiomyocyte nuclear homeostasis is maintained by a push-pull balance between microtubules and intermediate filaments at the nucleus [147]. Cortical microtubules, which are perpendicular to myofibrils, are involved in mechanotransduction and regulation of transmembrane protein or ion channels (for review, see [148]). It was shown that microtubule depolymerisation with colchicine or nocodazole increases the current amplitude and surface density of the potassium channel Kv1.5 in adult rat atrial myocytes [149]. Similarly, nocodazole increased or decreased potassium channel currents, leading to shorter action potential duration in adult rat ventricular myocytes [150]. On the other hand, the microtubule-stabilizing agent taxol reduced the density and the peak current amplitude of the sodium channel Nav1.5 in neonatal rat cardiomyocytes [151]. Thus, alterations of microtubule density impair ion flux in cardiomyocytes. Stretching of ventricular myocytes during diastole happens with every heartbeat as the ventricles fill with blood in the working heart. This physiological stretch of cardiomyocytes stimulates the reduced nicotinamide adenine dinucleotide phosphate oxidase 2 (NOX2) to produce reactive oxygen species (ROS) in a microtubule-dependent manner (named X-ROS signaling; [152]). ROS triggers a burst of Ca^{2+} sparks, which increases Ca^{2+} signaling sensitivity in healthy cardiomyocytes. Interfibrillar microtubules, in contrast to cortical ones, are longitudinally associated with myofibrils and provide viscoelasticity that helps to protect the cellular network against the permanent stress of cardiomyocyte contraction, with a behavior known as “buckling” (Fig. 5; [153]) Microtubules are important sensors for the response of cardiomyocytes to shear stress, providing resistance against stiffness [154].

Microtubules also provide tracks for intracellular transport of mRNAs [155, 156], proteins [157, 158], intercalated disk proteins [159], vesicles and organelles such as sarcoplasmic reticulum or mitochondria [160, 161] in cardiomyocytes. This transport is mediated by the anterograde and retrograde molecular motors kinesins and dyneins, respectively [162]. A recent body of evidence

indicates microtubules as essential rails to transport in a kinesin-1 (KIF5B)-dependent manner both mRNAs and ribosomes to specific sites to enable local translation and assembly of sarcomere components in adult rat ventricular myocytes [158]. Microtubule-associated protein 4 (MAP4) phosphorylation is a major regulator of microtubule dynamics in cardiomyocytes ([140] and for review, see [163]). Microtubules act as global and local integrators of the autophagy-lysosomal pathway. Along with the ubiquitin-proteasome system, they provide a major role in cardiomyocyte protein homeostasis (for reviews, see [164, 165]). Both autophagosomes and lysosomes are transported on microtubule tracks via kinesin and dynein, and the localization of lysosomes determined the rate of autophagosome-lysosome fusion [166]. Trafficking of autophagosomes on microtubules is necessary for effective fusion with lysosomes around the nucleus [167]. Compromised trafficking of autophagy components could lead to impaired autophagy-lysosomal pathway, as shown in human and mouse models of cardiomyopathy [168-170].

4.2. The detyrosination/re-tirosination cycle in healthy cardiomyocytes

Tubulin concentrations in neonatal heart is about 0.04% of total protein [144] or 0.4% of the soluble proteins [18] and about 0.01% in adult rat ventricular myocytes [171]. Nothing is known about the specificity of α -tubulin in the heart, while β -tubulins have distinct localizations in adult rat cardiomyocytes [172]. Proteomic analysis performed in cardiac tissue from 8 control human individuals showed that the α -tubulin pool is composed of 86% of α 1B and 10% of α 4A, while β 4B constitutes 80% of the β -tubulins [173].

The TCP activity was high in fetal rat heart until postnatal day 2 and then progressively decreased to adulthood [18]. Whereas tyrosinated tubulin fraction decreased gradually over time, detyrosinated α -tubulin level dipped at birth and then increased gradually until 2 weeks before declining to low levels (20% of total tubulin) in the adult rat heart. This suggests a specific role of detyrosinated tubulin in the early postnatal development of cardiomyocytes. The most important posttranslational modifications of tubulin in adult rat ventricular myocytes is detyrosinated α -tubulin, while acetylated and Δ 2 α -tubulins were found in lower amounts or absent [19]. These stable microtubules were not affected by treatment with isoprenaline, cholera toxin or forskolin. Prosser *and colleagues* have shown that detyrosinated tubulin binds to the intermediate filament desmin at the Z-discs of the sarcomere (Fig. 5; [174]), and the combination of desmin and tubulin detyrosination stabilize growing microtubules in cardiomyocytes [175]. Furthermore, microtubules modulate the transverse Young's modulus of cardiomyocytes, with microtubule detyrosination being associated with a stiffening of cardiomyocytes [176]. Phosphorylation of the microtubule-associated protein MAP4 reduces its affinity for tubulin and prevents stabilization of microtubules [177-179].

