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Abstract

The establishment of neuronal connectivity relies on the microtubule (MT) cytoskeleton, which provides mechanical support, roads for axonal transport and mediates signalling events. Fine-tuned spatiotemporal regulation of MT functions by tubulin post-translational modifications and MT-associated proteins is critical for the coarse wiring and subsequent refinement of neuronal connectivity. The defective regulation of these processes causes a wide range of neurodevelopmental disorders associated with connectivity defects. This review focuses on recent studies unravelling how MT composition, post-translational modifications and associated proteins influence MT functions in axon guidance and/or pruning to build functional neuronal circuits. We here summarise experimental evidence supporting the key role of this network as a driving force for growth cone steering and branch-specific axon elimination. We further provide a global overview of the MT-interactors that tune developing axon behaviours, with a special emphasis on their emerging versatility in the regulation of MT dynamics/structure. Recent studies establishing the key and highly selective role of the tubulin code in the regulation of MT functions in axon pathfinding are also reported. Finally, our review highlights the emerging molecular links between these MT regulation processes and guidance signals that wire the nervous system.

Key words

Microtubules; Axon guidance; Axon pruning; Microtubule-associated proteins; tubulin isotypes; tubulin posttranslational modifications.

Introduction

The nervous system governs vital functions as well as complex behaviours enabling animals to interact with their environment. The mature nervous system is composed of billions of neurons that are interconnected according to a highly precise map (*i.e.* the connectome). Fine-tuned regulation of these connections is crucial for the accurate functioning of the network. Neuronal connectivity is established during development by linked processes of axon specification, outgrowth/navigation, synapse formation and pruning of exuberant connections and undergoes limited remodelling in the mature central nervous system [1,2]. Consequently, ectopic, exuberant or imprecise connections can lead to major neurodevelopmental disorders [3–6]. A crucial step in the assembly of neuronal circuits lies in the ability of axons to accurately navigate towards their appropriate targets, which can be located over very long distances (*i.e.* more than one meter). During their road trip in the developing embryo, the distal tips of growing axons (*i.e.* the growth cone) undergo cycles of growth, pausing, turning or retraction behaviours, which are dictated by a myriad of physical,

mechanical and chemical cues present in their extracellular environment. Different classes of chemical cues are encountered by the growth cone along its journey such as permissive (*e.g.* Netrin-1) or adhesive molecules (*e.g.* CAMs), extra-cellular matrix components (*e.g.* laminin), anti-adhesive substrate-bound cues (*e.g.* Slits) and diffusible chemotropic cues (*e.g.* Semaphorins, morphogens, neurotrophic factors), which will elicit growth cone attraction or repulsion [2,7,8]. Importantly, several studies have demonstrated that the response of attraction versus repulsion is not due to the intrinsic property of a particular cue. Rather, it is due to the specific growth cone receptors and downstream effectors engaged, including cytoskeleton-associated proteins [9,10; Figure 1 and Figure 2].

While the repertoire of guidance cues and associated receptors wiring the nervous system has mostly been identified, the intracellular pathways that modulate axon responsiveness to guidance signals are far from being deciphered. Notably, how guidance signals are integrated and translated into the cytoskeleton remodelling that underlies growth cone mechanical behaviours remains largely unknown. Pioneer studies aiming at linking guidance signals to the cytoskeleton remodelling first focused on the actin cytoskeleton [11,12]. Since then, several cues have been shown to drive growth cone attraction, repulsion or collapse through RhoGTPase-mediated actin remodelling [for review, see 13–15]. By contrast, molecular links between guidance signals and the MT cytoskeleton have remained largely unstudied until recently. This is mostly due to the fact that unlike F-actin, MTs have only recently emerged as key driving forces and direct targets of guidance cues in axon pathfinding and pruning [16,17]. Indeed, several MT-interacting proteins have been identified as decisive players in these developmental processes *in vitro* and/or *in vivo* [18–27]. Furthermore, tubulin isoforms and posttranslational modifications have lately come out as key regulators of MT functions in the axon navigation processes influencing MT properties as well as the activity/MT binding affinity of MT-interacting proteins [28–31]. This review summarises the main experiments that have established MT remodelling as a key driving force in axon guidance and pruning and provides a detailed overview of the molecular mechanisms that tune MT properties and functionalities to steer developing axons or prune exuberant connections.

I. Neuronal microtubules: Key cytoskeletal elements with various properties and functionalities

Microtubules (MTs) are one of the major cytoskeletal components of eukaryotic cells and are essential for cell morphology, movement and intracellular trafficking. MTs are hollow 25 nm diameter tubes composed of polarised linear protofilaments built of head-to-tail assembled α- and β-tubulin heterodimers [32]. MTs are highly dynamic polymers that stochastically switch between polymerisation and depolymerisation, a process known as dynamic instability. This dynamic property is driven by GTP binding to the αβ-tubulin dimer and its hydrolysis into GDP. The transition from MT growth to shrinkage is called catastrophe, while the reverse reaction is termed rescue [33]. Since their discovery in 1950, MTs have emerged as critical structures in neuronal development and function, providing mechanical support and rails for axonal transport, as well as mediating key signalling pathways. The fine-tuned spatiotemporal regulation of MT structure, mechanical properties, dynamics and crosstalk with the actin cytoskeleton (F-actin), which is mediated by tubulin isotypes, posttranslational modifications and MT-associated proteins (MAPs), is decisive to build functional neuronal circuits [34]. This is highlighted by the increasing number of neurodevelopmental disorders (*e.g.* ASD, schizophrenia, lissencephaly...) caused by mutations in genes encoding the MT building blocks (*i.e.* tubulins), tubulin-modifying enzymes, or MT-regulatory proteins [35–37]**.**

Over the past decades, the emergence of novel imaging technologies with high spatiotemporal resolution (correlation EM microscopy, TIRF, super-resolution, expansion microscopy, motor

painting approach, cryoelectron tomography) has gradually added to the complexity of the neuronal MT architecture and function [38–44]. Unlike what was initially thought, the MT cytoskeleton is not a homogeneous network, but rather a highly mosaic array composed of several subpopulations of MTs. These subpopulations (i) are decorated by different posttranslational modifications (PTMs) and MT-associated proteins [MAPs; 34,38,45,46] and (ii) exhibit distinct structural/dynamic properties and functionalities. In axons, unlike dendrites, MTs are organised in a highly polarised manner with a fast-growing end (plus end) pointed outwards, towards the synapse, and a slow-growing end (minus end) pointed inwards, towards the soma. This typical polarity pattern of axonal MTs has recently been shown to rely on non-centrosomal MT nucleation, mediated by the augmin-γTuRC complex [47]. Furthermore, the axonal MT network is organised into longitudinal parallel bundles and consists of a mixed population of stable (*i.e.* long persistance) MTs decorated with distinct posttranslational modifications, including acetylation, detyrosination, glutamylation. While some of these PTMs influence the binding affinity and/or activity of several MAPs *in vitro* [46,48,49], the functional specificity of these different subtypes of stable axonal MTs remains mostly unknown under physiological conditions [50]. The distal tip of developing axons (*i.e.* the growth cone) is divided into three compartments, which are characterised by their specific composition and arrangements of cytoskeletal elements [51; Figure 1 and Figure 2]. The central domain (C-domain) exclusively contains stable and acetylated MTs. These polymers extend from the axon shaft and are locally restricted and bundled by an actin barrier (*i.e.* actin contractile arcs) present at the transition zone [T-zone; 52,53]. In this T-zone, the local coupling between MT growing plus ends and the actin retrograde flow drags MTs back into the C-domain, thereby inducing MT looping and bending. The peripheral domain (P-domain) of the growth cone is composed of an F-actin branched network called lamellipodia-like veils and long bundles of F-actin forming the filopodia. These F-actin-based structures are locally intermingled with a few individual MTs (also called pioneer MTs) that have escaped from the C-domain. In contrast to MTs that are constrained to the C-domain, MTs that penetrate the actin-rich P-domain are tyrosinated and highly labile [*i.e.* dynamic; ,52,53]. Notably, this subpopulation of MTs are often found in close apposition with F-actin bundles in filopodia, where their growing plus tips sometimes reach the growth cone margin. Their local exposition to extracellular cues makes them relevant candidates to integrate axon guidance signals [53–55].

The following sections will emphasise how the characteristic organisation and heterogeneity of the neuronal MT cytoskeleton are critical for its functions in axonal navigation.

II. Microtubules in axon guidance: the outside players

II.1 Instructive role of microtubules in growth cone steering

MTs, together with the actin cytoskeleton, are major determinants of the mechanical properties of growing axons. Despite this evidence, the role of MTs as driving forces of growth cone mechanical turning has long been disregarded compared to that of the actin cytoskeleton. Indeed, due to their highly dynamic and protrusive behaviours at the leading edge of the growth cone and their well-established role in cell migration, actin filaments (F-actin) have long held the leading (if not exclusive) role in growth cone steering processes. By contrast, MT remodelling was thought to occur in response to F-actin changes and to be required to consolidate the novel portion of the axon shaft and stabilise growth in the appropriate direction. MTs were first postulated to play an instructive role in growth cone steering decisions in the late 1990s. This hypothesis was based on live imaging studies from the Kirschner lab revealing that in advancing growth cones, a few individual MTs

dynamically explore the P-domain and that their growth orientation often precedes growth cone turning and predicts the steering direction [56,57]. Several groups subsequently showed that the asymmetric invasion and stabilisation of theses dynamic MTs in filopodia facing an adhesive/attractive cue or located on the opposite side of a repulsive cue is decisive to drive growth cone turning towards or away from the cue, respectively [58–61,62; Figure 1 and Figure 2]. The first experimental evidence establishing the instructive role of MTs in growth cone decision-making behaviours comes from the elegant work of Buck and Zheng in 2002. Their study shows that modulation of MT dynamics on one side of the growth cone by photo-uncaging a MT-stabilising (*i.e.* Taxol) or destabilising agent (*i.e.* Nocodazole) is sufficient to induce growth cone turning towards or away from the treated side, respectively [16]. These data have been strengthened by the work of several groups revealing that (i) asymmetric manipulation of MT-associated protein activity with MICRO-CALI techniques is sufficient to drive growth cone turning [63–65] and that (ii) MTs (more precisely, brain specific tubulin isoforms) can be direct targets of guidance cues in axon navigation processes [29]. However, it is worth noting that the instructive role of MTs in growth cone steering critically relies on their fine-tuned coupling to the actin network. The latter either acts as a barrier preventing premature MT invasion of the P-domain or as a guide facilitating MT growth and stabilisation within filopodia [62,66,67]. Notably, the myosin II-based actin retrograde flow, which is regulated by the RhoA GTPase, has been shown to play a significant role in restricting and bundling stable MTs within the C-domain [68]. It has further been shown to regulate dynamic MT growth alongside filopodia [53,55,69–71].

