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Microtubule Minus-End-Targeting Proteins

Review

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Microtubules are cytoskeletal filaments that are intrinsically polarized, with two structurally and functionally distinct ends, the plus end and the minus end. Over the last decade, numerous studies have shown that microtubule plus-end dynamics play an important role in many vital cellular processes and are controlled by numerous factors, such as microtubule plus-end-tracking proteins (+TIPs). In contrast, the cellular machinery that controls the behavior and organization of microtubule minus ends remains one of the least well-understood facets of the microtubule cytoskeleton. The recent characterization of the CAMSAP/Patronin/Nezha family members as specific 'minus-end-targeting proteins' ('-TIPs') has provided important new insights into the mechanisms governing minus-end dynamics. Here, we review the current state of knowledge on how microtubule minus ends are controlled and how minus-end regulators contribute to non-centrosomal microtubule organization and function during cell division, migration and differentiation.

Introduction

Microtubules are cytoskeletal filaments present in all eukaryotic cells, where they serve as rails for intracellular transport and are involved in controlling organelle positioning, cell shape, polarity and division. Microtubules are built from dimers of α- and β-tubulin that bind in a head-to-tail fashion to form protofilaments, which through lateral association organize into hollow tubes. Microtubules with 13 protofilaments are found in nature most frequently, but microtubules with 11 or 15 protofilaments also occur in certain cell types in mammals and invertebrates [1–4].

Microtubules assemble and disassemble from their ends, which can switch between phases of growth and shortening, a behavior termed dynamic instability [5]. This behavior is explained by the 'GTP cap' model. Both α- and β-tubulin bind to the nucleotide GTP, and, although the GTP bound to α-tubulin does not exchange, the GTP bound to β-tubulin can be hydrolyzed, and this process is stimulated by the formation of longitudinal contacts with the α-tubulin subunits in the microtubule lattice [2,6,7]. Therefore, while the microtubule lattice is built from GDP–tubulin, there is a cap of GTP-bound β-tubulin at the newly polymerized microtubule ends. Due to its higher stability, the GTP-bound microtubule cap inhibits microtubule disassembly [2,6,7]. When the stabilizing cap is lost, the microtubule switches to the depolymerizing state (a transition called catastrophe), whereas regaining the cap is associated with switching back to growth (a transition called rescue). All phases of microtubule growth dynamics are strongly regulated by a multitude of cellular factors, which in this way control the shape and density of microtubule arrays.

The two ends of each microtubule are structurally distinct, because α-tubulin is exposed at one end (termed the minus end), while β-tubulin is exposed at the other end (the plus end). In solutions of purified tubulin, both ends can grow and depolymerize, but the plus end grows faster and undergoes catastrophe more frequently [2,8]. When microtubules grown *in vitro* from purified tubulin are severed with a laser beam, the plus end rapidly depolymerizes, while the minus end is relatively stable and can resume growth [9,10]. In cells, the behavior of the two microtubule ends is also very different. The plus ends, which often extend towards the cell periphery, can display phases of rapid growth and shortening and thus explore the cellular space [6]. Alternatively, microtubule plus ends can be stabilized, with their dynamics reduced due to attachment to different cellular structures, such as mitotic kinetochores or the cell cortex [11,12]. In contrast, it is currently generally accepted that microtubule minus ends in cells never grow [13], possibly due to capping by specific factors, such as the γ-tubulin ring complex (γ-TURC) [14]. Microtubule minus ends are often densely clustered in central regions of the cell, where they can be stabilized through attachment to nucleation sites, such as the centrosome. Careful measurements of the dynamics of free microtubule minus ends generated by release from the centrosome or by breakage due to actomyosin-based retrograde flow revealed that these ends either are stable or undergo depolymerization [13,15–19]. Microtubule minus-end depolymerization can significantly contribute to microtubule turnover [20]. In cells with a centrosomally-centered microtubule system, such as CHO-K1 fibroblasts, microtubule minus ends depolymerize more frequently than in certain epithelial cells that have many non-centrosomal microtubules [15,21]. The balance between minus-end depolymerization and stabilization thus controls interphase microtubule organization: when free microtubule minus ends are depolymerized efficiently, the centrosome is the predominant microtubule-stabilizing site and the microtubule system becomes more radial. Importantly, in some differentiated cell types, such as epithelial cells or neurons, non-centrosomal microtubules predominate; they can be arranged into parallel or anti-parallel arrays (see below), an organization that strongly contributes to cell polarity. Understanding how such arrays are formed and maintained is a long-standing, unresolved problem.

In addition to their involvement during interphase, microtubule minus-end dynamics are also important for controlling the architecture of the mitotic spindle during cell division. The minus ends of spindle microtubules depolymerize at the poles, while their plus ends undergo net polymerization; as a result, spindle microtubules translocate towards the poles, a process termed poleward microtubule flux [22]. Depending on the species and the cell type, the poleward flux plays a role in spindle-length control and regulates different aspects of chromosome separation [23].

The behavior and distribution of the two microtubule ends, and also our knowledge of these two microtubule extremities, display a clear asymmetry. The plus ends, which are often easy to monitor because they are localized at low density in flat peripheral cell regions, have received a lot of attention. The studies of microtubule plus ends were strongly facilitated by the discovery of microtubule plus-end-tracking proteins (+TIPs) [24–26]: +TIPs form an interacting network at the core of which are the proteins of the EB family. EB proteins autonomously bind to the growing microtubule ends, which they can recognize by sensing the nucleotide-bound

