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The role of CKAP5 in mediating crosstalk between actin and microtubule cytoskeleton

Role CKAP5 v zprostředkování interakce mezi aktinovým a mikrotubulárním cytoskeletem

MASTER THESIS

Supervisor: RNDr. Zdeněk Lánský, Ph.D.

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Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Na části experimentů a jejich analýze se podílel Ján Sabó (Laboratoř Strukturních Proteinů, BTÚ AVČR), jehož podíl je pečlivě specifikován v textu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, dne 23.4.2024 Bc. Michaela Dujava Ždímalová

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M.D.Ž.

Abstract

Dynamic changes in cytoskeletal architecture are essential for many crucial events throughout lives of all cells. One of the examples is neuronal pathfinding, ensured by specialized axonal structures enriched in actin and microtubule cytoskeleton, known as neuronal growth cones. Growth cones act as motile sensors and navigators, as the tight regulation of their actin and microtubule cytoskeleton results in directed axonal outgrowth guided towards the proper targets, which is crucial for processes such as synaptogegnesis or regeneration. Recently, protein CKAP5 (previously described as a microtubule + tip polymerase) has been proposed to facilitate actin–microtubule crosstalk in growth cones, indispensable for their proper functioning. Here, we combine the power of *in vitro* reconstitution assays with total internal reflection fluorescence microscopy to explore the underlying mechanism of CKAP5 actions. Our findings confirm that CKAP5 also associates with actin filaments and indeed, recruits them to the microtubule lattice. Furthermore, we describe a remarkable behavior of dynamic system containing CKAP5 and both, microtubules and actin filaments, wherein actin bundles are templated along dynamic microtubules by CKAP5. Importantly, upon microtubule depolymerization, the corresponding actin bundle can persist and serve as a track for microtubule repolymerization, guided by CKAP5 which is accumulated at the microtubule + tip. Considering our *in vitro* results alongside indications from experiments in growth cones, we hypothesize that this mechanism could potentially contribute to growth cone dynamics. Specifically, exploratory microtubules may be guided along actin bundles in filopodia by CKAP5, thus consolidating growth cone movement.

Keywords

neuronal growth cones, cytoskeletal crosstalk, actin filaments, microtubules, CKAP5, *in vitro* reconstitution, TIRF microscopy

Abstrakt

Dynamické zmeny v usporiadaní cytoskeletu sú nevyhnutné pre mnoho dôležitých udalostí v živote buniek. Jedným z príkladov je navigácia rastu neurónov k ich správnym cieľom. K tomuto procesu slúžia špeciálne axonálne štruktúry známe ako neurónové rastové kužele, ktoré slúžia ako dynamické senzory a navigátory. Regulácia dynamiky ich aktínového a mikrotubulárneho cytoskeletu vedie k riadenému rastu axónov smerom k hľadaným cieľom, čo je kľúčové pre procesy ako tvorba synapsií alebo ich regenerácia. Proteín CKAP5 bol nedávno navrhnutý ako potenciálny regulátor komunikácie medzi aktínom a mikrotubulami v rastových kužeľoch, pričom táto komunikácia je nevyhnutá pre ich správne fungovanie. V tejto práci kombinujeme *in vitro* rekonštitučné experimenty s TIRF mikroskopiou za cieľom detailne preskúmať mechanizmus tejto interakcie. Naše výsledky demonštrujú, že CKAP5 (pôvodne známy hlavne ako mikrotubulárna polymeráza) je tiež schopný interagovať s aktínovými vláknami a navyše ich prepájať s mikrotubulami. Ďalej popisujeme pozoruhodné správanie dynamického systému obsahujúceho CKAP5, mikrotubuly a aktínové vlákna, kedy CKAP5 formuje aktínové zväzky pozdĺž dynamických mikrotubúl. Zaujímavé je, že aj po depolymerizácii mikrotubulu sa príslušný aktínový zväzok nerozpadá, ale pretrváva a slúži ako "koľajnica" pre opätovnú repolymerizáciu mikrotubulu, ktorý je po nej navádzaný pomocou CKAP5 lokalizovaného na + konci mikrotubulu. Navrhujeme, že tento mechanizmus je založený a poháňaný veľkým rozdielom v afinitách CKAP5 k rôznym komponentom cytoskeletu a môže zohrávať úlohu v neurónových rastových kužeľoch. Konkrétne, dynamické mikrotubuly môžu byť navádzané pri raste po aktínových zväzkoch vo filopódiách pomocou CKAP5, čím sprostredkujú konsolidáciu pohybu rastového kužeľa.

Kľúčové slová

neuronálne rastové kužele, medzi-cytoskeletálna interakca, aktín, mikrotubuly, CKAP5, *in vitro* rekonštitúcie, TIRF mikroskopia

List of Abbreviations

ADP	Adenosine Diphosphate
APC	Adenomatous Polyposis Coli
ARP2/3	Actin Related Protein 2/3
ATP	Adenosine Triphosphate
au	arbitrary unit
BRB80	Britton–Robinson Buffer
BRB80Tx	Britton–Robinson Buffer with Taxol
CDC42	Cell Division Control Protein 42
CKAP5	Cytoskeleton-Associated Protein 5
CLASP	Cytoplasmic Linker-Associated Protein
CLIP-170	Cytoplasmic Linker Protein 170
CRMP	Collapsin Response Mediator Protein
DAAM	Dishevelled-Associated Activator of Morphogenesis
DDS	Dichlorodimethylsilane
EB1,3	End Binding Protein 1 or 3
EGTA	Egtazic Acid
EMA/VASP	Enabled/Vasodilator-Stimulated Phosphoprotein
GAP	GTPase-Activating Protein
GDI	Guanine Nucleotide Dissociation Inhibitor
GDP	Guanosine-5'-Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GTP	Guanosine-5'-Triphosphate
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
IRM	Interference Reflection Microscopy
MAP1B	Microtubule-Associated Protein 1B
mDIA	mammalian Diaphanous
MIP	Maximum Intensity Projection
mNG	mNeonGreen
Msps	Mini spindles
PB	Polymerization Buffer
PBS	Phosphate-Buffered Saline nepouzite
PIPES	Piperazine-N,N-bis(2-Ethanesulfonic Acid)
RAC1	Ras-Related C3 Botulinum Toxin Substrate 1
RhoA	Ras Homolog Family Member A
ROCK	Rho-Associated Protein Kinase
TAB	TIRF Assay Buffer
TACC3	Transforming Acidic Coiled-Coil–Containing Protein 3
TIRF	Total Internal Reflection Fluorescence
TOG	Tumor Overexpressed Gene
XMAP215	*Xenopus* Microtubule-Associated Protein

Contents

1. Introduction

The developmental phase of all organisms is a critical period in their lifes. The development of the brain plays an essential role, as it is the central organ of the nervous system responsible for processing and integrating sensory information.

1.1 Neuronal growth cones

1.1.1 Function of the growth cones

During brain and nervous system development, or later during their regeneration, crucial events are formations of proper synaptic connections. To accomplish this mission, neuronal axons must travel to their appropriate targets often located at considerable distances, and identify them. For that purpose, a specialized transient structures are formed at the distal tips of axons — neuronal growth cones (see in Figure 1.1).

Their ability to guide the axonal outgrowth through the extracellular space primarily relies on chemotaxis [Kennedy et al. 1994] [Onesto et al. 2021*], as well as on interactions with neighboring cells [Rigby et al. 2020]. Malfunctioning in this process can lead to severe developmental or neurodegenerative diseases [Stephens et al. 2022*], which drives the motivation to understand the mechanism of growth cone functioning as deeply as possible.

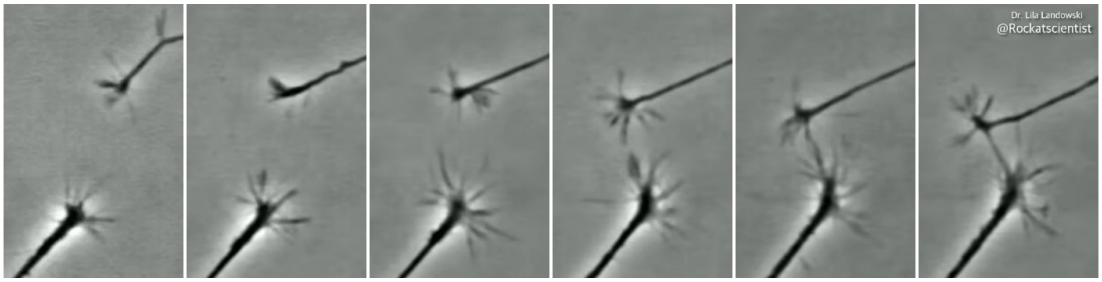


Figure 1.1: Two growth cones finding each other and creating connection in *in vitro* conditions. Timescale is 20 min. Original video by Dr. Lila Landowski [Landowski 2021].

The growth cones were first identified and named by Spanish histologist Santiago Ramón y Cajal in 1890 [García-Marín et al. 2009*]. While growth cones can arise on both dendritic and axonal tips, and several differences in their behavior have been described [Wang et al. 2014*] [Bott et al. 2020], the axonal growth cones were in the highest interest of the research from the beginning.

1.1.2 Operational pathway of the growth cones

The main focus of the growth cone research lies in elucidating the whole process of growth cone guidance. Similarly to other cellular activities, this phenomenon can be split into several key steps, all essential for achieving the desired outcome (illustrated in Figure 1.2).

Firstly, there are extracellular signals indicating changes or actions in the surrounding environment to which the cell can potentially react. In the case of growth

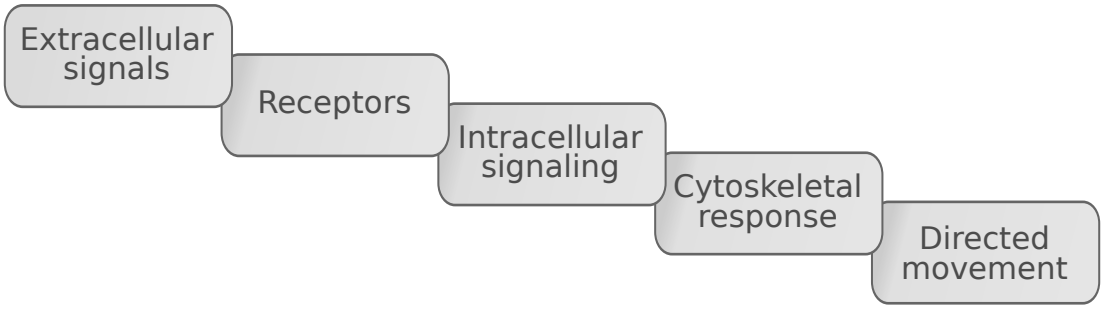


Figure 1.2: General cascade of transforming extracellular signals to the cellular response through intracellular signaling.

cones, extracellular signals can be categorized into two main groups: chemotropic diffusible cues and substrate-bound molecules. Diffusible cues comprise a highly diverse group of various types of molecules, such as netrins and semaphorins [Chilton 2006*], morphogenic and growth factors like Wnt (from "Wingless/Integrated") and BMP (Bone Morphogenetic Protein) [Zou and Lyuksyutova 2007*], or secreted transcription factors [Brunet et al. 2005] [Butler and Tear 2007*] and neurotransmitters [Mattson et al. 1988]. Substrate-bound molecules can be nonadhesive, such as ephrins or slits [Dickson 2002*], while adhesive molecules also significantly contribute to shaping the pathway for the growth cone. These include transmembrane cell adhesion molecules like cadherins [Maness and Schachner 2007*] or components of extracellular matrix such as laminin and fibronectin [Evans et al. 2007]. Interestingly, it was initially believed that some of these molecules are strictly attractive for the axonal outgrowth and the others act as repellents. However, the role of these guidance molecules turned out to be rather context dependent, meaning that the given molecule can act as an attractant with certain cofactors or for one type of axons, but it can have a repellent effect for another type of axons or in interplay with different molecules [Bartoe et al. 2006] (also reviewed in [Bashaw and Klein 2010*] [Tamariz and Varela-Echavarría 2015*]).

To elicit a cellular response, the chemotropic cue by itself is not satisfactory. The cell, or in this case the neuronal growth cone, must possess the ability to detect the cue in its vicinity. Typically, this task is fulfilled by receptors on the cytoplasmic membrane. The growth cones are not an exception, for each group of guiding cues mentioned above, functional receptor complexes have been identified on the axonal/growth cone membrane. For instance, receptor DCC (from "Deleted in Colorectal Carcinoma") for netrin, plexin for semaphorin, or robo for slit, reviewed in [Bashaw and Klein 2010*] [Laura Anne Lowery, Stout, et al. 2013].

Once chemotropic cues bind to receptors, they can trigger a diverse array of intracellular signaling pathways. The most extensively studied signaling pathway affecting the growth cone guidance is through Rho family of small GTPases, reviewed in [Laura Anne Lowery, Stout, et al. 2013] [Hall and Lalli 2010*]. Rho signaling was shown to be triggered by various chemotropic cues representing diverse situations encountered by the growth cone in its environment, hence it is highly abundant signaling there. Three main nodes of this signaling are the small G proteins RhoA (Ras Homolog Family Member A), RAC1 (Ras-Related C3 Botulinum Toxin Substrate 1) and CDC42 (Cell Division Control Protein 42), which exhibit a typical behavior of G proteins — switching between active state with bound

GTP (Guanosine-5'-Triphosphate) and inactive state following hydrolysis of GTP to GDP (Guanosine-5'-Diphosphate). Their upstream regulation is secured by GTPase-activating proteins (GAPs) catalyzing the hydrolysis of GTP to GDP, by guanine nucleotide exchange factors (GEFs) stimulating the exchange of hydrolyzed GDP for fresh GTP, and by guanine nucleotide dissociation inhibitors (GDIs) blocking a spontaneous activation [Schmidt and Hall 2002*]. Several specific GAPs and GEFs were identified to play a role in Rho signaling in growth cones, such as ephexin (a GEF activating RhoA in ephrin signaling) or α-chimerin (a GAP inhibiting RAC1 and CDC42 in netrin signaling). Additional examples are depicted in Figure 1.3.

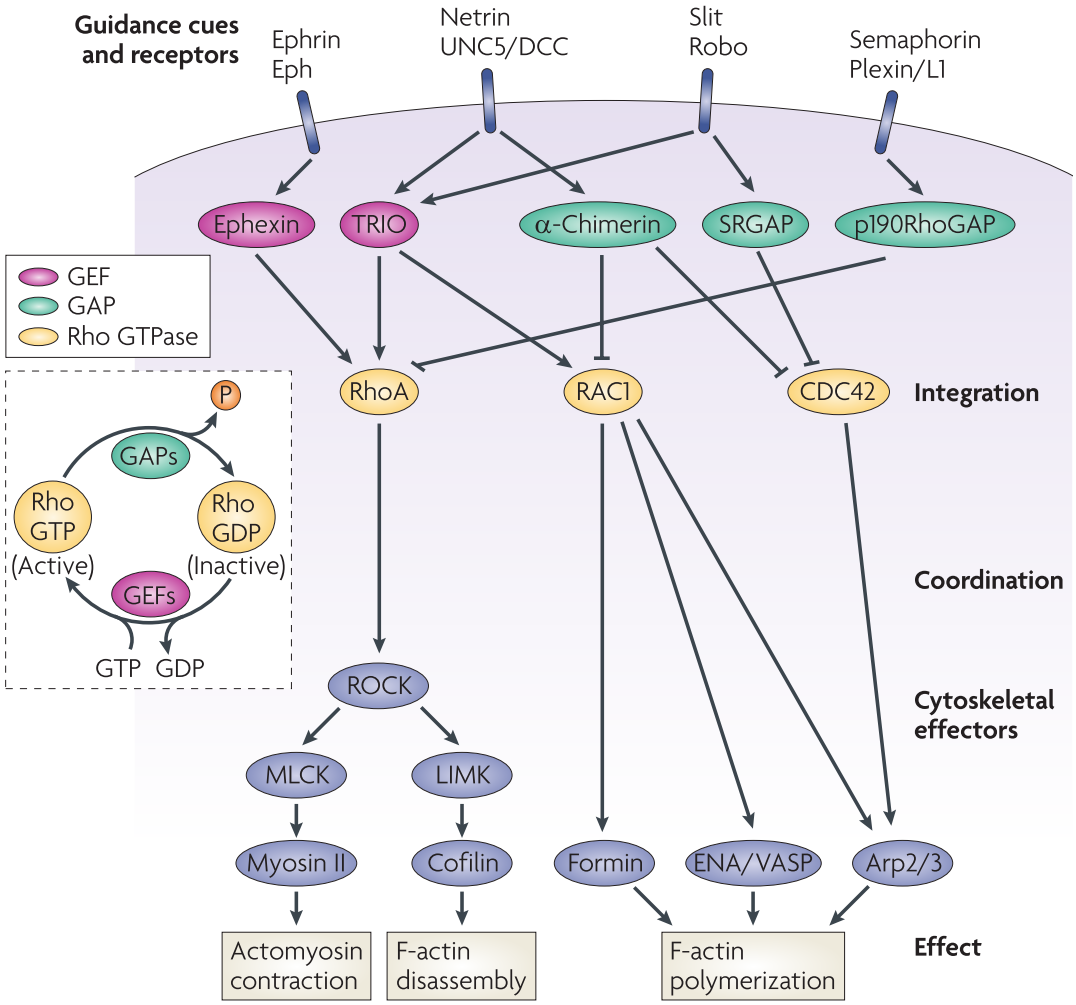


Figure 1.3: Simplified Rho signaling cascade in neuronal growth cones. Exemplary guidance cues and their receptors are depicted at the very top. Purple ellipses represent GEFs and green ones GAPs, while both of them are regulatory units of small G proteins with GTPase activity, shown as yellow ellipses. Cycle of active and inactive states of small G proteins regulated by GEFs and GAPs is depicted in the dashed rectangle on the left. Downstream effectors of small G proteins are shown as blue ellipses (kinases ROCK, MLCK (Myosin Light Chain Kinase) and LIMK (LIM-Domain-Containing Protein Kinase); a molecular motor myosin II; a depolymerizing factor of actin filaments cofilin; actin nucleators formin, ENA/VASP and Arp2/3). The overall effect of this cascade mainly results in regulation of actin dynamics. Adapted from [Laura Anne Lowery and David Van Vactor 2009*].

In the growth cones, RhoA mainly participates in inhibitory or repulsive signaling that impairs their motility. Its primary downstream effector is ROCK (Rho-

Associated Protein Kinase), which can through other components activate both actin disassembly or acto-myosin contractility utilized in a process such as growth cone retraction. On the other hand, RAC1 and CDC42 are associated with attractive or progressive signaling. Upon activation of their downstream effectors, nucleators of actin filaments, they induce rapid polymerization of actin filaments in activated regions (for more details, refer to Figure 1.3). Additionally, Rho signaling can also regulate microtubule dynamics both directly and indirectly [Daub et al. 2001] [Mimura et al. 2006].

Many other signaling molecules have been identified to play a role also in the growth cones, including protein kinase A with second messenger cAMP (cyclic Adenosine Monophosphate) [Cheng and Reese 1987] [Murray et al. 2009], 14-3-3 protein [Kent et al. 2010], atypical protein kinase C [Wolf et al. 2008], focal adhesion kinase [Robles and Gomez 2006], CaMKII (Calcium/Calmodulin–Stimulated Protein Kinase II) [Z. Wen et al. 2004], phosphatases [Ensslen-Craig and Brady-Kalnay 2004*], or the second messenger Ca^{2+} [Gomez and J. Q. Zheng 2006*] [Gasperini et al. 2017*], which was shown to have a broad regulatory function in growth cone motility based on its concentration changes. Phosphoproteomic analysis combined with bioinformatics has also suggested that another kinase group highly active in growth cones is MAPK (Mitogen-Activated Protein Kinases), especially JNK (c-Jun N-terminal Kinase) [Kawasaki et al. 2018]. However, the overall corresponding signaling pathways have not been well explored in the growth cones yet.

Noticeably from the paragraph about Rho signaling, the response of neuronal growth cones to signaling cues primarily relies on cytoskeletal rearrangements. The crucial effectors of this process are indeed actin filaments, microtubules and their associated proteins, which are altogether known to be highly dynamic and capable of generating forces. Synchronized changes in their structures and dynamics can be transferred to the cellular level, leading to the directed movement of the growth cones toward their proper targets, as will be discussed more throughout this thesis.

Although the research of growth cone guidance primarily focuses on the cytoskeleton, another phenomenon linked to growth cone functioning is vesicle transport, involving exocytosis and endocytosis and their regulation by SNARE proteins (Soluble N-ethylmaleimide–Sensitive Factor Activating Protein Receptor). Vesicle transport might play two roles in the growth cone guidance. Firstly, it could represent an additional layer of regulation, since internalization of receptors is one possible way of their desensitization [Piper et al. 2005] or redistribution e.g. to signalling endosomes [Zimmer et al. 2003]. While endocytosis was indicated in repulsive turning of the growth cones from chemorepellents like semaphorin 3A or myelin-associated glycoprotein [Tojima, Itofusa, et al. 2010] [Hines et al. 2010], exocytosis was shown to be involved in attractive outgrowth of cones [Tojima, Akiyama, et al. 2007], as well as in axon branching [Winkle et al. 2014] [Tojima, Itofusa, et al. 2014].

The second potential function of vesicle transport might be in regulating mechanical properties of the membrane and mechanical signals, since exo- and endocytosis can also influence the membrane tension. It was demonstrated, even though on mouse embryonic fibroblasts and not neurons, that during cell migration, the tension on the plasma membrane at the leading edge is significantly increased and as a consequence, the cell activates exocytosis in order to relieve that tension

[Gauthier et al. 2011]. However, to better understand this feedback mechanism, as well as the overall role of vesicular transport in the growth cone guidance, further investigation is necessary.

Altogether, guidance of axonal growth by growth cones comprises several executive phases, all of which are crucial for proper and successful functioning. However, this thesis is mainly focused on the cytoskeletal level and other stages will not be discussed more in detail.

1.2 Cytoskeleton

Since cytoskeleton is a crucial executor of the growth cone movement, this section is focused on introducing the cytoskeletal components themselves.

Cytoskeleton is an indispensable part of every cell needed for retaining its integrity and viability. It comprises a set of filamentous polymers (see Figure 1.4), which are regulated by their associated proteins and posttranslational modifications and can acquire various forms as networks, bundles or "highways".

Plethora of essential cellular processes are dependent on cytoskeleton. One of its well known functions is that cytoskeleton determines cell shape and helps to maintain it. However, cytoskeletal components can undergo fast dynamic structural changes potentially leading to generation of physical forces as pulling or pushing, which can be transferred to the cellular level and utilized in actions as division of the cell, vesicular trafficking or directed motility and migration of the cell. In consequence, dysregulation of cytoskeletal components often leads to cellular or tissue defects and broad spectrum of pathologies.

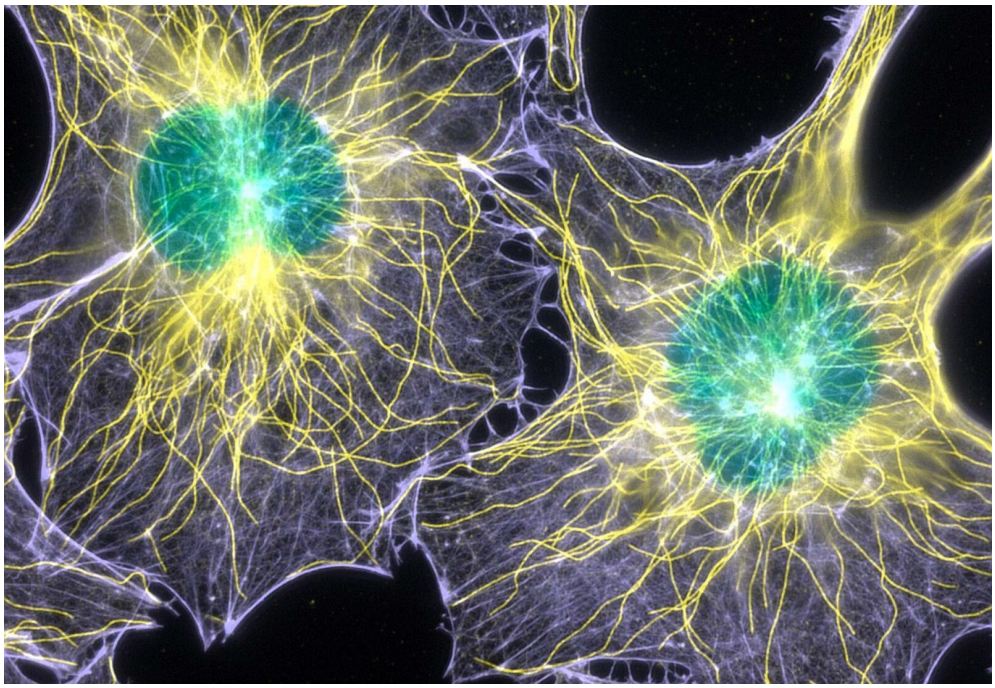


Figure 1.4: Fluorescently-labeled cytoskeleton in mouse fibroblasts. Microtubules shown in yellow are oriented from the nucleus (in turquoise) towards the periphery, while actin filaments (in light purple) form a meshed network. Magnification of the picture is 1000×, captured by Dr. Torsten Wittmann, Nikon Small World [Wittmann 2003].

1.2.1 Actin filaments

Actin is present in cells in two forms: as a small globular protein called G-actin, serving as an essential subunit of the filamentous polymer F-actin (also called actin filament). Structure of a single actin filament resembles a regular helix of two twisted strings of G-actins, with a diameter of 7 nm, being the thinnest of cytoskeleton filaments [Hanson and Lowy 1963] [Depue and Rice 1965].

