Chapter 1

Introduction: The eukaryotic cytoskeleton and actin-microtubule coordination

In this introductory chapter we first outline the general properties of the three eukaryotic cytoskeletal systems: actin, microtubules and intermediate filaments, and how they individually shape the cell interior. Thereafter, we focus on cytoskeletal cross-talk with a focus on actin-microtubule interactions. We highlight the mechanisms that allow cross-linking proteins and biochemical regulators to efficiently orchestrate the cytoskeletal architecture, and discuss how their activity is modulated by the basic mechanical and dynamical properties of microtubules, actin filaments and intermediate filaments. Finally, we provide an outline for this thesis.

1.1 The eukaryotic cytoskeleton

Cells, the basic unit of life, have the incredible ability to change shape, divide and move. These vital cellular functions are powered by a dynamic supramolecular assembly known as the cytoskeleton. Among others, the key roles of the cytoskeleton are: to power the drastic morphological changes required in cell division, growth and migration; to provide the cell with mechanical stability and integrity to interact with its environment; and finally, to function as an internal scaffold that organizes the cell interior, by providing tracks for the transport of molecules and vesicles.
In metazoans, the cytoskeleton is typically composed of three distinct protein polymers: microtubules (MTs), filamentous actin (F-actin), and intermediate filaments (IFs) (Fig. 1.1). Depending on cell cycle and cell type, the cytoskeleton can adopt a variety of higher-order architectures to better serve the task at hand. To this end, actin, microtubules and intermediate filaments rely on their individual mechanical properties and assembly dynamics, as well as on the activity of accessory proteins. Some of these proteins regulate the spatial organization of these systems, some their assembly dynamics, while others mediate cooperation across systems. All of these functions are equally required for proper cellular function.

In the following sections we summarize the main properties of microtubules, actin filaments and intermediate filaments; followed by a review on cytoskeletal cross-talk between F-actin and microtubules; and finally, we provide an outline for this thesis.

**Figure 1.1: The main components of the metazoan cytoskeleton.** Top, Fluorescence micrographs of CV-1 monkey fibroblastic kidney cells (left), PtK2 rat-kangaroo epithelial kidney cells (middle), and A-10 rat myoblastic thoracic cells in 2D culture immunostained with tubulin (left) and cyto-keratin (right) antibodies, or labeled with pEGFP-βActin (middle). DAPI staining (blue) marks the nucleus. Figures taken from [1]. Bottom, polarity, turn-over dynamics and persistence length $L_p$ of microtubules, actin filaments and intermediate filaments.
1.1.1 Microtubules

Microtubules are protein polymers that assemble through the longitudinal addition of GTP-bound $\alpha - \beta$ tubulin heterodimers (Fig. 1.2 a and b). The three main types of tubulin in eukaryotes are $\alpha$, $\beta$ and $\gamma$ tubulin, highly-homologous $\sim 55$ kDa GTP-binding proteins, with multiple isoforms that are cell- and tissue-specific [2, 3]. While the $\alpha - \beta$ tubulin heterodimers constitute the soluble unit for microtubule polymerization in cells, $\gamma$-tubulin is typically found in large protein assemblies that mediate microtubule nucleation [4–7].

Figure 1.2: Microtubule structure and dynamic instability. (a) Microtubules polymerize from the longitudinal assembly of $\alpha - \beta$ tubulin dimers. (b) The most typical tubulin assembly in cells is a 13-protofilament hollow tube in which lateral contacts are dominated by $\alpha - \alpha$ and $\beta - \beta$ tubulins, with a discontinuity called the microtubule seam in which $\alpha$ tubulins contact $\beta$ tubulins. (c) GTP hydrolysis allows microtubules to undergo dynamic instability [8], a process by which they transition between phases of growth and shrinkage, with transitions in between called catastrophes and rescues. Figure taken from [9].
In vitro, tubulin can assemble into a variety of structures [10], whose rate of polymerization depends on tubulin concentration, Mg\(^{2+}\) concentration, pH and ionic strength [11, 12]. In vivo, the predominant mode of tubulin assembly is a hollow cylindrical tube, with a diameter of \(\sim 25\) nm, composed of 13-protofilaments [13], (Fig. 1.2 b). In this type of microtubular architecture, commonly called B-lattice [14], longitudinal contacts primarily occur between \(\alpha\) and \(\beta\) subunits, while lateral contacts occur between \(\alpha - \alpha\) and \(\beta - \beta\) subunits [15]. This organization results in all protofilaments running parallel to the long axis of the microtubule, but also in a structural discontinuity (called the microtubule seam) where \(\alpha\) and \(\beta\) subunits contact laterally, in an A-lattice architecture [16]. It has been proposed that A-lattice type lateral bonds at the seam constitute the weakest structural feature in microtubules [17]. Recent in vitro work supports this notion, as reconstitutions of seeded microtubule polymerization with ectopic A-lattice seams revealed that these microtubules are more prone to depolymerization [18].