Despite the now well-established role of MTs as driving forces of axon guidance, the numerous actors that control MT remodelling and interplay with F-actin in navigating axons, as well as their specific mode of action and regulation by guidance cues are only starting to be deciphered. This is mostly explained by the technical challenge associated with the monitoring of these nanoscale structures with high spatiotemporal resolution in growth cones while locally applying guidance cue *in vitro* and above all in single-labelled axons navigating in a complex *in vivo* environment. However, over the past decade, the emergence of novel microscopy approaches such as super resolution microscopy (e.g. SIM, STED) and live TIRF imaging as well as of optically transparent vertebrate models with straightforward genetic manipulations (*e.g.* zebrafish and Xenopus models) have allowed several groups to overcome the above-mentioned limitations. This has contributed to provide novel pieces of information regarding the regulation of MT functions in axon guidance and targeting [22,24–26,72–74].

II.2 Microtubule-interacting proteins: key effectors of guidance signals in growth cone steering

In neurons, MT organisation, dynamics and cross-talk with F-actin are tightly regulated by a myriad of MT-interacting proteins. The vast majority of them, including MT plus-end tracking proteins (+TIPs), structural MAPs, MT-depolymerising and severing enzymes, or molecular motors, have been shown to play critical roles in axon extension. However, only few of them have been reported to influence growth cone steering *in vitro* or to ensure the fidelity of axon targeting *in vivo*. We will here focus on the different classes of MT-interacting proteins whose regulation of MT functions in neurons is crucial to evoke growth cone behaviours (Table 1). It is worth noting that even if these MT-interactors remain classified in different functional categories, data collected over the past years have gradually revealed their functional versatility in neuronal circuit wiring and homeostasis.

II.2.1 Plus-end tracking proteins as key signalling integrators in axon navigation

MT plus-end tracking proteins (+TIPs) bind the growing plus end of MTs, which can extend to the growth cone periphery where guidance receptors reside. This specific subcellular distribution combined with their ability to regulate MT dynamics, anchoring at the cell cortex and interplay with F-actin makes them ideal signalling integrators of axon guidance processes. CLASP proteins, for cytoplasmic linker protein (CLIP)-associated protein, were the first +TIPs to be implicated in axon navigation. In 2004, the *Drosophila* CLASP orthologue was identified as a downstream effector of the Slit/Robo repellent pathway and the Abelson (Abl) tyrosine kinase during midline crossing [19]. This new role in axon repulsion came as a surprise given the previously identified role of CLASP in MT rescue and stabilisation [75–78]. A later study, conducted during axon elongation, has nevertheless offered possible keys to the problem, reporting two distinct MT-binding activities for CLASP in nerve growth cones: both at MT plus ends and on the MT lattice [79]. While GSK3β inhibition induces CLASP binding to the MT lattice and impedes growth cone advance, CLASP plus-end activity enables axon elongation by stabilising MTs. Further supporting a dual CLASP binding activity, elevation of Abl activity in spinal cord growth cones has also been shown to delocalise CLASP away from MT plus ends, in association with slower growth cone advance [80]. Abl has moreover been found to bind and phosphorylate CLASP in response to extracellular signals, such as serum or PDGF. Finally, using biochemical assays as well as culture experiments, the authors suggest a role for the Abl-induced CLASP phosphorylation in CLASP-mediated MT-F-actin crosslinking. While such studies highlight CLASP as a modulator of MT dynamics downstream of guidance signals, the role of its plus-end binding activity – rather than its MT lattice activity – needs further characterisation, especially in a context of axon navigation (Figure 2C).

The adenomatous polyposis coli (APC) protein is another +TIP involved in axon navigation. Originally identified as a tumour suppressor mutated in colorectal cancers [81], APC has been reported in many studies to act in synergy with EBs (End-Binding proteins) to promote MT stabilisation, in addition to its role as a regulator of the Wnt/β-catenin canonical pathway [82–84]. Zhou and colleagues were the first to report a role for APC at MT plus ends in axon elongation, downstream of NGF and its GSK3β effector [69]. Since then, studies have involved APC in growth cone steering *per se*. Indeed, local MICRO-CALI inactivation of APC in one half of retinal ganglion cell growth cones is sufficient to regulate axon turning [64]. Wnt3a has also been shown to delocalise APC from MT plus ends in DRG sensory neurons, resulting in a MT growth directionality and lopping phenotype that resembles APC loss of function [85]. Similarly, APC2, an APC-like protein [86], is also important in the ephrin-A2-induced repulsive guidance of chick retinal axons [87; Figure 2D], and to promote *Drosophila* axon growth across the midline [27]. Of note, studies have implicated APC in the crosslinking of the MT and actin cytoskeleton during cell migration [88–91]. Given the importance of this crosstalk during axon navigation, future investigations will be of interest to assess its influence on axon pathfinding.

Importantly, a study in *Drosophila* has shown that Msps (minispindles), the fly ortholog of the +TIP and MT polymerase XMAP215, physically interacts with CLASP, and functions during axon guidance by antagonising both CLASP and Abl activity [92]. The authors propose a model in which CLASP and Msps converge in an antagonistic manner on the Abl signalling pathway to regulate midline crossing. A study in *Xenopus laevis* embryos further confirms this role for XMAP215 in axon guidance. Indeed, using SIM super-resolution microscopy and live imaging in cultured spinal cord explants, the Lowery lab reveals that XMAP215 promotes MT/F-actin interactions and is required to regulate growth cone morphology and dynamic behaviour. They further show that XMAP215 accurately mediates repulsion to the ephrinA5 guidance cue in an *in vitro* stripe assay [25]. Importantly, as for

CLASP, a dual mode of XMAP215 binding to MTs has been identified, on the MT lattice and at the MT plus ends in the growth cone [93]. Both modes of MT binding appear to regulate MT-F-actin coupling, in addition to the known XMAP215 MT polymerase activity at plus ends (Figure 2D). Understanding how these different XMAP215 functions are coordinated and synchronised by intracellular signalling pathways to regulate different aspects of axon outgrowth and/or pathfinding requires further investigations. It is likely that a phosphorylation-based regulation of XMAP215 by kinases acting downstream specific guidance cues such as Abelson may regulate its subcellular targeting and coupling with MTs and/or F-actin, as shown for CLASP and APC [53].

A known binding partner of XMAP215 is TACC3, a member of the family of proteins carrying a transforming acidic coiled-coil (TACC) domain. TACC3 was initially known as a centrosome-associated protein regulating MT growth and stability during mitosis, at least in part via its interaction with XMAP215 [94–97]. However, TACC3 has also been identified as a +TIP that colocalises with XMAP215 in vertebrate growth cones and is required to enhance the MT growth dynamics influencing axon outgrowth [98]. Since then, the same group has explored the role of TACC3 in axon steering. Erdogan and colleagues report that TACC3 depletion induces spinal motor axon misrouting in whole-mount *Xenopus* spinal cords. Consistently, TACC3 overexpression leads to an increased resistance to repulsive signal-induced growth cone collapse, such as Slit2 or ephrin-A5 [21,99; Figure 2C]. Interestingly, TACC3 phosphorylation by the Abl tyrosine kinase has also been involved in axon outgrowth [99]. Altogether, these results suggest that TACC3 could be acting in concert with XMAP215 to drive the MT remodelling required for growth cone responsiveness to specific repellent signals such as ephrin-A5. However, further investigations will be needed to confirm the physiological relevance of the TACC3-XMAP215 complex in a context of axon guidance.

Another +TIP, navigator-1 (NAV1), has recently been reported to play a role in axon navigation via its MT/actin crosslinking activity. NAV proteins were initially described as +TIPs regulating MT bundling and stabilisation [100,101]. Despite a few studies reporting misguided neuronal tracts in *C. elegans* or mouse mutant animals for Nav proteins [102,103], most studies have so far focused on a role for these +TIPs in axon outgrowth, and the precise cellular and molecular mechanisms involving Nav proteins in axon navigation have remained unexplored until recently. Sanchez-Huertas et al. have recently identified NAV1 as a MT-actin crosslinker required to promote MT persistence in the P-domain of exploring cortical growth cones and to elicit axon attractive turning in response to Netrin-1 gradients in Dunn chamber assays [26; Figure 1B]. More studies will be required to explore the role of other NAV proteins in axon pathfinding, and to decipher the signalling cascades that could regulate their activities.

Similarly to NAV proteins, the spectraplakin +TIPs have mostly been studied for their role in axon extension, despite a few reports mentioning guidance defects in loss of function models [104–107]. Interestingly, during axon outgrowth, as for many other +TIPs, a role for spectraplakins in MT-actin crosstalk has been described [108–110], as well as a dual MT lattice and plus-end binding activity [111]. Exploring the mechanisms that regulate this functional switch therefore appears of particular interest in a context of axon guidance. Finally, the *Drosophila* Shot spectraplakin has also been shown to support midline axon repulsion via its binding to the Krasavietz (Kra) translation inhibition factor [106], suggesting an additional role for +TIPs in mediating local translation during growth cone steering.

Surprisingly, while the core proteins of MT plus-end complexes (EBs) act as hubs locally recruiting many of the above mentioned +TIPs, functional evidence demonstrating their role in growth cone steering are still lacking. Nevertheless, three MT-interacting proteins critical

for the guidance of different populations of axons (MAP1B, Tau and Fidgetin-like 1) have been reported to regulate EB binding at MT plus ends through distinct mechanisms [22,112–114], suggesting a key role for these MT plus-end scaffolding proteins in axon guidance. Altogether, data from the past decade reveal the diversity of processes that +TIPs may coordinate at the cell cortex to promote steering events. They further highlight the importance of their fine-tuned subcellular targeting and activity for proper neuronal circuit development.

II.2.2 Structural MAPs in axon guidance: more than just MT stabilisers

Structural MAPs form a family of MT-interacting proteins whose characteristics have considerably evolved since their discovery in the 1970s [115,116]. Early on, structural MAPs have been defined as proteins that can be co-purified with MTs and that have the ability to promote the assembly and stabilisation of these polymers. However, during the past 20 years, the extensive characterisation of neuronal MAPs has gradually expanded their functional diversity, unravelling among others their roles as key MT/actin crosslinkers [24,117] and as signalling molecules in developing neurons [118].

MAP1B was the first MT stabilising protein involved in axon guidance *in vivo.* Its loss of function leads to white matter deficits in mice and humans, including corpus callosum agenesis [119,120]. MAP1B is highly expressed in growth cones where it is required for the Netrin-1-induced outgrowth of cortical axons [20], the guidance of DRG axons facing a non-permissive substrate [121], as well as for nitric oxide- or Draxin-evoked growth cone collapse [122,123]. Notably, MAP1B functions in axon guidance are critically dependent on its posttranslational modifications [124]. Asymmetric blockage of phospho-MAP1B activity in growth cones using MICRO-CALI approaches is sufficient to induce growth cone turning *in vitro* [63]. Consistently, GSK3β-mediated phosphorylation of MAP1B regulates the dynamics and spatial distribution of tyrosinated MTs in growth cones [125] and is necessary for both Netrin-1- and Draxin-evoked axon outgrowth and repulsion, respectively [20,122; Figure 1B and Figure 2F]. By contrast, MAP1B S-nitrosylation mediates nitric oxide-induced axon retraction [122; Figure 2E]. Furthermore, in developing axons, MAP1B was reported on the one hand to enhance the assembly rate of tyrosinated MTs [125] and on the other to negatively regulate EB binding at MT plus ends via EB sequestration [112]. While these independent studies might at first glance appear contradictory, they both converge on the same conclusion that physiological levels of MAP1B are critical to maintain MT dynamic growth and proper axon extension. Further challenging its role as a structural MAP, MAP1B has been suggested to influence MT/actin crosstalk [126]. However, whether MAP1B directly or indirectly (*i.e.* via its influence on EBs) modulates MT/actin interplay in growth cones and whether this emerging function of MAP1B is required for axon responsiveness to the Netrin-1/GSK3β or Draxin/GSK3β pathways remains to be assessed.