Different studies, including the human protein atlas (HPA) containing three transcriptome datasets, showed *VASH1* (18.2 normalized expression (NX)) to be substantially higher expressed than *VASH2* (2.8 NX) in the human heart, whereas in rats this discrepancy is less pronounced [180, 181]. The HPA database shows slightly higher level of *SVBP* mRNA (19.6 NX) than *VASH1* in heart muscle, which is in line with recent findings [180]. *TTL* is lower expressed in heart tissue (10.2 NX), however, expression values show a high variability between datasets in the HPA [181].

5. Dysfunction of the cycle leads to brain and heart diseases

A timeline related to this chapter is presented in [Fig. 6](#).

5.1 Alterations of the cycle in neurodegenerative diseases

Neurodegeneration refers to a progressive loss of structure and/or function of neurons, often beginning at the synaptic distal ends of axons, a phenomenon termed as ‘dying back neuropathy.’ The abnormalities in axon connectivity and synaptic function long precede somatic cell death. The balance between stable and dynamic microtubules is crucial for neuronal plasticity and survival, and its alteration leads to brain degeneration [182-184]. Dynamic microtubules are critical for the synapse both in the pre- and postsynaptic compartments (paragraph 3.3), and thus perturbation of intraspinal microtubules could be deleterious [116, 117]. Defects in cytoskeleton organization could also result in dendritic and axonal trafficking alteration and thus altered protein quality control [185] and toxic protein aggregation [183, 186], which are two prominent cellular features involved in neurodegenerative diseases. Perturbations of the de-tyrosination/re-tyrosination cycle, which is tightly linked to the microtubule dynamics, could thus be a key mechanism contributing to the neurodegenerative process [187-190].

Alzheimer’s disease (AD) is a progressive degenerative disease in which the main pathological features are cognitive impairment, β -amyloid plaques formed by toxic amyloid- β ($A\beta$) deposition, and neurofibrillary tangles formed by the excessive phosphorylation of the tau protein. Decreased synaptic density was correlated to the cognitive decline in AD [191]. In the early stages of the disease, soluble $A\beta$ plays critical synaptotoxic and neurotoxic roles. Recent studies showed that exposure of hippocampal neurons to oligomeric $A\beta$, which mimics conditions of the AD brain [192], promoted acute stabilization of dynamic microtubules giving rise to increased microtubule de-tyrosination mediated by mDia1 formin, and was also associated with tau-dependent spine loss [189, 193]. Such changes may then affect the affinity of microtubules for tyrosination-sensitive proteins, such as kinesins and CAP-Gly proteins (see paragraph 1.4 & 3.3) and contribute to dysfunction of multiple neuronal pathways.

Another pathogenic mechanism present in AD is the accumulation of a calpain-generated neurotoxic fragment of tau (τ_{45-230}) in the cytoplasm of degenerating neurons. This neurotoxic tau interfered in the interaction between full-length tau and microtubule and resulted in a transient increase followed by a significant decrease in tyrosinated tubulin [194]. The resulting imbalance between stable and dynamic microtubules affected anterograde and retrograde axonal transport and caused synapse loss and cell death in cultured hippocampal neurons [194]. Glucocorticoid is another major AD-causing factor which was shown, on the contrary, to lower acetylation (or detyrosination)/tyrosination ratio, and thus to destabilize microtubules in neuroblastoma Sh-SY5Y cells after a series of cellular events including autophagy inhibition [195]. In mice exposed to corticosteroid, the changes in microtubule dynamics ultimately resulted in the reduced trafficking of AMPA receptors and mitochondria into the synapse due to detachment of kinesin-1. Hence several AD-causing factors were linked to changes in the balance between tyrosinated and detyrosinated forms of tubulin, resulting in compromised neuronal functions.

Interestingly, in humans, reduced TTL expression and accumulation of non-tyrosinated tubulin (detyrosinated and $\Delta 2$) were recently associated to both sporadic and familial AD [119], in agreement with a previous study showing $\Delta 2$ tubulin accumulation in the hippocampi of post-mortem AD patients [42]. The TTL impairment was present from an early stage of the disease and was associated with increased amounts of non-tyrosinated tubulin especially in the entorhinal cortex, the first region to be affected in AD. Exposure of hippocampal neurons to oligomeric $A\beta$ reduced dynamic microtubule entry into spines and was correlated to spine pruning. Controlled TTL overexpression promoted the entry of dynamic microtubule into spine and prevented $A\beta$ -induced spine pruning. Thus, loss of re-tyrosination and accumulation of stable microtubules (composed of detyrosinated and $\Delta 2$ tubulins) were proposed to drive the AD pathology, and this might occur by perturbing invasion of dynamic microtubules into spines and through alteration of specific tracks for transport in the vicinity of the synapses [119].