The rate-limiting step in microtubule polymerization is nucleation [20], in which a \(\sim 6 - 15\)-mer unit first needs to form for microtubule polymerization to proceed [12, 21–24]. In vitro, the critical concentration for nucleation at room temperature and in the absence of co-factors ranges from \(\sim 20 - 40\) \(\mu\)M [25, 26]. This is comparable to the cytosolic tubulin concentration in a variety of eukaryotic cells [27], which helps explain why spontaneous microtubule nucleation is rarely (if ever) observed. Notwithstanding, microtubules constitute \(\sim 65\%\) of the total tubulin content in cells [28]. This balance suggests the existence of potent microtubule nucleators. Indeed, cells have evolved a variety of microtubule organizing centers (MTOCs) whose spatial distribution and activity are tightly regulated throughout the cell cycle [29]. The elemental unit for microtubule nucleation in cells is \(\gamma\)-tubulin. While in monomeric form it is a poor nucleator of microtubules [6], \(\gamma\)-tubulin is usually found in large (\(\sim 280 - 2,200\) kDa) protein assemblies (i.e. the \(\gamma\)-tubulin ring complex, \(\gamma\)-TuRC, and \(\gamma\)-tubulin small complex, \(\gamma\)-TuSC), which drive efficient microtubule nucleation [4–7]. Notably, the \(\gamma\)-TuRC is a ring-like structure with a diameter of \(\sim 25\) nm, which contains 13 vertically-staggered \(\gamma\)-TuSC units, and thus mimics the 13-protofilament architecture of microtubules [30]. The highest concentration of microtubule nucleating centers is found at the centrosome,
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which serves as the primary MTOC in most cells. During interphase, the centrosome is kept in tight association with the nuclear membrane, where it drives the polymerization of radial microtubule arrays from the cell center towards the cell edge. In contrast, during mitosis the duplicated-centrosomes migrate to opposite sides of the cell to drive the polymerization of the mitotic spindle. Additionally, non-centrosomal microtubule-nucleation and/or minus-end stabilization is a common feature in cells [31]. Notable examples include microtubule branching events driven by augmin at the mitotic spindle of animal and plant cells [32–35], microtubule nucleation and minus-end stabilization at the Golgi apparatus [36–39], microtubule minus-end capture at the apical membrane of polarized epithelial cells [40–43], and microtubule minus-end stabilization by the CAMSAP/Nezha/Patronin family of proteins [44, 45].

GTP-hydrolysis is tightly coupled to the polymerization dynamics and stability of microtubules [46–48]. However, hydrolysis is not required for polymerization, as slowly- (or non-) hydrolyzable GTP-analogs (i.e. GMPCPP and GMPPNP) can also promote microtubule growth [49, 50]. GTP hydrolysis (which occurs only at the β-tubulin part of the dimer [51]), is enhanced through polymerization [52], and results in destabilization of the microtubule structure. The manner by which GTP-hydrolysis destabilizes the microtubule has been the subject of extensive work [53]. For a long time the prevailing picture was that GDP-bound dimers are curved [54, 55], whereas GTP-bound dimers are straight [56]; consequently, it was postulated that GTP-hydrolysis would introduce strain in the microtubule lattice as the GDP-bound tubulin dimers tend to curl outwards. However, recent work has revealed that GTP and GDP-bound tubulin dimers have similar curvatures [57–59], and that straightening of the GTP-bound tubulin is induced only as it incorporates into the microtubule lattice [60, 61]. These observations are consistent with electron-microscopy studies which showed that polymerizing microtubules tend to flare outwards [54, 62, 63]. The precise conformational changes induced by GTP hydrolysis inside the microtubule have just been recently elucidated. Contrary to the previous picture, in which hydrolysis destabilized lateral tubulin contacts (which are weaker than their longitudinal counterparts [64]), it has been shown that hydrolysis and phosphate release are accompanied by a compaction of the longitudinal dimer-dimer interface, and a rearrangement of the α-tubulin subunits [65]. The ways by which these conformational changes introduce instability to the microtubule as a whole are yet to be elucidated.