During the past decades, particular attention has been paid to the Tau protein due to its implication in several neurodegenerative disorders. Tau is a structural MAP, which was first shown to crosslink protofilaments within MTs. Tau has then been shown to increase MT polymerisation, prevent MT shrinkage [127] and regulate MT network organisation [128]. Interestingly, as for MAP1B, Tau has been shown to reduce EB binding at MT plus ends [113,114], supporting an emerging key role for structural MAPs in fine tuning MT dynamics through the regulation of EB levels at MT plus ends. Although mainly studied for its role in neuronal homeostasis, Tau is highly expressed in the developing brain where it is required for Wnt5a-evoked cortical axon outgrowth and repulsive turning [72]. In this context, phosphorylation of the Tau MT binding domain at Ser262 by CAMKII (*i.e.* phosphorylation that impacts Tau binding to MTs *in vitro*) is required for the reorientation and bundling of

dynamic MTs that drive Wnt5a-induced axon extension and repulsive steering [72; Figure 2G]. Elie et al. have moreover revealed that Tau directly binds to F-actin, promotes MT/F-actin crosslinking and regulates the co-polymerisation of both filaments in cell-free reconstructed systems [117]. Consistently, using super-resolution STED microscopy, Biswas and colleagues have shown that Tau localises to growth cone regions characterised by an extensive crosstalk between these two networks (*i.e.* T-zone and filopodia). Its loss of function further impairs MT growth alongside F-actin bundles in growth cone filopodia [24]. Notably, by combining Dunn chamber guidance assays with live TIRF imaging of cytoskeletal dynamics, they show that Tau-mediated regulation of MT invasion in filopodia is critical for both Wnt5a-induced axon extension and directional turning [24; Figure 2G]. Interestingly, Tau has recently been shown to act in concert with the above mentioned +TIPs, XMAP215/Msps and EB1 to regulate MT polymerisation and bundling in developing axons, both *in vitro* and *in vivo* [129]. While these proteins have independently been involved in axon guidance, whether their cooperative role in the regulation of MT dynamics/organisation is required to drive the growth cone turn in response to specific extracellular cues remains to be confirmed.

MAP6 (also known as STOP) is a versatile regulator of neuronal connectivity that was first identified as a MT stabilising protein required for MT protection against cold- and nocodazole-induced depolymerisation [130,131]. Neuroanatomical analysis of MAP6 knockout brains reveals substantial hypoplasia of several commissures and axonal tracts, including the absence of the post-commissural part of the fornix [132–134]. Regarding this latter phenotype, MAP6 is required for the Sema3E-induced axon outgrowth of subicular neurons (*i.e.* axons that form the fornix). Notably, Deloulme and colleagues have unexpectedly revealed that the function of MAP6 as a downstream effector of Sema6E is independent of its MT-binding domains. Rather, via its N-terminal prolin-rich domain, MAP6 acts as a signalling hub promoting interactions between components of the Sema6E receptor complex and key downstream effectors during axon guidance [e.g. PI3K, Src; 130; Figure 1C]. However, they identify the MAP6-interacting MAP CRMP4 as the final effector of this Sema6E signalling pathways directly connecting the Sema6E receptor complex to the MT-cytoskeleton in extending subicular neurons [134; Figure 1C]. Interestingly, the same group has recently revealed another functional facet of MAP6 in neuronal connectivity. Indeed, neuronal activity-induced phosphorylation of MAP6 by CAMKII relocalises MAP6 to dendritic spines, where it locally binds to F-actin and stabilises this network to adapt dendritic spine morphology [135]. Since MAP6 also binds to F-actin in growth cones, it will be of particular interest to determine whether its ability to switch from one cytoskeleton to another in response to specific signalling pathways influences the targeting of specific axonal populations. Another important issue regards the relevance of its emerging role as a MT intraluminal protein (MIP) in a context of neuronal circuit wiring. It will be interesting to investigate whether the suggested ability of luminal MAP6 to relieve MT mechanical tension by promoting MT lattice torsion [136] increases the flexibility and resistance of navigating axons to compressive forces generated by the surrounding developing tissues.

Collapsin response mediator proteins **(**CRMPs) were first identified as signalling molecules, before being classified as structural MAPs based on the ability of a subset of their members to stimulate the MT assembly and stabilisation [118,137–139]. CRMP2 has long been described as a key effector of Sema-3A repulsive cues [140,141]. In its non-phosphorylated state, CRMP2 binds to tubulin dimers and promotes their polymerisation [137]. However, upon phosphorylation by the Sema-3A downstream kinases Cdk5 and GSK3β, CRMP2 dissociates from MTs, thereby allowing the MT remodelling underlying Sema3A-induced axon repulsion [142,143; Figure 2B]. Supporting these findings, CRMP2 loss of function has been shown to

mostly affect the pathfinding of Sema3A-sensitive axonal populations both in invertebrate [144,145] and vertebrate models, including peripheral nerves [140,146] and callosal axons [146]. By contrast, CRMP4 is required for Sema6E-induced axon outgrowth of subicular neurons [134; Figure 1C]. Supporting the non-overlapping role of CRMP2 and CRMP4 in axon guidance, knockdown approaches in the zebrafish have revealed that while CRMP2 regulates retinal axon outgrowth, CRMP4 promotes midline crossing, most likely downstream of Neuropilin 1 [147; Figure 1D]. Whether CRMP4-mediated regulation of growth cone decision-making behaviours at the zebrafish optic chiasm relies on its dual regulation of MT and actin dynamics in growth cones [148] needs to be clarified. Still in the zebrafish, CRMP2 has been identified as an effector of the Rac-GAP α2-chimaerin in ocular motor axon wiring and has been suggested to act in concert with the MT depolymerising enzymes stathmin 1/2 in this process [149; Figure 2B]. Interestingly, CRMPs have been identified as direct binding partners and potential regulators of the MT-severing spastin [150–152], supporting possible cooperative roles for CRMPs and MT destabilising proteins in neuronal circuit wiring.

Doublecortin (DCX) is a MT-associated protein enriched at the distal tip of developing neurites, which mutations cause lissencephaly, a neurodevelopmental disorder characterised by severe cortical malformations [153]. DCX has been classified among structural MAPs based on its ability to promote MT assembly through catastrophe inhibition and to induce MT bundling [154–156]. DCX is critical for cortical migration (Kappeler et al., 2006; Friocourt et al., 2007). However, its MT-related functions also seem to be required for later stages of neuronal development, including axon guidance. A recent study from the Winckler lab has revealed its role as a downstream effector of the intermediate filament protein Nestin in the regulation of growth cone morphology and sensitivity to Sema3A [157]. Indeed, Nestin increases growth cone responsiveness to Sema3A by facilitating DCX phosphorylation by the Cdk5 kinase through scaffolding activity. However, the molecular mechanisms involved remain to be clarified. The authors suggest that Nestin-dependent phosphorylation of DCX by Cdk5/p35 might release MTs from DCX stabilisation/protection by promoting DCX detachment from MTs as shown for CRMP2 downstream of a Sema3A/Cdk5/Nestin-independent pathway. This mechanism might thereby promote the MT remodelling required for Sema3A-evoked axon repulsion [157; Figure 2B].

Interestingly, among their emerging functions, some of the above-mentioned structural MAPs (*e.g.* Tau and CRMPs) influence the activity of MT severing or depolymerising enzymes in developing axons [150,151,157], which paves the way for future investigations of their cooperative or antagonistic roles in axon navigation processes.

II.2.3 MT severing/depolymerising enzymes: Engines that power the growth cone turn

MT severing enzymes including spastin, p60-katanin and fidgetin are nanomachines from the AAA+ superfamily [ATPases Associated with diverse cellular Activities; 157] that combine chemical (*i.e.* ATP hydrolysis) and mechanical forces to promote the extraction of tubulin dimers from MTs through tubulin unfolding [160]. Propagation of the nanodamages generated by these enzymes leads to MT severing. Based on this functional property, MT severers are critical regulators of MT mass, length and organisation in cells [161,162] and thereby of axon elongation and/or branching [158,163–166]. Nevertheless, their ability to rapidly breakdown taxol-stabilised MTs in cell-free systems [160,167] and to dismantle the MT network when overexpressed in non-neuronal cells [168–170] positioned them as relevant candidates to promote the asymmetric disassembly of MTs that drives the growth cone turn [52]. Using several genetic approaches combined with *in vivo* live imaging in zebrafish larvae, the Hazan team has recently uncovered the decisive and non-overlapping roles of p60-katanin [31] and

spastin main isoforms [M1 versus M61; 23] in zebrafish motor circuit wiring and larval locomotion. They show that loss of function of spastin main isoforms leads to highly specific pathfinding errors of rostral motor nerves, while p60-katanin deficiency mainly impairs dorsal motor nerve targeting (*i.e.* abnormal splitting). This group further reveals that the functional specificity of these severers in zebrafish axon guidance relies on their preference for specific populations of MTs and on their selective involvement in guidance signalling pathways. Indeed, they identify TTLL6-mediated MT-polyglutamylation as a selective regulator of p60-katanin activity in motor axon guidance [31; Figure 3B]. Moreover, they uncover a selective role for M1 and M61 spastin as a BMP signalling inhibitor and a Neuropilin-1 downstream effector, respectively [23; Figure 2B]. Although not investigated in these studies, the selective regulation of the p60-katanin and spastin MT-severing activity by structural MAPs [*e.g.* Tau or CRMP proteins, respectively; 143–145,155] might also underlie their specificity of action in axon pathfinding, as suggested by the overlapping motor neuron phenotypes associated with Sema3A [171], CRMP2 [172] and spastin M61 knockdown [23] in zebrafish embryos. Nevertheless, an important unsolved issue concerns the molecular mechanisms by which MT severers drive growth cone turning. Spastin and p60-katanin knockdown drastically reduce the number of dynamic growing plus ends in zebrafish motor neuron axons, a phenotype consistent with a lack of MT severing activity [165]. However, depending on whether newly generated ends are stabilised or not, MT-severing enzymes can either promote MT breakdown or on the contrary, increase MT mass [161,173]. Furthermore, the *Drosophila* orthologue of p60-katanin has been shown to have a MT plus-end depolymerising activity *in vitro*, in addition to its MT severing activity [174,175]. This dual function seems to be dependent on its concentration and might thereby underlie the dose-dependent effect of p60-katanin on motor axon targeting [31]. Finally, an elegant work from the Roll-Mecak lab - based on *in vitro* reconstructed systems - reveals that nanodammages generated by MT severers can be repaired through the incorporation of novel GTP-tubulin dimers. This suggests an additional role for these AAA+ machines in MT lattice renewal [176]. *In vivo* investigations in the zebrafish model, which offers the possibility to monitor cytoskeleton dynamics in navigating growth cones, should provide crucial information regarding the basic mechanisms governing this “Cut and Turn process”.