Abnormalities in the cycle were also noted in two other degenerative diseases: hereditary spastic paraplegia (HSP) and spinal muscular atrophy (SMA). HSP is an autosomal dominant neurodegenerative disorder, caused by gene mutations in *SPAST*, encoding the microtubule severing protein spastin, and characterized by corticospinal tracts degeneration. This degeneration leads to gradual axonopathy, distinguished by local axonal swellings and an impaired retrograde movement of organelles [196]. Interestingly, these phenotypic defects were observed in a region of the neurite, close to the growth cone, where there is a transition between stable, detyrosinated to dynamic, tyrosinated microtubules. It remains to be known whether these anomalies are solely due to *SPAST* mutation or observed in other neurodegenerative disorders as well. Interestingly, a preference of spastin for detyrosinated microtubules has also been suggested *in vitro* [197]. SMA is an autosomal-recessive neurodegenerative disorder, which primarily causes infant death affecting the lower motor neurons in

the spinal cord. SMA is caused by the loss of survival motor neuron (SMN) protein which damages the microtubule framework. SMN deficiency results in increased levels of microtubule-associated protein 1B (MAP1B) and TTL, leading to a reduction in α -tubulin detyrosination and therefore, less stable, more dynamic tyrosinated microtubules [198]. This ultimately causes impaired axonal transport of the mitochondria.

Besides, disturbance of the cycle was recently proposed to be involved in side effects of Parkinson disease treatment. Parkinson's disease is typically identified by degeneration of the dopaminergic neurons of the substantia nigra in the midbrain. L-3,4-dihydroxyphenylalanine (L-dopa) treatment is the most relevant therapy found till date; however, long-term treatment with L-dopa leads to L-dopa-induced-dyskinesia. One recent study figured out one of the possible reasons behind this phenomenon [73]. The authors found that L-Dopa can be irreversibly incorporated into the α -tubulin C-terminus by TTL (cannot be released by the TCP), and that this irreversibility alters the tyrosination state of tubulin in neurons, leading to reduced mitochondrial transport and ATP concentration in axons.

Thus, dysregulated microtubule tyrosination can prove to be quite detrimental and key in degenerative processes.

5.2 Alterations of the cycle in cardiac hypertrophy and heart failure

Cardiac hypertrophy is the response to pathophysiological increased hemodynamic load of the heart. Hypertrophy fails to be compensatory when the overload surpasses the growth capacity of cardiomyocyte to normalise stress or when the contractile performance of hypertrophied heart becomes less than that of normal myocardium. The contribution of microtubule network in cell growth and contractile dysfunction of hypertrophied adult cardiomyocytes was first suggested by Cooper 4th and colleagues in 1993 [199]. The authors found that right ventricular pressure overload inducing ventricular hypertrophy and dysfunction in cats is associated with an increase in the free and polymerized microtubule content in right ventricular myocytes. Colchicine, which binds to the β -subunit of the $\alpha\beta$ -tubulin heterodimer and thereby prevents its assembly into microtubules, corrected right ventricular myocyte dysfunction [200]. This finding was supported by the reverse experiment with taxol, inducing microtubule stabilization, which reduced cell shortening in control cat cardiomyocytes [199]. Further findings confirmed that microtubule depolymerisation normalised contractility in right papillary muscles of cats subjected to right pulmonary artery banding [201]. Taken together, these data suggested that cardiomyocyte function is inversely correlated with the microtubule content in hypertrophy. Further experiments showed that microtubule densification in

hypertrophy was mediated by MAP4 dephosphorylation on serine 924 (Ser924), decorating and stabilizing microtubules in the right ventricle of these cats [177, 178]. This pathological microtubule phenotype and cardiac dysfunction were reversed by the β -blocker propranolol [202], leading to the suggestion by the authors that in this model β -adrenergic stimulation activates p21-activated kinase and protein phosphatases that specifically dephosphorylate Ser924 of MAP4 and stabilize microtubules.