Both in vivo and in vitro, microtubules undergo cycles of fast polymerization and even faster depolymerization, with transitions in between that are commonly called catastrophes and rescues (Fig. 1.2 c). This property of microtubule growth has been
termed dynamic instability, and it allows microtubules to quickly remodel in order to explore the cell interior efficiently [8]. The parameters of dynamic instability are tightly regulated across cell types [66], during the cell cycle [67], and even between distinct regions in the cell [68]. In mitotic and meiotic spindles, microtubule turnover takes place in the order of tens of seconds [67, 69], whereas in interphase microtubules turn over in the order of minutes [70]. These differences result from regulated changes in the balance between dimer and polymer concentration through the activity of tubulin-sequestering proteins such as stathmin [71, 72], as well as regulation of microtubule polymerases and depolymerases [73–75]. An emerging picture, worth noting, is that slight changes in microtubule dynamic properties can have huge effects on the stability of microtubule assemblies [76, 77].

The dynamic instability of microtubule growth is intimately related to the fact that the transition from GTP to GDP-bound tubulins does not occur instantaneously but with some delay after tubulin addition, resulting in what has been termed a stabilizing GTP-cap at the microtubule tip [48, 78]. The nature and length of the GTP-cap has been controversial. In vitro experiments in which microtubule catastrophes were induced by tubulin removal revealed that the GTP-cap consists of up to ∼200 tubulin dimers [79]. In contrast, later studies with GMPCPP-tubulin-capped microtubules suggested that a GTP-cap of ∼13 tubulin dimers is sufficient to stabilize microtubules against depolymerization [80]. In vitro measurements of tubulin on and off dynamics at microtubule tips revealed that even during growth-phases, microtubules can lose many layers of tubulin dimers without undergoing a catastrophe, and that the extent of polymer loss during these tip fluctuations increases with tubulin concentration [81]. This observation suggests that the length of the stabilizing cap at microtubule tips is probably longer than a couple of dimers. Recent work suggests that the GTP-cap is more likely to be an intermediate in the GTP-hydrolysis cycle, which is precisely the structure that is recognized by end-binding proteins (EBs) [82–85]. EB proteins are master regulators of microtubule dynamics and organization in cells, both intrinsically [86], but also by recruiting microtubule-associated proteins to the microtubule plus-tip [87]. In vivo measurements of cap-length obtained by imaging the distribution of EB proteins at microtubule tips estimate that the average GTP-cap consists of ∼750 tubulin dimers, extending ∼0.5 μm from the microtubule tip [88].

Microtubules are about 100- and 1000-fold stiffer than F-actin and intermediate filaments respectively (Fig. 1.1). A variety of methods have been applied to measure the bending rigidity, $EI$, of microtubules, such as analysis of filament thermal fluctuations and probing with optical tweezers. In the absence of microtubule-stabilizing agents, the
bending rigidity of microtubules is $\sim 8 \text{ pN} \mu\text{m}^2$ [89–91]. The addition of the microtubule stabilizing agent taxol tends to soften microtubules $\sim 3 \text{ pN} \mu\text{m}^2$ [89, 91], whereas polymerization with BeF$^{-3}$ (which occupies the site of the cleaved $\gamma$-phosphate, and thus mimics the GTP-Pi intermediate [84, 89]) increases the bending rigidity to $\sim 29 \text{ pN} \mu\text{m}^2$ [89]. The increased bending rigidity of BeF$^{-3}$-stabilized microtubules suggests that the GTP-cap could have distinct mechanical properties from the rest of the microtubule, which could play a key role in the sensitivity of microtubule growth to force [92, 93]. The bending rigidity of microtubules corresponds to a persistence length in the order of millimeters, which is larger than the average eukaryotic cell size ($\sim 5 – 15 \mu\text{m}$ [94]). This helps explain why microtubules often seem straighter than actin filaments or intermediate filaments in cells, whose persistence lengths are at or below the average eukaryotic cell size (Fig. 1.1).