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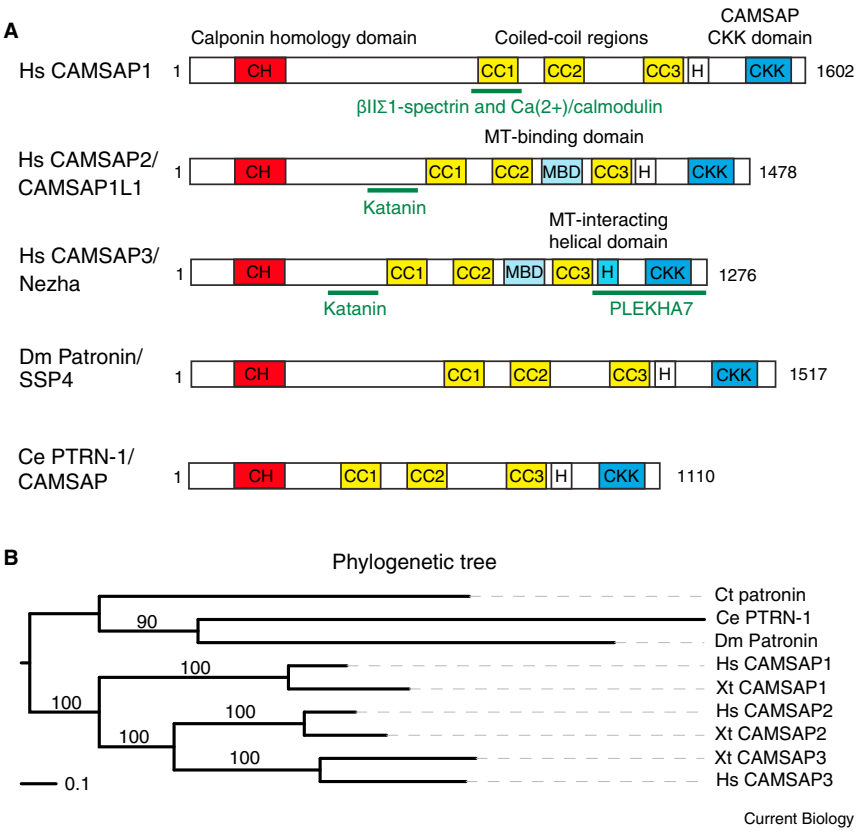

Figure 1. Phylogenetic tree and structure of the CAMSAP/Patronin/Nezha family members.

(A) Schemes of CAMSAP/Patronin/Nezha proteins from three different organisms are shown. The mammalian members include three homologues, CAMSAP1, CAMSAP2 (CAMSAP1L1) and CAMSAP3 (Nezha), while invertebrates have one family member, Patronin in *D. melanogaster* and PTRN-1 in *C. elegans*. The structural motifs are illustrated in the diagram. The family is characterized by the presence of an amino-terminal calponin homology domain (CH, red) and a carboxy-terminal CKK domain (also named DUF1781, blue), involved in microtubule minus-end binding. The middle part of the proteins contains three predicted coiled-coil regions (CC1–CC3, yellow). Note that CC2 is poorly conserved in worms. In CAMSAP2 and CAMSAP3 an additional microtubule-binding domain (MBD) is present in the linker region between CC2 and CC3: the MBD decorates and stabilizes the microtubule lattice but does not recognize the microtubule minus end by itself. A helical domain (H) between CC3 and CKK is present in all members, but it is only involved in microtubule stabilization and minus-end growth inhibition in CAMSAP3 [38]. The various blue boxes in the CAMSAP structures indicate the domains contributing to microtubule minus-end tracking, microtubule binding and/or microtubule stabilization. The green lines indicate the positions of the domains interacting with protein partners. Protein sequences with the following NCBI accession numbers were used for the drawings: CAMSAP1 (NP_056262), CAMSAP2 (NP_982284), CAMSAP3 (NP_001073898), Patronin (NP_788398), PTRN-1 (CCD66253). Hs: *Homo sapiens*; Dm: *Drosophila melanogaster*; Ce: *Caenorhabditis elegans*. (B) RAxML phylogenetic tree of selected CAMSAP/Patronin homologues from the animal kingdom. The numbers at the nodes show support values derived from a RAxML bootstrap analysis. Branch lengths are proportional to the number of substitutions per site (see scale bar). The following protein sequences were used for the phylogenetic tree analysis, NCBI accession numbers for Hs CAMSAP1 (AAI44083), Hs CAMSAP2 (Q08AD1.3), Hs CAMSAP3 (Q9P1Y5.2), Dm Patronin (AFA36631.1), Ce PTRN-1 (CCD66253.2), *Capitella teleta* (Ct) Patronin (ELU11545.1); UniProt accession numbers for *Xenopus tropicalis* (Xt) CAMSAP1 (F6TMK5), Xt CAMSAP2 (F6VFE3), Xt CAMSAP3 (F6X904).

state of β-tubulin [27,28]. The EBs — such as EB1 and EB3 [29,30] — and some of their binding partners serve as excellent markers of growing plus ends, greatly facilitating the analysis of microtubule plus-end dynamics. In contrast, microtubule minus ends are often strongly clustered, so that individual ends are difficult to discern by light microscopy. Until recently, the only factor known to specifically associate with microtubule minus ends was the γ-TURC, which nucleates microtubules and can cap their minus ends [14]. The localization of this complex can provide useful information about the distribution of minus ends; for example, it was used in combination with photoactivation to analyze the dynamics of microtubule minus ends in the spindle [31]. However, not all γ-TURCs may be active and attached to microtubules, and not all minus ends are capped with γ-TURC, so observations of γ-TURC are likely to be insufficient in describing the distribution and behavior of all microtubule minus ends.

Recently, a series of studies from different laboratories demonstrated that the members of the CAMSAP/Patronin/Nezha family specifically recognize microtubule minus ends and control their dynamics in different animal systems [32–34]. In this review, we discuss how the identification and characterization of these factors has advanced our understanding of microtubule minus-end dynamics and has helped to demonstrate the importance of microtubule minus-end regulation in the control of mitotic spindle length [34–36], epithelial cell migration and proper organelle distribution [37,38], and neuronal development and regeneration [39–42].