Similarly to other cytoskeletal filaments, actin filaments do not need any macromolecular complex for their assembly. Rather, G-actins can spontaneously self-assemble in the presence of ATP (Adenosine Triphosphate) when the G-actin concentration meets the critical threshold. The state between free G-actin and G-actin bound into the actin filament is regulated by ATPase activity of the subunits [Graceffa and Dominguez 2003]. In the ATP-state, the subunit is favored to remain in the filament, while the subunit in ADP-state (Adenosine Diphosphate) is more likely to get released. Also, the longer ATP–G-actin is associated in actin filament, the higher the probability it will be hydrolyzed to ADP and released. This leads to different association and dissociation dynamic of the G-subunits at both ends the filament [Wegner and Isenberg 1983] [Pollard and Borisy 2003*] (see the association and dissociation constants in Figure 1.5).

Differential kinetics at both ends, together with the fact that G-actin is not symmetrical [Kabsch et al. 1990] and interacts with other subunits in oriented way, results in the polarity of actin filament. The "older" end is termed minus or pointed end, while the elongating and faster polymerizing end is called plus or barbed end. When the dissociation rate at the pointed end is balanced with the association rate at the barbed end, a phenomenon called "actin treadmilling" occurs, manifesting as spatial travelling of the filament of a constant length.

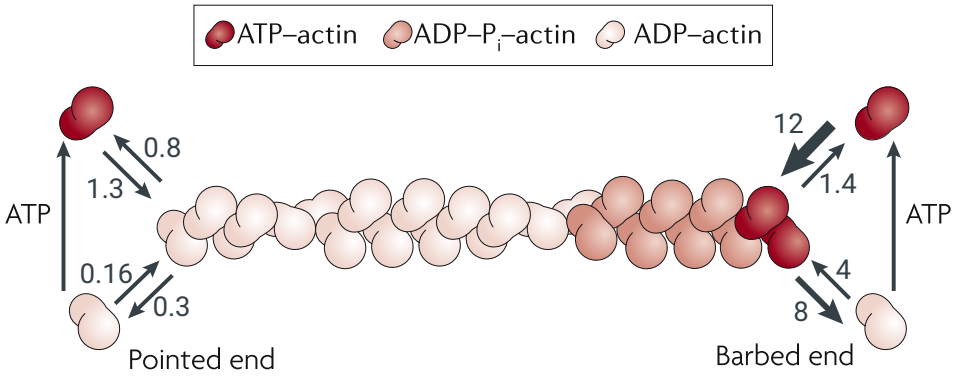


Figure 1.5: Scheme of actin filament depicting association (in $\mu M^{-1}s^{-1}$) and dissociation (in s^{-1}) rate constants at pointed and barbed end for G-actin in ATP or ADP state. Adapted and modified from [Pollard and Borisy 2003*] and [Li and Gundersen 2008*].

Besides ensuring a contractility of muscle cells, actin plays many other crucial roles in non-muscle cells, as for example in final stages of cell division by forming a contractile strangling ring [Schroeder 1972], in a material trafficking [Kübler and Riezman 1993] or in symmetry breaking during embryogenesis e.g. in *Caenorhabditis elegans* [Naganathan et al. 2014]. Outstanding function of actin filaments is driving a motility of many cells, which is an important research topic from a long time ago

[Kübler and Riezman 1993], and the growth cones are not an exception. Function of actin filaments in the growth cones will be further discussed in upcoming sections.

1.2.2 Microtubules

Microtubules are rigid [Gittes et al. 1993] hollow tubes with the largest diameter among all cytoskeletal filaments, measuring around 25 nM [Ledbetter and Porter 1963] [Burton et al. 1975]. Although they share many attributes with actin filaments, their structure is quite different. Microtubules also consist of smaller globular subunits, heterodimers of α- and β-tubulin [Nogales et al. 1998] [Löwe et al. 2001]. In the assembled state, tubulin dimers are bound head–to–tail to each other forming a straight protofilament [Cote and Borisy 1981]. This arrangement exposes α-tubulins at one end of the polymer, called minus end, and β-tubulin at the other, known as the plus end. Therefore, microtubules are similarly to actin filaments polar polymers with 2 distinct ends (see Figure 1.6).

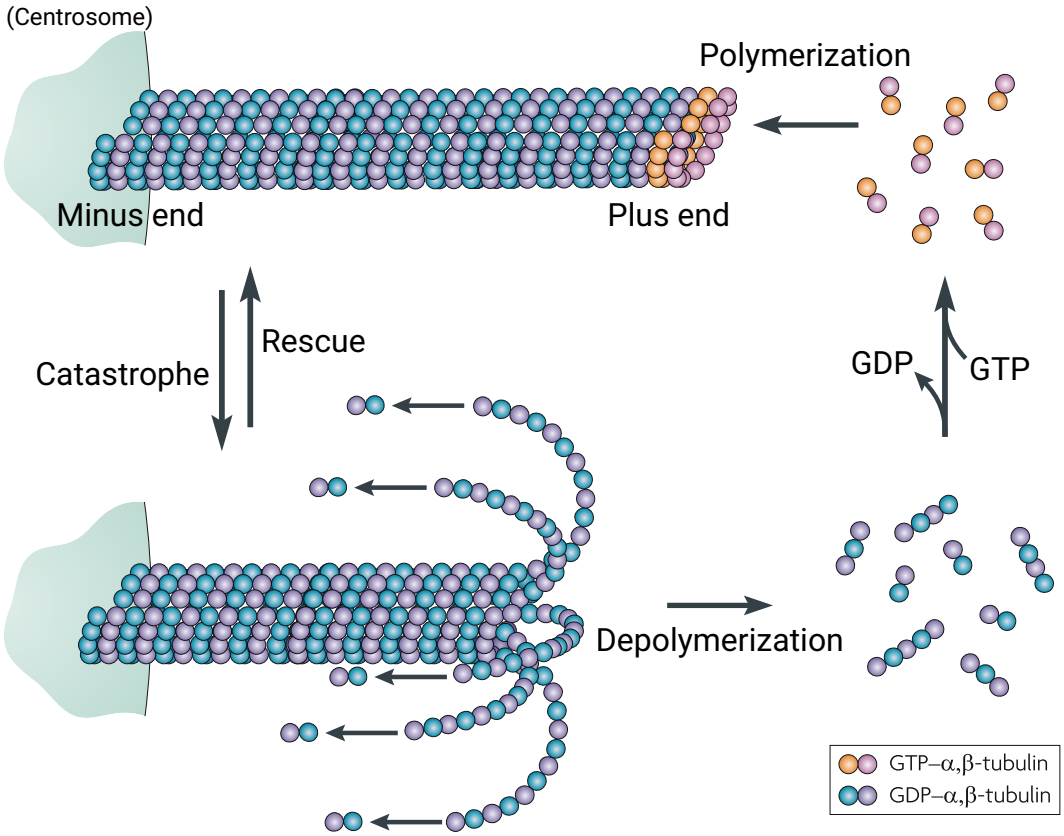


Figure 1.6: Scheme of microtubule instability. Catastrophe and rescue are switches between polymerization and depolymerization phases of microtubule. GTP-tubulin (yellow) elongates the + end of microtubule and forms so-called GTP-cap. Once the GTP-tubulins in GTP-cap hydrolyze GTP to GDP (blue), microtubule starts to disassemble from the end, releasing new pool of available tubulins. In cells, − end is usually anchored in a microtubule organizing center, here in centrosome (green). Adapted and modified from [Li and Gundersen 2008*] and [Lodish et al. 2020].

Protofilaments associate laterally together, usually in a number of 13 protofilaments for a canonical microtubule [Tilney et al. 1973], which was found in every subgroup of eukaryotes [Unger et al. 1990*], but the number can differ. Purified tubulin can

spontaneously give rise to microtubules with 9–16 protofilaments, while the ones with 14 protofilaments are the most abundant *in vitro* [Pierson et al. 1978].

The dynamics of microtubule self-assembly is driven by GTPase activity of β-tubulin, although α-tubulin contains a binding site for GTP, too [Nogales et al. 1998] [Geyer et al. 2015]. When the local concentration of tubulin dimers reaches critical concentration, microtubule seed nucleate. Tubulin dimers are added to the lattice in the GTP-state, but over time, GTP bound to β-tubulin is hydrolyzed to GDP. Hence, as microtubule grow, its lattice is converted to the GDP state and only the growing + end contains a GTP-area (termed GTP-cap), where fresh GTP-tubulins are added. The properties of tubulin dimer in GTP and GDP state vary, similarly to actin subunits. In the hydrolyzed GDP state, dimers are more prone to detach from the microtubule lattice. If the rate of adding new GTP-dimers to the plus end becomes slower than the hydrolysis of GTP to GDP on freshly added tubulins, microtubule growth halts, leading to depolymerization, termed catastrophe. However, depolymerization phase can switch back to polymerization phase, which is called rescue. These switches can occur relatively fast and this phenomenon, known as dynamic instability of microtubules, is crucial for the cellular functioning [Cote and Borisy 1981] [Tim Mitchison and Marc Kirschner 1984], reviewed in [Desai et al. 1999].

Microtubules steer many substantial cellular processes, such as separation of chromosomes during cell division [Hunt and McIntosh 1998] [Prosser and Pelletier 2017*], flagellar or cilliar motility [Ringo 1967] [McGrath et al. 2003], they serve as "highways" for driven intracellular transport of material [Hirokawa et al. 2010*], or they can cooperate with actin filaments to ensure cellular mobility, which is also the case in neuronal growth cones, as will be discussed throughout this thesis.

1.2.3 Intermediate filaments

Intermediate filaments display more differences compared to actin filaments and microtubules. Although they can also self-assembly, they do not bind ATP nor GTP. Long filamentous monomers assemble in a head–to–head manner into polar coiled-coil dimers, however, dimers subsequently form head–to–tail non-polar tetramers [Pauling and Corey 1953] [Steinert et al. 1976]. Those can further laterally associate giving arise to larger complexes and whole intermediate filaments of 10 nM diameter [Small and Sobieszek 1977], as can be seen in Figure 1.7.

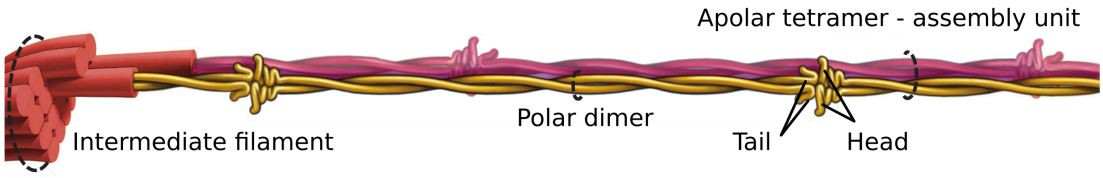


Figure 1.7: Scheme of dissected intermediate filament. Yellow and pink filaments represent polar dimers assembled to non-polar tetramers, which further associate into the whole intermediate filament. Adapted and modified from [Pollard, Earnshaw, et al. 2017].

Intermediate filaments do not exhibit such a dynamic behavior as actin filaments or microtubules. Once they are assembled, they excel in resisting to stress and tensile forces [Wagner et al. 2007] [Kreplak et al. 2008]. In cells, intermediate filaments take care

of maintaining the cellular shape, organization of its 3D structure by anchoring the organelles within the cellular space, but they also mediate a connection to extracellular matrix and neighboring cells through junctions (reviewed in [Herrmann et al. 2007*]).

Intermediate filaments turned out to be important also in regenerating and growing neurons, even though most of the research in this area is focused on microtubules and actin filaments due to their dynamics. After an injury, neurofilaments (one of the many types of intermediate filaments) were shown to be significantly enriched in regenerating neurons compared to the neurons unable to regenerate [Jacobs et al. 1997]. Neurofilaments were also identified directly in the growth cones [Chan et al. 2003]. Since the growth cones may be formed far away from the cell body (even hundreds of centimeters), researchers were interested in the question of how neurofilaments get there. Interestingly, they were shown to be actively and bi-directionally transported along microtubules in axons to the growth cones [Helfand et al. 2003] [Uchida and A. Brown 2004], but there are also indications that neurofilaments are being locally synthesized there [Tesser et al. 1986] [Baraban et al. 2013] [Jin et al. 2016]. However, their role in the growth cones is not well explored yet.

Functional studies have shown that some of the other types of intermediate filaments can be crucial for the growth cone functioning. For example, neurons with depleted peripherin were not able to extend and form growth cones [Helfand et al. 2003]. Another more thorough study demonstrated that nestin in neurons selectively acts as a scaffold protein for phosphorylation of DCX (Doublecortin) by CDK5/p35 complex (Cyclin-Dependent Kinase 5 with its neuron-specific activator p35), while DCX is a stabilizing factor for microtubules and its phosphorylation prevents it from interaction with microtubules [Bott et al. 2020]. Combined with another study showing that absence of DCX leads to decreased stability of microtubules and their higher protrusivity in growth cones [Jean et al. 2012], this could be a perfect example of how passive intermediate filament can affect dynamics of another cytoskeletal filament and further of the whole cellular structure.

1.2.4 Septins

Septins are the fourth and newest group of cytoskeletal polymers, identified to be expressed in all eukaryotes except for plants [Pan et al. 2007]. Even though they can bind GTP, it is not fully clear whether they can also hydrolyze it [Fischer et al. 2022], hence the mechanism of their self-assemly is not thoroughly understood yet. However, some studies indicate that also post-translational modifications as phosphorylation, sumoylation and ubiquitylation play roles in the self-assembly, reviewed in [Mostowy and Cossart 2012*].

There are three types of basic septin subunits expressed in cells, which then self-assembly into hetero-hexamers or even hetero-octamers [Sirajuddin et al. 2007] [Soroor et al. 2021]. Those further combine to form higher-order structures as non-polar filaments, bundles, rings or even “cages”, depicted in Figure 1.8.

So far, their function has been identified in creating scaffolds for other proteins [Matsuyama et al. 2002] [Joo et al. 2007], barriers in membranes to prevent diffusion of

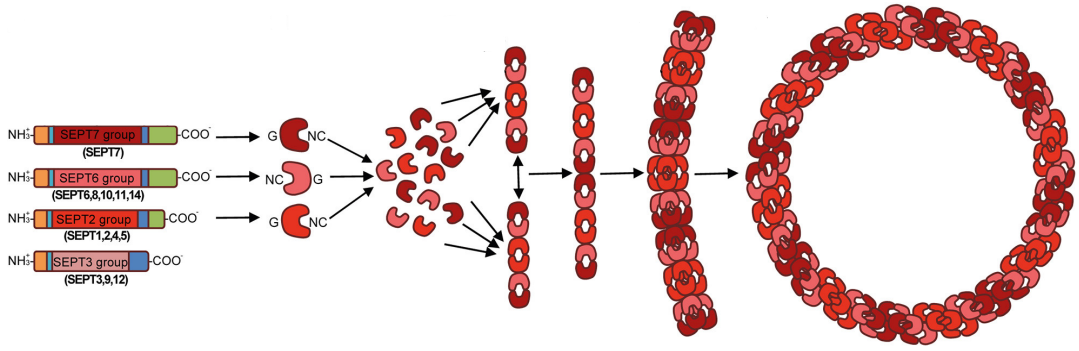


Figure 1.8: Scheme of possible structural arrangements rising from three basic septin units. Hetero-hexamers can further form e.g. bundles or rings. Adapted and modified from [Torraca and Mostowy 2016*]

other components [Caudron and Barral 2009], contributing to regulation of membrane rigidity [Gilden et al. 2012], defending cell against pathogens [Torraca and Mostowy 2016*] or interacting with microtubules and actin filaments regulating their behavior [Kremer et al. 2007] [Bowen et al. 2011].

Interestingly, septins were reported to play a role also in neuronal development of axons and dendrites. Namely, depletion of SEPT7 followed by rescue experiments shown that it is required for axono- and also dendrito-genesis, both *in vitro* and *in vivo* [Ageta-Ishihara et al. 2013]. The mechanism underlying this is thought to be based on a fact that SEPT7 acts as a scaffold for HDAC6 (Histone Deacetylase 6) to deacetylate tubulin [Matsuyama et al. 2002], leading to higher dynamics of microtubules, which is beneficial for neuritogenesis. In addition, another recent study showed that SEPT7 is required for protrusive morphology of growth cones of primary rat hippocampal neurons and that it colocalizes with microtubules overlapping with actin filaments in the growth cones. Moreover, recombinant SEPT2,6 and 7 were demonstrated to directly mediate an interaction between microtubules and actin filaments in *in vitro* reconstitution experiments, although authors have used quite a high concentration of tested proteins (500 nM), which can compromise a specificity of interactions [Nakos et al. 2022].

Nevertheless, since the main functions of growth cones — searching the surrounding, sensing the cues around them and guiding the growth of neurits — require high mobility of the whole structure, main focus of the cytoskeletal research of the growth cones lies on actin filaments and microtubules. Therefore, intermediate filaments and septins will not be discussed further much more.

1.3 Cytoskeleton as a driver of growth cone movement

1.3.1 Structure of the growth cones

The growth cone itself consists from three conceptual parts — the central domain, transition zone and peripheral domain [Forscher and Smith 1988], reviewed in [Cammarata et al. 2016*]. Each of these regions has its specifics in terms of structure

and actions taking place there (see Figure 1.9). In the peripheral domain, actin filaments predominate forming two main structures: oriented parallel actin bundles that give rise to filopodia and meshed actin networks between them, creating lamellipodia-like areas [Schaefer et al. 2002]. Microtubules are primarily situated in the central domain near the end of axon shaft, where they are tightly bundled [Bray and Bunge 1981]. The transient zone comprises actin arcs hindering the microtubule growth [Schaefer et al. 2002], even though recent studies indicate that the transient zone might not be present in the growth cones formed in 3D environment [Santos et al. 2020]. However, individual microtubules can overcome these obstacles and dynamically polymerize by their + ends towards the periphery, in which case they are called exploratory microtubules. In the periphery, microtubules meet actin bundles and can align with them while continuing to grow. This crosstalk enhances the ability of filopodia to dynamically steer from the growth cone and to palpate and sense the surrounding environment.

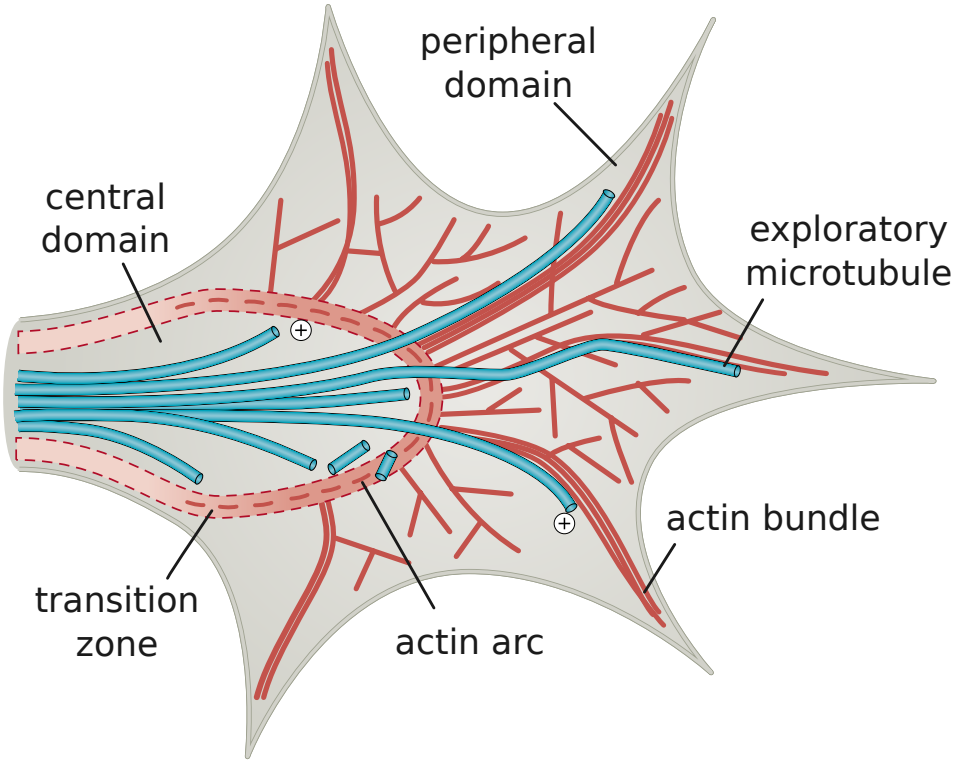


Figure 1.9: Structure of a growth cone. The central domain contains stable, bundled microtubules, the transition zone is formed of actin arcs obstructing microtubule polymerization and the peripheral domain is composed of actin bundles in filopodia and actin networks in lamellipodia-like structure. Individual microtubules polymerizing towards the periphery and shrinking back are called exploratory microtubules. Adapted and modified from [Dogterom and Koenderink 2019*]

In contrast to other microtubules in cells, microtubules in axon shaft and growth cones are not anchored in centrosome, but rather transported from the cell body towards the periphery and to form tightly bundled highways along the long axon shafts [Dent and Gertler 2003*]. However, alternative microtubule organizing centers can take over the role of centrosomes and anchor/cap the microtubules on the way [Wilkes and Moore 2020*].

1.3.2 Mechanical basis of growth cone movement

To move forward, the growth cones undergo three stages of action repeatedly: protrusion, engorgement and consolidation (see Figure 1.10), which were first described by observing large growth cones of *Aplysia californaca* [Goldberg and Burmeister 1986], reviewed in [Laura Anne Lowery and David Van Vactor 2009*] [Dent, Gupton, et al. 2011*]. Each of these stages is driven by specific cytoskeletal changes at particular locations within the growth cone. Preceding protrusion, exploratory microtubules dynamically inspect region of the peripheral domain [P. C. Letourneau 1983].

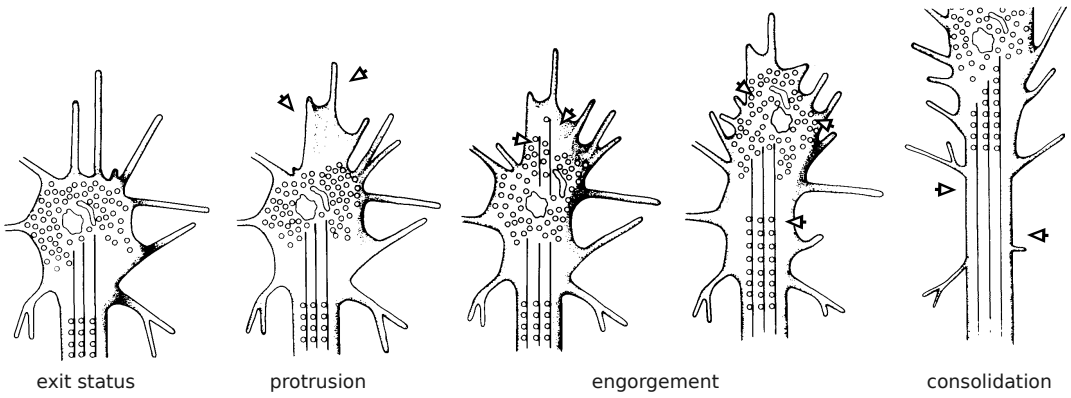


Figure 1.10: Three conceptual phases of growth cone progression: protrusion characterized by extension of filopodia and lamellipodia; engorgement when microtubules invade further into the growth cone pushing the central zone; consolidation distinguished by forming of the new part of axon shaft. Arrows point to the location of a given action. Adapted and modified from [Goldberg and Burmeister 1986]

Protrusion itself is mainly driven by abrupt actin polymerization upon activation of actin nucleators [Korobova and Svitkina 2008] and by actin-mediated substrate adhesion [Paul C. Letourneau and Shattuck 1989]. As a result, growth cone edge extends ahead by extension of filopodia and lamellipodia-like structures.

The engorgement phase is typically characterized by progression of the central domain, hence by invasion of microtubules associated with actin arcs [P. C. Letourneau 1983] closer to the relocated growth cone edge, probably driven by built up acto-myosin tension between adhesion sites and actin arcs [A. C. Lee and Suter 2008]. It is accompanied by the arrival of membranous organelles and vesicles to the peripheral domain, mediated by microtubule-associated transport.

The result of subsequent consolidation stage is the transformation of the former growth cone area into the cylindrical axon shaft filled with oriented bundled microtubule highways [Suter and Forscher 2000*], complemented by actin filament depolymerization at the growth cone neck. Alongside these phases, growth cones also exhibit responses such as turning or pausing, similarly based on cytoskeletal rearrangements. Some of the key players regulating these cytoskeletal changes will be discussed in following section.

Another two phenomena connected to actin filament dynamics have been described in growth cones. One of them is the turnover of actin filaments at the periphery, where their polymerization together with progressive actin treadmilling is balanced

by retrograde actin flow. This flow is driven by contractility of myosin II, as well as by pushes from the growth cone edge when actin filaments encounter with it [Medeiros et al. 2006]. This process not only facilitates the recycling of actin material, but also provides a tool for regulation of the growth cone movement. For instance, when the rates of actin polymerization and treadmilling exceed the retrograde flow rate, the protrusion of growth cone can arise, however if these rates are balanced, the protrusion does not occur [Suter and Forscher 2000*], reviewed in [Laura Anne Lowery and David Van Vactor 2009*] [Dent, Gupton, et al. 2011*].

Interestingly, it was shown that microtubules can be coupled to actin filaments undergoing retrograde flow, leading to the reversal oft heir advancement [A. C. Lee and Suter 2008]. The coupling is mediated by actin–microtubule crosslinkers and can be regulated by uncoupling factors [Grabham et al. 2007].

Another phenomenon, also observed during the protrusion phase (at least *in vitro*), involves adhesive binding to the substrate mediated by actin filaments in filopodia. This process resembles a molecular "clutch" as it mechanically couples growth cone membrane receptors bound to the adhesive substrate with anchored actin filaments, thereby promoting forward protrusion by inhibiting actin retrograde flow and providing support against the substrate [Suter and Forscher 2000*]. This mechanism was first proposed by Mitchison and Kirschner in 1988 [T. Mitchison and M. Kirschner 1988*] and has been supported by many findings afterward, e.g. [Bard et al. 2008] [Shimada et al. 2008]. However, recent studies suggest that the situation *in vivo* differ from *in vitro* conditions, as growth cones likely do not rely on adhesion to generate supportive forces in 3D environments [Santos et al. 2020], reviewed in [Alfadil and Bradke 2023*].