Fidgetin-like 1 (fignl1) belongs to the same subfamily of AAA+ engines as spastin and p60-katanin. However, unlike its extensively studied homologues, fignl1 functions in the nervous system had never been investigated until the pioneer study from Fassier et al. in 2018. Their work identifies this MT-associated ATPase as a critical player in zebrafish motor axon pathfinding [22,73]. The authors show that fignl1 controls growth cone morphology and steering behaviours at guidance choice points through the regulation of MT plus-end dynamics. Using *in vitro* systems, they have unexpectedly discovered that unlike its related proteins, fignl1 does not sever MTs but rather regulates MT plus-end composition and dynamics. Indeed, alternative translation of the fignl1 transcript generates two isoforms with distinct functionalities. The full-length protein displaces EBs from MT plus ends in an ATP-dependent manner. By contrast, the N-terminal truncated variant exhibits an F-actin-dependent cortical MT depolymerising activity [22]. Through this functional versatility, fignl1 may concomitantly tune +TIP-dependent MT behaviours (*i.e.* MT growth directionality, MT/F-actin crosstalk and MT anchoring at the cell cortex) and promote MT dynamics in the P-domain to drive the growth cone turn. By taking advantage of the zebrafish model, the authors were able to monitor MT dynamics in navigating axons of live fignl1-deficient larvae. The *in vivo* MT phenotype they describe, including the sustained stability of MTs, their aberrant invasion and growth directionality in the P domain, as well as the increased MT growth duration in filopodia, fits with the above-mentioned hypothesis [22]. Deciphering how the actin network influences the MT-depolymerising activity of fignl1 and

connecting this process to guidance signals should shed new light on the regulation of the MT/actin crosstalk underlying axon guidance.

Another family of MT-destabilising enzymes that has recently been involved in axon guidance is the stathmin family. Stathmins promote MT depolymerisation by decreasing the concentration of free-tubulin through tubulin dimer sequestration [177]. While stathmins have long been identified as critical regulators of axon extension [178,179], Carretero-Rodriguez et al. have unravelled a role for stathmin1/2 as downstream effectors of the Rac-GAP alpha2-Chimaerin required for zebrafish oculo-motor axon fasciculation and guidance [149]. Further work will be required to define the role and regulation modes of stathmins downstream of this signalling protein.

Altogether, these studies emphasise the key role of alternative translation in multiplying the functional diversity of MT-destabilising enzymes in directed axon outgrowth. This conclusion is strengthened by the recent work of Atkins and colleagues, uncovering an additional role for the MT AAA+ machine fignl1 in the regulation of molecular motor motility and its decisive contribution to axon guidance [73; *See § I.2.4*].

II.2.4 Molecular motors: Microtubule sliding and bidirectional cargo transport

Navigating growth cones need to be provided with a diverse set of building and navigation material (lipids, membrane receptors, mRNAs, mitochondria…) in order to accurately respond to their environment in a spatiotemporally regulated manner. It is therefore generally assumed that molecular motors are required for axon navigation. Confirming this idea, various animal models carrying mutations affecting molecular motors or altering kinesin binding sites on specific tubulin isoforms have been associated with guidance defects [180–184]. However, the precise mode of action and molecular mechanisms through which molecular motors may control growth cone turning responses remain poorly characterised.

A first set of studies reports a role for molecular motors in MT sliding [for review, see 185] and asymmetric invasion of the growth cone during turning events [186–189]. In a model proposed by Baas and colleagues, extracellular guidance cues signal to the growth cone molecular motors in order to regulate this MT sliding process. In this model, dynein counters myosinII-driven forces to instruct MT invasion of the P-domain on the turning side of the growth cone. On the opposite side, kinesins 5 and 12 antagonise dynein-driven forces, thereby locally restricting MTs to the C domain [190]. These synchronised force balances between dynein and myosin II on the one hand, and dynein and kinesins on the other, could ensure the polarised MT sliding required for the growth cone to turn.

Importantly, other forms of axonal transport, such as receptor-containing vesicle trafficking, are also starting to emerge in a context of axon navigation. First, it has been shown that axonal transport of different cargoes can be activated by extracellular guidance cues, such as TrkB anterograde axonal transport downstream of NGF [191,192] or retrograde transport of a Plexin A4-TrkA downstream of Sema3A [193]. Selective links between specific molecular motors and cargo complexes are also just starting to emerge in a context of axon guidance. For example, in mouse embryonic cortical axons, Netrin-1 regulates axon initiation, branching and targeting by promoting anterograde transport of the Myosin X molecular motor in a KIF13B-dependent manner [194; Figure 1B]. Similarly, Saez and colleagues have linked the kinesin-1 motor to the axonal transport of cannabinoid type-1 receptors, a process required for mouse corticofugal axon pathfinding [195]. Alternatively, some studies have focused on adaptor/regulatory proteins of known molecular motors. The ATPase fignl1 has been shown in this context to restrict dynein-based retrograde transport velocity through dynein coupling to the opposite polarity-directed motor Kif1bβ thereby fine tuning bidirectional vesicular

trafficking in vivo in navigating axons of zebrafish embryos. Importantly, pharmacological inhibition of the dynein motor rescues the pathfinding defects associated with fignl1 loss of function, confirming the key role of fignl1 as a dynein speed limiter required for accurate axon targeting [73]. The kinesin-1 adaptor proteins FEZ1 (fasciculation and elongation protein zeta 1) and Calsyntenin 1 have also been involved in axon pathfinding events. Calsyntenin1 regulates Robo1 and Frizzled3 trafficking during midline crossing and longitudinal axon guidance, respectively [196]. By contrast, FEZ1 binds to Neuropilin1 (Nrp1) and DCC and regulates the response of rat hippocampal axons to Sema3A and Netrin-1, respectively [197].

Importantly, such studies highlight the variety of cargoes that can be loaded via different adaptor complexes onto distinct sets of molecular motors. Further studies will be required to increase our knowledge on the possible combinations and how their precise synchronisation in space and time is required to instruct accurate growth cone turning.

II.3 Emerging key role of the tubulin code in axon navigation

As mentioned above (§ I.), our vision of the MT network has considerably evolved across the past decades. It is now fully accepted that the MT cytoskeleton is a highly mosaic network composed of mixed populations of MTs that differ from one another based on their composition (*i.e.* the nature of the tubulin isoforms that compose the polymer) and the posttranslational modifications (PTMs) that decorate the tubulin dimers [48,49]. Both elements establish the tubulin code (*i.e.* by analogy to the histone code), which is starting to emerge as a highly selective regulator of MT functions in axon guidance.

II.3.1 Tubulin isoforms as direct targets of guidance cues in growth cone steering

Tubulin dimers are the building blocks of the eukaryotic MT cytoskeleton and are formed through chaperone-assisted folding and assembly of one α- and one β-tubulin isoform. The tubulin gene family has gradually expanded across evolution, from 2α- and 1β- tubulin genes in yeast to 9 α- and 9 β-tubulin genes in humans [198]. Functional specification of tubulin isotypes is supported by (i) their different spatiotemporal expression patterns, (ii) their selective incorporation into specialised populations of MTs (*e.g.* axonemal, axonal...), (iii) their critical and non-overlapping roles in the development/homeostasis of particular tissues/cells, and above all (iv), the broad spectrum of human disorders caused by mutations in tubulin genes [i.e. Tubulinopathies; for review: 48,49,198,199]. Notably, mutations in genes encoding the neuronal-specific β-tubulin isoforms, TUBB2 and TUBB3, are responsible for brain malformations in humans associated with axon guidance defects including ocular nerve hypoplasia, dysgenesis of the corpus callosum, anterior commissure and corticospinal tracts [181,184,201,202; Figure 3]. Direct binding of TUBB3 to DCC or UNC5C receptors has been shown to couple Netrin-1 signalling to MT dynamics in axon guidance [28,29]. In this context, Netrin-1 stimulation promotes DCC or reduces UNC5C interaction and colocalisation with polymerised TUBB3 in primary neuron growth cones and locally influences MT dynamics (Figure 3A). Importantly, TUBB3 knockdown or pathogenic mutations impair these processes and thereby affect Netrin-evoked growth cone attraction or repulsion *in vitro* (Figure 1B & Figure 2H), as well as the pathfinding of spinal axon populations sensitive to Netrin-1/DCC or Netrin-1/UN5C signalling *in vivo* [28,29,202,203; Figure 3B]. Interestingly, the TUBB2 E421K pathogenic mutation (*i.e.*; located in the C-ter tubulin tail), responsible for commissural axon pathfinding errors in mice and humans, affects the recruitment of the MT depolymerising motor Kip3p at MT plus ends and increases MT stability in yeast [184]. These effects strikingly contrast with other TUBB2 pathogenic variants, which drastically impair tubulin heterodimerisation and cause brain malformations

without guidance defects [205]. These data demonstrate the critical role of residues lying in the C-terminal tail of tubulin isotypes for their functional specification. Further investigations will be required to clarify how these mutations impact growth cone MT behaviours and responsiveness to guidance cues. An attractive hypothesis is that through their sequence specificity, tubulin isoforms generate specific signalling hubs on MTs selectively recruiting guidance receptors, downstream kinases and MT-regulatory proteins that will act in concert to locally tune MT dynamics and thereby axon behaviours.

Altogether, these studies emphasise how the genetic diversity of MT structural subunits influences MT functions in axon guidance. The functional specification of tubulin isoforms has been shown to arise at transcriptional, translational and posttranslational levels [200]. Nevertheless, despite their striking degree of homology, tubulin isotypes exhibit a significant divergence in their C-terminal tail, which is subjected to extensive PTMs, suggesting a prominent role for PTMs in the functional specification of tubulins. This hypothesis is strengthened by the above-mentioned work of Jaglin et al. and the recent work of Zheng and colleagues, uncovering a novel α-tubulin isotype in *C.elegans* which, unlike other α-tubulin isoforms, destabilises MTs and lacks potential sites for polyamination and polyglutamylation [206]**.**

II.3.2 Tubulin posttranslational modifications: a selective code that tunes critical MAPs in axon guidance

With the exception of acetylation, which occurs on the luminal Lysine 40 of α-tubulin, PTMs consist in the covalent addition of amino acids with different physio-chemical properties (*e.g.* glutamate, glycine, tyrosine) on the outer surface of MTs, and more specifically on the acidic C-terminal tail of tubulins. Tubulin PTMs modulate MT properties and/or tune the activity of MT-interacting proteins at molecular and cellular scales [48,49]. However, the physiological role of these modifications remains largely unknown, especially in a context of neural circuit wiring where MTs are key driving forces for axon guidance. Since excellent reviews describing the tubulin PTM observed in neurons and their influence on neuronal MT functions have recently been published [45,46], we will here focus on the very few studies that directly involve tubulin PTMs in axon pathfinding.