CAMSAP/Patronin/Nezha Family Proteins are Specific Microtubule Minus-end Regulators

The CAMSAP/Patronin/Nezha family is characterized by the presence of the signature domain CKK (which stands for C-terminal domain common to CAMSAP1, KIAA1078 and KIAA1543, and is also known as domain of unknown function DUF1781) [33] (Figure 1A). CAMSAP homologues are present in the genomes of all sequenced eumetazoa (animals with tissues) [33]; furthermore, the CKK domain can be found in the sequenced genomes of diverse unicellular organisms, suggesting an ancient evolutionary origin. In worms, overexpression of the CKK domain is necessary and sufficient to rescue the function of the Patronin homologue during axon regeneration, supporting its important function [42]. At the amino terminus, CAMSAPs/Patronin have a calponin homology (CH) domain, homologues of which are present in numerous other cytoskeletal regulators and can mediate interactions with either actin or microtubules [43]. The function of the CH domain in CAMSAPs is currently unknown, as

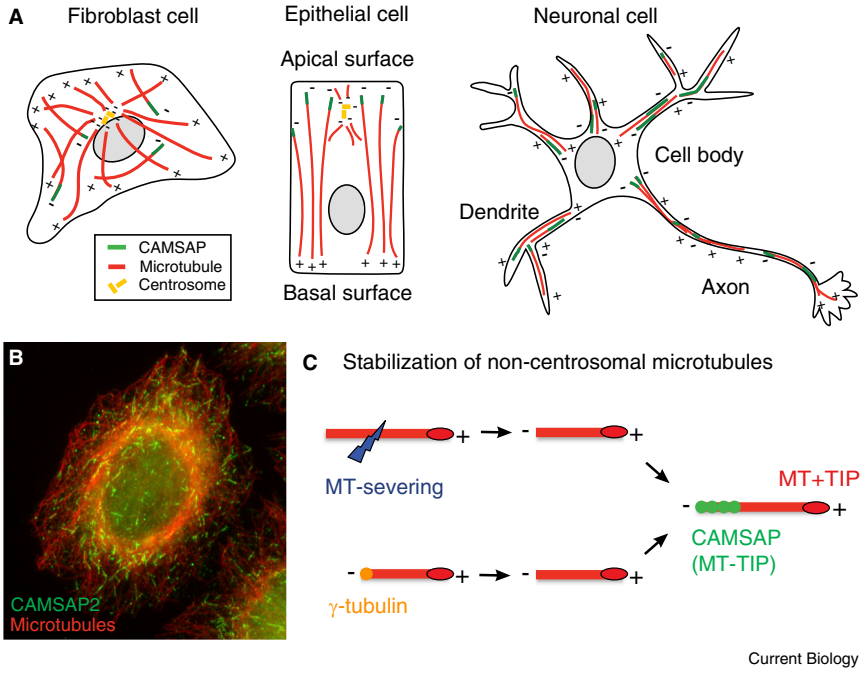

Figure 2. Centrosomal and non-centrosomal microtubule arrays in various cell types.

(A) Cultured fibroblasts have a radial array of microtubules organized around the centrosome as well as non-centrosomal microtubules with CAMSAP2-decorated minus ends distributed in the cytoplasm. In polarized epithelial cells, the centrosome is located close to the apical surface, and microtubule arrays are organized along the apical–basal axis, with microtubule minus ends enriched at the apical surface; CAMSAPs are likely involved in their stabilization. Mature (polarized) neurons lack a single microtubule-organizing center and contain CAMSAP2-labeled non-centrosomal microtubule arrays in both axons and dendrites. (B) In cultured HeLa cells, CAMSAP-decorated microtubule stretches are distributed throughout the cytoplasm. Note that cultured cells with radially organized microtubules have a significant proportion of CAMSAP2-labeled non-centrosomal microtubules. (C) Non-centrosomal microtubules can be generated by two distinct mechanisms: enzymatic severing or mechanical breaking of pre-existing microtubules (blue) or local nucleation at non-centrosomal sites by γ-TURC (orange). CAMSAPs (green) mediate stabilization of newly generated microtubule minus ends, forming sites of non-centrosomal microtubule plus-end outgrowth (red ovals).

it does not associate with any cytoskeletal structures [33,34,38]. The middle part of the proteins contains predicted coiled-coil (CC) regions interspersed with regions that are predicted to be unstructured (Figure 1A).

The mammalian members of the family, CAMSAPs (calmodulin-regulated spectrin-associated proteins), include three homologues, CAMSAP1, CAMSAP2 (KIAA1078/CAMSAP1L1) and CAMSAP3 (KIAA1543/Nezha), which have diverged from each other during vertebrate evolution [33] (Figure 1B). CAMSAP3, which was initially named Nezha after a Chinese deity, was the first family member shown to be able to recognize and bind microtubule minus ends [32]. A whole-genome screen in *Drosophila* S2 cells has identified the only fly member of this family (initially named Ssp4 for short spindle phenotype 4) as a regulator of spindle length [36]. A subsequent study, in which the protein was renamed as Patronin (after the Latin *patronus*, meaning 'protector'), directly demonstrated that the protein can associate with free microtubule minus ends and inhibit microtubule minus-end disassembly by kinesin-13 depolymerases in interphase and mitotic cells as well as in an *in vitro* system with purified proteins [34]. The *in vitro* work was performed using stabilized microtubules, on which Patronin localized to the outermost tips of the minus ends, suggesting that it might act as a capping factor. This idea appeared to be consistent with studies in mammalian epithelial cells, which showed that CAMSAP2 and CAMSAP3 stabilized the ends of non-centrosomal microtubules [37].