1.3.3 Regulators of actin dynamics in the growth cones

To achieve dynamic rearrangements of actin filaments as described above, many associated proteins interact with the filaments and reversibly alter their structure. This section is dedicated to introduce some of them, which where also recognized in a context of the growth cone regulation (summarized in Figure 1.11), reviewed in [Dent, Gupton, et al. 2011*] [Omotade et al. 2017*].

- Actin nucleators and elongators — Arp2/3, formins, Ena/VASP. They promote axonal outgrowth by locally enhancing actin polymerization upon activation by small GTPases RAC1 or CDC42 (depicted in Figure 1.3), utilized, for example, in the protrusion phase. Arp2/3 (Actin Related Protein 2/3) is a complex capable of nucleating a new actin filament from an already existing one, thereby creating a branch. Arp2/3 operates widely in lamellipodia-like regions, but also in filopodia, since its depletion led to defective progression of both structures [Korobova and Svitkina 2008]. Additionally, it is required for the proper guidance by growth cones [Norris et al. 2009]. On the other hand, formins nucleate actin filaments *de novo* and also participate in its elongation phase by continuously associating with the actin + end, actively adding new G-actin subunits to it. One of the formins operating in growth cones is DAAM (Dishevelled-Associated Activator of Morphogenesis), primarily involved in filopodia formation [Matusek et al. 2008]. The last mentioned protein, Ena/VASP (Enabled/Vasodilator-Stimulated Phosphoprotein), acts as an

elongator of already nucleated filaments, enriched in tips of filopodia and leading lamellipodia, while it is highly expressed in developing brain [Lanier et al. 1999]. It is an important player in various signaling pathways and as demonstrated in several organisms, it is required for proper guidance ability of the growth cones [Wills et al. 1999] [Bouchard et al. 2004] [Chang et al. 2006].

- Actin destroyers — cofilin, mical. Cofilin severs actin filaments, which can result in regulated actin disassembly (as depicted in Figure 1.3 upon RhoA activation). However, cofilin severs actin in a way that generates new + ends prone to polymerization, thus if they are not capped, it can also lead to enhanced actin polymerization [Ghosh et al. 2004]. In the growth cones, cofilin was also shown to play a role in growth cone turning events [Marsick et al. 2010]. Mical depolymerizes actin filaments through oxidation in a response to plexin–semaphorin repellent signaling [Terman et al. 2002] [Hung et al. 2010]. Together, these two factors demonstrate that regulated destruction of the cytoskeleton is also important for growth cone to react to emerging changes in the environment and allowing its rebuilding.

- Monomer binders — profilin, thymosin-β. Another group of actin-associated proteins interacts with G-actin subunits. Profilin bound to G-actin promotes the exchange of ADP for ATP, helping it to get ready for polymerization [Mockrin and Korn 1980][Goldschmidt-Clermont et al. 2017]. Therefore, it is a crucial component of actin turnover. Mutation of the profilin homolog in chickens resulted in the arrest of axon growth before reaching their targets or in bypassing them [D. V. Vactor et al. 1993] [Wills et al. 1999]. Conversely, thymosin-β sequesters G-actin, preventing its polymerization. It has been found to be enriched in growth cones [Roth et al. 1999] and to regulate neurite outgrowth [Kesteren et al. 2006], as well as it was suggested to be involved in the regeneration of some neurons [Roth et al. 1999]. Both profilin and thymosin-β are enriched in lamellipodia-like regions in growth cones, contributing to actin turnover [Paavilainen et al. 2004*].

- Motor proteins — myosin IIA, myosin IIB. Molecular motors can bind to several actin filaments and contract them in respect to each other. For example, myosin II is involved in retrograde flow in growth cones, driving it by generating shear and compressive stresses based on contraction of the actin network in the peripheral and transition zone [Lin et al. 1996] [Medeiros et al. 2006]. Myosin II was also studied in the context of the chemorepellent semaphorin 3A, unrevealing its biphasic response. After exposure, myosin IIA is transferred away from the growth cone, leading to the destabilization of actin network and inducing its collapse, while myosin IIB appears to remain associated with actin filaments in the growth cone and subsequently drives a contractile retraction from the repellent [J. A. Brown et al. 2009].

- Bundling factors — fascin, filamin. Fascin is in general one of the crucial abundant crosslinking protein in filopodia, forming parallel actin bundles there and holding them together [Sasaki et al. 1996] [Aratyn et al. 2007]. This seems to be the case in growth cones as well, as depletion of its activity in growth cones of *Helisoma* snail and in *Droshopila* led to significantly disrupted morphology of the cones lacking bundles, as well as to improper navigation

[Cohan et al. 2001] [De Arcangelis et al. 2004]. Another actin bundling protein localized in growth cones is filamin [Paul C. Letourneau and Shattuck 1989], which was also shown to be involved in growth cone guidance and progression [L. Zheng et al. 2011].

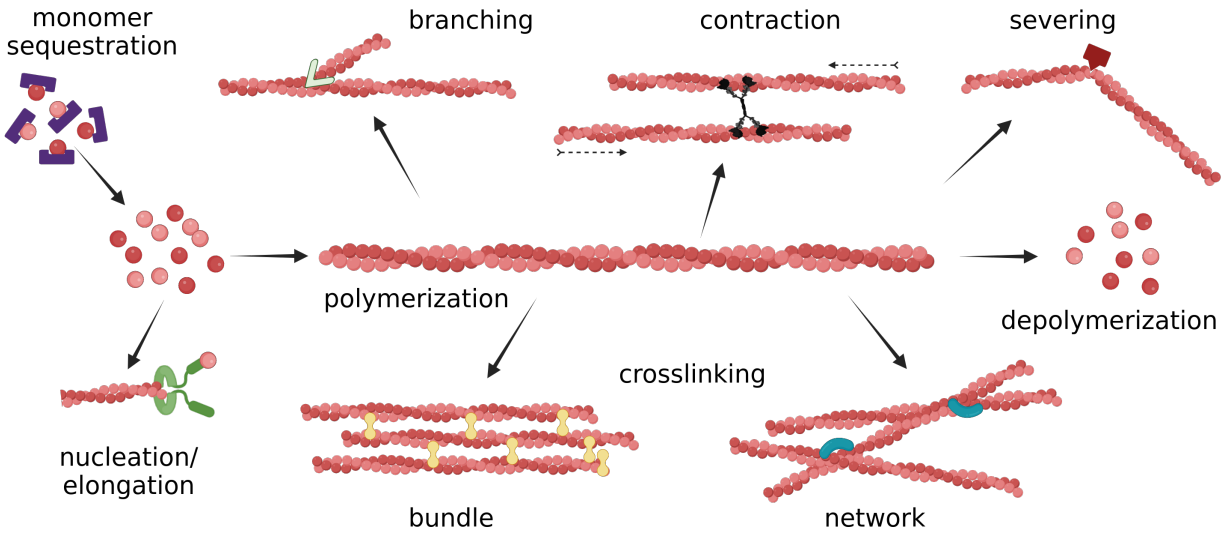


Figure 1.11: Summary of effects of various actin binding proteins. Created in BioRender.

1.3.4 Regulators of microtubule dynamics in the growth cones

Initially, researchers were primarily focused on studying the actions of actin filaments in the growth cones, since actin is a main driver of cell motility and migration also in many other cells. However, the importance of microtubules for growth cone functioning became evident, as growth cone can not progress forward without them [Tanaka et al. 1995].

Indirectly, microtubules and their associated proteins along the axon shaft and in the central domain of the growth cones serve as highways and regulators for bidirectional transport [Uchida and A. Brown 2004] [Chamberlain and Sheng 2019*], supplying the distant periphery with necessary material and cargo. Furthermore, microtubules can also serve as a scaffold for signaling molecules, such as for Src kinase family at adhesion sites [Suter, Schaefer, et al. 2004] or for components of the Rho signaling [Stehbens and Wittmann 2012*], participating also in the regulation of signaling pathways involved in motility.

Microtubules also play a direct role in growth cone movement by utilizing their characteristic feature — dynamic instability. Exploratory microtubules dynamically grow towards the periphery and back, actively exploring the environment [P. C. Letourneau 1983]. They can consolidate and stabilize filopodia, with a substantial role in growth cone turning when changing the direction of movement [Challacombe et al. 1997] [Buck and J. Q. Zheng 2002].

Similarly to actin filaments, the dynamics of microtubules is additionally regulated by a huge palette of associated proteins. Here, a few of those recognized

in regulation directly in growth cones are briefly introduced (summarized in Figure 1.12).

- Microtubule stabilizers — MAP1B, CRMP and Tau. These proteins bind to microtubule lattice and stabilize it. Both MAP1B (Microtubule-Associated Protein 1B) and CRMP (Collapsin Response Mediator Protein) family were identified as key components in axonal outgrowth and growth cone turning, as indicated by their activation at the side of the turn, supporting the hypothesis that microtubules are key players when it comes to consolidation of the new direction [Del Río et al. 2004] [Higurashi et al. 2012] [Khazaei et al. 2014]. For MAP1B, an upstream signaling cascade was also revealed, as it facilitates attractive turning upon netrin signaling [Del Río et al. 2004]. The tau protein is well-known for stabilizing microtubules in axon shafts, however, it is enriched in growth cones too [Black et al. 1996]. A recent study with living growth cones has demonstrated that tau organizes microtubules in the central domain, since tau knockdown manifested in abolished steering of exploratory microtubules through the transition zone. It also caused their misalignment with actin bundles in peripheral domain, altogether leading to disrupted outgrowth and, similarly to MAP1B, growth cone turning [Biswas and Kalil 2018].

- Microtubule cutters — katanin and spastin. Both katanin and spastin are microtubule severing proteins and axonal outgrowth is sensitive to their cellular levels. Overexpression of these proteins leads to reduced axonal outgrowth [Karabay et al. 2004] [Riano et al. 2009], as one would expect when increasing destruction of microtubules. However, knocking them down causes similar phenotype, as microtubules become overly stabilized. In addition, overexpression of spastin increases axonal branching [Yu et al. 2008], indicating its involvement in this mechanism, and a very recent preprint suggests katanin to affect the growth cone guidance as well [Martin et al. 2024].

- Motor proteins — kinesins and dyneins. These motors are known for their ATP dependent walking along microtubules. Kinesins walk towards the + end of microtubules, hence anterogradely towards the growth cone, while dynein walking is oriented towards the − ends, thus retrogradely towards the neuron body. The primary role of these motors is to actively deliver cargo material to the desired locations, such as the kinesin KIF1B (Kinesin Family Member 1B), which is known to transport mitochondrias [Nangaku et al. 1994], or KIF2A, which was shown to mediate transport of nonsynaptic vesicles to the growth cone, necessary for membrane extension [Noda et al. 1995] [Morfini et al. 1997]. Nevertheless, motors also have another roles in the growth cones. In transition zone, active kinesin 5 was suggested to inhibit microtubules progress to the peripheral domain in an interplay with actin arcs. When it was experimentally inhibited, growth cones exhibited no turning events from the substrate that they usually would have turned away [Nadar et al. 2008]. Similarly, kinesin 12 was shown to impact turning and navigation of the growth cones too [Liu et al. 2010]. Interestingly, some of the motors can associate with + tips of microtubules, such as kinesin 13 family or dynein. Members of kinesin 13 family are known to depolymerize microtubules from their + ends by coupling ATP hydrolysis with GTP-tubulin removal [Desai et al. 1999] [Kinoshita et al. 2006]. Their activity needs to be tightly regulated in growth cones, since their

high activity results in the decay of axon, while their low activity allow too many microtubules to polymerize towards the periphery of the growth cone, causing microtubule looping and halting progression [Homma et al. 2003] [Morii et al. 2006] [Poulain and Sobel 2007]. Dynein seems to have a similar function to kinesin 5, as its depletion results in the disrupted turning ability of the growth cones. It was demonstrated that dynein, as well as kinesin 5, generates antagonistic forces to myosin-II actions during the turning process, but the overall mechanism is not yet fully understood [Myers et al. 2006] [Pfenninger et al. 2003].

- Microtubule + tip proteins — CLASP, APC, EB1/3. These 3 players represent a group of proteins that track the + ends of microtubules, which can act as processive polymerases, depolymerases or connectors with other cellular structures (e.g. focal adhesions). CLASP (Cytoplasmic Linker-Associated Protein) appears to be involved in various processes in growth cones, such as supporting outgrowth, but as well as in its inhibition [Hur et al. 2011], depending on its phosphorylation state. It also participates in axon guidance regarding turning events [H. Lee et al. 2004], while supporting the growth of + tips of exploratory microtubules. These various roles might result from CLASP's regulation by different signaling pathways, as CLASP was shown to be a downstream effector of GSK3 (glycogen synthase kinase-3) [Owen and Gordon-Weeks 2003], but also to be regulated by Abl kinase [Engel et al. 2014], reviewed in [Cammarata et al. 2016*]. Similarly, APC (Adenomatous Polyposis Coli) was shown to participate in growth cone turning while being enriched and activated at the site of emerging turn [Koester et al. 2007], regulated by Wnt signaling [Purro et al. 2008]. However, interaction between APC and + tips of exploratory microtubules is facilitated downstream of the nerve growth factor (NGF), a signaling molecule for axonal outgrowth [Zhou et al. 2004]. Finally, EB1/3 (End Binding Protein 1 or 3) act as a + tip scaffolding protein. Many + tip associated proteins do not interact with the microtubule tip directly but rather through the Eb1/3 protein [Honnappa et al. 2009], which is able to localize to + tips autonomously [Bieling et al. 2007]. This mediation of the interaction is indispensable for the functioning of many + tip proteins, hence crucial also in growth cones.

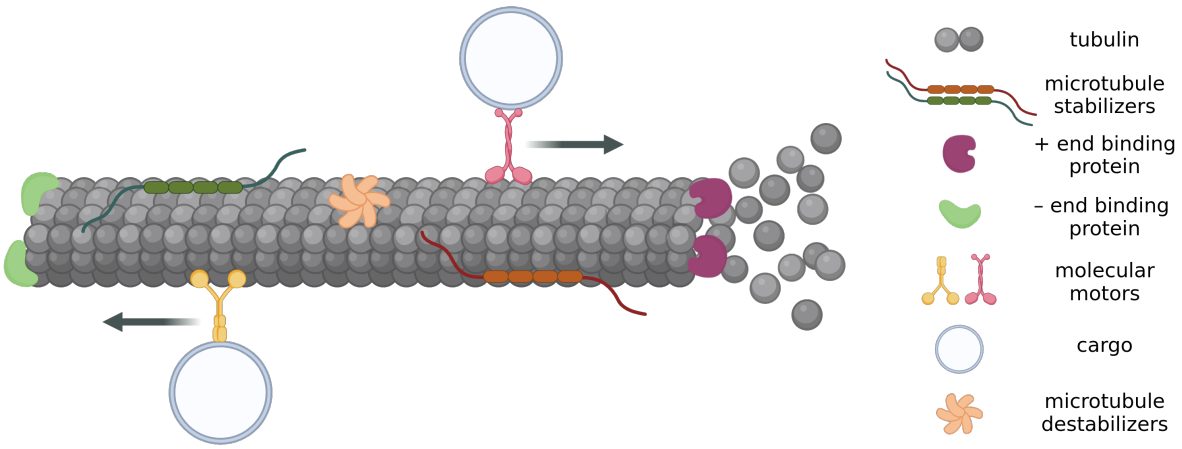


Figure 1.12: Summary of microtubule associated proteins. Created in BioRender.

In addition to microtubule associated proteins, posttranslational modifications of tubulin, such as acetylation or detyrosination, can also regulate microtubule dynamics in the growth cones [Dent and Gertler 2003*] and affect neuronal regeneration [Rodemer et al. 2020*].

1.3.5 Mediators of inter-cytoskeletal crosstalk

Besides the regulation of microtubule and actin filament dynamics alone, their interplay is becoming more and more in the focus of research interest. As already mentioned above, microtubules can serve as scaffolds for Rho signaling molecules, which can be released upon microtubule depolymerization and regulate actin dynamics. Apart from this indirect interplay, microtubules can also interact with actin filaments physically. For example, in the growth cones, properly advancing and functioning filopodia seem to require a synchronized interplay between both actin bundles and microtubules.

Similarly, microtubules were also identified as activators of site-directed actin assembly in newly proposed structures in growth cones — interpodia, actin-based protrusions initiated exclusively within lamellipodia-like regions [Beckerle 2017]. While these are only two of many examples of actin–microtubule interplay, researchers have successfully identified certain mediators of the inter-cytoskeletal crosstalk within growth cones, which will be discussed in this section (depicted in Figure 1.13), reviewed in [Cammarata et al. 2016*] [Dogterom and Koenderink 2019*].

- Actin–microtubule crosslinkers — spectraplakins. A protein family, whose members are described as binders of both microtubules and actin filaments, mediating interlink between them [Leung et al. 1999] [Kodama et al. 2003] [Applewhite et al. 2010]. A special attention is given to the ACF7 member, or its homolog named "Short-stop" or "Shot" in *Drosophila*, since its deletion is associated with axons that "stop-short" of their final target, indicating its role in axonal extension and guidance [D. V. Vactor et al. 1993]. ACF7 can bind to microtubule lattice in order to bundle and stabilize them, however, it can also associate with the microtubule + end through interaction with EB1. ACF7, in both modes of microtubule binding, can simultaneously execute an interaction with actin filaments, which was shown to be crucial for axonal outgrowth too, specifically for successful filopodia formation and proper microtubule organization [Sanchez-Soriano et al. 2009] [Alves-Silva et al. 2012].

- Microtubule associators — Tau, CRMP5. Protein Tau, originally recognized as a microtubule associated protein, was recently suggested to participate also in actin–microtubule crosstalk, as it colocalizes with the actin–microtubule interface in the peripheral domain of growth cones. More importantly, knock-down of Tau prevents microtubule intrusion into the periphery and disrupts their alignment with actin bundles in filopodia [Biswas and Kalil 2018]. An *in vitro* study substantiates tau's ability to crosslink microtubules with actin filaments, organizing the assembly of both polymers simultaneously. Specifically, tau was shown to facilitate the polymerization of single microtubule aligned with existing actin bundle, as well as to guide actin polymerization along microtubule [Elie et al. 2015]. Additionally, CRMP5, which localizes to growth cones and promotes their development, has been demonstrated to interact

with both tubulin and actin cytoskeleton too. However, whether it is able to mediate their crosstalk and what is its exact function remain unclear [Gong et al. 2016].

- Actin nucleators — formins. Increasing evidence reveals that microtubules can influence localization of actin nucleation. Namely, the actin nucleator formin mDIA (mammalian Diaphanous) has been shown to also directly bind to the microtubule lattice [Ishizaki et al. 2001] [Bartolini et al. 2008] as well as to microtubule + tip through the interactions with EB1 [Y. Wen et al. 2004], APC or CLIP-170 (Cytoplasmic Linker Protein 170), microtubule + tip tracking proteins. This leads to actin nucleation coupled to microtubule tip [Lewkowicz et al. 2008] [Henty-Ridilla et al. 2016] [Juanes et al. 2017]. While this mechanism has been confirmed by both *in vitro* and *in vivo* results and seems to be conserved [Jaiswal et al. 2013], studies demonstrating it in neuronal cells are lacking so far.

- Actin bundling protein — drebrin. Drebrin, a protein primarily known for decorating actin filaments and bundling them together. However, it is capable of association with microtubule + tip through a mediator EB3, while it guides the polymerization of microtubules to filopodia, probably along actin bundles. Functional studies have demonstrated drebrin's crucial role in neuritogenesis [Geraldo et al. 2008] [Worth et al. 2013].

- Microtubule + tip proteins — CLASP, APC. Several studies highlight the major role of microtubule + tip proteins in the actin–microtubule crosstalk located in growth cones. One of the first microtubule + tip protein studied in this context was CLASP, as it was shown to undergo retrograde flow and to directly bind to actin filaments [Tsvetkov et al. 2007]. CLASP depletion in *Xenopus* spinal cord neurons had a negative impact on the morphology of actin network, microtubule progression to filopodia and the overall axonal outgrowth [Marx et al. 2013]. A recent *in vitro* study explored the concomitant biding of CLASP to microtubules and actin filaments in more detail [Rodgers et al. 2023], demonstrating its ability to facilitate dynamic crosstalk between these components. Similarly, APC, another + tip protein involved in growth cone guidance, was shown to also bind actin filaments *in vitro* [Moseley et al. 2007]. In growth cones, APC-enriched microtubule tips were observed to predominantly localize to the actin-rich areas at the periphery [Zhou et al. 2004], and strikingly, they were shown to induce formation of branched actin networks for targeted membrane protrusion of growth cones [Efimova et al. 2020]. Additional microtubule + tip proteins, such as EB1/3, TACC3 (Transforming Acidic Coiled-Coil–Containing Protein 3) or CLIP170, have also been implicated to play a role in navigating microtubule growth along actin filaments, suggesting a substantial contribution to actin–microtubule crosstalk by this group of proteins (reviewed in [Bearce et al. 2015*]).

Taken together, it appears that many more and diverse proteins can physically couple microtubules with actin filaments than initially believed, indicating the potential existence of antagonist players. The uncoupling process can be regulated by simple dissociation and by reducing the local concentration of available units, or actively through sequestering or uncoupling factor. One example of such a factor is LIS1 protein, which was demonstrated to uncouple dynamic microtubules from

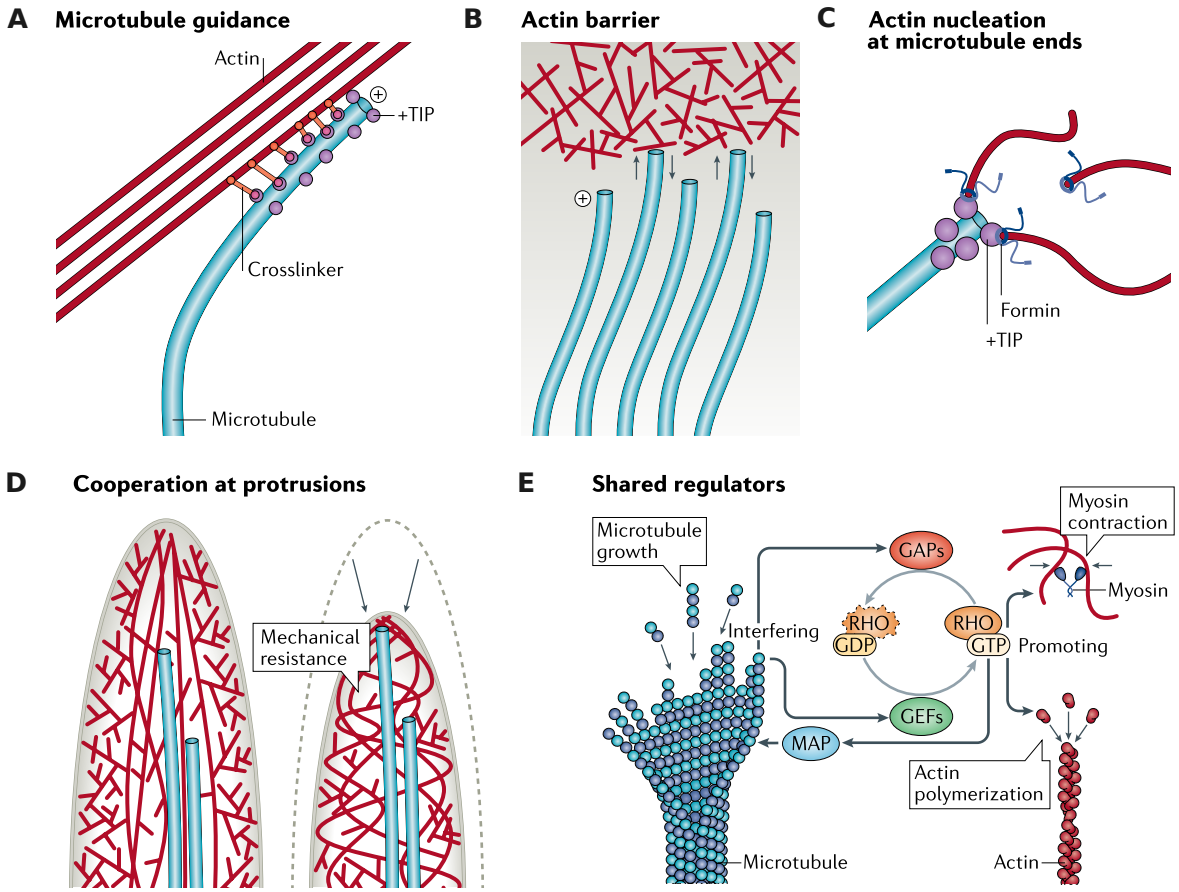


Figure 1.13: Summary of various types of actin–microtubule crosstalk in growth cones. **A** Microtubule guidance along actin bundles by microtubule + tip proteins and actin–microtubule crosslinkers. **B** Actin barrier in the transition zone halting microtubule growth (in an interplay with e.g. kinesin 5). **C** Nucleation of actin filaments from microtubule + tip through formins. **D** Cooperation of microtubules with actin bundles in filopodial protrusion. **E** Shared Rho regulators affecting both actin and microtubule dynamics. Adapted and modified from [Dogterom and Koenderink 2019*]

actin filaments, especially from retrograde flow, through interaction with dynein [Grabham et al. 2007]. However, this uncoupling has positive effect on growth cone advance and steering, since prevention of microtubules being bound to retrograde flow allows them to steer towards the periphery and filopodia [Myers et al. 2006].

Another promising candidate from the microtubule + tip protein group has been suggested to regulate the interaction between microtubules and actin filaments in the growth cones. Given the focus of the research underlying this thesis on this protein, a separate subsection dedicated to it follows.