The first demonstration of altered tubulin detyrosination/re-tyrosination cycle was described in a cat model of pressure overload right ventricular hypertrophy [43]. The authors showed a marked accumulation of detyrosinated and $\Delta 2$ tubulins, associated with microtubule stabilization. Successively, it was shown in a rat model of congestive heart failure via thoracic aortic stenosis that both α - and β -tubulin protein levels were markedly elevated and particularly in the free, soluble fraction, and this was associated with a marked increase in detyrosinated tubulin amount without alteration in tyrosinated tubulin level [203]. Specifically, detyrosinated tubulin was detected only in the polymerized tubulin fraction in both groups, whereas the $\Delta 2$ tubulin was detected in both fractions. The $\Delta 2$ tubulin level was higher and lower in the free and polymerized tubulin fractions in heart failure, respectively. In neonatal rat cardiomyocytes, phenylephrine, which is a selective $\alpha 1$ -adrenergic receptor agonist acting on β -adrenergic receptors at high concentration, promoted hypertrophy and reorganization of the cytoskeleton, including the accumulation of sarcomeric proteins, total/detyrosinated α -tubulin, and desmin [204]. Adenosine or 2-chloroadenosine, likely through its antiadrenergic effects, normalized the level of total and detyrosinated α -tubulin but did not affect desmin level. Recent findings indicate that the molecular motor kinesin-1 is involved in the relocation of mRNA and ribosomes along microtubule tracks towards cell periphery in phenylephrine-induced hypertrophy of rat ventricular myocytes [158].

The role of microtubule and intermediate filament network in cardiac diseases has gained interest in recent years. Specifically, enhanced density of desmin, total and/or detyrosinated α -tubulin was observed in several forms of human heart failure, hypertrophic cardiomyopathy (HCM) and in mouse models of HCM (Fig. 5; [145, 173, 174, 205, 206]). Similarly, total and detyrosinated α -tubulin was accumulated in cardiomyocytes isolated from the *mdx* mouse model of Duchenne muscular dystrophy [142]. Accumulation of detyrosinated tubulin increased cardiomyocyte stiffness of the microtubule and intermediate filament network in heart failure [174, 207]. Recent data showed 28-50-fold higher mRNA level of *VASH1* than *VASH2* in human heart failure (dilated, ischemic and hypertrophic cardiomyopathies), lower *VASH2* mRNA level in HCM and higher *VASH1* mRNA level in dilated cardiomyopathy than in non-failing heart samples, while expression of *SVBP* did not differ between human cardiac samples [180]. In HCM, higher levels of total and detyrosinated α -tubulin were

found in septal myectomy from several patients carrying sarcomeric gene mutations but not in sarcomere-negative patients [173]. Other proteins involved in microtubule stability are accumulated in HCM and several forms of human heart failure such as $\alpha 4A$, which is the only tubulin translated in its detyrosinated form, and MAP4 [173, 205]. Unphosphorylated MAP4 (at Ser914 and Ser1046 sites in mice) binds to microtubules. MAP4 is phosphorylated by the microtubule-affinity regulating kinase 4 (MARK4), which is expressed in the brain and heart [208] and located in a striated manner in cardiomyocytes [209]. MARK4-triggered MAP4 phosphorylation induced conformational changes, which alter MAP4 binding to microtubules and thereby microtubule dynamics (for review, see [210]). Li *and colleagues* elegantly added a piece in the puzzle, by showing that acute myocardial infarction in *Mark4*-deficient mice resulted in reduced accumulation of (dephosphorylated) MAP4, desmin, VASH2 and detyrosinated tubulin, associated with preserved ejection fraction when compared to wild-type mice [209]. These data suggest that MARK4, by inducing MAP4 phosphorylation, conformation change and microtubule detachment, facilitates the access of the VASH2-SVBP complex on the polymerized microtubule, thereby promoting α -tubulin detyrosination and reduction in cardiomyocyte contractility [209].

In cardiomyocytes from human failing myocardium, treatment with parthenolide (a non-specific inhibitor of TCP [90, 100]) restored 40-50% of the lost contractile function, while in non-failing myocytes the effect was much less pronounced. Similarly, treatment with parthenolide reduced the detyrosinated α -tubulin level by 40% and markedly increased contractility of adult mouse HCM cardiomyocytes [173]. These effects are maybe not direct on the TCP, because a recent study showed that parthenolide did not affect the VASH1-SVBP activity *in vitro*, but rather reduced detyrosination by destabilizing microtubules by tubulin adduction [211], suggesting a similar mechanism of action as colchicine [144]. Importantly, treatment with parthenolide was shown to affect Ca^{2+} handling, by decreasing Ca^{2+} transient amplitude and increasing Ca^{2+} decay, indicating off-target effects of the drug [205]. Studies in non-cardiac cells have also revealed inhibitory effects of parthenolide on DNA (cytosine-5)-methyltransferase 1 (DNMT1) or nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B), with a lower EC_{50} compared to the effect on detyrosination [212, 213], advising caution when considering parthenolide as a treatment for increased detyrosinated microtubule. More recently, it was described that *TTL* overexpression or *VASH1* knockdown markedly improves contractility and reduces stiffness in human failing cardiomyocytes (Fig. 5B; [180, 205]). Similarly, *Ttl* overexpression or parthenolide treatment markedly improved cardiomyocyte contractility after myocardial infarction in mice [209].