Tubulin tyrosination was the first PTM to be involved in axon guidance. Following the incorporation of tubulin dimers in protofilaments, the C-terminal tyrosine residue of α-tubulins is removed by the tubulin carboxypeptidases vasoinhibins (VASH1/2), assisted by its regulatory subunit, the small vasoinhibin binding protein [SVBP; 207,208]. This tyrosine residue is subsequently re-added on free tubulin dimers by the tubulin-tyrosine ligase [TTL; 209,210]. This tubulin detyrosination/tyrosination cycle has been shown to be critical for axon specification and outgrowth *in vitro* [207,211,212], as well as for neuronal connectivity *in vivo*. Indeed, TTL knockout mice exhibit several axonal tract defects including a disrupted cortico-thalamic loop [211] and aberrant navigational behaviours of olivary fibres at the floor plate [30]. Moreover, SVBP knockout mice (*i.e.* equivalent of VASH1/2 knockout mice) show axonal tract agenesis associated with connectivity defects [213]. At the cellular and molecular levels, TTL-deficient neurons show abnormal growth and branching behaviours at guidance choice points or on specific substrates. They also exhibit enlarged growth cones with defective polarisation of F-actin protrusions and mislocalisation of several cytoskeleton regulators, including the CLIP-170 +TIP and the Myosin IIb motor [30,211]. Importantly, TTL loss of function does not directly impact MT dynamics [214]. Thus, tubulin tyrosination may act as a local signal within growth cones for the recruitment of key mediators of the MT/actin crosstalk underlying axon steering (Figure 3B). Investigating the role of the α4A-

tubulin isotype, a natural source of detyrosinated tubulin in neurons [214,215], during nervous system development should provide additional key insight into the contribution of the tubulin tyrosination/detyrosination cycle to axon pathfinding.

An additional tubulin PTM that has recently been involved in neuronal circuit wiring is polyglutamylation. Tubulin polyglutamylation is the most abundant tubulin PTM in neurons. It consists in the enzymatic addition of glutamate side chains on tubulin C-terminal tail glutamate residues. This modification is catalysed by enzymes from the Tubulin Tyrosine Ligase Like family [TTLL; 217,218] and removed by enzymes from the Cytosolic Carboxypeptidase family [CCP; 219,220]. Each TTLL has an enzymatic specificity (*i.e.* generation of short or long glutamate side chains) and a substrate preference for α- versus β-tubulin [221]. Tubulin polyglutamylation has been shown to tune the binding affinity and/or activity of molecular motors [222–224], structural MAPs [225,226] and MT-severing enzymes [227–230] *in vitro.* It has therefore emerged as a potential regulator of axon guidance. A recent study from Ten Martin and colleagues provides the first *in vivo* evidence that tubulin glutamylases act in a highly selective manner to tune the activity of MT severers during neuronal circuit wiring [31]. TTLL6 and TTLL11 are two tubulin glutamylases enriched in the zebrafish developing spinal cord [231], which catalyse the addition of long glutamate side chains on the C-terminal tail of α-tubulins [218]. Using loss of function and rescue experiments, Ten Martin et al. show that despite their strong similarities, both glutamylases have non-overlapping key roles in zebrafish motor axon pathfinding. Notably, the authors reveal that TTLL6-mediated polyglutamylation specifically boosts p60-katanin activity in navigating axons to control their targeting [31]. However, TTLL6 and TTLL11-deficient larvae also exhibit motor neuron migratory and axon guidance defects that resemble those described in spastin mutants [23,31]. These data suggest a concerted action of TTLL6 and TTLL11 in the fine tuning of spastin activity in motor circuit wiring (Figure 3B).
In conclusion, the emerging key roles of tubulin isoforms and PTMs in the establishment of neuronal connectivity emphasise the need to use multi-scale and system approaches in order to crack the tubulin code that regulates MT functions in developing axons. Notably, investigating the role of other PTMs present in neurons such as tubulin acetylation and phosphorylation, respectively shown to influence p60-katanin severing activity [232] or DCC/TUBB3 interaction [233], will be of particular interest.

III. Microtubules in axon pruning

After reaching their terminal targets, many developing axons, including motor and sensory axons, initially form exuberant collaterals and supernumerary synapses. While a subset of them is stabilised to form the mature terminal axonal arbour, misplaced branches and synapses are pruned. Pruning comes in diverse manifestations that differ in morphology and extent as well as in their initiation signal. While in some cases, axonal pruning is initiated by growth cone exposure to conventional guidance cues, it can also be induced by the lack of sufficient trophic support or activity-dependent competition mechanisms. In some species such as in insects undergoing metamorphosis, large scale axon remodelling is induced by intrinsic genetic programs [234,235]. In all cases, obvious changes of the MT cytoskeleton architecture have been reported as a common and early event in axon pruning. Such MT remodelling has been described in various systems: the axonal branch elimination in the *Drosophila* mushroom body during metamorphosis, the selection of the single axon that innervates each muscle fibre at the mammalian neuro-muscular junction or the neurotrophin-dependent pruning of sensory axons [17,236,237]. Importantly, abnormal refinement of exuberant connectivity is likely to explain subtle connectivity defects at the origin of

neurodevelopmental disorders including schizophrenia, autism spectrum disorders, attention deficit hyperactivity disorders. Consistently, causal mutations in a few MT-regulatory proteins have been reported in these disorders [238]. However, although postulated as key players in axon pruning for many years, the precise contribution of MTs and their regulatory proteins to this developmental process is only starting to be deciphered.

III.1 MT remodelling as a driving force of axon pruning

During the past decades, several lines of evidence have underpinned MT breakdown as an early event required for axon pruning. In 2002, Eaton and colleagues revealed MT loss as an early cellular process in the stereotyped pruning of *Drosophila* neuromuscular junction supernumerary synapses, preceding the removal of presynaptic markers [239]. Supporting this idea, Watts et al. subsequently showed that during *Drosophila* metamorphosis, branches subjected to pruning in the mushroom body lose their tubulin content before any other reported change in their protein content including actin, presynaptic proteins or adhesion molecules. These observations establish MT remodelling as one of the earliest molecular changes occurring in axonal branches to be pruned [236]. Consistently, motor axons retreating from mammalian neuromuscular junctions are largely devoid of MTs [240]. Further supporting MT disassembly as an early and evolutionary conserved feature of axon pruning, Brill and colleagues have recently revealed that MTs are preferentially fragmented in motor axon branches undergoing retraction. Indeed, the authors show that the density of EB3-positive MT growing plus ends is reduced in branches to be pruned. Notably, this loss of MT polymerisation is correlated with a reduction in the MT mass and occurs before organelle evacuation [17]. Importantly, they further demonstrate that in this system, epothilone B-induced stabilisation of MTs delays synapse removal, demonstrating that MT loss is required for efficient axonal branch pruning [17]. This need to disassemble the MT network to drive axonal branch elimination is supported by the work of Maor-Nof and colleagues [237]. Using an *in vitro* assay based on NGF deprivation-driven DRG axon elimination, they show that the lack of NGF induces a reduction in DRG axon MT mass. The latter is correlated with a reduction in posttranscriptional modifications characteristic of stable MTs (*i.e.* detyrosination, acetylation and glutamylation). Notably, the authors demonstrate that pharmacological stabilisation of MTs with paclitaxel prevents NGF deprivation-induced changes of MT dynamics and posttranslational modifications in DRG axons, as well as their subsequent degeneration. Overall, these pharmacological studies underpin MT breakdown as a common intrinsic driving force of sensory and motor axon pruning occurring downstream of diverse initiation signals, such as neurotrophic factor deprivation or activity-based competition, respectively. However, despite this evidence, the intracellular signalling events and MT-interacting effectors that selectively drive MT disassembly in response to these pruning initiation signals remain largely unknown.

III.2 MT-regulatory proteins required for axon pruning

Several MT-regulatory proteins involved in dendritic pruning have been identified [for review, see 241]. However, the driving mechanism of axon pruning might largely differ from the one underlying dendritic branch elimination. Indeed, some MT-interactors involved in this latter process fail to influence axon remodelling. A relevant example comes from the work of Lee et al. showing that during *Drosophila* metamorphosis, the MT severer katanin-like 1 is required to drive the pruning of *da* neuron dendrites, while it is dispensable for the elimination of γ neuron axonal branches in the mushroom body [242]. This report highlights the difficulty to extend the mechanisms involved in dendritic pruning to axonal branch-specific elimination processes without experimental investigations.

Studies identifying MT-regulatory proteins required for axon pruning are scarce. However, a few modulators of MT dynamics have recently emerged as key players in this process (Table 1)**,** including the MT-severing enzyme spastin. Spastin knock-out mice exhibit a delayed elimination of polyinnervated neuromuscular junctions, highlighting the involvement of spastin in motor axon branch pruning [17,243]. An elevated MT content is found in delayed retreating motor axons of spastin-deficient mice, confirming the key contribution of the spastin severing activity to the MT cytoskeleton breakage required for efficient axon pruning [17; Figure 4A]. Interestingly, the loss of MT mass (especially polyglutamylated and stable MTs) reduces local membrane tension [244] and is therefore predicted to promote endocytosis, another driving process in neurite pruning [245,246]. Based on its molecular and functional links with the ESCRT-III (Endosomal Sorting Complex Required for Transport III proteins) proteins Ist1 and CHMP1B in the endocytic pathway [247–249], it is most likely that spastin drives axon pruning by coordinating MT disassembly with membrane remodelling.

Spastin is also part of a set of MT-destabilising proteins screened by Maor-Nof et al. to identify the molecular mechanisms underlying MT breakage in NGF-deprivation-induced DRG axon pruning. The screened proteins include the four members of the stathmin family, the MT severers katanin and spastin, as well as the MT-depolymerising motor Kif2A. Among these candidates, only the knock-down of Kif2A protects DRG axons from NGF deprivation-induced degeneration *in vitro*. The involvement of Kif2A in axon pruning is supported by the hyperinnervation of the skin by sensory axons in Kif2A knock-out animals [236; Figure 4B].

While MT regulators involved in axon pruning mentioned so far exclusively comprise MT-destabilising proteins, two recent studies have uncovered a critical role for CRMP structural MAPs in a subset of axon refinement models [146,250]. This observation is consistent with their implication in neurodevelopmental disorders associated with subtle connectivity defects [251]. Extensive anatomical analysis of neuronal connectivity in CRMP2 knockout mice has led Ziak and colleagues to uncover a critical role for CRMP2 in Sema3F-induced pruning of the infrapyramidal bundle of hippocampal mossy fibres and cortico-collicular axons. These observations are consistent with the CRMP2 requirement for Sema3F-induced axon repulsion *in vitro* [146]. By contrast, although CRMP2 also mediates Sema3A-induced axon collapse *in vitro* [142,143], the Sema3A-dependent pruning of hippocampo-septal axons is not affected in CRMP2 knockout mice [146], suggesting a selective role for CRMP2 as a Sema3F effector in axon pruning *in vivo* (Figure 4C). In line with this observation, Nakanishi et al. have uncovered a non-overlapping role for CRMP2 and CRMP4 in the axon pruning of mossy fibres in the mouse hippocampus and reveal a selective epistatic interaction between the Sema3F receptor, Neuropilin-2 [252] and CRMP2 in this process [250]. The counter intuitive role of these MT stabilising proteins in MT breakdown-driven axon pruning suggests that CRMP proteins might not directly operate on MTs in this process. Rather, they may be acting as key signalling hubs promoting interactions between components of the Semaphorin or Neuropilin receptor complex and downstream effectors such as MT-destabilising enzymes (*e.g.* stathmins, spastin). Addressing this issue will be of particular interest in the field.