1.4 Protein CKAP5 and its role in the growth cones

Structural basis of CKAP5

Protein CKAP5 (Cytoskeleton-Associated Protein 5, also called ch-TOG) is a highly conserved microtubule-associated protein across all eukaryotes [Sophie Charrasse et al. 1998] [Matthews et al. 1998] [Cullen et al. 1999] [Nakaseko et al. 2001] [Whittington et al. 2001]. It is composed of C-terminal domain and 5 TOG (Tumor Overexpressed Gene) domains located N-terminally [Al-Bassam et al. 2007] [Fox et al. 2014a] (depicted in Figure 1.14), altogether forming elongated and quite flexible protein [Cassimeris, D. Gard, et al. 2001]. Each TOG domain consists of six α-helical HEAT repeats folding to a "paddle" like structure [Al-Bassam et al. 2007]. However, the number of TOG domains can differ from 2 to 5 among the protein family depending on organism [Ohkura et al. 2001*]. For instance, *C. elegans* homolog ZYG-9 has 3 TOG domains, yeast homolog Stu2p contains only 2 of them [Breugel et al. 2003], while *Xenopus* homolog XMAP215 is structurally extremely similar to human CKAP5, having also 5 TOG domains.

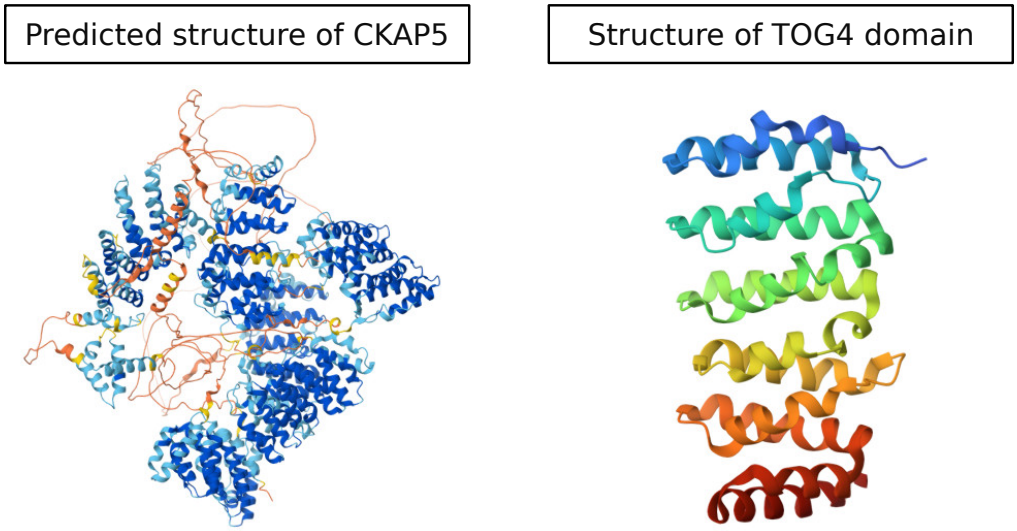


Figure 1.14: Predicted structure of human CKAP5 by AlphaFold on the left. A model of a structure of human TOG4 domain based on X-ray diffraction results with a resolution 1.90 Å on the right [Fox et al. 2014a]. Adapted from ["AF-Q14008-F1 — Computed Structure Model of Cytoskeleton-Associated Protein 5" 2021] and [Fox et al. 2014b], respectively.

Xenopus homolog of CKAP5, XMAP215, was identified as first of this family, and it was isolated from *Xenopus* oocytes in 1987 [D. L. Gard and M. W. Kirschner 1987]. Therefore, many studies were performed with XMAP215, but the main structural and functional features apply also for human version CKAP5 identified 3 years later [Sophie Charrasse et al. 1998]. This initial study characterized it as a factor which 10-fold increases the growth rate of microtubules *in vitro*. Indeed, CKAP5 was later confirmed to act as a processive polymerase for and was categorized as a microtubule + tip protein, due to its predominant localization [Brouhard et al. 2008]. In contrast to other + tip proteins, CKAP5 is able to autonomously track the + tip of the microtubule and does not need a help of mediator as EB1 or

EB3 [Brouhard et al. 2008], although it can functionally cooperate with other + tip proteins in cells to enhance the effect on microtubule dynamics, for example with CLASPs, SLAIN1/2 or TACC3 [L. A. Lowery et al. 2010] [Vaart et al. 2011] [Nwagbara et al. 2014].

When it comes to mechanism of how CKAP5 is managing to add tubulins to the microtubule ends and what are the functions of different part of this protein, researchers were leading discussions about several models. Initial studies with *Xenopus* homolog XMAP215 were suggesting that it binds several tubulin dimers at once [Kerssemakers et al. 2006] [Slep and Vale 2007], which was also in alignment with confirmed data that each individual TOG domain is sufficient to interact with tubulin dimer [Al-Bassam et al. 2007].

However, it was later shown that XMAP215 rather form a $1:1$ complex with tubulin [Brouhard et al. 2008] and that each TOG domain in the protein has slightly different structure and also a purpose [Fox et al. 2014a]. A study exploring yeast homolog Stu2, containing 2 TOG domains, provided a model of microtubule polymerization facilitated by TOG domains [Ayaz et al. 2012]. It has shown that TOG1 binds free tubulin dimers with higher affinity, while TOG2 is more prone to bind to microtubule lattice. However, these binding interactions also affect a conformation of tubulin dimers, which in return affect the interactions back. Specifically, when TOG1 binds to a free tubulin dimer, it causes a slight conformational change of the tubulin, which leads to lower affinity of TOG1 to that tubulin, hence it will release it and hand over to TOG2 domain, which will incorporate it to the lattice at the growing end.

In XMAP215, the basic region responsible for microtubule lattice binding is between TOG4 and TOG5 domains, while TOG1 and 2 similarly fulfill a polymerase activity [Currie et al. 2011] [Widlund et al. 2011]. On the other hand, function of C-terminal domain is less conserved across the TOG/XMAP215 family. Yeast homolog operates as a homodimer and C-terminal domain has a form of coiled-coil ensuring the dimerization [Breugel et al. 2003], while homologs in higher eukaryotes function as monomers and C-terminal domain is responsible for interaction with γ tubulin, being involved in nucleation of microtubules [Thawani et al. 2018].

Cellular functions of CKAP5

CKAP5 is quite an abundant protein expressed in almost all tissues [S. Charrasse et al. 1995] ["Tissue Expression of CKAP5" 2024]. Initial functional studies recognized its crucial role in cell division, since the perturbation of its expression led to abnormally short mitotic and meiotic spindles (in *Xenopus* [Tournebize et al. 2000], *Saccharomyces cerevisiae* [Severin et al. 2001] and *Drosophila* [Goshima et al. 2005]), hugely disorganized spindle poles and spindle morphologies (in *C. elegans* [Matthews et al. 1998], *S. pombe* [Garcia et al. 2001] and HeLa cells [Gergely et al. 2003] [Cassimeris and Morabito 2004]) and disturbed interactions between microtubules and cell cortex [Whittington et al. 2001] [Hestermann and Gräf 2004]. The aberrant functioning of CKAP5 was further identified as a driver of various cancers [Olson et al. 2011] [Kemp et al. 2013] [Chanez et al. 2015]. Interestingly, very recent study proposes silencing of *ckap5* gene as a potential cancer therapy, since its silencing resulted in lethality of chemo-resistant ovarian cancer cells. Even though CKAP5 is widely expressed

in many tissues which could cause difficulties with targeting of the treatment, cancer cells are much more sensitive to this type of therapy, due to their genetic instability [Chatterjee et al. 2023]. Hence, CKAP5 might be attractive also from medical point of view.

Surprisingly, a novel function of CKAP5 was uncovered too — its involvement in neuronal growth cones. First study suggesting it was published in 2010 [L. A. Lowery et al. 2010]. In this study, a *Drosophila* homolog of CKAP5, known as Msps (from "Mini spindles"), was identified through genetic and proteomic screening as an interactor of CLASP, a microtubule + tip protein already associated with axon guidance at that time. The authors further investigated a direct involvement of Msps in axon guidance, and indeed, *msps* mutants manifested deficiencies in this process [L. A. Lowery et al. 2010], as well as significant malformations in the overall axonal morphology and their outgrowth [Hahn et al. 2021] (see Figure 1.15).

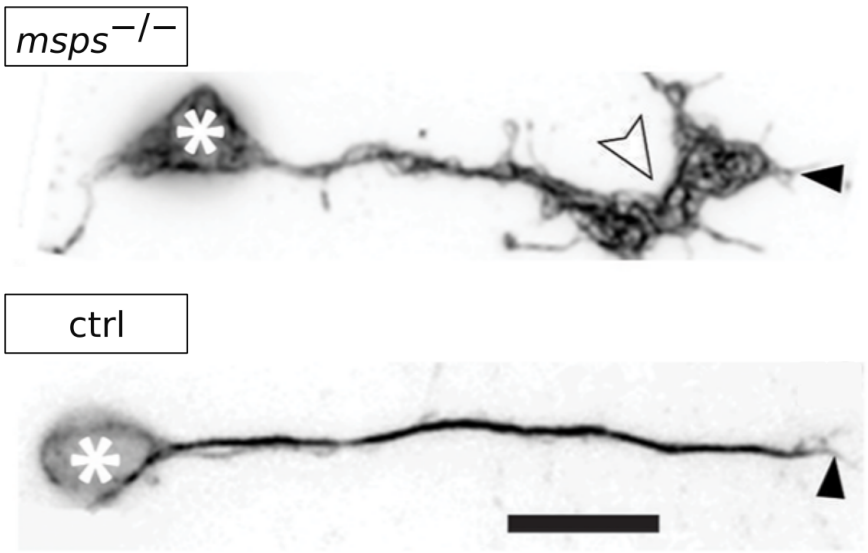


Figure 1.15: Comparison of a normal *Drosophila* embryonic primary neuron and the one with $msps^{-/-}$ knockout. Note the disturbed morphology of axon and axonal tip in knockout condition. Black arrows point to axonal tips, white arrows point out abnormal microtubule curling. Scale bar is 15 µm. Adapted and modified from [Hahn et al. 2021].

A more detailed functional analysis of this polymerase in growth cones was conducted using neuronal explants from *Xenopus*, thus considering XMAP215, a much closer homolog to mammalian CKAP5. It was elucidated that the disrupted axonal outgrowth observed after XMAP215 knockdown (and *msps* knockout) is attributed to actomyosin retraction [Laura Anne Lowery, Stout, et al. 2013]. Further examination focused on behavior of the growth cones with XMAP215 knockdown condition has revealed their prolonged pausing while traveling compared to the normal growth cones (see Figure 1.16), alongside with increased growth cone area and filopodia length [Slater et al. 2019]. Exactly this phenotype was previously assigned to decreased microtubule dynamics in the growth cones [Tanaka et al. 1995] [Kalil et al. 2000], which aligns with XMAP215 functioning.

Furthermore, assays testing the growth cone ability to guide their growth using chemorepellent demonstrated impaired guidance ability in knockdown condition, as twice as many of these growth cones were observed to trespass the applied repellent,

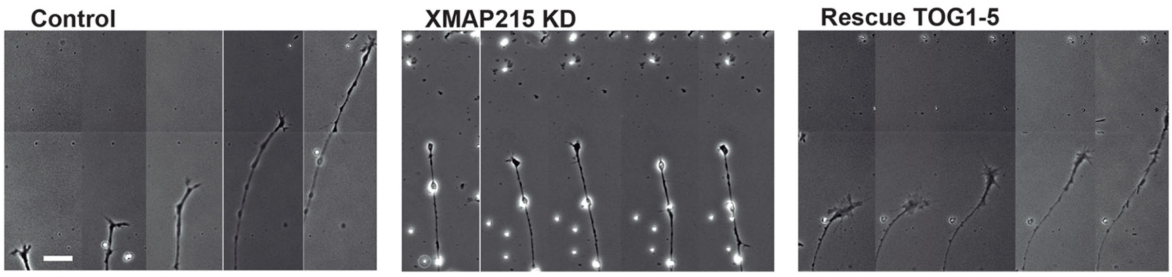


Figure 1.16: Comparison of mobility of representative axons and their growth cones in 3 different conditions — control, XMAP215 knockdown, rescue. Growth cones with reduced XMAP215 expression were more often pausing for a longer time. Rescue with TOG1-5 domains of XMAP215 was able to repair the phenotype back to normal mobility. Scale bar is 25 µm, time range is 150 min. Adapted from [Slater et al. 2019].

underscoring the importance of a physiological level of XMAP215 for a proper guidance [Slater et al. 2019]. Importantly, rescue experiments were able to repair both morphology and the guidance ability of *Xenpous* growth cones, confirming that the observed phenotypes are associated with XMAP215 functioning.

One initial study has presented a slightly contradictory and unexpected results, since they have observed increased microtubule velocity specifically in the *Xenopus* growth cones after depleting the XMAP215 expression [Laura Anne Lowery, Stout, et al. 2013]. But further experiments have shown that a major contributor to the measured + end velocity change was overall microtubule translocation/sliding, rather than microtubule polymerization. In normal growth cones, more microtubules were undergoing retrograde movement coupled to actin retrograde flow, while XMAP215 knockdown tipped the scales in favor of anterograde microtubule transfer. However, this finding indicated a previously unknown role for XMAP215 in affecting microtubule translocation within the growth cone, possibly by mediating a crosslink with actin filaments [Bearce et al. 2015*].

Experiments with high-resolution structured illumination microscopy on fixed *Xenopus* growth cones provided more detailed insights. Knocking down XMAP215 disrupted microtubule organization, resulting in more looped microtubules than splayed ones [Slater et al. 2019], while splayed microtubules are a signature of advancing growth cones [Dent, Callaway, et al. 1999] [Hendricks and Jesuthasan 2009].

Simultaneously, overall number of exploratory microtubule making it through the transition zone was higher in the knockdowns, but much fewer were able to reach filopodia [Slater et al. 2019]. This finding support the indication that XMAP215 might couple the microtubule tips with the retrograde flow of actin filaments in normal situation, hence suppression of its activity would lead to increased microtubule progression through the transition zone. However, once microtubules got over the transition zone, the lack of positive enhancing effect of XMAP215 maybe starts to manifest, and they are not able to reach the filopodia. The fact that XMAP215 indeed has a positive effect on microtubules was confirmed by its overexpression leading to increased number of microtubules successfully arriving to filopodia at the periphery [Slater et al. 2019].

Furthermore, authors were more curious about the possible interplay of micro-

tubules with actin filaments, hence they have also inspected the colocalization of both filaments in the growth cones. Strikingly, the percentage of microtubules colocalizing to actin filaments at the growth cone periphery was significantly decreased in XMAP215 knockdown condition (see Figure 1.17), while their proper alignment was enhanced by overexpressed XMAP215. In addition, a spin-down assay has revealed that XMAP215 is able to directly interact with actin filaments alone too, suggesting a potential role of XMAP215 in mediating a crosstalk between microtubules and actin filaments [Slater et al. 2019]. However, a molecular mechanism of this interplay remains to be elucidated, together with uncertainty whether XMAP215 can mediate it independently of other + tip partners.

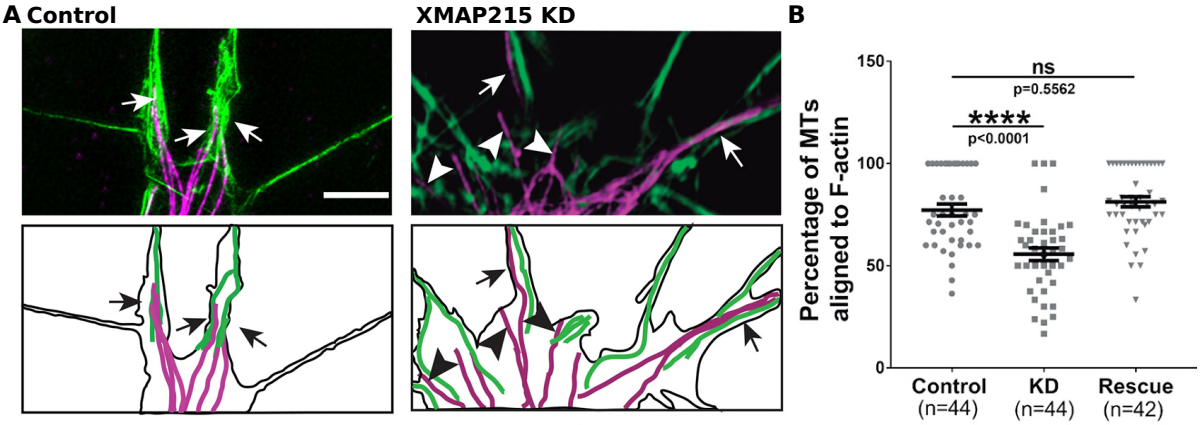


Figure 1.17: **A** Micrographs of actin filaments (green) and microtubules (purple) in growth cones of *Xenopus* explants captured using high-resolution structured illumination microscopy. Microtubule signal overlaps with actin filaments in a normal condition (left), while knockdown of XMA215 (right) impairs their colocalization. Arrows point to microtubules aligned with actin filaments, while arrowheads point to exploratory microtubules misaligned with actin filaments. **B** Percentage of microtubules aligned to actin filaments in different scenarios (significance was tested by one-way ANOVA test). The scale bar is 3 µm. Adapted from [Slater et al. 2019].

2. Aims of the Thesis

Following the studies suggesting a novel role of microtubule + tip polymerase, CKAP5, in facilitating crosstalk between microtubules and actin filaments crucial for neuronal growth cones, the overarching aim of this thesis was to elucidate the underlying mechanism of such crosstalk. To address this objective, we took advantage of *in vitro* reconstitution experiments employing purified human CKAP5 in combination with TIRF microscopy. The specific objectives leading to the overarching aim were as follows:

(1) Clarify how CKAP5 interacts with actin filaments alone.

(2) Compare the interactions of CKAP5 with actin filaments and with microtubules.

(3) Mechanistically explain CKAP5 mediated actin–microtubule crosstalk.

(4) Shed light on how CKAP5 affects architecture and dynamics of the inter-cytoskeletal system.

3. Materials and Methods

Contents of these methods are part of a published paper [Sabo et al. 2024].

3.1 Materials

If not specified otherwise, common chemicals were purchased from Sigma Aldrich, suitable for "cell culture" or "molecular biology".

REAGENT	IDENTIFIER	SOURCE
Chemicals		
GTP	`Cat#NU-1012`	Jena Bioscience
GMPCPP	`Cat#NU-405`	Jena Bioscience
Paclitaxel (Taxol)	`Cat#17191`	Sigma Aldrich
Dichlorodimethylsilane (DDS)	`Cat#440272`	Sigma Aldrich
Pluronic F127	`Cat#P2443`	Sigma Aldrich
Methylcellulose	`Cat#M0512`	Sigma Aldrich
Antibodies		
Goat Anti-Biotin	`Cat#B3640`	Sigma Aldrich
Proteins and peptides		
Biotin-labeled tubulin	`Cat#T333P`	Cytoskeleton
Unlabeled rabbit muscle actin	`Cat#AKL99`	Cytoskeleton
Human recombinant Profilin-1	`Cat#PR02-A`	Cytoskeleton
Biotin-phalloidin	`Cat#B7474`	Thermo Fisher Scientific
Enzymes		
Benzonase	`Cat#70664`	Novagen
Protease inhibitor cocktail	`Cat#34044100`	Roche Diagnostics GmBH
HRV 3C protease	`Cat#71493`	Merck Milipore
Cell lineages and virus strains		
Sf9 insect cells	`Cat#11496015`	Thermo Fisher Scientific
FlexiBAC	`DefBacFur+(H133)`	Lemaitre et al. [2019]
Other materials		
NiNTA agarose resin	`Cat#XF340049`	Thermo Fisher Scientific
Amicon Ultra-15	`Cat#UFC910024`	Merck

Table 3.1: Materials used in this research.

3.2 Methods

3.2.1 Protein expression and purification

To approach the aims of this project, we chose *in vitro* reconstitution assays combined with total internal reflection (TIRF) microscopy as main methods. For this purpose, the protein of the interest needs to be fluorescently labelled, expressed, and purified from a cell culture.

CKAP5 expression

Human version of the protein CKAP5 (CKAP5; GenBank: `NM_001008938.4`) was expressed with a fluorescent tag mNeonGreen at its C-terminus and with 6×His tag. To express the protein construct, Sf9 insect cells were chosen for their ability to provide a spectrum of posttranslational modifications, offering a favorable compromise between bacterial and mammalian cells. The whole procedure of expression was performed according to the previously described baculovirus FlexiBac system (DefBac DNA) [Lemaitre et al. 2019]. To infect the cells, 8 ml of P2 baculovirus stock was added to the cell culture at a ratio of 1: 100 (P2 virus to cell culture). Infected cells were then incubated at 27 °C with moderate shaking at 120 RPM. Harvesting was performed after 72 h by centrifugation at $300 \times g$ for 10 min. Cells were subsequently resuspended in PBS and snap-freezed in liquid nitrogen for storage.

CKAP5 purification

For purification, cells were defrosted and resuspended in Lysis buffer (see Table 3.2a). Cell lysis was achieved by centrifugation at $70000 \times g$ for 1 hour at 4 °C (to maintain the protein in a cold environment to prevent it from its degradation). In order to perform HisTrap purification, the lysate was then incubated with agarose NiNTA beads (Thermo Scientific) for 2 hour in a "cold room" (4 °C) while slowly rotating. Subsequently, the column was washed with 2×25 ml of Wash buffer (see Table 3.2b) to get rid of nonspecifically bound proteins, thanks to higher concentration of imidazole compared to the previous (Lysis) buffer. The protein of interest (CKAP5) was released by cleaving the 6×His tag off during overnight incubation of the beads with PreScission protease (HRV 3C protease, Merck Milipore) in a "cold room" at 4 °C. The ratio used was 1 µg of protease/100 µg of protein. After incubation, cleaved CKAP5 was collected and concentrated using an Amicon filter tube (Merck) with pores sized to allow only molecules smaller than 100 kDa to pass through. Finally, the concentration of purified protein was calculated from absorbance measured on NanoDrop ND-1000 spectophotometer (Thermo Scientific) at wavelengths of 280 nm and 506 nm. Prior to storing the protein in −80 °C freezer, it was snap-freezed in liquid nitrogen.

Tubulin purification

To perform *in vitro* reconstitution assays, purified subunits of cytoskeletal polymers, in this case of microtubules and actin filaments, are needed, too. Tubulin dimers were purified from porcine brains, which were provided by a local abattoir and

Lysis buffer	pH = 7.5
Na–phosphate buffer	50 mM
Imidazole	30 mM
Glycerol	5 %
KCl	300 mM
$MgCl_2$	1 mM
Tween-20	0.1 %
β-mercaptoethanol	10 mM
ATP	0.1 mM
Benzonase	0.63 U/ml
Supplemented with	
Protease inhibitor cocktail	1×

(a) Contents of Lysis buffer

Wash buffer	pH = 7.5
Na–phosphate buffer	50 mM
Imidazole	50 mM
Glycerol	5 %
KCl	300 mM
$MgCl_2$	1 mM
Tween-20	0.1 %
β-mercaptoethanol	10 mM
ATP	0.1 mM

(b) Contents of Wash buffer

Table 3.2: Contents of buffers used for CKAP5 purification.

processed within 4 hour after death of the animal. Isolation of the tubulin was carried out according to previously described method containing two rounds of polymerization–depolymerization phases in a high-molarity PIPES buffer [Castoldi and Popov 2003] [Gell et al. 2011].

Tubulin labeled with biotin was purchased from Cytoskeleton (#333P) and mixed with purified unlabeled tubulin in a ration 1 : 50, respectively.

Lyophilized unlabeled G-actin from rabbit muscles (#AKL99) was also purchased from Cytoskeleton, and it was resuspended, aliquoted and stored according to manufacturer's directions.

3.2.2 Experiment preparation

Since cytoskeletal filaments can self-assemble also *in vitro*, their reconstitution and polymerization is considerably simple.

Actin filament assembly

To initiate the polymerization of actin filaments, an aliquote of stored G-actin purchased from Cytoskeleton was mixed with Polymerization buffer (see Table 3.3) to achieve a final concentration 400 µg/ml. To prevent unwanted preliminary depolymerization of actin filaments, the Polymerization buffer was supplemented with phalloidin-rhodamin in a final concentration of 10 µM. While phalloidin is a drug isolated from toadstools, which covalently binds to actin filament lattice and stabilizes/"fixes" it, rhodamin labels it fluorescently. Polymerization in this mix was running overnight at 4 °C. Subsequently, polymerized and stabilized actin filaments were used for experiments for one month, typically with additional dilution described below.

Polymerization buffer (FA)	pH = 7.8
Tris-HCl buffer	5 mM
$CaCl_2$	0.2 mM
KCl	50 mM
$MgCl_2$	2 mM
ATP	1 mM

Table 3.3: Contents of Polymerization buffer for actin assembly.

Microtubule assembly

There are two most frequently used methods to stabilize microtubules: either by a drug named taxol, which was originally isolated from yew tree, or with the unhydrolyzable analog of GTP — GMPCPP (Guanosin-5'-(α, β)-Methylene-Diphosphonate).

Taxol-stabilized microtubules were polymerized from 4 mg/ml biotinylated tubulin (in a ratio of 1 : 50) in the Polymerization mix (see Table 3.4) at 37 °C for 30 min. After polymerization, microtubules were diluted and stabilized in BRB80Tx buffer (see Table 3.5). In order to get rid of any remaining unpolymerized tubulin, stabilized microtubules were centrifuged at 18000 × g for 30 min (Microfuge 18 Centrifuge, Beckman Coulter). The supernatant was discarded and the pellet containing microtubules was resuspended in BRB80Tx. Microtubules were stored at a room temperature and used for one week, while storing and imaging of taxol-microtubules was always done in a presence of taxol.

Polymerization mix 1	
BRB80 buffer	
DMSO	5 %
$MgCl_2$	4 mM
GTP	1 mM

Table 3.4: Contents of Polymerization mix 1 for taxol-stabilized microtubules.