However, at high expression levels CAMSAP2/3 and Patronin decorated and bundled microtubules [32,34,37], which argued in favor of lateral microtubule association. Furthermore, staining of endogenous CAMSAP2 and CAMSAP3 showed that they form distinct (~0.5–2 μm) stretches rather than dots at the ends of non-centrosomal microtubules [37,38] (Figures 2A,B). The underlying mechanism was clarified by reconstitution experiments with dynamic microtubules, which showed that CAMSAPs do not cap microtubule minus ends, but instead are specifically recruited to the growing microtubule minus ends [38,44]. The behavior of the three mammalian CAMSAP proteins is different: while CAMSAP1 only transiently associates with and essentially 'tracks' the growing minus end, CAMSAP2 and CAMSAP3 are stably deposited on the microtubule lattice generated by minus-end polymerization [38,44] (Figures 3A,B). Through this process, CAMSAP2 and CAMSAP3 form stretches of decorated microtubule lattice, which are highly stable and resistant to depolymerization from both ends [37,38]. The behavior of CAMSAPs is thus to some extent reminiscent of EBs and EB-dependent +TIPs, in that they are recruited to the growing microtubule end, where they associate with the side of the microtubule lattice. We propose to call the proteins that specifically recognize the microtubule minus ends without capping them '–TIPs', for microtubule minus-end-targeting proteins.

Importantly, there are significant differences between +TIPs, such as EBs, and –TIPs, such as CAMSAPs/Patronin. While EBs recognize the nucleotide-bound state of β-tubulin and do not distinguish between the plus end and the minus end [27,45], CAMSAPs/Patronin are specifically recruited only to the growing minus ends [38,44]. In contrast to EBs, CAMSAPs/Patronin can also form a dot at the outermost tip of the microtubule minus end when it is not growing [32,34,44]. Another important difference relates to the protein dynamics at the microtubule tip: while EBs and their partners exchange rapidly at the growing microtubule ends, CAMSAP2 and CAMSAP3 are deposited stably, and exchange only slowly, if at all, after they are bound [38,39]. It is currently unclear whether CAMSAPs and EBs physically interfere with each other's binding to microtubules; at least for CAMSAP1, it was observed that it can accumulate at

the growing microtubule minus end simultaneously with EB3 [38].

The major function of CAMSAP2 and CAMSAP3 is to generate strongly stabilized microtubule lattices that can serve as 'seeds' for microtubule outgrowth [37–39]. In addition, CAMSAP2 and CAMSAP3 have a strong effect on microtubule dynamics: they specifically inhibit microtubule minus-end polymerization and catastrophes [38,44]. This property likely explains why the polymerization of free microtubule minus ends in animal cells has been overlooked until now: due to the presence of CAMSAPs, microtubule minus ends grow much more slowly than could be expected on the basis of the tubulin concentration. However, careful analysis demonstrated that minus ends in cells do undergo tubulin addition, although this process is slow [38]. In agreement with the microtubule minus-end growth-suppressing role of these proteins, the depletion of Patronin in *Drosophila* S2 cells facilitated detection of microtubule minus-end growth events with the EB1 marker [34]. The –TIPs CAMSAPs/Patronin are thus important regulators that determine the behavior of free, non-centrosomal microtubule minus ends.

The capacity to recognize, track or decorate microtubule minus ends is determined by the sequences located in the carboxy-terminal half of the proteins. In mammalian CAMSAPs, the minimal region that shows specific, albeit weak microtubule minus-end binding, is a single CKK domain [38]. For CAMSAP1, robust microtubule minus-end tracking is observed with a protein fragment that includes the CKK and the CC3 domain with the preceding linker sequence [38]. A similar fragment of Patronin also tracks polymerizing microtubule minus ends and affects their growth; however, it is the unique unstructured linker region with the adjacent carboxy-terminal CC domain, and not the CKK, which seems to be responsible for the minus-end specificity [44]. The ability of CAMSAP2 and CAMSAP3 to decorate and stabilize the microtubule lattice is due to the presence of an additional strong microtubule-binding domain (MBD) in the linker region between CC2 and CC3. CAMSAP3 possesses another microtubule-interacting helical domain between CC3 and CKK, which makes it the most potent microtubule stabilizer and minus-end growth inhibitor among the three mammalian CAMSAPs [38] (Figure 1A). Interestingly, CAMSAP3 is expressed as multiple splice isoforms, some of which lack CC1, CC2 and MBD [46] and thus likely have reduced microtubule affinity.

The microtubule minus-end specificity and the microtubule-stabilizing properties of CAMSAPs/Patronin thus appear to depend on the combined activities of several microtubule-binding domains. Since the proteins form a dot at the minus end of non-dynamic microtubules [32,34], they might recognize some feature associated with the exposed α-tubulin. It is possible that this interaction, which does not block the addition of another tubulin subunit, can be strengthened by additional contacts with the microtubule lattice and/or interactions with other CAMSAP/Patronin proteins. Another possibility is that, similar to the model currently proposed for the Stu2p/XMAP215/Dis1 polymerases [47], a part of the protein (e.g. the CKK domain) recognizes some lateral microtubule-end-specific feature, such as curved tubulin, and the additional minus-end specificity results from the relative position of microtubule lattice-binding regions that extend in the plus-end direction. Structural cryo-electron microscopy studies with CAMSAP-decorated