Overall, a diversity of MT-regulatory proteins are involved in axon pruning, with functional specificities that might rely on the neuronal subtype considered and/or on the nature of the initiating signal (*e.g.* activity-dependent mechanisms, trophic deprivation or axon guidance cues). Deciphering the signalling events underlying the selective regulation of MT-associated proteins downstream of specific axon pruning signals, as well as their contribution to the MT breakdown that drives axon remodelling, should provide essential information on the basic mechanisms governing neuronal connectivity refinement.

Conclusion

Our understanding of the microtubule function as a driving force of axon guidance and pruning has considerably expanded and evolved in recent years, as highlighted by the numerous studies reported here and the complementary review from Sanchez-Huertas and Herrera that was published during the final writing steps of the present article [253]. These emerging studies reveal not only the importance of the microtubule composition and posttranslational modifications in fine tuning MT functions in neuronal circuit wiring, but also of the functional versatility of the numerous MT interactors in such process. This extensive intracellular diversity contrasts with the relatively limited number of identified extracellular cues that wire the nervous system. It therefore highlights how decisive the tubulin code and the diversity/versatility of MT-interactors are in expanding the range of axon guidance behaviours and underlying neuronal connections that can be controlled by a limited repertoire of extracellular cues. The versatility of a given MT interactor may be dependent on the neuronal subtype in which it is expressed (which defines the pool of genes expressed), the diversity of the upstream intracellular signalling events by which it is recruited to the MTs, its binding partners, but also the subpopulations of MTs (dependent on the tubulin isoforms and MT modifying enzymes expressed in this neuronal cell type) with which it interacts. Adding to this complexity, different sets of MT interactors with cooperative or antagonistic functions may be acting concomitantly downstream of a single guidance molecule to regulate different aspects of MT dynamics/organisation required for axon steering or pruning. With this in mind, dissecting the MT regulatory networks that adapt the axon responsiveness to extracellular signals, rather than focusing on individual MT-associated effectors, will represent a major challenge in the field to gain a more comprehensive and realistic understanding of the basic intracellular mechanisms underlying neuronal circuit wiring and refinement. Tackling these issues will require the combined use of global transcriptomic and proteomic approaches, as well as multigenic functional analyses (such as the one recently published by Hahn and colleagues [129]). Similarly, moving towards model organisms enabling *in vivo* live imaging of MT dynamics and genetic/optogenetic manipulation of MT regulatory proteins in navigating growth cones or retracting axons will be crucial to dissect MT functions in a physiological context, where crosstalks between several chemical but also physical and mechanical extracellular signals may influence MT functions.

Legends to figures:

Figure 1: MT-regulatory proteins that drive the growth cone turn in response to attractive/permissive guidance cues and their associated molecular mechanism(s).

(A) Asymmetric invasion and stabilisation of MTs within growth cone filopodia facing an attractive/permissive cue drives the growth cone turn. **B**, **C** and **D** are higher magnifications of the boxed region in **A** and depict the molecular effect(s) of Netrin-1 (**B**), Sema3E (**C**) or Sema3D/3E (**D**) on the MT cytoskeleton through different MT-regulatory proteins. While the boxed region in A gives an example of the growth cone region where the depicted mechanisms occur, it is important to bear in mind that these mechanisms may also be functioning in other growth cone regions, such as the transition zone or the central domain. Molecular mechanisms are schematised according to the literature cited in Table 1. Full arrows indicate the direct effect of the attractive/permissive cue on a specific receptor and its downstream effectors. Dotted arrows represent an indirect effect of the cue on a given effector through a receptor that remains unidentified. MAP: microtubule-associated protein; MTs: microtubules.

Figure 2: MT-regulatory proteins that drive the growth cone turn in response to repulsive/non permissive guidance cues and their associated molecular mechanism(s).

A. Asymmetric destabilisation of MTs on the growth cone side facing a repulsive/ non permissive cue drives the growth cone turn. **B**-**H** are higher magnifications of the boxed region in **A** and depict the different effectors engaged downstream of Sema-3A (**B**), Slit/Slit2 (**C**), EphrinA2/A(**D**), NO (**E**), Draxin (**F**), Wnt5A (**G**) and Netrin-1 (**H**) to drive the MT remodelling required for growth cone repulsion. While the boxed region in A gives an example of the growth cone region where the depicted mechanisms occur, it is important to bear in mind that these mechanisms may also be functioning in other growth cone regions, such as the transition zone or the central domain. Molecular mechanisms are schematised according to the literature cited in Table 1. While full arrows indicate the direct effect of the repulsive/non-permissive cue on a specific receptor and its downstream effectors, dotted arrows represent an indirect effect of the cue on a given effector through a receptor that remains unidentified. MAP: Microtubule-Associated Protein; MTs: Microtubules; NO: Nitric Oxide.

Figure 3: Emerging key role of the tubulin code in axon guidance. Diagram depicting our current knowledge of the tubulin code impact on axon guidance from a single molecule scale (left hand panels) to a physiological one (right hand panels). (**A**) Selective involvement of tubulin isotypes in axon guidance pathways. β-II (light pink sphere) and β-III tubulin (dark pink sphere) isotypes control the pathfinding of specific populations of mammalian axons through their selective interaction with MT-regulatory proteins or axon guidance receptors(respectively) and their subsequent modulation of MT dynamics. (**B**) Posttranslational modifications of the C-terminal tail of α-tubulin (*e.g.* tubulin tyrosination and polyglutamylation) tune the activity of specific MT-regulatory proteins, thereby influencing MT-driven growth cone steering. (**A**-**B**) Molecular mechanisms are schematised according to the literature cited in Table 1. (Left hand panels) The C-terminal tyrosine residue of α-tubulin is indicated in blue, while glutamic acids subjected to polyglutamylation are shown in grey. Polyglutamate side chains generated by TTLL6 or TTLL11 are shown in yellow and red, respectively. (Middle panels) Tubulin C-terminal tails that protrude from the MT lattice are represented in grey in the absence of key PTMs. Dotted arrows indicate MT polymerisation events. Purple arrow represents Myosin IIb displacement on F-actin (red filament). Boxes are higher magnifications of the adjacent left hand dotted panel. Polymerising and depolymerising microtubules are depicted as in Figures 1 and 2. CA: callosal axons; CSA: corticospinal axons, OMA: oculomotor axons; FB: forebrain; MB: midbrain; HB: hindbrain; SC: spinal cord; FP: floor plate; DRG: dorsal root ganglia; DREZ: dorsal root entry zone; PCN: precerebellar nuclei; HM: horizontal myoseptum; MyoIIb: myosin IIb; TTL: tubulin tyrosine ligase; TTLL: tubulin tyrosine ligase like; Kinesin depol: Kinesin depolymerising motor; ABP: Actin binding protein.

Figure 4: MT-regulatory proteins that contribute to the MT breakage required for axon pruning. (**A**) Activity-dependent retraction of supernumerary axons in polyinnervated neuromuscular junctions relies on the spastin MT-severing activity. (**B**) NGF deprivation-induced pruning of DRG axons requires the MT depolymerising motor Kif2A (**C**) Sema3F-evoked elimination of exuberant axonal branches involves the structural MAP CRMP2. Since CRMP2 mostly stabilises MTs, Sema3F might detach CRMP2 from MTs, thereby favouring their depolymerisation. Whether phospho-CRMP2 acts as a signalling hub promoting the interaction between Sema3F receptor complex components and MT-destabilising enzymes remains to be addressed. **Table 1: Summary table of the MT regulators involved in axon guidance and pruning.**

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Conflict of Interest

The authors declare no conflict of interest.

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Author contribution

MA, XN and CF wrote the manuscript and designed the figures. All authors approved the submitted version.