BRB80(Tx)	pH = 6.9
PIPES buffer	80 mM
$MgCl_2$	4 mM
EGTA	1 mM
Potential supplement	
paclitaxel	10 µM

Table 3.5: Contents of BRB80Tx buffer for storage of taxol-stabilized microtubules.

GMPCPP-microtubules were prepared using the exactly same procedure as taxol-stabilized microtubules, but with different buffers. Polymerization was performed

in the presence of GMPCPP instead of GTP, see Table 3.6. After polymerization and centrifugation, the pellet with microtubules was resuspended in BRB80 (without taxol), refer to Table 3.5. The microtubules were also kept at room temperature and utilized for one week.

Polymerization mix 2	
BRB80 buffer	
$MgCl_2$	1 mM
GMPCPP	1 mM

Table 3.6: Contents of Polymerization mix 2 for GMPCPP-stabilized microtubules.

Preparation of experimental chambers for TIRF microscopy

All experiments were conducted in chambers constructed as described here. Two glass coverslips were assembled together by melting 5 thin strips of parafilm in between them at 60 °C for approximately 1 min, resulting in 4 chambers of similar volume, as depicted in Figure 3.7. Coverslips were beforehand cleaned in "piranha" solution (composed of one part 30 % hydrogen peroxide and 2.5 parts 95 % sulfuric acid) and silanized with 0.05 % dichlorodimethylsilane (DDS, Sigma). Upon assembly, the chambers were incubated as described below based on a specific experiment and secured into a coverslip holder for the microscope. Volumes of solutions flushed in were always at least 4-fold higher than the volume of experimental chamber, to achieve proper equilibration of the entire chamber.

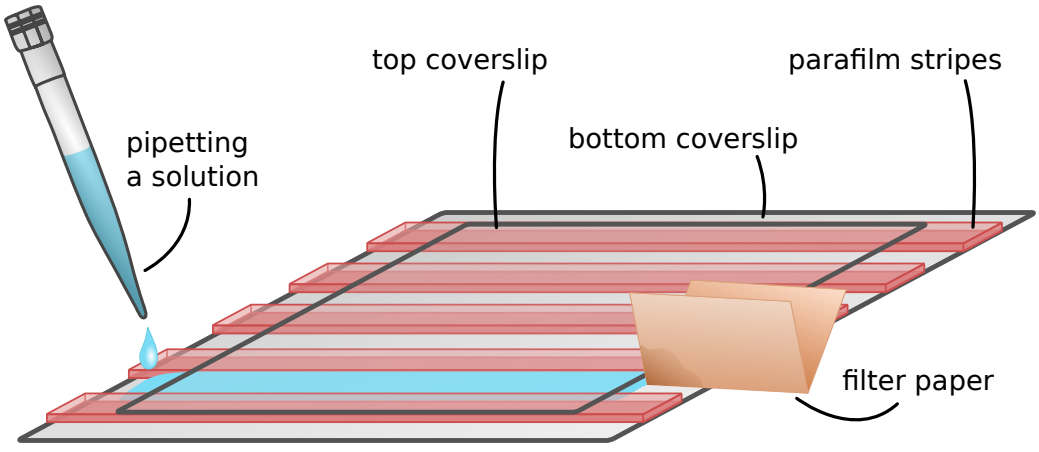


Figure 3.7: Scheme of chamber assembly. Two glass coverslips assembled together by thin strips of melted parafilm. Solutions are flushed in using vacuum (not shown in the picture) or by filter paper.

Each chamber was used for only one experiment, since re-using it could compromise its properties and thus affect the behavior of tested proteins. All experiments were quantified by pooling data from multiple chambers from multiple coverslips and from experiments performed on at least two different days.

3.2.3 TIRF microscopy and IRM

TIRF microscopy

Total internal reflection fluorescence microscopy (TIRF) is an advanced microscopy technique pioneered by E. J. Ambrose in 1956 [Ambrose 1956] and further developed by D. Axelord [Axelrod 1981]. It is based on total internal reflection, which occurs at the interface of two media with different refractive indexes. If a beam of light (laser) travels from a medium with a lower refractive index (oil) to a medium with a higher refractive index (glass coverslip) at an angle greater than the critical angle, the whole beam is reflected back towards the first medium (oil), with none of it refracting through the coverslip (see Figure 3.8). However, an evanescent wave is created, which can excite fluorofores in very close proximity to the coverslip surface (around 100–200 nM), as it exponentially decays in intensity with the distance. The fact that only fluorophores within a very thin plane are excited offers several advantages. The most crucial advantage is a dramatic improvement in signal–to–noise ratio and, consequently, spatial resolution. Considering the requirements, advantages and constraints of this method, it is best suited for observing cellular events at the plasma membrane or even dynamics of single molecules, for example in *in vitro* reconstitution assays with cytoskeletal components.

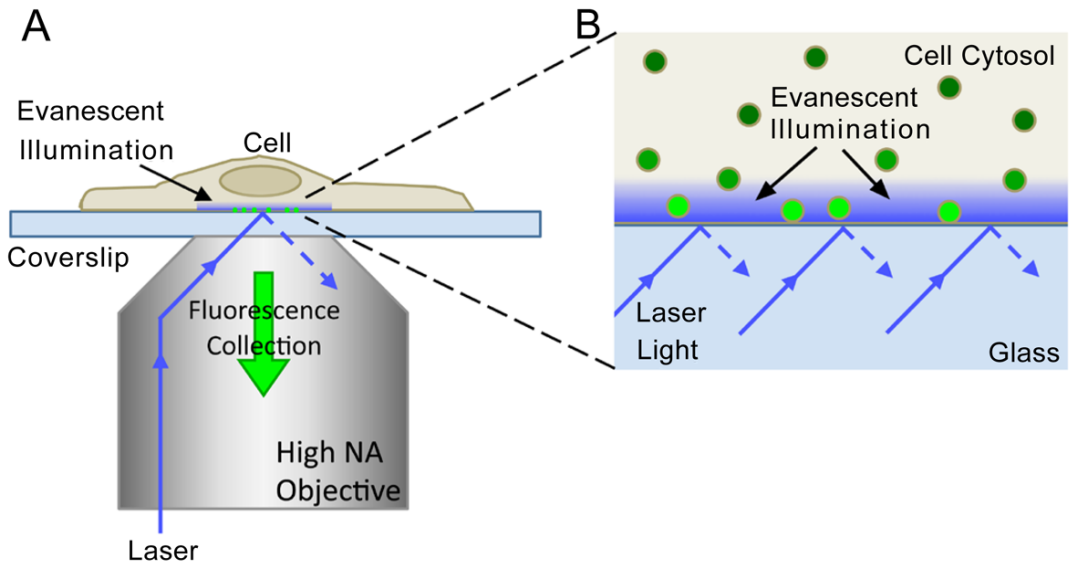


Figure 3.8: Scheme depicting TIRF microscopy basis. Laser beam comes through the objective with high numerical aperture and immersion oil (not depicted) to the coverslip surface at the angle larger than critical. Hence, the total internal reflection of the beam occurs, but it creates en evanescent wave illuminating fluorophores located very near above the coverslip surface. Emitted fluorescence is collected through the same objective. Adapted from [Kisler et al. 2013].

IRM

Interference reflection microscopy (IRM) is an optical microscopy technique used to visualize events at the coverslip surface too. In contrast to the TIRF, it does not require fluorescent labeling of the observed objects, providing the possibility to study them in a native state. The method is based on interference effects of reflected light from the coverslip and specimen such as constructive or destructive

interference, while the intensity of the signal depends on the proximity of the object to the coverslip surface (see Figure 3.9). The technique was developed by A. S. Curtis in 1964 [Curtis 1964] and quite recently, studies have demonstrated that microtubules are ideal objects for being observed by IRM [Mahamdeh et al. 2018].

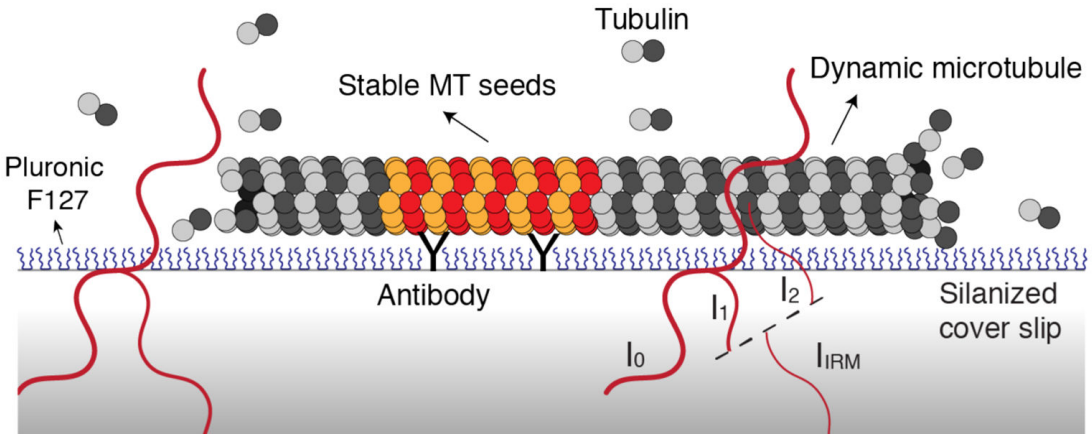


Figure 3.9: Scheme depicting IRM basis. Light reflected from the water–microtubule interface interferes with light reflected from the water-glass interface, which results in visual contrast between microtubule and background. The contrast depends on microtubule–surface distance. Light comes from epi-illumination. Adapted from [Kuo and Howard 2022].

Microscope setup

All experiments were carried out using an inverted microscope (Nikon-Ti E and Nikon-Ti2 E) controlled be Nikon NIS Elements software, v5.20. The objective type used was for oil immersion with magnification of 60× and a numeric aperture 1.49 (Apo TIRF, Nikon). Two different cameras were utilized for these experiments: the sCMOS Hamamatsu Orca Flash 4.0 LT or PRIME BSI (Hamamatsu Photonics, Teledyne Photometrics, respectively). However, when quantification of fluorescent signals was required for analysis, all experiments for given assay were performed using the same microscope and the same camera. Imaging of microtubules was conducted using IRM, as mentioned above, with an epifluorescent lamp (either LED or xenon discharge lamp). Fluorescently labeled proteins (CKAP5, actin filaments) were imaged by periodically switching between the filter cubes for mNeonGreen and rhodamin channels or by using a quad band set (405/488/561/640). All assays were performed at room temperature.

3.2.4 Data acquisition — *in vitro* reconstitution assays

Experiments were performed within a time frame of several months and no data was excluded from the study.

Bundling of stabilized actin filaments by CKAP5

Chambers prepared as described above were subsequently treated with 1 % Pluronic (F127 diluted in PBS, Sigma) for 30 min in order to block the glass surface. Subsequently, chambers were flushed with TIRF assay buffer (abbreviated TAB, see Table 3.10), supplemented with methylcellulose (Sigma) of a final concentration

of 0.2 %. Phalloidin-stabilized actin filaments, prepared as described above, were diluted 10× (final concentration 40 µg/ml)) in TAB with methylcellulose and mixed together with CKAP5 to achieve its final tested concentration (1 nM, 2 nM, 4 nM, 10 nM or 40 nM CKAP5). In case of the control experiment, CKAP5 was left out. The mixture of actin filaments and CKAP5 was immediately introduced to the chamber and imaged for 10 min with 5 min intervals.

TIRF assay buffer	pH = 7.4
HEPES buffer	50 mM
EGTA	1 mM
Glycerol	5 %
$MgCl_2$	2 mM
KCl	50 mM
DTT	10 mM
Tween-20	0.1 %
BSA	0.5 mg/ml
Mg-ATP	1 mM
D-glucose	20 mM
Glucose oxidase	0.22 mg/ml
Catalse	0.22 mg/ml
Potential supplement	
Methylcellulose	0.2 %

Table 3.10: Contents of TIRF assay buffer (TAB).

Affinity measurements of CKAP5 to microtubules and actin filaments

Chambers prepared as described above were subsequently incubated for 5 min with anti-biotin antibodies diluted in PBS to a concentration of 20 µg/ml. Afterward, the chamber surface was blocked by incubating them with 1 % Pluronic (F127 in PBS, Sigma) for 30 min.

Measurement of CKAP5 affinity to microtubules
Biotinylated taxol-stabilized microtubules were flushed into the chamber and allowed to bind to the anti-biotin antibodies on the surface for approximately 30 sec. Unbound microtubules were then flushed out the chamber with BRB80Tx. Subsequently, TAB supplemented with 10 µM taxol was introduced into the chamber for pre-incubation. The following steps, common to all affinity measurements, are described below.

Measurement of CKAP5 affinity to single actin filaments
Biotin-phalloidin (Thermo Fischer Scientific), dissolved in methanol and further diluted in PBS to 1 µM concentration, was introduced into the experimental chamber and allowed to adhere to the anti-biotin antibodies for 2 min. Afterward, phalloidin-stabilized actin filaments diluted 100× (final concentration 0.4 µg/ml) in TAB were flushed in and further allowed to attach to biotin-phalloidin for

approximately 1 min. Free actin filaments were washed away from the chamber with TAB.

Measurement of CKAP5 affinity to actin bundles
The chamber was incubated with 1 µM biotin-phalloidin in the same way as described in the previous case. To measure the affinity to actin bundles, pre-formed actin bundles were needed. This was achieved by incubating diluted phalloidin-stabilized actin filaments of a final concentration of 20 µg/ml with 10 nM CKAP5 for 5 min in the chamber. Pre-formed actin bundles landed and attached to the surface through biotin-phalloidin, and the chamber was then thoroughly washed out by TAB to remove remaining CKAP5 from the solution.

The subsequent steps were the same for all filaments attached to the surface as described above. Increasing concentrations of CKAP5 (1, 2, 4, 10 and 40 nM) in TAB were introduced one by one into the experimental chamber, incubated for 2 min before capturing several images of different spots from the currently tested condition. One set of experiments containing the whole range of concentrations was conducted in one chamber for one type of filaments. Note that all affinity measurements were conducted in the absence of methylcellulose.

Crosslinking of single actin filaments to microtubules

Chambers were prepared in the same manner as for microtubule affinity measurement — biotinylated taxol stabilized microtubules were attached to the coverslip surface via anti-biotin antibodies. Single short actin filaments were prepared from longer phalloidin-stabilized actin filaments (polymerization described above) by shredding them by repeated mixing of the solution through a pipette tip pressed to the bottom of a microtube. These short actin filaments were then diluted to the final concentration of 0.4 µg/ml. A mixture of short single actin filaments and CKAP5 at a final concentration of 4 nM in TAB was introduced into the chamber and imaged for 10 min with 5 min interval. In a control experiment, CKAP5 was omitted. Note that these experiments were performed without methylcellulose in the buffer, thus only microtubules were attached to the surface by antibodies and actin filaments were allowed to diffuse and freely land to the surface.

Templating of prevailing actin bundles on dynamic microtubules by CKAP5

Chambers were prepared as for microtubule affinity measurement described above, whereby the surface was coated with anti-biotin antibodies. Biotinylated GMPCPP-stabilized microtubules were polymerized as described previously and broken into short seeds by repetitive mixing through the pipette tip pressed against the bottom of the microtube. These seeds were then introduced to the chamber and incubated for 30 s before flushing out any unbound seeds with BRB80. Afterward, the BRB80 in the chamber was exchanged for the Polymerization buffer (abbreviated PB, see Table 3.11). To promote the dynamics of microtubules, free tubulin and GTP in the solution are needed, thus GTP was contained in the PB. Further, a mixture of 10 µM tubulin, 10x diluted phalloidin-stabilized actin filaments (final concentration of 40 µg/ml) and 200 nM CKAP5 in the PB

was introduced into the chamber, and microtubule dynamics was initiated. These experiments were imaged for a duration of 20 min with 5 s interval.

Polymerization buffer (MTs)	
BRB80 buffer	
DTT	10 mM
Tween-20	0.1 %
Casein	0.5 mg/ml
Mg-ATP	1 mM
GTP	1 mM
D-glucose	20 mM
Glucose oxidase	0.22 mg/ml
Catalse	0.22 mg/ml
Supplemented with	
Methylcellulose	0.2 %

Table 3.11: Contents of Polymerization experimental buffer for microtubules (PB).

3.2.5 Analysis

All microscopy data were analyzed using `ImageJ 2.3.0/1.53q (FIJI)` [Schindelin et al. 2012] and `Matlab (R2020b)`. In general, `StackregJ` plugin (kindly provided by Jay Unruh at Stowers Institute for Medical Research in Kansas City, MO) was applied on videos with significant drift to compensate it.

All fluorescent intensities used for calculations were first corrected for the background signal in corresponding channel. The background was measured from the same areas, but outside the objects of the interest.

Kymographs and Maximum Intensity Projections

Kymographs were generated using `ImageJ` from manually drawn segmented lines along microtubules or actin bundles, and further processed by plugins `KymoResliceWide` or `KymographBuilder`.

To generate MIP (Maximum Intensity Projections), a segmented line along a microtubule/actin filament/actin bundle was also manually drawn and then the `Maximum Intensity Projections` function was applied.

Bundling of stabilized actin filaments by CKAP5

Regarding the bundling of stabilized actin filaments by CKAP5, regions exhibiting significantly increased intensity in the actin channel were identified as bundles, while these regions simultaneously overlapped with high intensity in CKAP5 channel. Various quantifications were conducted to explore the nature of these bundles.

Fluorescent density of CKAP5 was in case of separated bundles determined from 1 µm long regions at their center by measuring the intensity from CKAP5 channel, 5 min after mixing the actin filaments with CKAP5, and then referring it to the area in µm^2. Considering conditions with higher concentrations of CKAP5 in the chamber, when the actin networks were nearly fully bundled and bundles were interconnected, the density of CKAP5 was determined from regions in the middle of bundles in-between branches. To compare it with the density of CKAP5 on actin filaments in non-bundles regions, identical 1 µm selections were placed randomly by hand outside the bundles and analyzed. The density of CKAP5 was plotted against the actin filament count per bundle and against the concentration of CKAP5.

Actin filament count per bundle was estimated by dividing the signal of actin intensity in the bundle area by the intensity of a single actin filament. The intensity of a single actin filament was determined as the mean of intensities of multiple single actin filaments on the given day of experiments. The actin filament count per bundle was then plotted against the concentration of CKAP5, and it was also used to calculate CKAP5 density per one actin filament (a ratio of CKAP5 density within the bundle to the estimation of actin filament count in the given bundle).

Coefficient of variation was shown to be a handy parameter to illustrate the degree of network bundling [Higaki et al. 2020]. It was calculated as the ratio of the standard deviation to the mean of overall fluorescent intensity of the actin channel, 5 min after mixing actin filaments with CKAP5. A higher coefficient of variation indicates greater bundling of the network, as there are more pronounced differences in intensity within the channels. This parameter was plotted against the concentration of CKAP5.

Bundle density was calculated by dividing the total length of actin bundles by a given area (field of view). The lengths of actin bundles was automatically detected by `RidgeDetection` plugin in `ImageJ`, based on CKAP5 signal (considering the high-density regions). In the case of separate actin bundles, the lengths were summed. Bundle density was plotted as a dependency on CKAP5 concentration.

Affinity measurements of CKAP5 to microtubules and actin filaments

Determination of CKAP5 affinities was based on measuring its intensities along the microtubule/actin filament/actin bundle after a 2 min incubation with the currently tested concentration of CKAP5, with the CKAP5 concentration gradually increasing during the experiment. Lines drawn along the tested filaments were always of the same width for the specific type of filament and utilized for all concentrations. For microtubules undergoing depolymerization due to activity of CKAP5, the lines were always adjusted to cover only the microtubule lattice.

Once the intensities were measured and the background signal was subtracted, they were transformed into densities by referring them to the area in µm^2. However, since the density of CKAP5 depends on the actin filament count, as shown in Figure 4.1 **D**, the density of CKAP5 in actin bundles was divided by the actin filament count per bundle estimated as described above. The dependency of CKAP5 density on CKAP5 concentration was then plotted for each type of

filament and fitted with the equation $Y = \frac{\mathrm{Max}_{\mathrm{SB}} \cdot X}{K_d + X}$, where X represents CKAP5 concentration, Y is CKAP5 density, $\mathrm{Max}_{\mathrm{SB}}$ denotes the maximum specific binding of CKAP5 and K_d is its binding constant.

Crosslinking of single actin filaments to microtubules

To calculate the density of short single actin filaments on microtubules, MIP were generated from 10 min long videos with 5 s intervals. The positions of microtubules were manually detected and marked using the `Segmented Line` tool in `ImageJ`. The density of actin filaments was subsequently calculated from the intensity in actin channel in the MIP along the marked microtubules and referred to their area in µm^2.

Templating of prevailing actin bundles on dynamic microtubules by CKAP5

Prior to analyzing the percentage of microtubules with actin bundles present at the microtubule + end, the density of actin bundles at the microtubule + tips was evaluated (not shown in this thesis, done by Ján Sabó). This density was measured from a 1 µm area, which was manually selected ahead of microtubule + end at the spots occupied by microtubules before their depolymerization. The `Fixed Length Line Tool` was used for this purpose.

The ability of CKAP5 to form actin bundles along stabilized microtubules was demonstrated by various experiments and their analysis (not shown in this thesis, [Sabo et al. 2024] Figure 4). However, the focus of this analysis was to showcase CKAP5 ability to template prevailing actin bundles on dynamic microtubules. Therefore, a microtubule with actin filament bundle at the + end was included to the percentage statistic as positive event only if the microtubule was dynamic while recruiting actin filaments and if the actin bundle was formed during the depolymerization phase. A microtubule + tip was counted as the one "following actin bundle" if the tip covered with CKAP5 colocalized with the signal of actin bundle for at least 1 min during polymerization or depolymerization of the microtubule.

4. Results

Functional analysis of CKAP5 in growth cones of *Xenopus* neuronal explants suggested CKAP5 as a potential mediator of actin–microtubule crosstalk (Introduction, Section 1.4). Here, we present data contributing to elucidating the underlying mechanism of such interaction facilitated by CKAP5.

Following data are published in [Sabo et al. 2024].

4.1 CKAP5 autonomously bundles actin filaments

Firstly, we aimed to test whether CKAP5 can autonomously interact with actin filaments and what kind of interaction it would be. To achieve this, we conducted an *in vitro* experiment using fluorescently labeled pre-polymerized actin filaments along with purified CKAP5-mNeonGreen. A network of actin filaments was introduced into the experimental chamber together with 4 nM CKAP5, and the experiment was visualized over time using TIRF microscopy. The same procedures and conditions were followed for control experiment, with a key difference — the absence of CKAP5 in the chamber.

The present interaction between CKAP5 and actin filaments was immediately evident upon starting the experiment. As visible in Figure 4.1 **B** (left panel), CKAP5 accumulated in regions with high actin density, leading to filament bundling. For better visualization of the dynamics of CKAP5 recruitment to the actin filaments in early stages, see the kymographs in Figure 4.2. Conversely, we did not observe any bundling of actin filaments in the control experiment, confirming that CKAP5 alone is indeed an inducer of actin bundling Figure 4.1 **B** (right panel).

During this experiment, we noticed that CKAP5 was not uniformly distributed across the actin network after reaching a steady state at the specified concentration. Quantitative analysis revealed a significantly higher density of CKAP5 in regions of actin bundles compared to non-bundled areas, while the presence of CKAP5 in non-bundled areas is nearly negligible (see Figure 4.1 **C**). Further examination demonstrates that CKAP5 density in the central parts of bundles increases linearly with the number of actin filaments in the bundle (see Figure 4.1 **D**). However, the density of CKAP5 per one actin filament within the bundle remains relatively constant as the bundle size increases (refer to the graph in Figure 4.1 **E**).