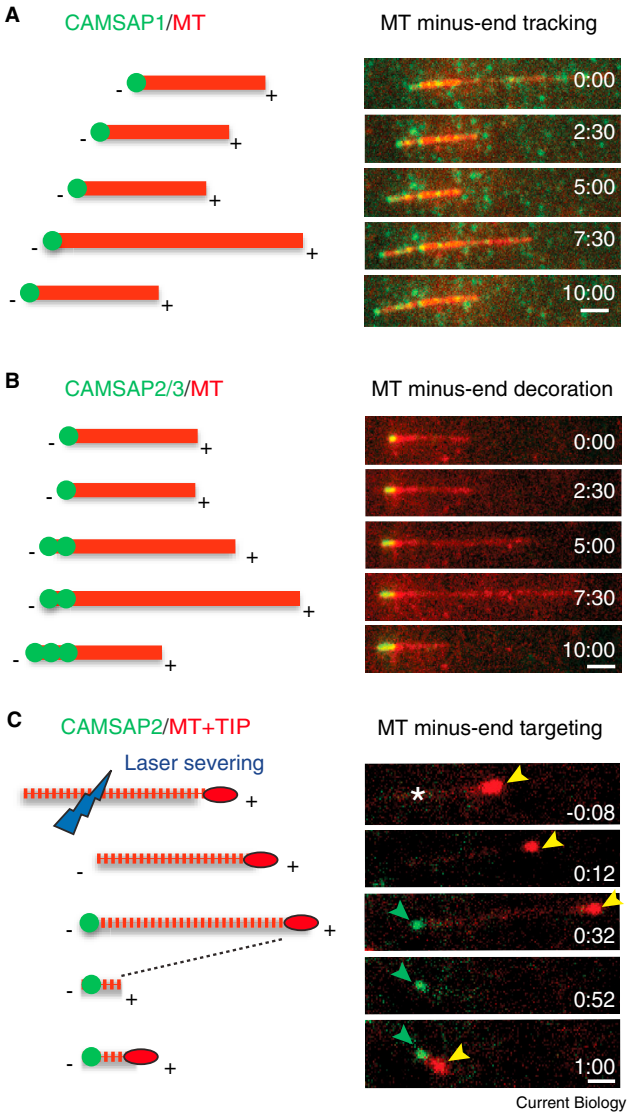

Figure 3. CAMSAP family members associate with growing microtubule minus ends and differentially regulate microtubule dynamics.

(A,B) Schematic representations and images illustrating the different behavior of full-length CAMSAP1 (A, green) and CAMSAP3 (B, green) on rhodamine-labeled dynamic microtubules (red). In this *in vitro* assay, microtubules are grown in solution of porcine brain tubulin from GMPCPP-stabilized microtubule seeds attached to a functionalized glass surface and observed by total internal reflection fluorescence microscopy (TIRFM). Purified GFP–CAMSAP1 (A) dynamically tracks growing microtubule minus ends, representing an example of a minus-end tracking protein. Purified GFP–CAMSAP3 (B) and CAMSAP2 (not shown) are stably deposited on the growing microtubule minus ends; they also reduce the microtubule minus-end growth rate. Modified from [38]. (C) Schematic representations and images illustrate the association of CAMSAP2 with microtubule minus ends in COS-7 cells. Laser-based microsurgery, in which a focused laser beam severs an individual microtubule, was used to generate new microtubule ends. The images are from TIRFM time-lapse recording of a COS-7 cell expressing a microtubule plus-end marker mCherry–microtubule+TIP (a dimeric version of an EB-binding peptide derived from the spectraplakin MACF2, red) and GFP–CAMSAP2 (green). Upon laser-induced severing (asterisk), GFP–CAMSAP2 is rapidly recruited to the newly generated microtubule minus end. Note the microtubule plus-end outgrowth observed from the newly formed CAMSAP2 cluster (arrows). Modified from [39].

lattices and analysis of the binding cooperativity of CAMSAPs to microtubules will help to shed light on this issue.

CAMSAPs/Patronin Stabilize Minus Ends of Non-centrosomal Microtubules in Interphase and Mitosis

The fact that CAMSAPs require microtubule minus-end polymerization to form a stabilized microtubule stretch suggests that they act independently of the minus-end nucleating and capping factor γ-TURC. In line with this view, CAMSAPs are promptly recruited to free microtubule minus ends generated by laser-induced severing [38,39] (Figure 3C), and functional analyses have shown that the primary function of CAMSAPs is to stabilize the ends of microtubules that are not embedded in the centrosome [37–39]. While the centrosomes are regarded as the major microtubule-organizing centers in most cultured mammalian cells, the situation is actually more complex. In some lines of cultured cells, such as CHO, COS-7 and MRC5-SV, a centrosomally-centered microtubule aster is indeed predominant, while in many others, such as HeLa, a single focus of microtubule minus ends is impossible to distinguish (Figure 2B). Importantly, even in cells that have a well-developed radial microtubule system, not all microtubule minus ends are attached to the centrosome. If the latter were the case, the distribution of microtubule staining intensity would decrease hyperbolically from the centrosome to the cell periphery [38]. In reality, such microtubule distributions are rarely observed, indicating that a significant proportion of microtubule minus ends is not connected to the centrosome and is instead associated with other structures, such as, for example, the Golgi apparatus [48], or just lies free in the cytoplasm.

The depletion of CAMSAP2/CAMSAP3 strongly reduced the number of non-centrosomal microtubules, suggesting that the stability of a significant proportion of free microtubule minus ends is dependent on CAMSAPs [37,38,49]. This view was directly confirmed by laser-induced severing experiments, which showed that, while in control cells newly generated microtubule minus ends often pause, they mostly depolymerize after CAMSAP depletion [38]. It is thus tempting to speculate that the concentration and activity of CAMSAPs can determine the proportion of non-centrosomal microtubule minus ends and thus the radial or non-radial organization of the microtubule array.

CAMSAP-stabilized microtubule minus ends can be generated soon after nucleation, provided that γ-TURC is released from the minus ends. Indeed, nocodazole washout experiments demonstrated that CAMSAP-stabilized microtubule minus ends appeared in the pericentrosomal area soon after the initial nucleation from the centrosome [38]. Alternatively, the minus ends generated by enzymatic severing or mechanical breaking of microtubules can be stabilized by CAMSAPs (Figure 2C). It is likely that both pathways are active, given that CAMSAP stretches can be distributed randomly throughout the cytoplasm, while the centrosome and the Golgi apparatus seem to be the major microtubule nucleation sites (Figures 2A,B). Experiments in epithelial cells and neurons support the independent roles of γ-TURC and CAMSAPs in microtubule formation and maintenance, although it is possible that they act sequentially to nucleate and stabilize microtubules, respectively [37,39].

As described above, in mammalian cells CAMSAP stretches are completely lost following nocodazole-mediated microtubule depolymerization and emerge only after the microtubule network is restored [38]. However, in insect cells, which have acentrosomal interphase microtubule arrays [50–52], Patronin-positive foci seem to participate in the initiation of microtubule outgrowth after disassembly and are associated with the centrosomal factors SAS-4 and SAK [34]. The absence of Patronin leads to the very frequent microtubule release from the nucleation sites and the ensuing abundance of depolymerizing microtubule minus ends in the cytoplasm. Microtubule minus-end depolymerization in these cells is driven by the kinesin-13 family depolymerase KLP10A, which accumulates at the shrinking ends [34]. The major function of Patronin in insect cells is thus to protect the minus ends from kinesin-13 activity.