Concluding remarks

Neurons and cardiomyocytes are highly differentiated cells in which tubulin de-tyrosination and re-tyrosination are very active. These cells contain similar proportions in their contents of α 1A/B (having a C-terminal Tyr) and α 4A (bears no Tyr) tubulins, with α 1A/B being majorly represented. In addition to tyrosinated and de-tyrosinated tubulins, neurons contain cycle-derived Δ 2- and Δ 3-tubulin variants, which are poorly represented in healthy cardiomyocytes. Pools of all these variants are highly regulated in both types of cells and their imbalance recently emerge as candidate mechanisms involved in human diseases. In both AD brain degeneration and heart failure, a marked accumulation of de-tyrosinated microtubules were observed, and increasing expression of TTL has been shown to be protective. In cultured neurons, controlled TTL overexpression protected neurons against A β -induced degeneration related to synapse loss in AD. In human or mouse models of cardiac disease, overexpression of TTL improved cardiac function. In addition, deletion of VASH1 or MARK4 (which limit VASH2 activity on microtubule) have been shown to be protective for the cardiac tissue. It would be of interest to identify upstream pathways (other than MARK4-MAP4 for cardiomyocytes) that fine tune de-tyrosination of microtubules and regulate function in these cells. In mature neuron, decrypting the downstream molecular relays after invasion of the synapses by dynamic, tyrosinated microtubule would also be of great interest to understand the role(s) of penetrating microtubule in synapse function. Comprehension of the links between the cycle, microtubule dynamics and molecular motor trafficking in cells has progressed recently. However, these mechanisms should be precisely examined in healthy and diseased neurons and cardiomyocytes.

De-tyrosination/re-tyrosination modification of α -tubulin is more complex than what was considered in the seventies when it was discovered: three enzymes were already identified including TTL and the two VASH-SVBP complexes, and (an)other de-tyrosinating enzyme(s) remain(s) to be discovered. No other tissue than brain was described having enriched TCP (de-tyrosinating) activity to be considered for a biochemical approach as used by Moutin *and colleagues* [83]. The haploid genetic screening strategy employed by Brummelkamp *and colleagues* is however very promising [84].

Future studies are needed to understand the functioning and regulation of all the involved enzymes. Specifically, several questions remain regarding the VASH-SVBP enzymes. *VASH1* expression is generally much higher (about 6-times) than *VASH2* in tissues. Is there also differences in the mode of action between *VASH1* and *VASH2*? Concerning regulation, a residue of TTL, and acknowledging the Phosphosite Plus database, certain residues of VASH and SVBP deemed as phosphorylation sites can be examined. Their potential phosphorylation might affect the interaction between VASH and SVBP, between the complex and some cellular partners, or the mode of binding to microtubules, and consequently the activity of the enzyme complex. In addition, are there any partners involved in the regulation of VASH-SVBP activity in cells?

Finally, since excessive tubulin detyrosination levels are associated with human diseases, the precise structural findings and regulation modes of detyrosinating enzymes would subsequently aid in the designing of small molecule inhibitors or drugs to regulate their activity and prevent deleterious effect of excessive detyrosination.

Acknowledgment

This work was supported by the Leducq Foundation, Research grant n° 20CVD01 (to LC and MJM), by INSERM, CNRS, University Grenoble Alpes, and grant SPEED-Y n° ANR-20-CE16-0021 (to MJM). Part of the salaries of CS and SRR were from a collaborative program between Servier laboratories and MJM team. Figures 2, 4 and 5 were created with Biorender.com. We thank Sung Ryul Choi for help in preparing Fig. 3.