To further investigate the nature of actin bundling induced by CKAP5, we performed the same experiment as mentioned above, but with increasing concentrations of CKAP5, while maintaining the amount of actin filaments in the chamber approximately constant. Representative images of the experiments for each concentration in Figure 4.3 **A**, captured after 5 min when steady-state was reached, demonstrate two things. Firstly, concentration of CKAP5 below 2 nM is insufficient to initiate actin bundling. Secondly, in the presence of higher concentrations of CKAP5 (here 10 and 40 nM) the separate bundled domains merge into

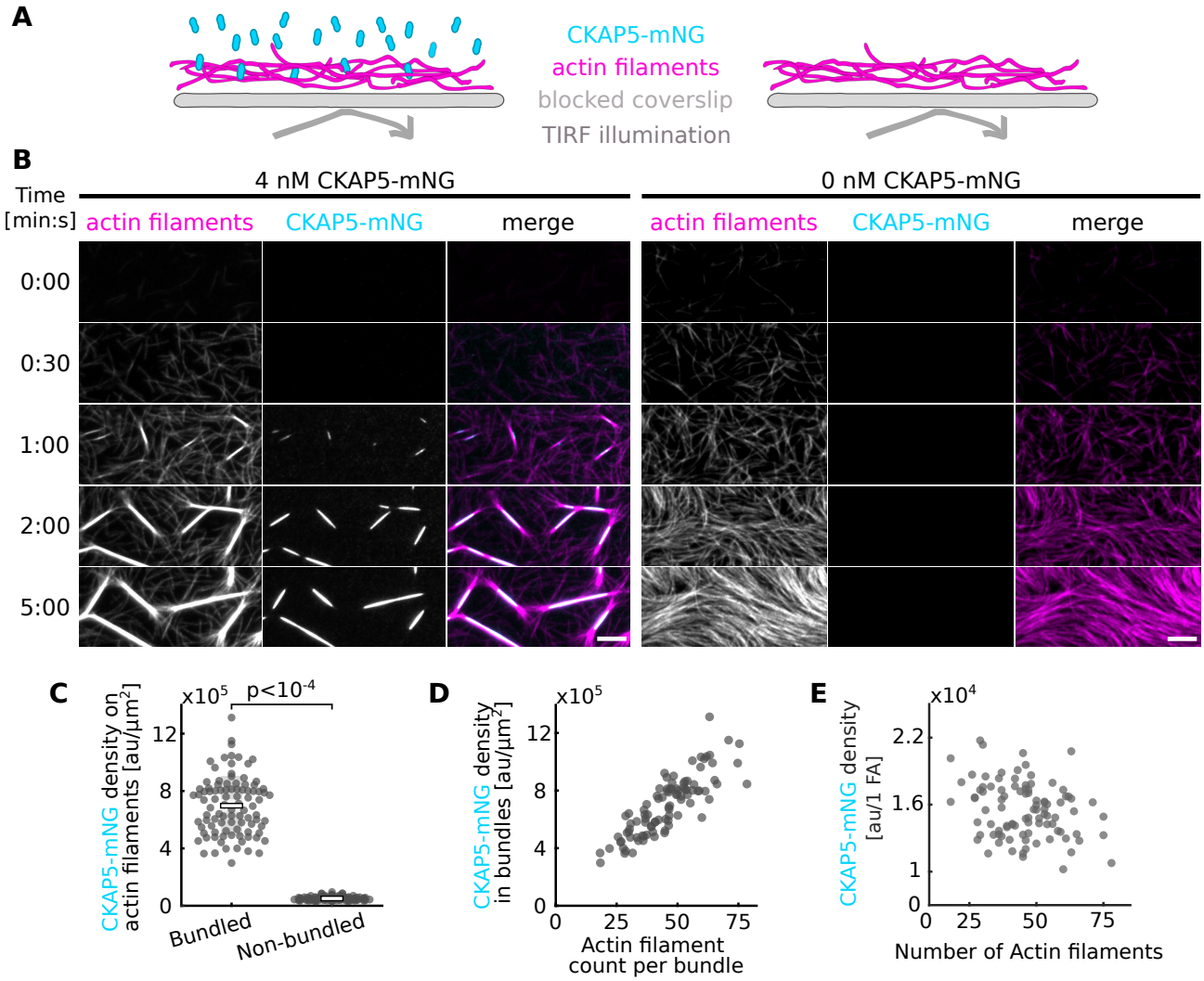


Figure 4.1: **A** Scheme of the *in vitro* experiment with free actin filaments and CKAP5-mNG (left) and control experiment with CKAP5 absent (right). **B** Time-lapse images of the experiment with 4 nM CKAP5 (left) and of the control experiment without CKAP5 (right). Note that bundles of actin filaments are formed only in the presence of CKAP5. Stabilized actin filaments by rhodamin-phalloidin are in magenta, CKAP5 in cyan, scale bar is 5 µm. To bring actin filaments to the surface for TIRF imaging, 0.2 % methylcellulose was used in both types of experiments. **C** The density of 4 nM CKAP5 after 5 min in the bundled area was $(7.0 \pm 2.0) \cdot 10^5$ au/µm^2, whereas at the actin filaments in non-bundled area it was $(0.52 \pm 0.14) \cdot 10^5$ au/µm^2. Mean and SD values were calculated from $n = 95$ in 3 experiments for both cases. Two-sided t-test resulted in a $p < 10^{-4}$. **D** Correlation of CKAP5 density in actin bundles and count of the actin filaments per bundle. Evaluated after 5 min incubation with 4 nM CKAP5, $n = 95$ actin bundles in 3 experiments. **E** Dependency of CKAP5 density per one actin filament on the number of actin filaments in the actin bundle ($n = 95$ actin bundles in 3 experiments).

larger continuous bundles, resulting in the bundling of the entire network. Both phenomena are also indicated by the coefficient of variation of the intensity in actin channel, depicted in the graph in Figure 4.8 **B**. The higher the coefficient, the more variable the intensity within the actin channel, indicating a more bundled network. We can notice that the coefficient of variation for 1 nM CKAP5 is almost identical to that for 0 nM CKAP5, and further that with increasing concentration, the coefficient also increases. A similar trend also applies for the density of bundles per unit area in the graph in Figure 4.3 **C**.

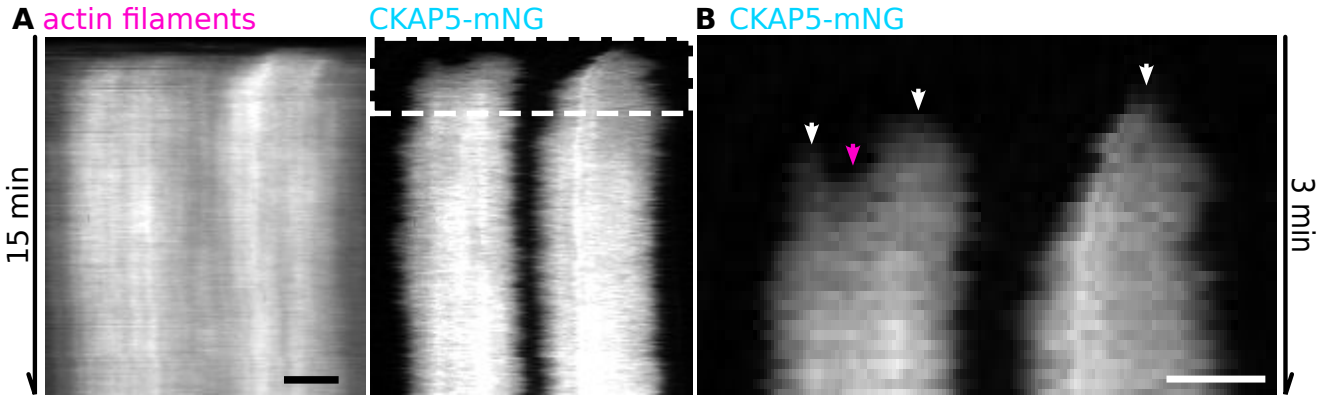


Figure 4.2: **A** Kymographs generated from a line drawn along separate actin bundles. Actin filament channel on the left, CKAP5 (4 nM) channel in the middle. Scale bar represents 2 µm, timescale 15 min. **B** Zoomed in region of the CKAP5 signal in **A**, illustrating dynamics of the CKAP5 recruitment to the actin filaments leading to their bundling. White arrows point at the initiating points followed by spreading of the CKAP5 boundaries to the width, while magenta arrow indicates merging of two separate CKAP5 domains. Scale bar is 2 µm, timescale is 3 min. *Similar experiments were performed by Ján Sabó for his dissertation thesis.*

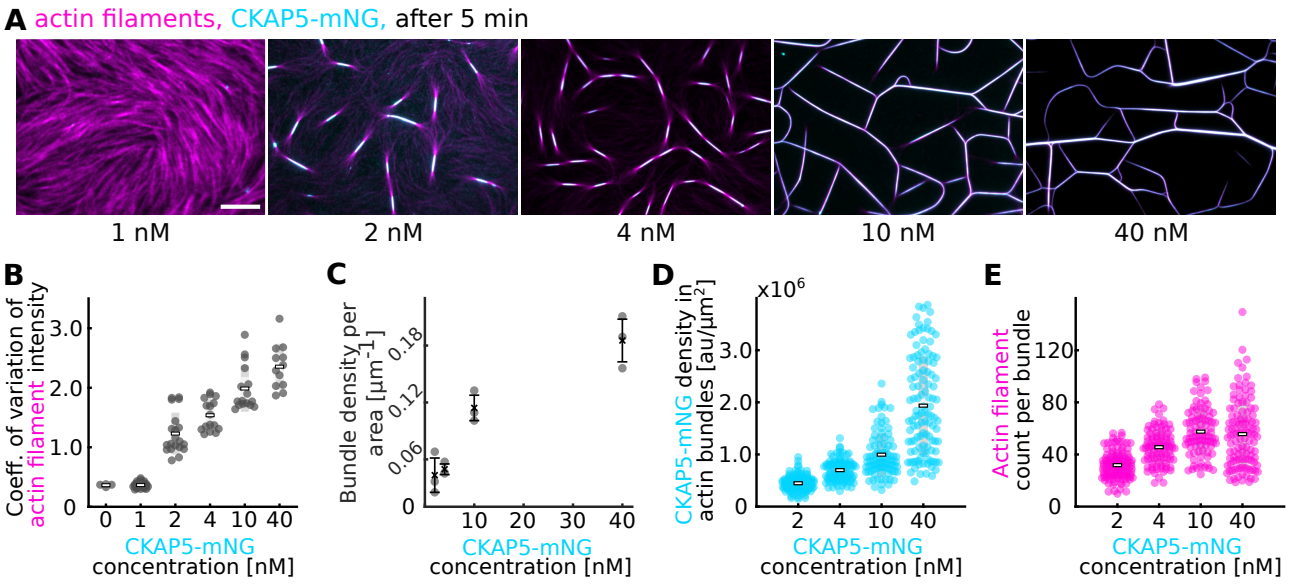


Figure 4.3: **A** Images from experiments with increasing concentrations of CKAP5-mNG (cyan) and constant amount of actin filaments stabilized with rhodamin-phalloidin (magenta), always captured after 5 min of incubation. Scale bar is 5 µm. **B** Coefficient of variation of the intensity in actin filament channel was (0.36 ± 0.02), (0.36 ± 0.05), (1.2 ± 0.3), (1.5 ± 0.3), (2.0 ± 0.4) and (2.4 ± 0.4) for CKAP5 concentration of 0, 1, 2, 4, 10 and 40 nM, respectively. Mean and SD values were calculated from $n = 4$, 24, 20, 16, 16 and 12 fields of view in 3 experiments for each condition, respectively. **C** Actin bundle density per given area was (0.040 ± 0.018), (0.050 ± 0.006), (0.110 ± 0.013), and (0.19 ± 0.23) for CKAP5 concentration of 2, 4, 10 and 40 nM, respectively. Mean and SD values were calculated from $n = 3$ for each condition from 3 different experiments. **D** Dependency of CKAP5 density in actin bundles on the concentration of CKAP5. Mean and SD were $(0.45 \pm 0.14) \cdot 10^6$, $(0.70 \pm 0.20) \cdot 10^6$, $(1.00 \pm 0.42) \cdot 10^6$, and $(1.90 \pm 0.93) \cdot 10^6$ for CKAP5 concentration of 2, 4, 10 and 40 nM, respectively. Values were calculated from $n = 117$, 95, 91 and 113 bundles, respectively, from 3 experiments for each concentration. **E** The count of actin filaments in the bundle was on average (32 ± 10), (46 ± 13), (58 ± 18), and (56 ± 26) for CKAP5 concentration of 2, 4, 10 and 40 nM, respectively. Mean and SD values were calculated from the same number of events as in **D**.

Transitioning from inspecting the networks as whole to inspecting individual bundles, we can observe from the graph in Figure 4.3 **D** that the density of CKAP5 in the bundles increases with the increasing concentration of CKAP5 in the experiment, as expected. The dependency of the count of actin filaments in the bundles on the CKAP5 concentrations shows the same trend. However, when comparing the last two highest concentrations of CKAP5 (10 and 40 nM), we notice that the number of actin filaments in the bundles is roughly the same, suggesting that most of the actin filaments in the chamber were already bundled, leaving no other available actin filaments for additional bundling (Figure 4.3 **E**).

Altogether, these results demonstrate that CKAP5 interacts with actin filaments directly and promotes their bundling.

4.2 CKAP5 binds to actin filaments with lower affinity than to microtubules

Interaction of CKAP5 with microtubules has already been well explored, as mentioned in the Introduction (Section 1.4). Therefore, our next objective was to compare the interaction of CKAP5 with microtubules to its interaction with actin filaments, especially in terms of affinities.

To measure the affinity of CKAP5 to microtubules, stabilized microtubules were immobilized on the coverslip surface using antibodies and visualized using IRM technique (see Methods, Section 3.2.3). Subsequently, increasing concentrations of CKAP5 were introduced step by step into the chamber. Each concentration was incubated for 2 min in the chamber before evaluating the intensity of CKAP5 along the microtubule lattice (see the representative image of the experiment in Figure 4.4 **B**). The affinity curve for microtubules is depicted in the graph in Figure 4.4 **C**, with $K_d = (2.0 \pm 0.3)$ nM and $[\mathrm{Max_{SB}}] = (5.40 \pm 0.20) \cdot 10^5$ au/µm^2.

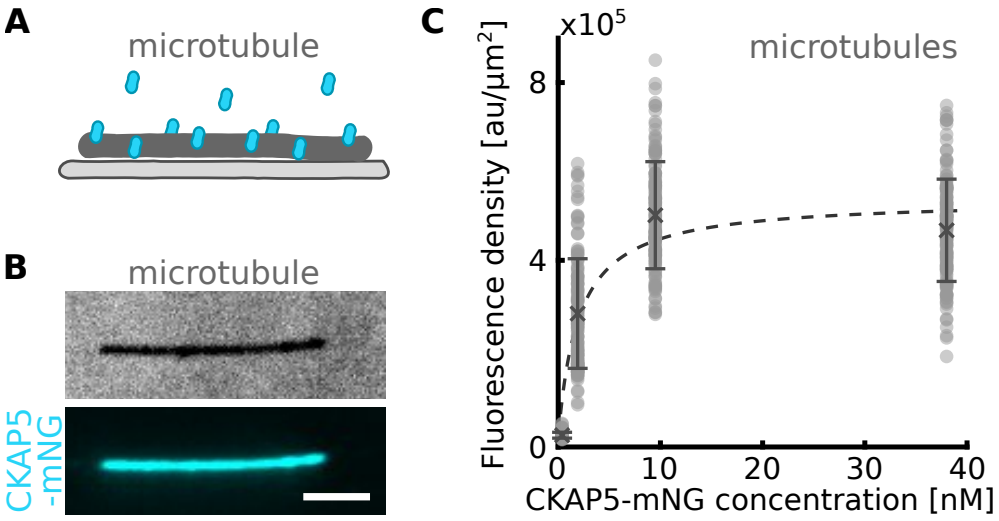


Figure 4.4: **A** Scheme of the affinity experiment with stabilized microtubules attached to the coverslip and incubated with increasing concentrations of CKAP5-mNG. **B** Still image from the experiment taken after 2 min incubation with the highest concentration of CKAP5 tested (40 nM). Microtubule (gray) fully covered with CKAP5 (cyan). Scale bar is 2 µm. **C** Affinity binding curve of CKAP5 to microtubules with $K_d = (2.0 \pm 0.3)$ nM and $[\mathrm{Max_{SB}}] = (5.40 \pm 0.20) \cdot 10^5$ au/µm^2. Calculated from $n = 132$ for 3 experiments.

To inspect the affinities of CKAP5 to actin filaments, we decided to split this matter into two conditions: the affinity of CKAP5 to single actin filaments and its affinity to preformed actin filament bundles. The experimental steps were the same as for microtubule affinity experiments. Surprisingly, the signal of CKAP5 on single actin filaments was nearly negligible, making it impossible to determine the parameters of the binding curve (see Figure 4.5 **C**).

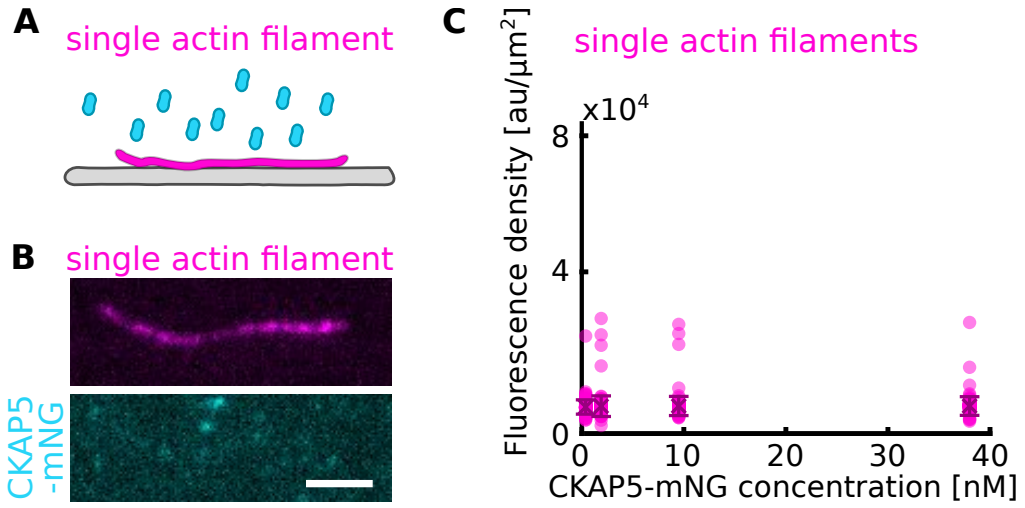


Figure 4.5: **A** Scheme of the affinity experiment with single actin filaments attached to the coverslip using biotin-phalloidin and incubated with increasing concentrations of CKAP5-mNG. **B** Still image from the experiments taken after 2 min incubation with the highest concentration of CKAP5 tested (40 nM). No colocalization of single actin filament (magenta) with CKAP5 (cyan) signal was visible by eye. Scale bar is 2 µm. **C** Affinity binding curve to single actin filaments, K_d and $\mathrm{Max_{SB}}$ could not be determined, $n = 196$ events in 3 experiments.

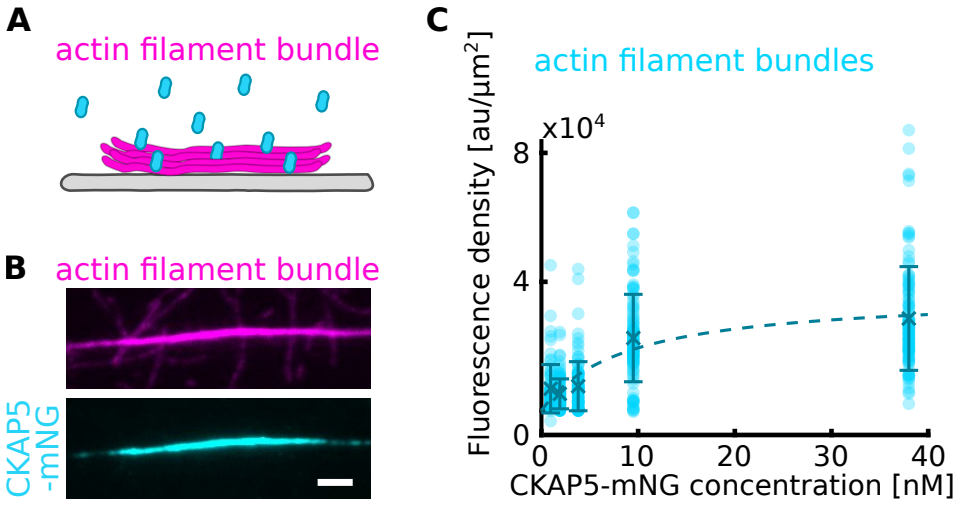


Figure 4.6: **A** Scheme of the affinity experiment actin bundles attached to the coverslip using biotin-phalloidin and incubated with increasing concentrations of CKAP5-mNG. **B** Still image from the experiment taken after 2 min incubation with the highest concentration of CKAP5 tested (40 nM). Preformed actin bundle (magenta) covered with CKAP5 (cyan). Scale bar is 2 µm. **C** Affinity binding curve of CKAP5 to preformed actin bundles with $K_d = (8.6 \pm 2.5)$ nM and $[\mathrm{Max_{SB}}] = (0.36 \pm 0.04) \cdot 10^5$ au/µm^2. Calculated from $n = 104$ from 3 experiments.

In contrast, CKAP5 exhibited relatively strong localization and binding to preformed actin filament bundles, although with a lower affinity compared to microtubules (see the graph in Figure 4.6 **C** and Figure 4.7). The binding curve for preformed actin bundles shows $K_d = (8.6 \pm 2.5)\,\mathrm{nM}$ and $[\mathrm{Max_{SB}}] = (0.36 \pm 0.04) \cdot 10^5\,\mathrm{au/\mu m^2}$. For the process of preformation of actin bundles, refer to Methods (Section 3.2.4).

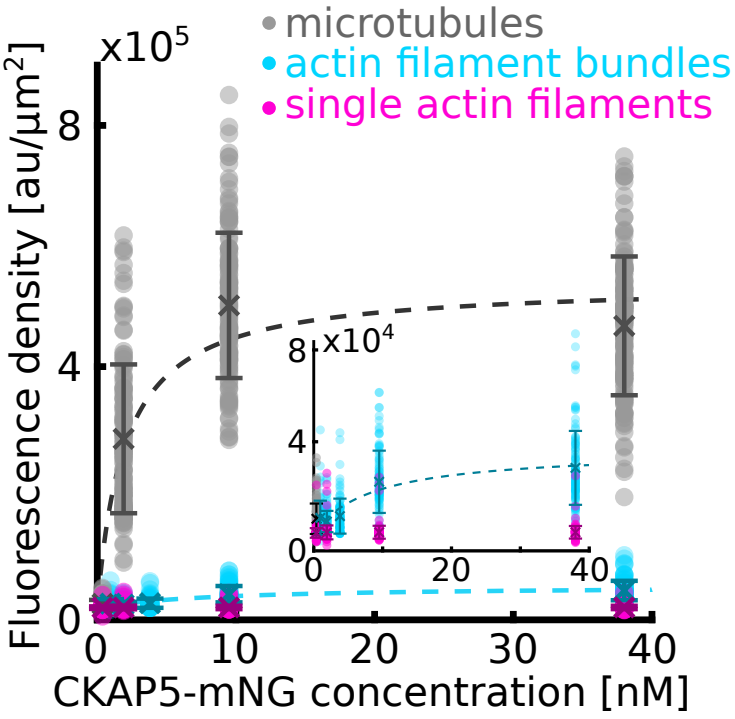


Figure 4.7: All graphs combined to one for easier comparison.

4.3 CKAP5 facilitates crosstalk between actin filaments and mictrotubules

In previous sections, we confirmed the interaction of our purified CKAP5 with both microtubules and actin filaments. Next, we wanted to investigate whether CKAP5 could independently facilitate crosstalk between these two polymers. To test this, microtubules were attached to the coverslip surface using antibodies, followed by the introduction of CKAP5 and short actin filaments into the chamber. Unlike in previous experiments, there were no antibodies against actin filaments or methylcellulose to bring them to the surface, allowing the actin filaments to remain free within the solution.

A snapshot from the experiment (Figure 4.8 **B**, left) illustrates that while microtubule is entirely covered with CKAP5, the signal of actin filaments also localizes along the microtubule lattice. To better exhibit the happening over time, I am providing kymographs generated from a line along the microtubule lattice (see Figure 4.8 **C**, left). The kymographs demonstrate that upon introduction of CKAP5 and actin filaments to the chamber, CKAP5 almost immediately covers

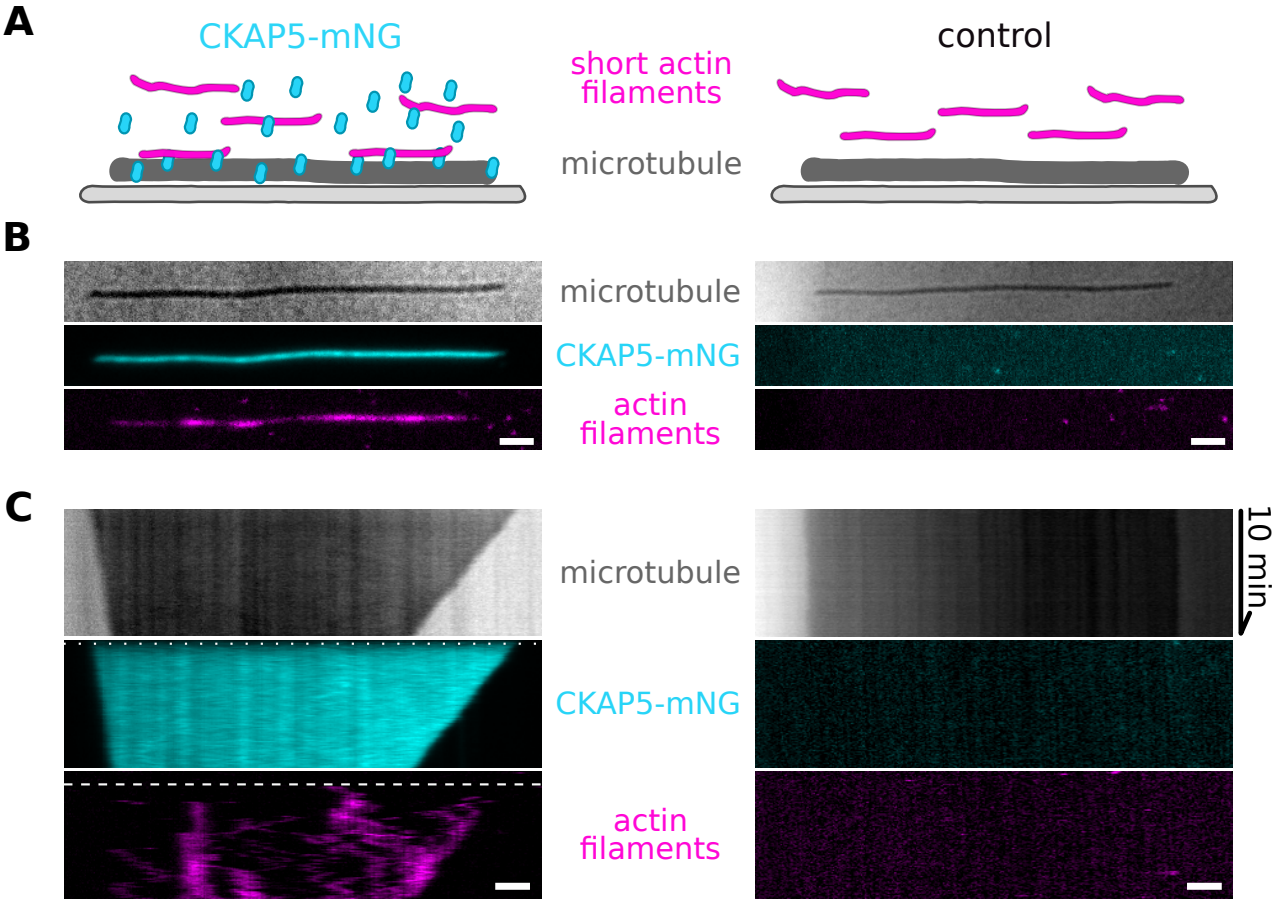


Figure 4.8: **A** Scheme of the experiments. Stabilized microtubules immobilized on the coverslip using antibodies in a presence of short shredded actin filaments and CKAP5 (left) or in the absence of CKAP5 (right, control experiment). **B** Maximum intensity projections of microtubule (gray) covered with CKAP5 (cyan) and crosslinked actin filaments (magenta) on the left. On the right, maximum intensity projections of all channels from control experiment. Scale bars are 2 µm. **C** Kymographs generated from the exemplary microtubules in B. There is immediate CKAP5 binding to the microtubule lattice (dotted line) followed by crosslinking of actin filaments to the microtubule (dashed line) on the left, whereas on the right in the control experiment, actin signal does not colocalize to the microtubule lattice in the absence of CKAP5. Scale bars are 2 µm, timescale is 10 min.

the whole microtubule at the given concentration. Subsequently, actin filaments are recruited to the microtubule lattice after a short time, where they bind and exhibit clear interaction with the microtubule. Notably, the binding of short actin filaments to the microtubule facilitated by CKAP5 has a diffusive character (see Figure 4.8 **C** left, Figure 4.9 **B** in detail.)