The antagonism between the –TIPs CAMSAPs/Patronin and the microtubule depolymerases manifests itself not only in interphase but also in mitosis. In fly cells, Patronin depletion results in shorter bipolar spindles, and this defect can be rescued by co-depletion of KLP10A [34,36]. Acute inhibition of Patronin by antibody injection in *Drosophila* embryos revealed that Patronin-mediated stabilization of microtubule minus ends against KLP10A-dependent depolymerization resulted in spindle elongation in anaphase B [35]. Modeling predicted that an increased activity of Patronin relative to KLP10A could be sufficient to cause the observed spindle elongation [35]. Whether CAMSAPs have similar functions in mammalian cells is currently uncertain, because the depletion of CAMSAP2 has no impact on cell division; CAMSAP2 dissociates from microtubules in prophase due to extensive phosphorylation, and its reassociation is observed only in telophase [38]. CAMSAP3 also seems to bind to microtubules predominantly in interphase [53]. Among the mammalian CAMSAPs, CAMSAP1 is thus the most likely candidate for a mitotic regulator.

Another factor that can act antagonistically to CAMSAPs is the microtubule-severing enzyme katanin. Katanin interacts with a short part of the linker between the CH and CC1 domains of CAMSAP2 and CAMSAP3 [38] (Figure 1A). Katanin plays a role in limiting the length of CAMSAP2-decorated microtubule stretches, and the katanin-binding region of CAMSAP2 is important for this process [38]. Katanin might contribute to disassembly of CAMSAP-decorated microtubule stretches or limit their growth, by either cutting them or depolymerizing them from the ends. Other severing enzymes might perform similar functions [54,55], with fidgetin being a particularly interesting candidate because of its minus-end preference [56].

Functions of CAMSAPs/Patronin in Epithelial Cells

As discussed above, in cultured mammalian epithelial cells, the depletion of CAMSAPs causes a significant loss of non-centrosomal microtubules [37,38,49]. The depletion of CAMSAP2 and CAMSAP3 resulted in a reduction of microtubule density, mildly increased microtubule growth rate and longer EB1 comets, in line with the elevated pool of soluble tubulin [37,38]. Further, CAMSAP depletion inhibited cell migration in monolayer wound healing assays [38], in line with previous observations, which showed that non-centrosomal microtubules are required for efficient cell migration [57]. The underlying mechanism is not clear, but might involve the Golgi-associated population of non-centrosomal microtubules, which is required for polarized cell migration [58]. Further, changes in the microtubule cytoskeleton such as a decrease in microtubule density or an altered

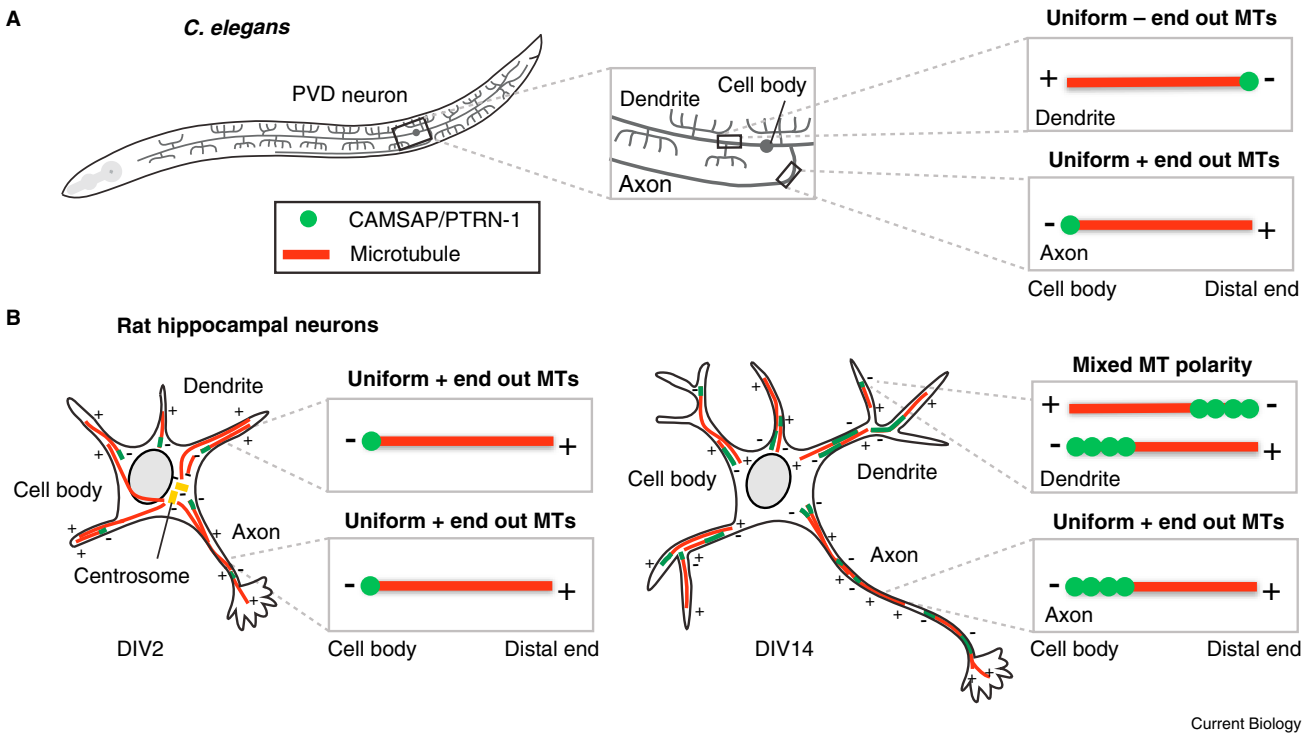

Figure 4. Microtubule organization and CAMSAP/Patronin distribution in vertebrate and invertebrate neurons.