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Protein	Models/Systems	Links with guidance cues	Functions in axon guidance	References	Figure
Plus-end tracking proteins					
CLASP	*Drosophila* nervous system	Slit/Robo	Downstream effector of Abl in Slit-mediated repulsion at the midline, regulation of MT-F-actin crosstalk	[19; 79; 80]	Fig. 2C
TACC3	*Xenopus* spinal neurons	Slit2	Regulation of MT dynamics involved in steering behaviours	[21]	Fig. 2C
APC2	Chick retinal axons	Ephrin-A2	Regulation of MT stability and retinal axon pathfinding; +TIP function?	[27; 94]	Fig. 2D
APC	Mouse DRG neurons	Wnt3a	Required for Wnt3a-induced axonal remodelling, regulation of MT plus-end growth directionality in growth cones	[92]	-
NAV1	Mouse cortical neurons	Netrin-1	Instructs growth cone steering, promotes MT perduration in the P-domain, MT-F-actin crosslinking	[26]	Fig. 1B
XMAP215	*Xenopus* embryonic neural tube explants *Drosophila* nervous system	EphrinA5 midline repellent cues ?	Regulation of growth cone morphology, response to the EphrinA5 repellent cue, MT-F-actin crosslinking; Growth cone repulsion from the midline by antagonising CLASP and Abl activity	[25; 81] [80]	Fig. 2D -
Structural MAP					
MAP1B	Mouse forebrain commissural axons	Netrin-1/DCC; Draxin/DCC/Gsk3β	Netrin-1- and Draxin-evoked axon outgrowth and repulsion, respectively Promotes tyrosinated MT dynamics, reduces EB-binding at plus ends, regulates MT-Factin crosstalk (?) in growth cones	[20; 122] [111; 124; 125]	Fig. 1B & 2F
	DRG axons	Non permissive substrate	Abnormal steering behaviour when facing a non-permissive substrate, regulation of growth cone MT dynamics (?)	[120]	-
	DRG axons	Nitric oxyde (NO)	Required for NO-induced growth cone collapse; MT effects ?	[121]	Fig. 2E
MAP6/STOP	Mouse subicular neurons	Sema6E	required for Sema3E-induced attractive outgrowth; Signalling hub protein	[131]	Fig.1C
Tau	Mouse cortical neurons	Wnt5a	Required for Wnt5a-evoked axon outgrowth and repulsive turning; MT invasion, remodelling and reorientation in the P-domain; MT-F-actin crosslinking	[24; 72] [116]	Fig.2G
CRMP2	Mouse peripheral nerves and callossal axons Zebrafish oculo motor axons	Sema3A/Cdk5 ?/ α2-Chimaerin	Downstream effector of Sema3A required for axon repulsion; phosphorylation-induced detachment from MTs, permissive for MT breakdown Downstream effector of the RhoGAP α2-Chimaerin; concerted regulation of MT dynamics with stathmin1/2	[141; 142; 145] [148]	Fig.2B
CRMP4	Zebrafish retinal axons	Neuropilin-1?	Required for midline crossing of retinal axons; MT effects (?)	[133; 146]	Fig.1D
DCX	Mouse cortical neurons	Sema3A/Nestin/Cdk5	Regulation of growth cone morphology and sensitivity to Sema3A; MT breakdown through phosphorylation-induced detachment from MTs (?)	[156]	Fig.2B
MT severing/depolymerizing enzymes					
M1 spastin	Zebrafish spinal motor neuron axons	BMPs	Controls rostrally-projecting axon pathfinding through BMP inhibition; MT dynamics (?), BMP receptor trafficking (?)	[23]	-
M61 spastin	Zebrafish spinal motor neuron axons	Neuropilin-1	Downstream effector of Neuropilin-1, controls steering behaviours at guidance choice points , Promotes growth cone MT dynamics (?)	[23; 164]	Fig. 2B
p60-Katanin	Zebrafish spinal motor neuron axons	?	Required for proper dorsal motor nerve pathfinding/targeting, Promotes MT dynamics in growth cones?	[31; 164]	Fig.3B
Fidgetin-like 1	Zebrafish spinal motor neuron axons	?	Controls growth cone morphology and steering behaviours at choice points, Regulates MT-plus-end composition and dynamics; Regulates MT-F-actin crosstalk (?)	[22]	-
Stathmin 1/2	Zebrafish oculo motor nerves	Sema3A?/α2-Chimaerin	Required for α2-Chimaerin-dependent ocular motor axon pathfinding/targeting; Concerted regulation of MT dynamics with CRMP2?	[148]	Fig.2B
Molecular motors/regulatory proteins					
kinesin-5	Rat primary neurons; *Xenopus* neural tube explants	NGF ?	Antagonises dynein-mediated forces to prevent MT invasion in the P-domain on the opposite of the growth cone turning side	[186; 188]	-
kinesin-12	Rat primary neurons	?	Restricts MT bidirectional transport in the axon, as well as MT invasion of the growth cone P-domain; Regulates growth cone morphology via its interaction with the actin cytoskeleton (?)	[187]	-
Fignl1/KIF1bB (kinesin-3)/bicd1 complex	Zebrafish spinal motor neuron axons	?	Controls rostral motor nerve pathfinding; Dynein speed limiter; regulates bidirectional axonal transport of Rab3 vesicules	[73]	-
Kif13B (kinesin-3)	Mouse cortical neurons	Netrin-1	Controls Myo X anterograde transport (later required for DCC transport), Required to promote axon inititiation and branching/targeting in a Netrin-1 dependent manner	[193]	Fig.1B
kinesin-1	Mouse cortical and hippocampal neurons	?	Regulates mouse growth cone remodelling and corticofugal axon pathfinding by mediating CB1R anterograde axonal transport	[194]	-
Tubulin isoforms					
TUBB2	Mouse/ human cortical neuron axons, ocular nerves	?	Required for proper axon pathfinding; regulates MT dynamics and MT-depolymerase targeting at MT plus ends	[183]	Fig.3A
TUBB3	Mouse/chicken commissural neurons DRG axons	Netrin-1/DCC Netrin-1/UNC-5	Controls Netrin-1/DCC-induced axon attraction of commissural neurons, promotes MT dynamics in growth cones Required for Netrin-1/UNC5-evoked axon repulsion and DRG axon pathfinding; promotes MT dynamics in growth cones	[28] [29; 202]	Fig.3A
Tubulin modifying enzymes					
TTL	Mouse cortical/hippocampal axons and olivary fibers	?	Required for proper navigational behaviour of olivary fibres at the floor plate and cortico-thalamic loop formation; Regulates growth cone morphology; recruitment of MT-F-actin crosstalk mediators in growth cones	[30; 210]	Fig.3B
TTLL6	Zebrafish spinal motor neuron axons	?	Required for proper dorsal nerve pathfinding/targeting; regulator of p60-Katanin activity	[31]	Fig.3B
TTLL11	Zebrafish spinal motor neuron axons	?	Required for proper rostral nerve pathfinding/targeting, concerted regulation of spastin activity with TTLL6?	[31]	Fig.3B

Protein	Models/systems	Axon pruning mechanism	Functions in axon pruning	References	Figure
MT severing/depolymerizing enzymes					
spastin	Mouse neuromuscular junction	activity-dependent competition	Elimination of supernumerary synaptic connections; MT severing	[17]	Fig.4A
KIF2A	Mouse KO/ primary cultures	NGF deprivation	Control of skin inervation; MT depolymerisation	[236]	Fig.4B
Structural MAP					
CRMP2	Mouse hippocampus and visual cortex	Sema3F	Branch-specific elimination; MT dynamics/ disassembly	[145]	Fig.4C

Table 1

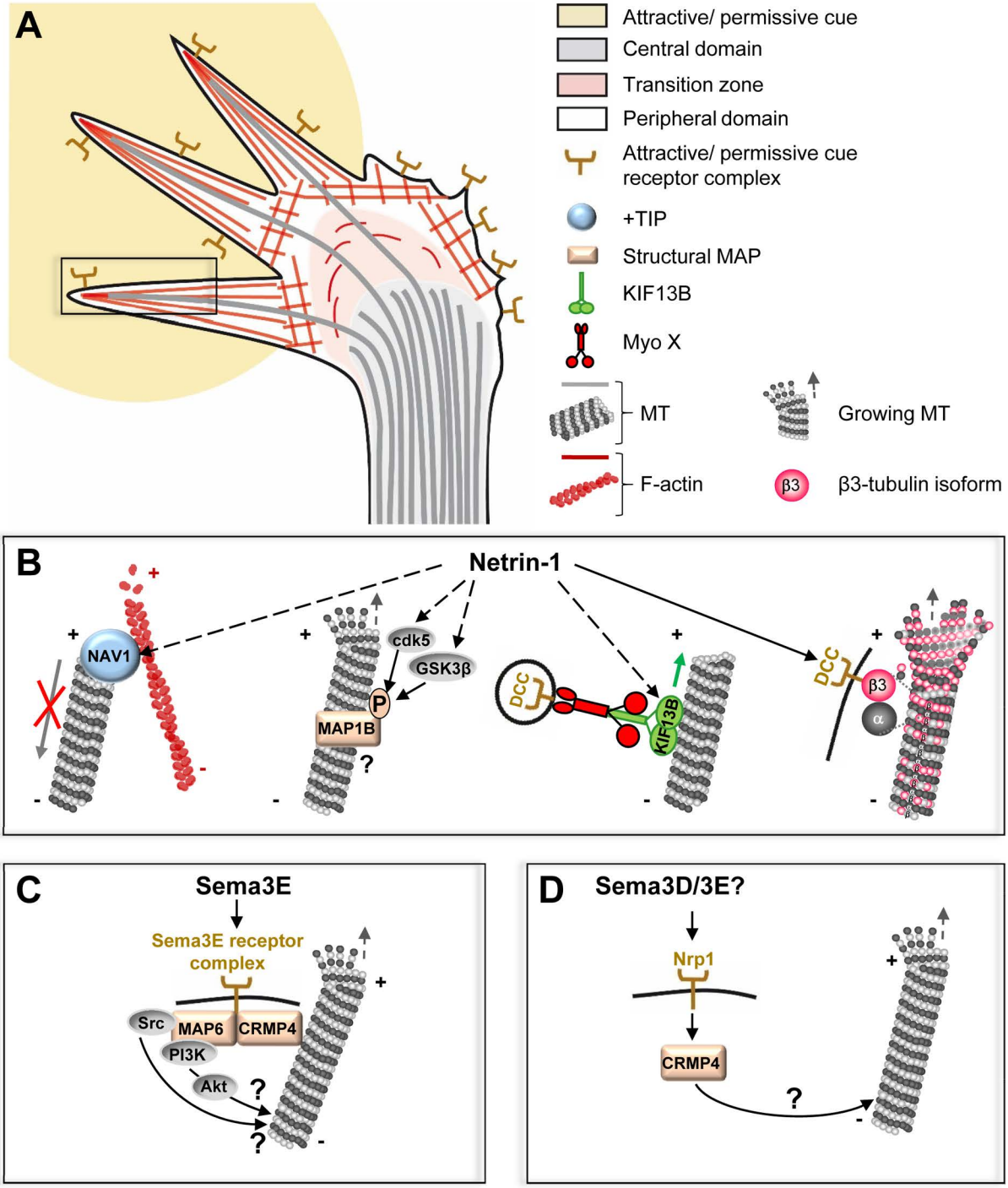

A
Attractive/ permissive cue
Central domain
Transition zone
Peripheral domain
Attractive/ permissive cue receptor complex
+TIP
Structural MAP
KIF13B
Myo X
MT
Growing MT
F-actin
β3
β3-tubulin isoform
B
Netrin-1
NAV1
cdk5
GSK3β
P
MAP1B
?
DCC
KIF13B
DCC
β3
α
C
Sema3E
Sema3E receptor complex
Src
MAP6
CRMP4
PI3K
Akt
?
?
D
Sema3D/3E?
Nrp1
CRMP4
?