As a control, the same assay was conducted without CKAP5 present in the chamber. In this case, we did not detect any actin filaments localizing to the microtubule lattice at all (see Figure 4.8, right). A comparison of densities of actin filaments along the microtubule lattice in the presence of CKAP5 or in its absence, depicted in Figure 4.9 **A**, implicates a significant difference, confirming the observations described above. Combined, these results manifest that CKAP5 is a sufficient factor in facilitating crosstalk between actin filaments and microtubules.

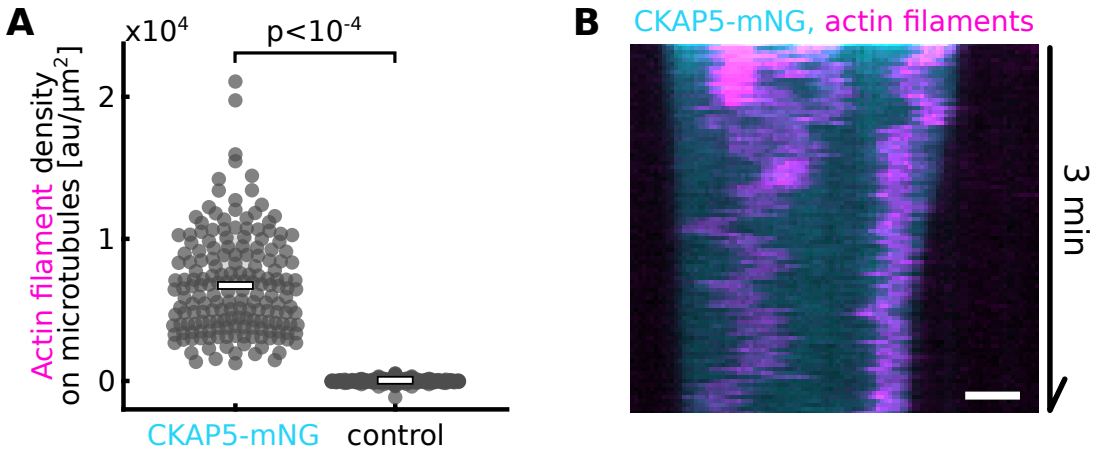


Figure 4.9: **A** Actin filament density along microtubules in the presence of CKAP5 was $(6.7 \pm 3.5) \cdot 10^3$ au/µm^2 ($n = 182$ microtubules in 2 experiments). In CKAP5 absence, the actin filament density was $(-0.0054 \pm 0.0150) \cdot 10^3$ au/µm^2 ($n = 199$ microtubules in 2 experiments). Two-sided t-test resulted in $p < 10^{-4}$. **B** Kymograph showing diffusion of short actin filaments (magenta) along microtubule shaft covered with CKAP5 (cyan). Scale bar is 1 µm, timescale 3 min.

4.4 Dynamic microtubules template persisting actin bundles by CKAP5

Lastly, we were curious to explore the dynamic behavior of the whole system. To approach this, short stabilized microtubule seeds were immobilized on the coverslip surface and alongside CKAP5 with longer actin filaments, free tubulin was introduced to the chamber too. This setup allowed microtubules to nucleate from stabilized seeds and undergo polymerization and depolymerization phases, mimicking exploratory microtubules in the growth cones. The experiment offered several interesting observations, as depicted by the kymograph of an exemplary microtubule and snapshots in Figure 4.10 **B** and **C**.

Firstly, as microtubule initiated polymerization, actin filaments were recruited and crosslinked to its lattice in the presence of CKAP5. Secondly, when microtubule approached depolymerization phase, it became evident that crosslinked actin filaments were forming a bundle along the microtubule shaft. Surprisingly, when microtubule underwent depolymerization upon catastrophes, the actin bundle did not disassemble in most cases but rather prevailed even in regions where the microtubule was no longer present (see Figure 4.10 **B** and graph in Figure 4.11 **B**). Lastly, during microtubule rescue and repolymerization, the microtubule followed the path of the actin bundle and grew in its direction.

The control experiment was performed in the same way as the dynamic experiment, but CKAP5 was absent from the chamber. Note that polymerization and depolymerization of microtubule is slower in the case of control experiment (indicated by kymograph in Figure 4.11 **A**) compared to the polymerization and depolymerization of microtubule in the presence of CKAP5 (in Figure 4.10 **B**). More importantly, actin filaments are not recruited to the microtubule lattice and do not form bundles along them in the control experiment.

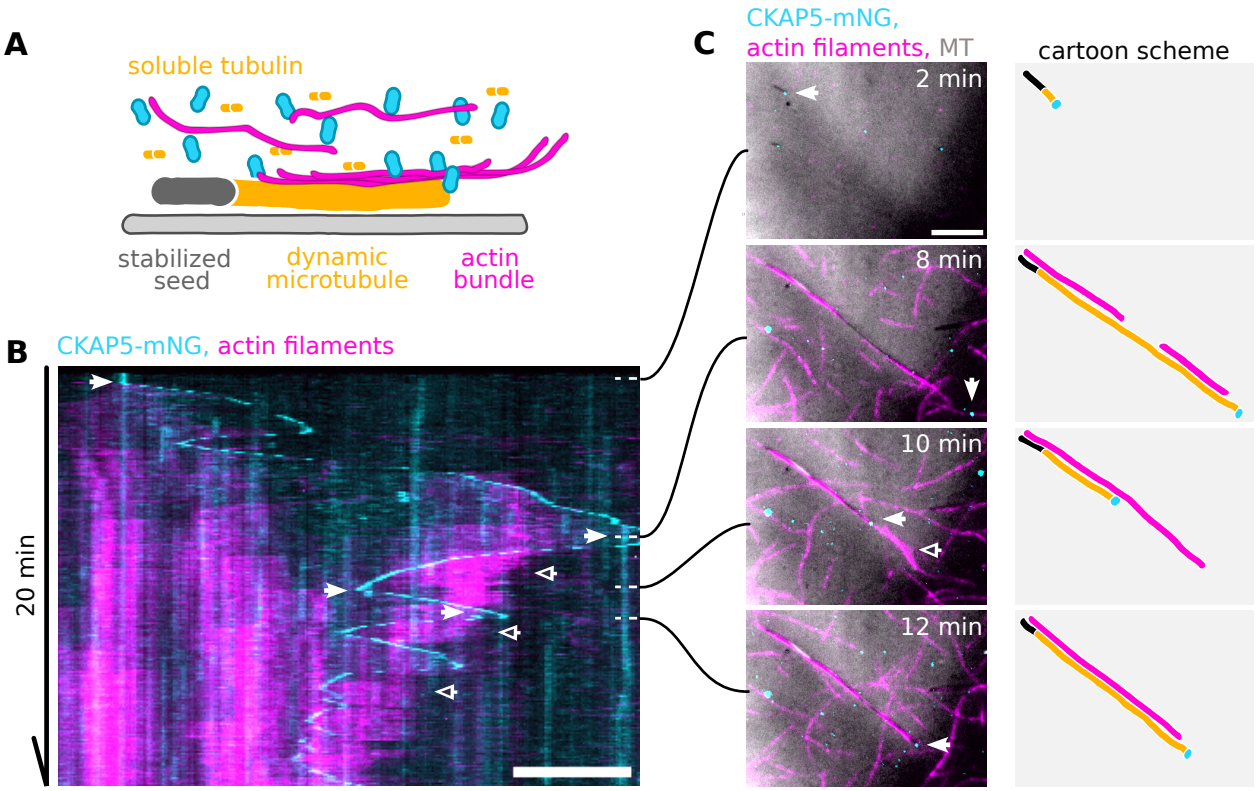


Figure 4.10: **A** Scheme of the experiment. Microtubule seeds stabilized with GMPCPP were attached to the coverslip using antibodies. Mix of longer stabilized actin filaments, CKAP5 and tubulin dimers were free in the solution. **B** Kymograph generated from an exemplary microtubule. CKAP5 signal (cyan) localizes mainly to the + tip of microtubule and tracks it as microtubule undergoes several catastrophes and rescues. Actin filaments (magenta) localizes to the microtubule lattice. Note that after a microtubule catastrophe, actin bundle is exposed and prevails (marked with hollow arrows). Scale bar is 5 µm, timescale is 20 min. **C** Time-stamps and cartoons of significant events which the exemplary microtubule (gray/yellow) undergoes together with bundled actin filaments (magenta) and CKAP5 (cyan, its intensity is adjusted for better portrayal). Filled arrows points to + end of microtubule with enriched CKAP5. Scale bar is 5 µm.

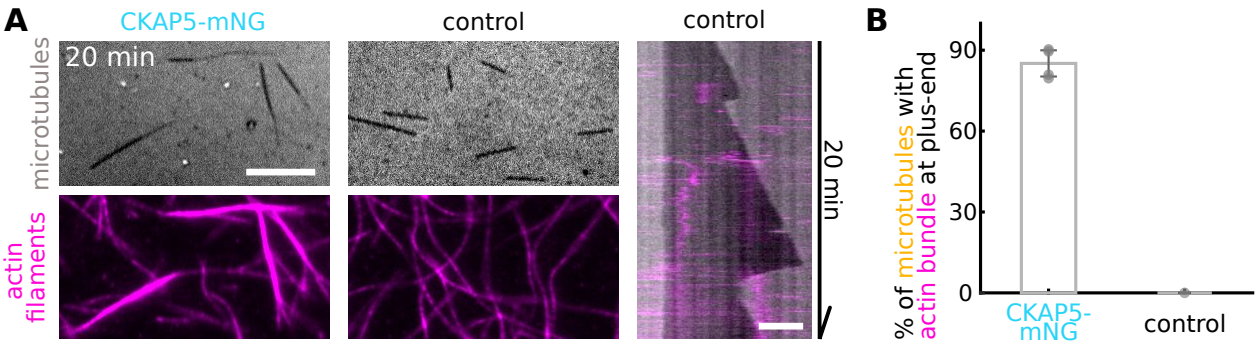


Figure 4.11: **A** Representative images of experiment with dynamic microtubules and actin filaments with (left, scale bar is 5 µm) or without (middle) CKAP5 present. Note that there is no bundling of actin filaments templated by microtubules in the control experiment in the absence of CKAP5 (middle). Kymograph (right) generated from an exemplary microtubule (gray) from the control experiment. Scale bar is 2 µm, timescales are 20 min. **B** Percentage of microtubules which had prevailing actin bundle at their + tip formed was (85.0 ± 4.9) % in the presence of CKAP5 and 0 % in its absence. Mean and SD values were calculated from $n = 160$ microtubules in 4 experiments and from $n = 130$ microtubules in 3 experiments, respectively. *These data were analyzed by Ján Sabó.*

As indicated above, repolymerizing microtubules predominantly followed the preformed actin bundles in their direction (refer to the graph in Figure 4.12 **B**). One particularly striking example is shown in Figure 4.12 **A**. At the beginning, there are two microtubules, one at the top and second one in the bottom (dark signal in the IRM channel), while also an actin bundle is prolonged from the top microtubule. The microtubule originating from the bottom begins polymerizing straight upwards over time, but upon encountering the actin bundle, it abruptly changes its growth direction and polymerizes diagonally towards the top microtubule, demonstrating a clear example of the microtubule + tip following the preformed actin bundle.

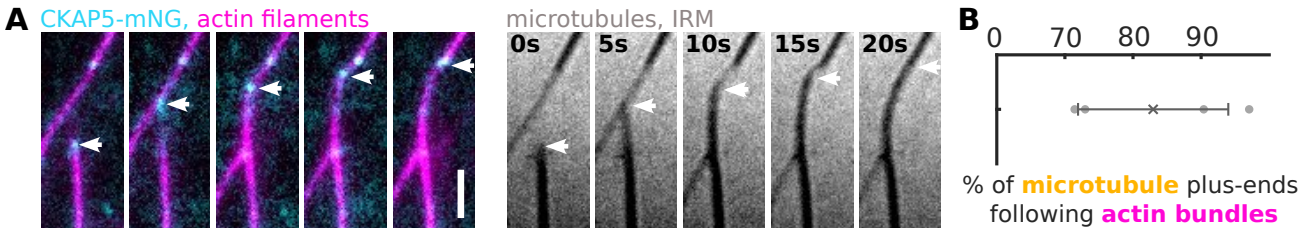


Figure 4.12: **A** Example of growing + tip with enriched CKAP5 of the microtubule being guided along preformed actin bundle. Actin (magenta) and CKAP5 (cyan) channel on the left, microtubules (gray) visualized using IRM on the right. Arrows point to + tip of growing microtubule, scale bar is 2 µm. **B** Percentage of growing microtubule + tips following prevailing actin bundles in the presence of CKAP5 was (83 ± 11) %. Mean and SD values were calculated from $n = 165$ microtubules in 4 experiments. *These data were analyzed by Ján Sabó.*

To sum it up, these experiments reveal that microtubules can template prevailing actin bundles in the presence of CKAP5, while these actin bundles can subsequently serve as guidance tracks for microtubule growth upon their possible catastrophes.

5. Discussion

Research supporting this thesis was focused on uncovering the mechanism of interaction between CKAP5, microtubules and actin filaments. CKAP5 is a well-known processive polymerase for microtubules, predominantly associating with microtubule + tips [Brouhard et al. 2008]. While its function was previously mainly linked to regulating and stabilizing the mitotic spindle during cell division [Tournebize et al. 2000] [Gergely et al. 2003], emerging studies implicate CKAP5 in the context of neuronal growth cones, too [L. A. Lowery et al. 2010] [Laura Anne Lowery, Stout, et al. 2013] [Slater et al. 2019] [Hahn et al. 2021]. Authors of these studies observed significant detrimental changes in the morphology and functionality of growth cones with knocked-down CKAP5. High-resolution microscopy images indicate that the issue with CKAP5 knockdowns lies in the misalignment of exploratory microtubules with actin bundles at the periphery [Slater et al. 2019]. In addition, they indicate that CKAP5 can also interact with actin filaments alone [Slater et al. 2019]. Altogether, these findings have suggested CKAP5 as a potential mediator of crosstalk between microtubules and actin filaments in the growth cones, yet the molecular mechanism of this process remained unclear.

Nature of the interaction between CKAP5 and actin filaments

Here in this thesis, we at first directly confirm that CKAP5 is able to autonomously interact with actin filaments (Figures 4.1 and 4.3), as it induces rapid bundling of *in vitro* reconstituted actin networks. Interestingly, at lower concentrations of CKAP5 (2 and 4 nM), the bundles formed stable distinct domains and CKAP5 was not evenly distributed throughout the network (Figure 4.3). The formation of a single bundle typically started at a nucleation point and then propagated outward from the edges until reaching equilibrium state (Figure 4.2), occasionally resulting in merging of adjacent bundles. It would be intriguing to explore the puzzling question of what determines the location of nucleation point of CKAP5 and why the bundles arise at some spots rather than others, leaving certain regions of the network non-bundled. Whether this occurs stochastically or via another mechanism remains unresolved.

On the other hand, at higher concentrations of CKAP5 (10 and 40 nM), the network was continuously bundled throughout the entire experimental chamber after reaching equilibrium (Figure 4.3). In earlier phases, long bundles in close proximity often underwent zipping events, which in consideration with the dynamics of single bundle formation from a nucleation point (described above) suggest a cooperative bundling mechanism facilitated by CKAP5.

Another intriguing observation is that only 2 nM CKAP5 is sufficient to induce the formation of quite appreciable bundles (Figure 4.3), indicating that CKAP5 has a high affinity to actin filaments. However, our affinity measurements towards single actin filaments gave us exactly opposite results. While CKAP5 binds extremely weakly to single actin filaments in our assay, it binds to preformed actin bundles with much higher affinity (Figures 4.5 and 4.6). These outcomes indicate that CKAP5 interacts with actin filaments through a different mechanism compared

to other crosslinkers of actin filaments, such as fascin, α-actinin or anillin, which exhibit strong binding to single actin filaments alongside their crosslinking activity [Winkelman et al. 2016] [Kučera et al. 2021].

Actin–microtubule crosslinking abilities of CKAP5

Our curiosity about whether CKAP5 could mediate an interaction between microtubules and actin filaments, as suggested by previously published data from *Xenopus* growth cones [Slater et al. 2019], was addressed by experiments shown in Figure 4.8. Corresponding kymographs clearly show the recruitment of short actin filaments to the microtubule lattice in the presence of CKAP5. Moreover, these kymographs offer additional engaging observations. Firstly, CKAP5 crosslinks single actin filaments to the microtubule in a diffusive manner, hence actin is not tightly bound and immobilized at the microtubule lattice (see Figure 4.9 **B**).

Secondly, even though these microtubules were taxol-stabilized, we consistently observed their relatively fast depolymerization compared to the control experiment (visible in kymographs in Figure 4.8). This aligns with the previously reported unexpected function of XMAP215 (CKAP5 homolog) that, besides being a processive microtubule polymerase, it can also drive the depolymerization of microtubules [Breugel et al. 2003] [Shirasu-Hiza et al. 2003] [Brouhard et al. 2008]. Hence, we confirm this behavior for human CKAP5 as well. The switch between these two functions is probably dependent on the local concentration of free tubulin, since CKAP5 and XMAP215 have different, in fact much higher, affinity to tubulin compared to microtubules [Spittle et al. 2000] [Brouhard et al. 2008] [Widlund et al. 2011]. Therefore, if there is no free tubulin near microtubule + end which might be incorporated into it by CKAP5, CKAP5 bound to the microtubule is likely ripping out the tubulins from the microtubule end to reach the more preferred state, overcoming even the taxol-stabilization effect.

Another fascinating observation from these experiments is that while none of the tested CKAP5 concentrations (2–40 nM range) were sufficient to exhibit effective binding of CKAP5 to single actin filaments (Figure 4.5), only 4 nM CKAP5 was adequate to recruit single actin filaments to the microtubule lattice. Therefore, we hypothesize that the density of CKAP5 is probably a crucial factor in enabling interaction of CKAP5 with single actin filaments, which again points to the direction of cooperative behavior of CKAP5 for actin binding. In this case, microtubules can be considered as a scaffold securing high density regions of CKAP5, further utilized by actin filaments for their binding. Combining these results with the finding that affinity of CKAP5 to microtubules is remarkably higher than to actin filaments, we propose a possible activated crosslinking mechanism where CKAP5 initially binds rapidly to microtubules, and actin filaments are subsequently recruited to its lattice once a sufficient density of CKAP5 is achieved.

The phenomenon of crosslinking actin filaments to microtubules facilitated by CKAP5 was further inspected in a more complex system as part of this project by a colleague Ján Sabó, motivated by the observation that not only CKAP5 recruits short single actin filaments to the microtubule lattice, but also several longer actin filaments at once [Sabo et al. 2024, Figure S2]. Hence, 10 nM CKAP5

was introduced to a network of actin filaments with incorporated taxol-stabilized microtubules. Interestingly, formation of actin bundles was induced, but exclusively at locations containing microtubules covered with CKAP5 [Sabo et al. 2024, Figure 4], despite the fact that this concentration of CKAP5 effectively bundled almost the entire actin network lacking microtubules, as shown in Figure 4.3. This finding further supports our proposed mechanism described in the previous paragraph and indicates that different affinity of CKAP5 to various components of cytoskeletal filaments regulates and fine-tunes the behavior of the whole system.

Following the preceding experiment, exciting results were obtained when we aimed to answer a question what would be the fate of actin bundles templated by microtubules in the network if the corresponding microtubule would disassemble. Upon exchanging the buffer in the experimental chamber to the one lacking the microtubule stabilizer taxol, microtubules depolymerized as the taxol bound to them diffused into the buffer due to its high turnover rate [Yvon et al. 1999] [Díaz et al. 2003]. Surprisingly, some of the actin bundles disassembled immediately after the depolymerization of their corresponding microtubule, while others were able to persist in the absence of the microtubule in a range of several minutes, a depicted in Figure 5.1 **A**, published in [Sabo et al. 2024]. Note, that both types of actin bundles were observed at the same concentration of CKAP5 (10 nM) in the same experimental chamber, hence what else could determine whether the actin bundle will persist or disassemble? As it turned out, the determinant factor for the persistence of actin bundles after microtubule depolymerization under these conditions was not only the concentration of CKAP5, but also the amount of actin filaments recruited to the bundle before microtubule depolymerization (see Figure 5.1 **B**).

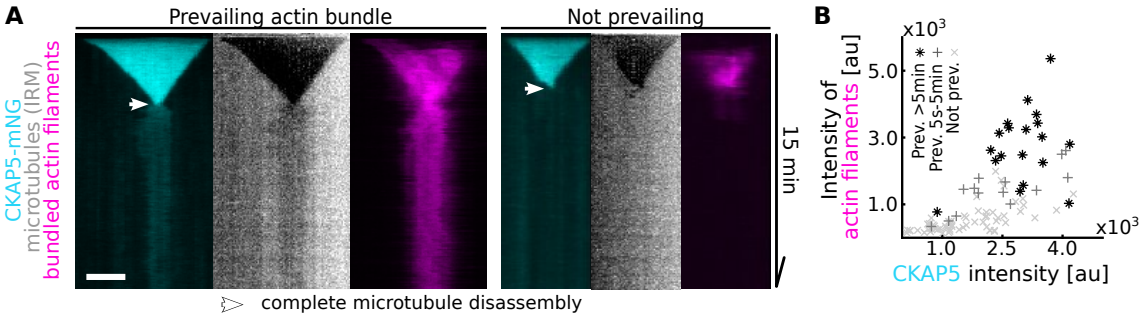


Figure 5.1: **A** Representative kymographs depicting two different populations of actin bundles templated by microtubules in actin–microtubule network. On the left, actin bundle (magenta) persists upon microtubule depolymerization (gray) with colocalized enriched CKAP5 (cyan). On the right, the exemplary actin bundle disassembles right after microtubule depolymerization. Arrows point to complete microtubule depolymerization. Scale bar is 2 µm, timescale is 15 min. **B** Graph of the correlation of actin intensity and CKAP5 intensity of actin bundles templated by microtubules. Actin intensity was measured at the time of microtubule disassembly, CKAP5 intensity was quantified 30 s before this event. Each point represents one actin bundle, while asterisks and plus signs represent prevailing actin bundles after the microtubule disassembly and crosses denote non-prevailing actin bundles. Adapted from [Sabo et al. 2024], experiments conducted by Ján Sabó.

Coming back to the initial experiments with only actin filaments networks without microtubules (Figures 4.2 and 4.3), this discovery, combined with affinity measurements showing that CKAP5 has extremely low affinity to single actin

filaments compared to actin bundles (Figure 4.7), suggests that inducing the interaction of CKAP5 exclusively with actin filaments leading to actin bundling requires a sufficient local concentration of actin filaments as well. This finding also supports the notion of a cooperative mechanism of actin bundling by CKAP5, as discussed earlier. Nevertheless, this experiment demonstrates that microtubules in combination with CKAP5 can template formation of persisting actin bundles and thereby organize the architecture of the entire network.

Does orientation matter?

One might question the relevance of all these findings to the growth cones, given that the actin bundles in filopodia exhibit a parallel orientation and microtubule growth is oriented relatively to the actin bundles too, which are not conditions replicated in the experiments presented here. The orientation of actin filaments in the network was random, as well as actin filaments could have been recruited to the microtubule lattice in both orientations. Nevertheless, the questions of whether CKAP5 can induce the bundling of parallel-oriented actin filaments and whether it can mediate an interaction between microtubules and oriented actin filaments, were addressed as part of this project, too [Sabo et al. 2024, Figure 3]. To ensure parallel orientation of actin filaments, small beads were coated with formin mDia1 (actin nucleator and elongator) in a defined manner through antibodies, allowing the formin domains FH2 responsible for actin nucleation [Pruyne et al. 2002] [Zigmond 2004*] to remain free. Formins nucleate and elongates actin filaments from G-actin subunits exclusively at the barbed ends [Pruyne et al. 2002] [Zigmond 2004*], hence the barbed ends were enriched at the beads, while the pointed ends of actin filaments were distant (see Figure 5.2). Upon addition of 10 nM CKAP5 to dynamically growing actin filaments, immediate bundling was visible and confirmed by plotting the coefficient of variation [Sabo et al. 2024, Figure 3C]. Interestingly, bundling has been reproducibly starting in the area of less dense pointed ends and spread towards the more dense barbed ends (from periphery towards the center containing the bead). These experiments confirm that CKAP5 is capable of bundling parallel-oriented actin filaments and that the bundling process is independent of the polarity of actin filaments.