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Figures legends

Figure 1: Discovery of the detyrosination/re-tyrosination cycle, its enzymes and significance. EM, electron microscopy; KO, knock-out; MT, microtubule; SVBP, small vasohibin binding protein; TCP, tubulin carboxypeptidase; TTL, tubulin tyrosine ligase; VASH, vasohibin

Figure 2: Schematic representation of the detyrosination/re-tyrosination cycle. (A) Schematic representation of $\alpha\beta$ tubulin dimer with their C-terminal tails. The last 4 amino acids of α tubulin C-terminal tail are represented. Tubulin carboxypeptidases (TCPs, VASH1 or VASH2 associated with SVBP) removes the C-terminal tyrosine residue (Y, in magenta) of α -tubulin to generate detyrosinated tubulin. Detyrosinated tubulin can be re-tyrosinated by the tubulin tyrosine ligase (TTL) that adds a tyrosine residue at the glutamate C-terminal (E, in green). Detyrosinated tubulin cans also exit the cycle

by removal of the penultimate glutamate residue (E, in green) by cytosolic carboxypeptidases (CCPs), generating $\Delta 2$ -tubulin. **(B)** Schematic representation of modified microtubules and the enzymes of the cycle acting on microtubules or soluble dimers. TCPs preferentially acts on tyrosinated microtubules (magenta) to generate de-tyrosinated microtubules (green), that are markers of stable microtubules in cells. After de-tyrosinated microtubules depolymerize, TTL acts on soluble dimers. When these soluble dimers polymerize, they form new tyrosinated microtubules (magenta) that are markers of dynamic newly formed microtubules in cells. In long-lived de-tyrosinated microtubules, CCPs generates $\Delta 2$ -microtubules (red). Most of the α -tubulins are neosynthesized with a C-terminal tyrosine. $\alpha 4$ -tubulin is the only tubulin that is neosynthesized without the C-terminal tyrosine. Since $\alpha 4$ -tubulin is predominantly non-tyrosinated in brain [102], we may assume that it polymerizes into de-tyrosinated microtubules. Figure adapted from [114].

Figure 3: VASH-SVBP complex structure and interaction with microtubule. (A) Surface representation of the structure of the complex between the core domain of VASH2 (white) and SVBP (gray). Key residues of VASH for α -tubulin tail contact and, in the active site, for tubulin de-tyrosination are highlighted in orange. A cluster of highly conserved basic residues on the surface of VASH2, whose mutation in alanine led to reduced binding to microtubule and loss of de-tyrosination in cells, is highlighted in purple. R137 in VASH2 (R148 in VASH1) is part of interface 1 in B; H257 in VASH2 (H268 in VASH1) is part of interface 2 in B. Adapted from [93]. **(B)** Schematic representation of the VASH-SVBP-microtubule complex with the three proposed interfaces of binding and the C-terminal tubulin substrate recognition indicated. Adapted from [101].

Figure 4: Significance in mature neurons of the three major pools of α -tubulin resulting from the de-tyrosination/re-tyrosination cycle. Schematic representation of de-tyrosinated (deTyr, green), tyrosinated (Tyr, magenta) and $\Delta 2$ (red) microtubules whose orientation is represented with a «+» at the microtubule +end. Effectors of the cycle, such as molecular motors (with their specific neuronal cargos) and CAP-Gly +end proteins, are represented in the color of the microtubule to which they are sensitive. **Inbox 1: dendrites** contain mixed-polarity microtubules composed of Tyr tubulin at their outer region and deTyr and $\Delta 2$ tubulin at their inner region; deTyr-sensitive kinesins-1 and 2 transport GABA(A), AMPA and NMDA receptors [129, 130, 135]. **Inbox 2:** Tyr microtubules from the dendritic shaft can transiently enter into **dendritic spines**. Tyr-sensitive kinesin-3 transport synaptotagmin vesicles into the spines [136]. Tyr-sensitive CAP-Gly proteins could have the following roles in spines: CLIP-170 and p150-Glued could interact with growing end of microtubules [57]; CLIP-170 could increase microtubule dynamics and favors actin polymerization [26, 137]; P150-Glued could initiate the retrograde dynein-dependent transport out of the spines [54]. **Inbox 3: axonal microtubules** have

uniform polarity and are mainly composed of stable deTyr and $\Delta 2$ tubulins. Tyr-sensitive kinesin-5 transport microtubules and kinesin-3 transport vesicles [48, 127]. DeTyr-sensitive kinesin-1 transports mitochondria and TrkB [126, 133]. **Inbox 4: axonal boutons** are hotspots of microtubule remodeling and delivery of synaptic vesicles driven by the Tyr-sensitive kinesin-3 [127]. Kinesin-3 also transports TrkB at the axon distal part [126]. Figure adapted from [114].