(A) Schematic representation of the *C. elegans* sensory PVD neurons showing a cell body, two highly branched dendrites and an axon that extends into the ventral nerve cord. The two boxes on the right indicate the organization of microtubules (red) and the distribution of CAMSAP2 (green) in the axon and dendrites. PVD neurons have a uniform 'plus-end-out' microtubule polarity in axons and uniform 'minus-end-out' microtubule polarity in dendrites. The GFP-tagged CAMSAP protein PTRN-1 localizes as puncta along neuronal processes [40–42], which most likely represent microtubule minus ends. (B) Schematic representation of cultured hippocampal neurons during early development (day *in vitro* 2 (DIV2), stage 2/3, after neuronal polarization) and after differentiation (DIV14, stage 4/5, mature neurons) showing a cell body, several dendrites and a single axon. During neuronal development, the centrosome loses its function as a microtubule-organizing center [74]. The two boxes at the right indicate the organization of microtubules (red) and the distribution of CAMSAP2 (green) in the axon (plus-end-out) and dendrites (mixed). The size of the CAMSAP2-positive structures changes during neuronal development: CAMSAP2-positive clusters (>1 μm and <10 μm) are present in young hippocampal neurons at DIV2, whereas long CAMSAP2 stretches (>10 μm) are abundantly present in mature neurons at DIV 14 [39].

microtubule modification pattern might have indirect effects on the cell architecture and the actin cytoskeleton, for example, through the activation of Rho GEF-H1, which is inhibited by association with microtubules [49,59].

When epithelial cells differentiate and polarize, the proportion of non-centrosomal microtubules increases, and they can become tethered to the apical cortex or to cell–cell junctions [60–65] (Figure 2A). One potential function of CAMSAPs in epithelial cells could be to stabilize microtubules at the apical side and attach them to cell junctions. In Caco2 cells, CAMSAP3 links microtubule minus ends to the apical-most part of the zonula adherens, a cadherin-based cell–cell junction, through the interaction with PLEKHA7 (Pleckstrin homology domain-containing family A member 7), which binds to p120-catenin [32]. PLEKHA7 binds to the same carboxy-terminal part of CAMSAP3 that binds to microtubules [32] (Figure 1A). The fact that this interaction can attach microtubules to the zonula adherens suggests that both interactions can occur simultaneously, although this possibility was not directly tested. The CAMSAP3–PLEKHA7 complex is required for the accumulation of the minus-end directed kinesin-14 KIFC3 at the zonula adherens and for the integrity of this cell–cell junction [32].

CAMSAP3 was found to be prominently expressed in the organ of Corti, where it was named Marshalin [46]. The supporting cells of the organ of Corti are a specialized type of epithelial cell and contain extremely dense microtubule bundles that originate from both centrosomal as well as non-centrosomal, cortical microtubule-organizing centers [46,66]. CAMSAP3/Marshalin was enriched at all non-centrosomal microtubule-organizing sites, suggesting that it might be required for their formation. Interestingly, microtubules in these cells are composed of 15 protofilaments: it would be interesting to know whether the expression of specific isoforms of CAMSAP3 contributes to this unusual microtubule structure [46].

Functions of CAMSAPs/Patronin in Neuronal Cells

Another polarized cell type where non-centrosomal arrays play a very prominent role is neurons (Figure 2A). Most differentiated neurons have one long axonal process and several branched dendrites, which strongly depend on microtubules for their integrity and organelle transport [67,68]. In axons, microtubules are typically arranged with their plus ends facing away from the cell body, while in dendrites a mixed microtubule polarity is observed in mammalian neurons, and predominantly 'minus-end-out' orientation can be found in invertebrate neurons [69–71] (Figure 4). When mammalian neurons start to differentiate, the centrosome acts as an active microtubule-organizing center, but over time its

activity is shut down and, while the centrioles persist and may serve as a basal body for cilia formation [72,73], the centrosome-dependent microtubule organization is lost [74]. This conclusion is supported by the analysis of microtubule organization using super-resolution microscopy, which revealed a loss of radial microtubule organization around the centrosome during development [39]. Importantly, electron microscopy studies have shown that microtubules are often free at both ends and that the average microtubule length is much shorter than the length of a neuronal process [75–77]. Based on all these findings, it is strongly believed that differentiated neurons have many non-centrosomal microtubules and that microtubule minus ends are distributed throughout the whole cell [70].

The localization and function of CAMSAPs/Patronin was analyzed in detail in mammalian and in *Caenorhabitis elegans* neurons [39–42]. In cultured mammalian hippocampal neurons, CAMSAP2 — the predominant CAMSAP family member in this cell type — is distributed as small puncta and clusters at early developmental stages and as long (>10 μm) stretches in more mature neurons [39]. The worm homologue, PTRN-1, is distributed as small puncta throughout neuronal processes [40–42]. The depletion of CAMSAP2 or mutation of PTRN-1 reduced microtubule density, but had no effect on microtubule orientation [39,41,42]. Interestingly, structural microtubule abnormalities were observed in some cells that normally have 15-protofilament microtubules [41]. CAMSAP family members are thus important determinants of microtubule density and stability in neurons, a conclusion supported by the synergistic effects between PTRN-1 loss and microtubule destabilization by colchicine in developing worms [41].