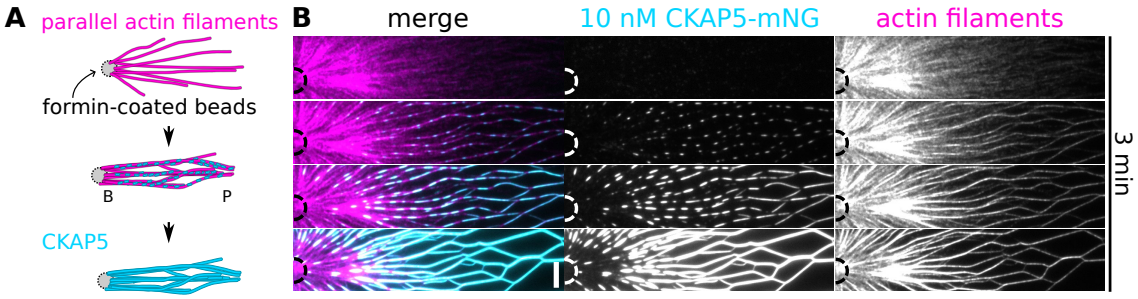


Figure 5.2: **A** Scheme of the experimental arrangement for bundling of parallel actin filaments by CKAP5. Beads coated by formin mDIA via His-tag antibodies were initiating points for actin filament assembly in parallel orientation, thanks to the nature of formin nucleation activity. B marks barbed ends of actin filaments, while P denotes pointed ends. **B** Representative snapshots of the experiment: 10 nM CKAP5 (cyan) profusely bundles oriented actin filaments (magenta). Dashed line marks position of the bead. Scale bar is 2 µm, timescale is 3 min. Adapted from [Sabo et al. 2024], experiments conducted by Ján Sabó.

To examine whether the crosslinking of microtubules to actin filaments is affected by the orientation of microtubules to parallel-aligned actin filaments, we took the advantage of the setup of previous experiment. Actin filaments were dynamically grown from formin-coated beads in the presence of randomly oriented microtubules bound to the surface [Sabo et al. 2024, Figure 3]. The orientation of microtubules was determined by the speed of depolymerization caused by CKAP5, with the + ends depolymerizing significantly faster. Upon introducing 2 nM CKAP5 to the experimental chamber, the crosslinking of actin filaments to all microtubules covered with CKAP5 was clearly visible. Further analysis revealed that the ability of CKAP5 to crosslink actin filaments oriented by their pointed ends towards + ends of microtubules is not significantly different from its ability to crosslink actin to microtubules in the opposite orientation [Sabo et al. 2024, Figure 3F].

Combined, these results confirmed that CKAP5 is able to bundle actin filaments regardless of their polarity as well as crosslink actin to microtubules regardless of their mutual orientation. This suggests that CKAP5 should be able to operate also in growth cones, where actin filaments in bundles are parallel-oriented and microtubules also interact with them in predetermined orientation (+ tips of exploratory microtubules aligning towards the distant barbed ends of actin bundles, pointing towards the growth cone periphery).

CKAP5 orchestrates dynamic inter-cytoskeletal crosstalk

In contrast to our experiments described above, exploratory microtubules in growth cones are dynamically unstable, undergoing frequent catastrophes and rescues. To approach a more realistic scenario, we observed a behavior of a system containing stabilized actin filaments, CKAP5, short GMPCPP-stabilized microtubule seeds, and free tubulin. Notably, CKAP5 was predominantly enriched at microtubule + tips tracking their dynamics (visible in Figure 4.10 **B**), which is in agreement with previously published *in vitro* data for its *Xenopus* homolog XMAP215 [Brouhard et al. 2008]. On the other hand, CKAP5 bound to the microtubule lattice quite sparsely compared to experiments with stabilized microtubules, despite a relatively high concentration of CKAP5 (200 nM). The reason for this was the presence of free tubulin in the chamber, as CKAP5 has an even higher affinity to tubulin than to microtubules. Hence, we believe that most of the CKAP5 was occupied by interaction with the free tubulin.

Remarkably, despite this, actin filaments were recruited to the microtubule lattice in the presence of CKAP5 (Figures 4.10 and 4.11). During depolymerization of the dynamic part of the microtubule, crosslinked actin filaments were transported together with the microtubule tip closer to the base of the microtubule, forming a larger actin bundle. Most importantly, these actin bundles were able to persist and did not disassemble even after microtubule catastrophe (Figure 4.10). In a return, when the microtubule started to polymerize back, its + tip with enriched CKAP5 was guided along the preformed actin bundle and followed the bundle in its direction in most cases (Figure 4.12). These observations indicate that the + tips of dynamic microtubule are crucial for the crosstalk, and that the enriched density of CKAP5 at the + tip of microtubule is sufficient to secure such a dynamic mutual influence *in vitro*. Therefore, our results contribute to discussions regarding whether CKAP5 needs to cooperate with another players,

like for example EB1 or CLASP, to mediate crosstalk between microtubule + tips and actin filaments [Cammarata et al. 2016*], clearly showing that CKAP5 is able to ensure this interaction autonomously (at least *in vitro*).

Other players in the scope

After all, we do not expect CKAP5 to be sufficient for fully regulating and executing actin–microtubule crosstalk also in neuronal growth cones, as there are likely additional executors responsible for integrating all the signaling guidance cues into a response. As stated in the Introduction (Section 1.3.5), numerous other proteins are implicated in actin–microtubule crosstalk within growth cones, including spectraplakins, CLASP, APC, Tau, mDia1 or even septins (Section 1.2.4). Therefore, it appears that many more proteins initially considered to be only microtubule- or actin-associated can directly mediate crosstalk between them.

In addition, these proteins likely cooperate in various ways, as indicated by a recent study proposing that EB1, Tau and Msps (*Drosophila* homolog of CKAP5) collaborate in regulation of microtubules in growth cones. When this cooperation was disrupted, microtubules did not elongate straight but rather grow in a curly, non-functional manner outside the pathway [Hahn et al. 2021]. Nevertheless, the overall interplay among all contributors that regulate growth cone guidance and their specific roles await further specification, which will require comprehensive additional research.

However, a current hot topic considering actin–microtubule crosstalk involves microtubule + tip proteins, suggesting that besides CKAP5, other proteins from this group might play a substantial role in it. Two relevant studies with observations similar to ours regarding CKAP5 are discussed further.

CLASP2, a microtubule + tip protein containing TOG domains as well, previously implicated in neuronal development, cell division and migration (reviewed in [Lawrence et al. 2020*]), was recently reported to crosslink actin filaments with microtubules in *in vitro* reconstitution assays [Rodgers et al. 2023]. Even though the authors used a relatively high concentration of purified CLASP2 in all assays, (100 nM), they demonstrated actin filament organization templated by microtubules based on their interaction facilitated by CLASP2, indicating behavior similar to CKAP5. Additionally, they showed that a single TOG domain (with ser-arg region) is capable of providing the inter-cytoskeletal interaction.

Another comprehensive study has brought results even more similar to ours, using engineered protein TipAct. Although TipAct is composed of different domains compared to CKAP5, it localizes to microtubule + ends via EB3, exhibits relatively low affinity to actin filaments, but can stably associate with actin bundles [López et al. 2014, supplement]. Hence, TipAct shares properties very similar to CKAP5. The authors demonstrated that TipAct can mediate an interaction between microtubules and actin filaments and, furthermore, that TipAct enriched at the dynamic microtubule + end guides its growth along actin bundles. Exactly as we observed with CKAP5 (Figure 4.12), the authors have also provided examples of redirecting microtubule growth along actin bundles positioned differently in a respect to the microtubule, emphasizing the guiding ability of TipAct. Additionally, they made a striking reveal that the dynamic microtubule + end enriched with

TipAct can capture and transport single actin filaments, and even pull or stretch them when actin is partially bound to the coverslip. Considering the overall similarities between the behavior of CKAP5 and TipAct despite their different structural compositions, we hypothesize that this type of actin–microtubule crosstalk might be executed by various palette of proteins capable of tracking microtubule + end as well as of binding actin filaments. Therefore, the mechanistic insights provided by our results gathered when inspecting CKAP5, might be relevant for also other interactors.

The nature of TipAct's actions led the same group to explore its effects more deeply from a different perspective. Recently, they published a study focusing on analyzing the forces driving the transport of single actin filaments by microtubule + ends mediated by TipAct over large distances (range of µm) [Alkemade et al. 2022]. Utilizing a combination of *in vitro* reconstitution assays, computer simulations, theoretical modeling, and force measurements by optical tweezers, they identified two antagonistic forces arising from the binding of the crosslinker at the overlap of actin filaments and microtubule:

(1) a condensation force in the range of 0.1 pN caused by the crosslinker attempting to maximize overlap between microtubule and actin filaments,

(2) a friction force along the microtubule lattice.

Therefore, they have described a compelling example of a passive crosslinker generating forces, adding a new member to the emerging field of such crosslinkers. However, other previously described passive crosslinkers generating forces towards cytoskeletal filaments were only for actin–actin or microtubule–microtubule interactions, such as anillin contracting actin filaments [Lansky et al. 2015] [Wierenga and Wolde 2020] [Kučera et al. 2021].

The authors speculate that this transport mechanism might be utilized in cells, e.g. for translocating actin filaments to the periphery of migrating cell to enhance the migration. Based on the all similarities between TipAct and CKAP5 behavior, it would be intriguing to test if CKAP5 could also ensure such translocation and confirm whether it generates similar forces. Even more thrilling would be to observe this phenomenon in living cells.

CKAP5 promotes actin–microtubule colocalization directly in growth cones

While our primary approach to exploring the mechanism of dynamic interaction between microtubules and actin mediated by CKAP5 was based on *in vitro* reconstitution, we complemented it with additional experiment in *Xenopus* neuronal explants as part of this project (published in [Sabo et al. 2024]). The experiment was kindly conducted by our collaborators, the Lowery lab from Boston Medical Center. As outlined in the Introduction (Section 1.4), previously published data demonstrated that XMAP215 (CKAP5 homolog) knockdown in *Xenopus* neurons resulted in a misalignment of exploratory microtubules with actin filaments in growth cones [Slater et al. 2019], which we aimed to further investigate.

By combining RNAi (RNA interference) approach with high-resolution structured illumination microscopy (SIM), we examined the intensity of actin filaments at

the + ends of exploratory microtubules, which indeed exhibited a significant decrease in the knockdown condition (depicted in Figure 5.3 **A**, **B**). On the other hand, the intensity of actin filaments in the growth cone veils remained unaffected in knockdown condition. This finding once again suggests that the tips of microtubules are the drivers of dynamic actin–microtubule crosstalk mediated by CKAP5. Moreover, we have shown colocalization of all three components — microtubules, actin filaments and XMAP215 — in fixed growth cones for the first time (Figure 5.3 **C**). Combined, these data underscore the involvement of CKAP5 in the formation of dense actin structures in the growth cones, colocalizing with microtubules. However, to elucidate the overall contribution of CKAP5 to the crosstalk mechanism, much more data, preferably from non-fixed cones, are needed.

Summary and future objectives

In summarizing the function of CKAP5 as can be understood by far, experiments with growth cones clearly show that CKAP5 is required for their proper morphology and functioning, and they also indicate that part of CKAP5's role involves facilitating inter-cytoskeletal crosstalk. Our study of *in vitro* reconstituted systems demonstrated that CKAP5 could also be sufficient to ensure the dynamic behavior and mutual organization of microtubules with actin filaments, very similar to those occurring in the growth cones — forming stable actin bundles (in filopodia), enhancing microtubule dynamics and facilitating microtubule growth coupled with actin bundles. I find it remarkably impressive that essentially only three passive components are enough to achieve such complex dynamic behavior, fine-tuned only by concentrations and various affinities between the components.

Considering all our *in vitro* results described thus far and their implications for growth cones, it led us to formulate the following hypothesis: exploratory microtubules might potentially also template the position of actin bundles at the growth cone periphery, while these actin bundles might then serve as a memory for depolymerized microtubules and navigate them back to the same protrusion when undergoing a rescue, if needed. Anyhow, to substantiate this hypothesis, many more diverse experiments would have to be conducted, mostly from living neurons.

Now that we have investigated what CKAP5 is capable of, our next step is to explore which part of CKAP5 is responsible for which function. So far, TOG1 and TOG2 has been shown to ensure polymerase activity, all TOG1-5 domains to bind tubulin dimers and that binding to the microtubule lattice is mediated by link between TOG4 and TOG5 [Widlund et al. 2011]. We would like to further unravel which part of CKAP5 is responsible for interaction with actin filaments exclusively and which part mediates actin–microtubule crosstalk. Although it was implicated that the C-terminal domain of XMAP215 (CKAP5 homolog) is unnecessary for actin–microtubule alignment in the *Xenopus* growth cones and there are indications that TOG5 might play a crucial role in their mutual alignment [Slater et al. 2019], further exploration and confirmation through additional experiments is needed. To clarify it, we will use different constructs lacking various domains to produce shorter versions of the protein and test it in our *in vitro* assays.

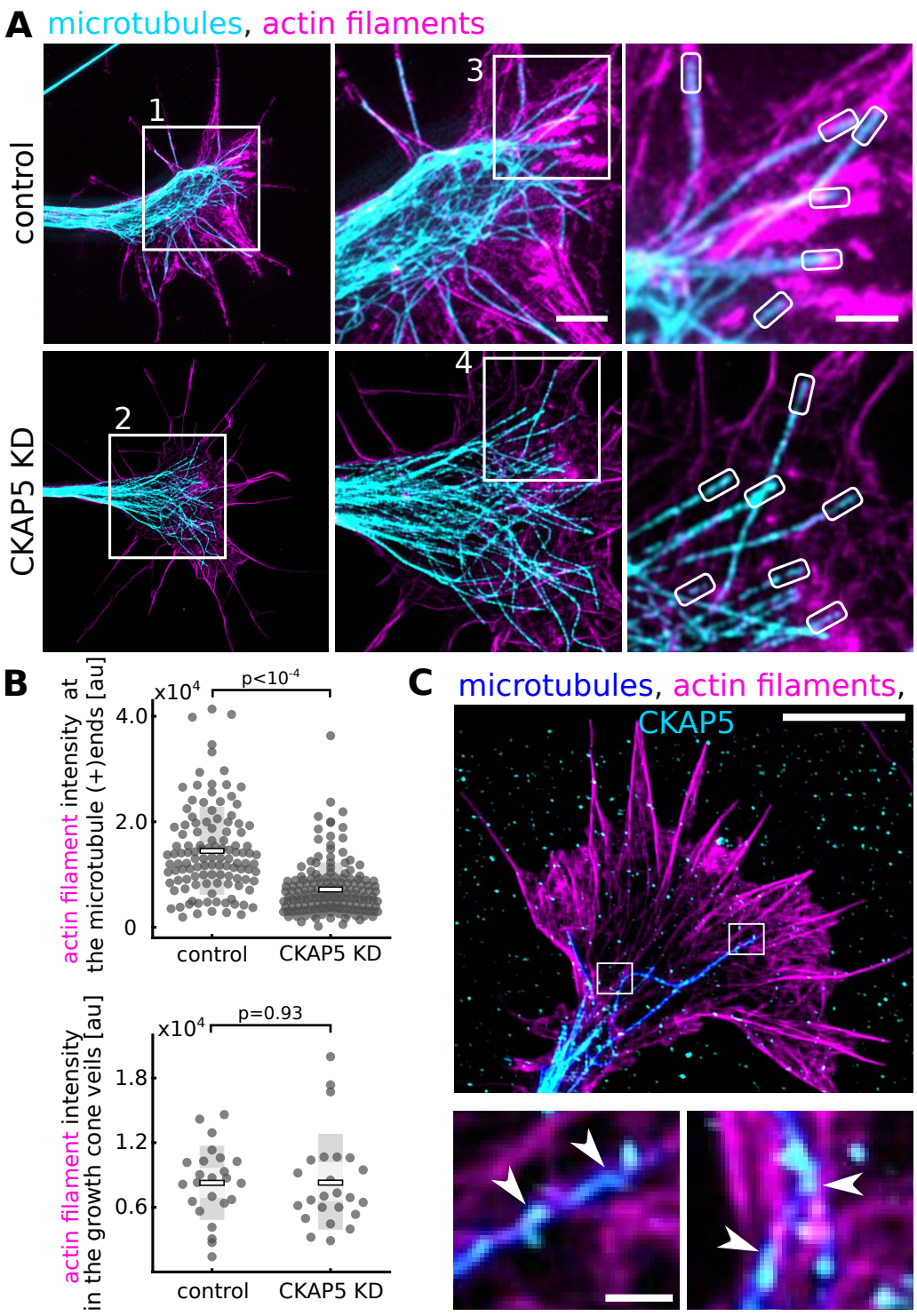


Figure 5.3: **A** Representative growth cones of *Xenopus* neuronal explants from control group and CKAP5 knockdown condition. Small boxes in the last images mark the areas of actin intensity quantification on microtubule + tips. Immuno-labeled actin filaments are in magenta and microtubules in cyan, while CKAP5 is not labeled here. Scale bars are 2 and 1 µm, respectively. **B** The intensity of actin filaments at the microtubule + ends is significantly lower in CKAP5 knockdown condition (upper graph, two-sided t-test, $p < 10^{-4}$), while the intensity of actin filaments in the growth cone veils is not affected by the knockdown (bottom graph, two-sided t-test, $p = 0.93$). **C** Colocalization of microtubules (blue), CKAP5 (cyan) and actin filaments (magenta) in the neuronal growth cones. Scale bars are 5 (top) and 0.5 µm (bottom). Adapted from [Sabo et al. 2024], experiments conducted by Paula G. Slater.

Further, it would also be interesting to explore upstream regulation of CKAP5 in neuronal growth cones, since this area remains uncovered. Even though there are indications that CKAP5 activity can be tuned by phosphorylation, it was studied in the context of cell cycle [Vasquez et al. 1999].

Regarding the future objectives of overall growth cone research with living neurons, it is important to acknowledge that the majority of current understanding of growth cone movement comes from experiments conducted in 2D environments. However, emerging evidence reveal that the mechanism of actin-based cell motility differs in 2D and 3D environments, not only in general [Galarza et al. 2020*], but also specifically in the case of growth cones [Santos et al. 2020] [Alfadil and Bradke 2023*]. For instance, growth cones traveling in 3D environment appear to be smaller and less splayed. They exhibit higher number of filopodia, while lacking lamellipodia-like structures. Their cytoskeletal architecture also slightly differs, as the transition zone seems to be lacking, resulting in higher number of exploratory microtubules protruding to the periphery, which is in line with the observed increased number of filopodia and would underscore the importance of actin–microtubule crosstalk in growth cones.

Given the integral relationship between morphology and function, the mechanism of movement might differ for 3D growth cones as well. Specifically, stiffness of the substrate surrounding the neurons seems to be a possible modulator of the motility mechanism, as the pulling by adhesion is a strategy favorable in stiff substrates, but not in the soft ones, which is the case in central nervous system [Alfadil and Bradke 2023*]. Importantly, an initial pioneer study has already demonstrated that the movement of growth cones in 3D is not reliant on adhesion and the classical "clutch" mechanism [Santos et al. 2020]. This opens the door for hypothesizing whether "ameboid" migration, based on enhanced actin severing by cofilin for clearing the pathway for microtubules towards the periphery and filopodia, is more suitable for growth cone movement in 3D [Alfadil and Bradke 2023*], as also suggested by the pioneering study [Santos et al. 2020]. Strikingly, the "3D growth cone morphology" — characterized by an increased number of filopodia and the absence of adhesive structures — was also observed *in vivo* in transparent *Drosophila* larvae [Clarke et al. 2020]. Therefore, it is clear that the shift from 2D to 3D studies is inevitable for better mimicking the *in vivo* scenario. It will be compelling to follow the upcoming findings clarifying these differences.

Conclusion

Researchers have been long intrigued by the mechanism of axonal pathfinding, motivated by the fact that its failure can result in serious neurodegeneration or developmental disruptions. As reviewed in Introduction, this process relies on growth cone movement, which is regulated by integrating signals from chemotropic cues in the environment and executed by cytoskeletal rearrangements generating forces transferred to the cellular level. While the prominent roles of actin and microtubules has been extensively studied and several models of their actions were proposed, the emerging concept of their crosstalk is currently a topic of research interest.

Our study was focused on one potential mediator of such crosstalk, the protein CKAP5, an abundantly expressed and conserved microtubule + tip polymerase. Recent findings have highlighted CKAP5's importance in shaping growth cone morphology and successful guiding function, while vaguely indicating its role in mediating crosstalk between exploratory microtubules and actin cytoskeleton at the growth cone periphery. Our aim was to investigate CKAP5's abilities to facilitate such crosstalk at the molecular level and propose the potential mechanism. To address this objective, we found *in vitro* reconstitution studies combined with TIRF microscopy to be the most suitable.

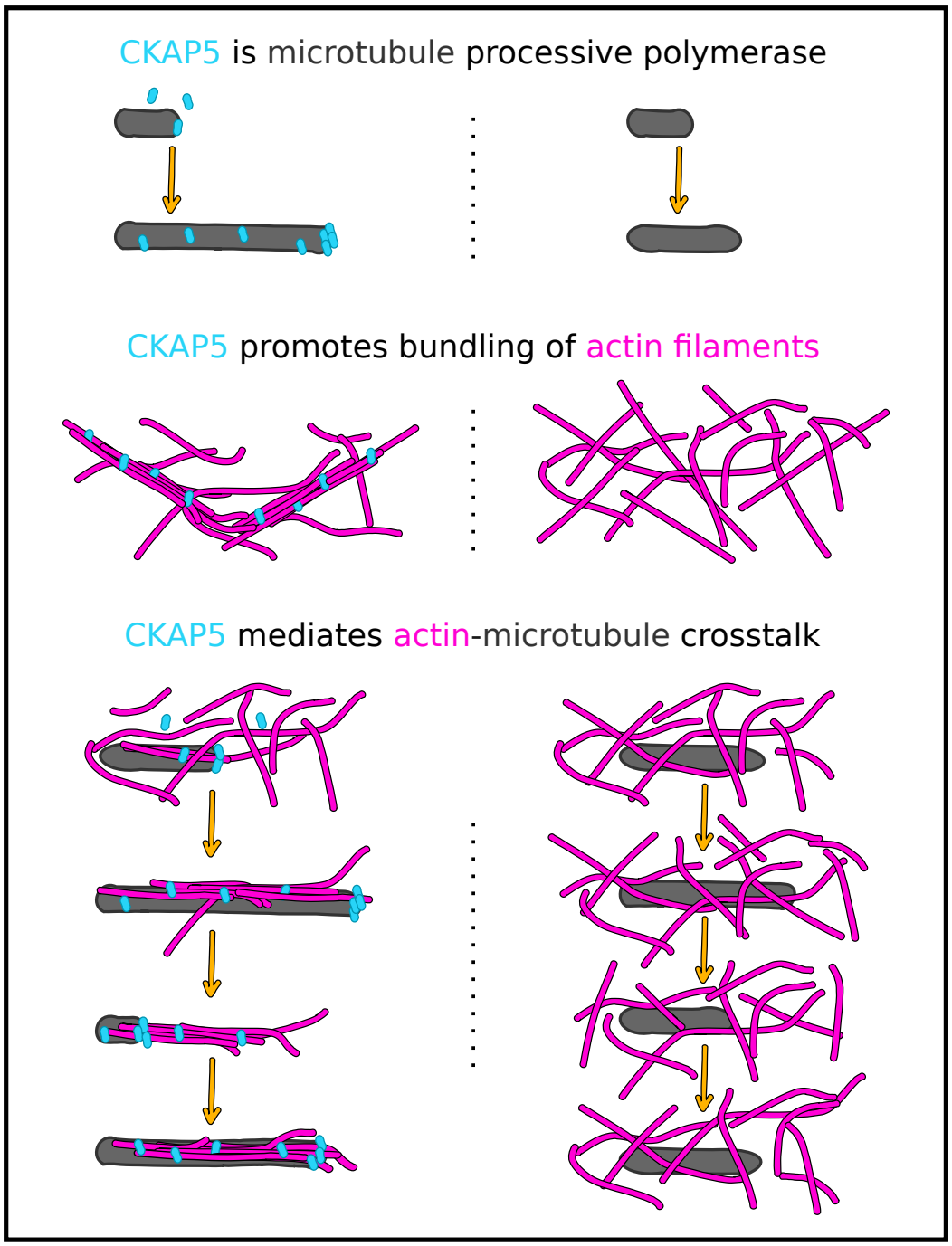
We confirm here that CKAP5 can interact with actin filaments alone, as previously suggested by spin-down assay, and we extend this knowledge by demonstrating its bundling capability. Further, we for the first time directly show that CKAP5 recruits single actin filaments to microtubules, thereby autonomously facilitating their crosstalk, regardless of their orientation or polarity. Interestingly, we observed a striking reorganization of architecture of actin–microtubule networks facilitated by CKAP5, wherein substantial actin bundles were formed at the spots occupied by microtubules decorated with CKAP5, even at CKAP5 concentration insufficient for its interaction solely with actin filaments (Discussion, Chapter 5).

When mimicking a scenario more relevant for growth cones, dynamic microtubules could also template actin bundles, even though CKAP5 was mostly enriched at their + tips in this case. Remarkably, during microtubule catastrophes, templated actin bundles were able to persist and subsequently serve as tracks for microtubule repolymerization, as they were guided along the actin bundles by CKAP5 at their tips. We propose that the mechanism underlying this dynamic interaction is fine-tuned by differential affinities of CKAP5 for various cytoskeletal components, and we hypothesize, that CKAP5 is able to generate forces despite being a passive crosslinker, as indicated by recent findings published by another group concerning a protein very similar to CKAP5.

Altogether, we presented results indicating that CKAP5 can autonomously ensure the dynamic rearrangements of cytoskeleton *in vitro*, very similar to those happening at the growth cone periphery between exploratory microtubules and actin bundles in filopodia. However, as stated in Introduction and Discussion, many more proteins, especially microtubule + tip proteins, were to some extent indicated in actin–microtubule crosstalk in growth cones.

Clarifying CKAP5's contribution and understanding inter-cytoskeletal crosstalk in growth cones will require further studies, ideally also in living growth cones within 3D environments to achieve conditions relevant for *in situ* state.

Graphic summary



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