Figure 5: Sarcomeres and cardiomyocyte in health and disease and impact of reduced detyrosination. (A) Schematic representation of two cardiac sarcomeres delineated by Z-disks during relaxation (left panels) and contraction (right panels) in healthy conditions (upper panels), heart failure (middle panels) and after *TTL* overexpression in heart failure (lower panels). The sarcomeres and microtubules (detyrosinated in green and tyrosinated in purple) are shown with putative stiff anchors (orange stars) of detyrosinated microtubules at the desmin filament. During contraction, the distance between anchors is reduced, which induces a sinusoidal buckling of microtubules under load. In heart failure, there is a marked accumulation of desmin and detyrosinated microtubules, leading to stiffness of the cardiomyocyte. *TTL* overexpression reduces detyrosination and thus the interaction of microtubules to the stiff anchors, allowing stronger contraction and better relaxation. Adapted from [174]. (B) Schematic representation of healthy (upper panel) and failing (lower panel) cardiomyocyte. In heart failure, there is densification of microtubules and/or accumulation of detyrosinated and $\Delta 2$ tubulin. Treatment of failing cardiomyocytes with *TTL* overexpression or knockdown of vasohibin 1 (*VASH1*; [180]) reduced microtubule detyrosination.

Figure 6: Impact of the cycle in neurodegeneration and heart diseases. A β , toxic amyloid- β ; AD, Alzheimer's disease; HCM, hypertrophic cardiomyopathy ; HSP, Hereditary spastic paraplegia; SMA, Spinal muscular atrophy.

Table: Main features of TTL and SVBP deficiencies in mice.

SVBP KO mice/ SVBP Knock-down*	TTL KO mice
Tubulin content	
High Tyr tubulin Low deTyr tubulin Low $\Delta 2$ tubulin	Low Tyr tubulin High deTyr tubulin High $\Delta 2$ tubulin
Neurodevelopmental phenotypes in mice	
Not lethal, reach adulthood, fertile [112] Adults [112] : Reduction of brain white matter and microcephaly Embryos [88] : Cortical neuron migration defects*	Lethal after birth [106] Embryos [106] : Blurred organization of cortical layers Enlarged ventricles Disruption of cortico-thalamic loop
Behavioral defects	
Mild hyperactivity Lower anxiety Impaired social behavior [112]	In heterozygous TTL KO : Impaired spatial working memory Short-term recognition memory defects [119]
Neuronal morphology alterations	
Neuronal differentiation delay* Decreased axonal length [88, 112] Increased dendritic arborization [112]	Accelerated neuronal differentiation Increased axonal length [27, 106] Increased axonal branching Increased microtubule stability [27]
Molecular pathways affected	
	Defective interaction with CAP-Gly proteins (CLIP-170, CLIP-115, p150-Glued) [57] Reduced interaction with kinesin-13 depolymerizing motors (MCAK and KIF2) [27] Defective recruitment of Myosin II due to Rac 1 over-activation [109]
Other known molecular effectors of Tyr and deTyr microtubules	
Tyr Kinesin-2 motor (KIF3C) [29] Kinesin-3 motor (KIF1A) [49, 50] Kinesin-5 motor (KIF11) [48] Dynein-dynactin p150Glued [54]	deTyr Kinesin-1 motor (KIF5) [47-49] Kinesin-2 motor (KIF17) [52] Kinesin-7 motor (CENP-E) [53]

Abbreviations used: deTyr, detyrosinated; Tyr, tyrosinated. *refers to SVBP knock-down.

Discovery of tyrosination on α tubulin : an RNA-independent, ATP-dependent enzyme can add Tyr to the C-terminus of α -tubulin [1, 3]

Discovery of a non-tyrosinable, brain tubulin variant [31]

α -tubulins are generally encoded with a C-terminal Tyr preceded by two Glu. The primary modification is therefore removal of Tyr. Later on, TUBA4 & TUBA8 with no Tyr or Phe instead of Tyr were described [9]

The non-tyrosinable variant lacks the C-terminal Tyr and Glu residues. It will be named $\Delta 2$ tubulin [13, 25, 35]

Identification of tubulin tyrosine ligase (TTL) by cloning [60]

Identification of functional role of the cycle, its link to cancer: TTL is suppressed during tumor growth [214]

First reader of the modification identified: CAP-Gly proteins bind tyrosinated microtubule [57]