In mammalian neurons, loss of CAMSAP2 had a strong effect both on axon formation as well as on the extension and branching of dendrites, indicating that the protein acts in all neuronal compartments [39]. It was important not only at early stages of neural development, e.g. during axon specification and neuronal polarization, but also during neuronal remodeling, such as dendrite growth induced by brain-derived neurotrophic factor [39]. The importance of CAMSAP2 function in the brain is further supported by the identification of the corresponding gene as a genetic trait locus for epilepsy [78,79]. It should also be noted that the relative importance of the three mammalian CAMSAPs might be different in various cell types of the nervous system because, while CAMSAP1 depletion had no strong effect in hippocampal neurons, it did affect neurite formation in cultured cerebellar granule cells as well as in PC12 cells. This function is possibly related to the ability of CAMSAP1 to interact with spectrin and calmodulin through its CC1 region (Figure 1A) [80]. CAMSAP1 is also highly expressed in astrocytes and has been proposed to be a useful astrocytic lineage marker [81], so it might have a specific function in this cell type.

In worms, loss of PTRN-1 caused defects in neurite morphology and synaptic vesicle localization [40–41], and also affected the axonal regeneration after injury [42]. An important player in the signaling pathway that is upregulated during neuronal regeneration is the DLK-1 MAP kinase, and complex interplay between PTRN-1 function and the DLK-1 pathway was observed [40–42]. Interestingly, one of the downstream targets negatively regulated by DLK-1 is the microtubule depolymerase kinesin-13 KLP-7 [82], which suggests that, similar to other systems, microtubule stability in neurons is regulated by the balance of activities at the microtubule minus ends, a view supported by studies of axon regrowth [42]. It will be interesting to test the role of CAMSAPs in other regeneration models, such as spinal cord injury [83].

In spite of defects in neuronal morphology, *ptrn-1* null worms displayed grossly wild-type growth and development, indicating that the bulk of neuronal functions can still be carried out in the absence of this protein and that alternative microtubule minus-end stabilizing factors must exist [40,41]. This is consistent with the observation that in cultured mammalian neurons CAMSAP2 forms intense stretches in the cell body and proximal dendritic regions [39], but is more difficult to detect in distal dendrites, where, based on the anti-parallel microtubule organization, microtubule minus ends should also be located. It is possible that some abundant neuronal microtubule-associated proteins (MAPs) that bind along the whole microtubule length may compensate for the absence of CAMSAPs/Patronin.

Potential Diversity of Microtubule Minus-end Regulators

In addition to γ-TURC, CAMSAP/Patronin family proteins have emerged as important players at microtubule minus ends. It is currently unknown whether γ-TURC and CAMSAPs account for stabilization of all microtubule minus ends in animal cells, or whether there are any other proteins that would have similar or overlapping functions. An interesting player in the organization and stabilization of the minus ends of non-centrosomal microtubules is ninein, which relocalizes from the centrosome to apical sites during epithelial cell polarization [63,64]. However, this protein likely acts through an interaction with γ-TURC [84], as no direct binding to microtubule minus ends has been reported. Another important factor needed for non-centrosomal microtubule formation is augmin, a microtubule-associated hetero-octameric protein complex, which can bind the sides of microtubules, recruit γ-tubulin and promote the centrosome-independent nucleation of new microtubules (see [85,86] and references therein). Yet another well-studied microtubule regulator that plays an important role in microtubule minus-end organization in the spindle is NuMA [87]. Interestingly, NuMA concentrates at the minus ends of kinetochore fibers that are not attached to the spindle pole, both when they are formed naturally or through photoablation [88,89]. It is assumed that this happens due to minus-end-directed transport by cytoplasmic dynein; however, NuMA has its own microtubule-binding domain [90], which might potentially contribute to the minus-end localization. Other proteins acting at the centrosome and mitotic poles [91,92] might also need to be examined for potential microtubule minus-end binding. An interesting candidate is the RanGTP-regulated factor MCRS1, which accumulates at the minus ends of kinetochore fibers in *Xenopus* extracts [93]. However, *in vitro* this protein showed no minus-end preference, suggesting that additional factors might confer the minus-end specificity [93].

Among microtubule motors, cytoplasmic dynein and kinesin-14 family members walk to microtubule minus ends and are strongly involved in their organization during mitosis in conjunction with MAPs [31,88,89,94–97]. These motors might exert some effects on microtubule minus-end dynamics, a possibility that has not been investigated. Such regulation might be similar to the plus-end-directed kinesins, some of which can strongly regulate microtubule plus-end dynamics [98,99]. Further, as mentioned above, microtubule

depolymerases of the kinesin-13 family and severing enzymes are important players in microtubule end regulation, irrespective of whether they have a specific preference for the minus ends.

While γ-TURC components are conserved in yeast and plants [100], CAMSAPs/Patronin are not. Plants lack centrosomes but contain extensive arrays of microtubules that strongly depend on the nucleation from the lattice of other microtubules as well as severing for their maintenance and reorganization [100,101], indicating that both γ-TURC-capped and free microtubule minus ends should be present throughout the cell. It would be interesting to know whether functional counterparts of CAMSAPs/Patronin exist in plants and fungi.

Conclusions

While detailed analyses of the dynamics of microtubule plus ends and their associated proteins have uncovered a multitude of regulatory mechanisms, microtubule minus ends were often treated in a way somewhat akin to the 'dark side of the moon' — the hemisphere that had never been seen from Earth until humans were able to send spacecraft around it. The discovery of factors that specifically recognize free microtubule minus ends (–TIPs), combined with systematic application of photoablation, photoactivation and photobleaching assays as well as super-resolution microscopy is rapidly changing this situation. Recent studies showed that the regulation of microtubule minus-end dynamics in cells is more similar to that of the plus ends than previously thought: for example, specific stabilizing factors of the CAMSAPs/Patronin family can be recruited to the growing minus ends and protect them against depolymerizing enzymes, similar to +TIPs at the microtubule plus ends. Importantly, loss-of-function studies in both vertebrate and invertebrate systems have shown that additional microtubule minus-end regulating factors are likely to exist. Identification of these factors and quantitative analysis of the relative contribution of different microtubule minus-end regulators in different systems will be essential to achieve a complete mechanistic description of cellular microtubule networks, such as neuronal microtubule arrays or the mitotic spindle. Moreover, understanding the fundamental principles of microtubule dynamics and stability will be important for the development of novel strategies to treat cancer or injuries and diseases of the nervous system [102,103].

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