Figure 1

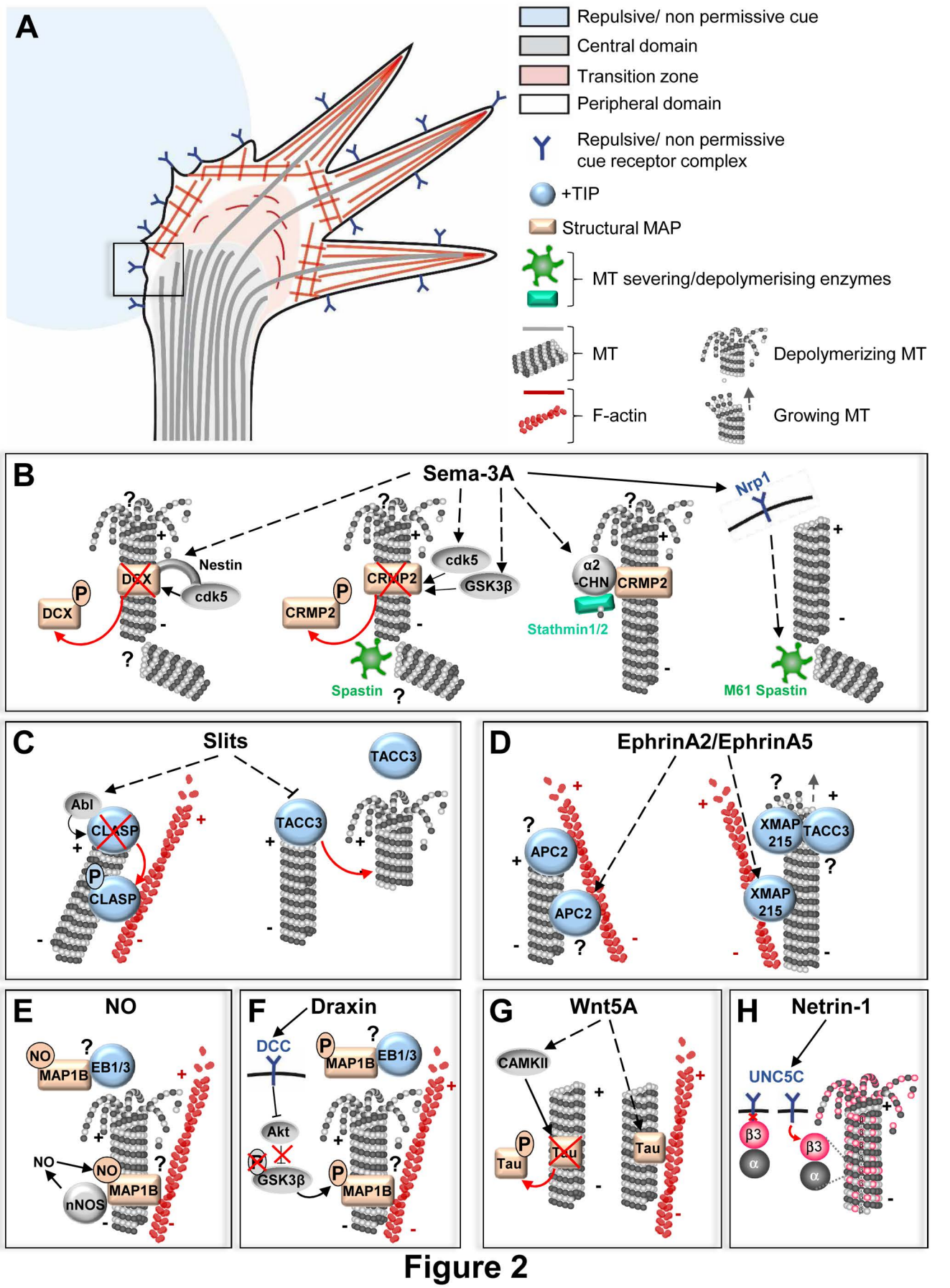

A
Repulsive/ non permissive cue
Central domain
Transition zone
Peripheral domain
Repulsive/ non permissive cue receptor complex
+TIP
Structural MAP
MT severing/depolymerising enzymes
MT
Depolymerizing MT
F-actin
Growing MT
B
Sema-3A
Nrp1
Nestin
DCX
cdk5
P
DCX
CRMP2
cdk5
GSK3β
P
CRMP2
Spastin
?
α2
-CHN
CRMP2
Stathmin1/2
M61 Spastin
C
Slits
TACC3
Abl
CLASP
P
CLASP
TACC3
D
EphrinA2/EphrinA5
APC2
APC2
XMAP
215
TACC3
XMAP
215
E
NO
NO
MAP1B
EB1/3
NO
NO
nNOS
MAP1B
F
Draxin
DCC
P
MAP1B
EB1/3
Akt
P
GSK3β
P
MAP1B
G
Wnt5A
CAMKII
P
Tau
Tau
Tau
H
Netrin-1
UNC5C
β3
α
β3
α

Figure 2

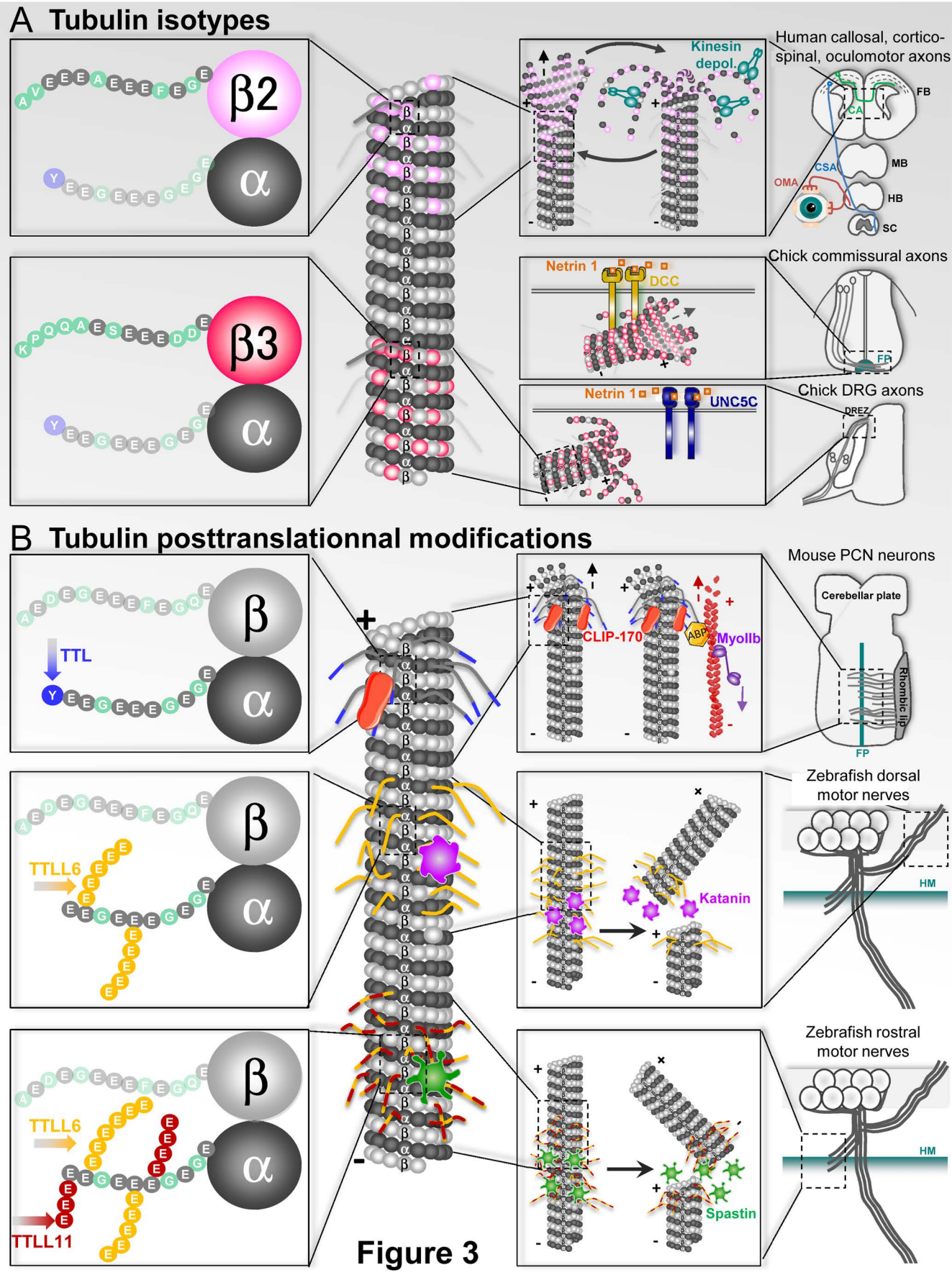
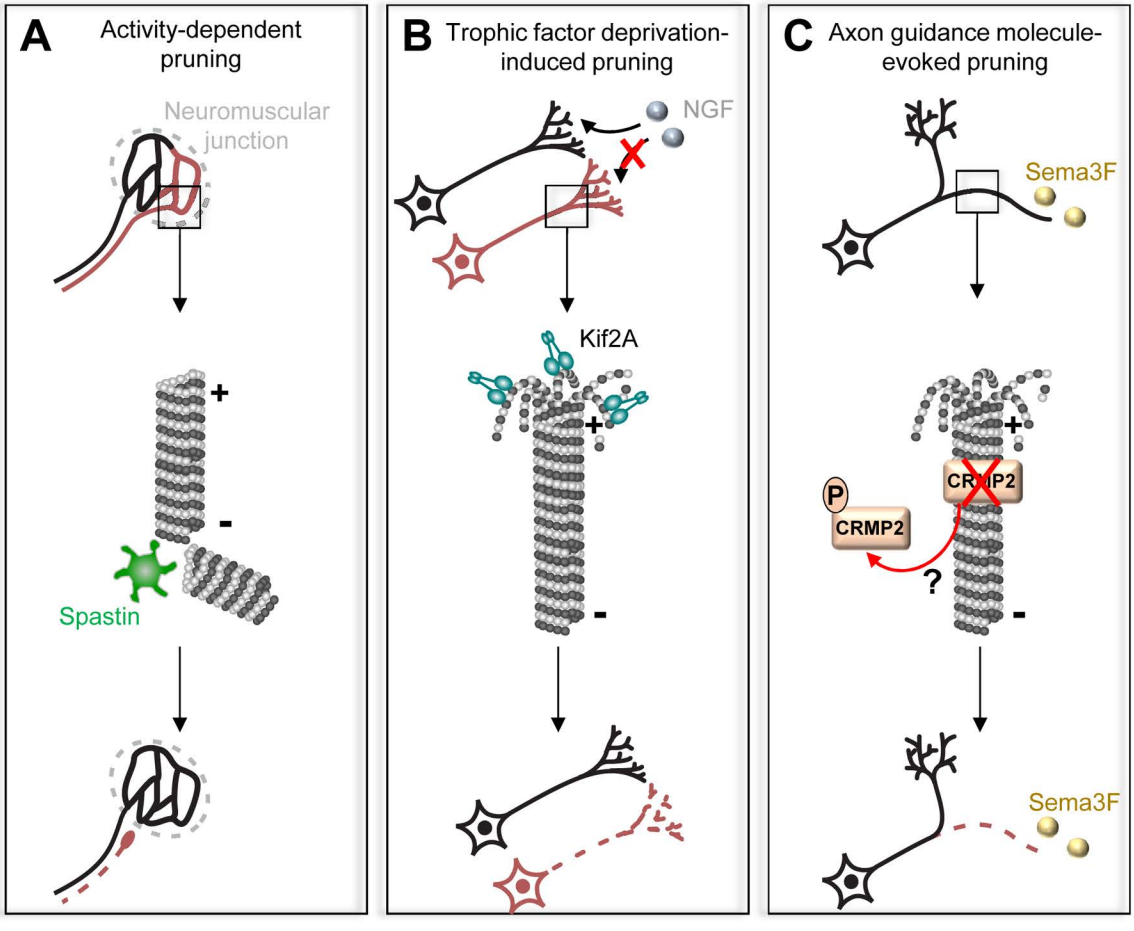

A Tubulin isotypes
β2
α
Kinesin depol.
Human callosal, cortico-spinal, oculomotor axons
FB
CA
CSA
MB
OMA
HB
SC
β3
α
Netrin 1
DCC
Chick commissural axons
FP
Netrin 1
UNC5C
Chick DRG axons
DREZ
B Tubulin posttranslationnal modifications
β
α
TTL
CLIP-170
ABP
MyoIIb
Mouse PCN neurons
Cerebellar plate
Rhombic lip
FP
TTLL6
Katanin
Zebrafish dorsal motor nerves
HM
TTLL6
TTLL11
Spastin
Zebrafish rostral motor nerves
HM

Figure 3


A
Activity-dependent pruning
Neuromuscular junction
+
-
Spastin
B
Trophic factor deprivation-induced pruning
NGF
Kif2A
+
-
C
Axon guidance molecule-evoked pruning
Sema3F
+
P
CRMP2
?
-
Sema3F