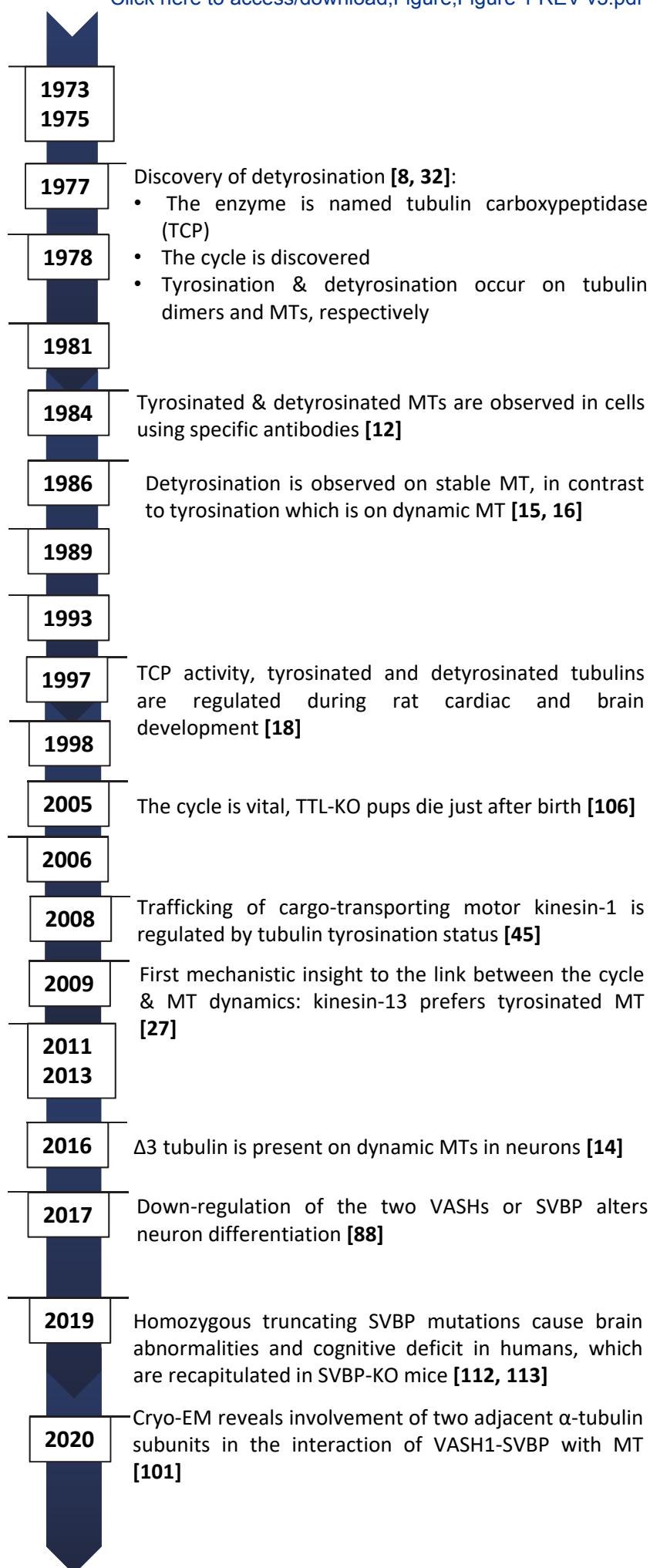
Resolution of TTL crystal structure:

- Binding sites on free tubulin are masked in MT
- Explains why $\Delta 2$ tubulin cannot be tyrosinated
- By sequestering free tubulin, TTL prevents MT assembly [67, 68]

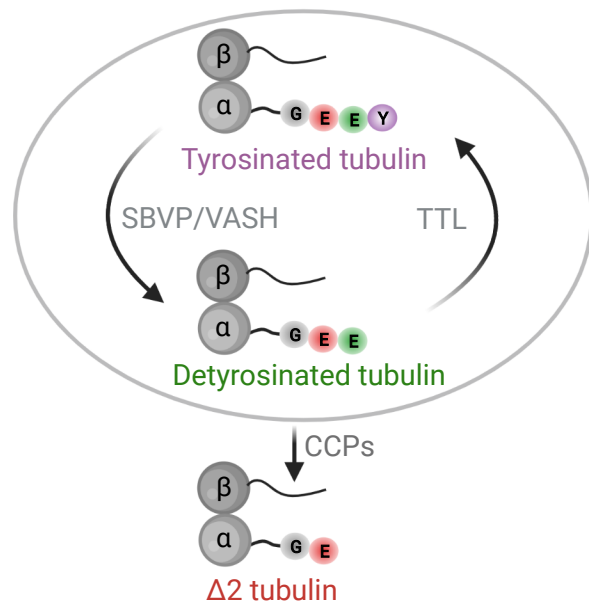
Identification of TCPs (chemical proteomics & haploid genetic screening): the VASH-SVBP complexes [88, 89]

VASH-SVBP complex structure is solved [93, 96-100]

- Identification of catalytic triad as Cys/His/Ser in VASH
- SVBP is a chaperone and an activator of VASH
- First contacts involved in MT binding are identified



A



B