### 1.1.2 EB and the family of microtubule plus-tip interacting proteins (+TIPs)

End-binding (EB) proteins were first identified as binding partners of the C-terminus of adenomatous polyposis coli (APC), and hence their name [95]. They are ubiquitously expressed proteins with homologues in all eukaryotes. EBs are small ($\sim 35$ kDa) dimeric proteins [96], which contain an N-terminal microtubule-binding domain (MTBD) made up of two calponin homology (CH) domains [97], a coiled-coil region which mediates dimerization, and two unstructured and negatively charged C-terminal tails that regulate their affinity for the microtubule lattice [98] (Fig. 1.3 a). Furthermore, EBs can adopt an auto-inhibited conformation through associations between the N-terminal MTBD and the C-terminal tails, which is thought to be relieved by binding to their many interacting partners in cells [99]. There are three main members of the EB family in mammals: EB1, EB2 and EB3. Both EB1 and EB3 autonomously recognize a feature of the microtubule growing end where they quickly exchange, displaying a localization pattern commonly termed the EB comet [86, 100] (Fig. 1.3 b). In contrast, EB2 tends to localize along the length of microtubules, with no clear preference for their growing ends [86]. Perhaps the most striking feature of EB1 and EB3 is not that they can autonomously localize at growing microtubule ends, but that, by doing so, they can enhance the dynamicity of microtubule polymerization. Both in vivo and in vitro studies have revealed that EB1 and EB3 promote faster microtubule polymerization [86, 101–104], while also induce catastrophes by accelerating the transition from GTP- to GDP-tubulin [100]. The microtubule-binding domain of EB lies at the interface between four tubulin dimers.
(except at the seam), in direct contact with the exchangeable GTP-binding site of β-tubulin [84]. *In vitro* studies revealed that EB recognizes an intermediate in the GTP-hydrolysis cycle, as it strongly binds to microtubules polymerized with the slowly-hydrolyzable GTP analog GTPγS, or with GTP and BeF$^{-3}$, which occupies the position of the hydrolyzed γ-phosphate ion after its release [83, 89]. Contrary to previous belief [82], EB does not bind microtubules polymerized from GMPCPP with high-affinity. Given the intermediate curvature of GMPCPP-stabilized microtubule protofilaments [105], which lies between that of the straight polymer-incorporated tubulin, and the curved dimeric tubulin, it is now thought that GMPCPP rather mimics the end-most structure of microtubule tips, where GTP has not yet been hydrolyzed [53]. This is the region where the potent microtubule polymerase XMAP215 binds, whereas EB localizes slightly behind [100, 106].

![Figure 1.3: EB protein structure and localization in cells.](image)

(a) Domain architecture of EB molecules: N-terminal microtubule-binding domain (MTBD) made up of two calponin homology (CH) domains [97], unstructured linker regions, coiled-coil region which mediates dimerization, EB-homology (EBH) domain which mediates interactions with SXIP-motif containing proteins [107], unstructured and negatively-charged C-terminal tails that regulate EB’s affinity for the microtubule lattice [98], and also mediate interactions with CAP-Gly-domain containing proteins. Interactions between the C-terminal tails and the MTBD mediate intramolecular inhibition [99]. (b) Fluorescence live-cell imaging micrograph showing the representative localization of mTFP1-labeled EB3 in MDCK dog epithelial kidney cells in culture. Figures taken from (a) [9] and (b) [108].

Coupled to their role as regulators of microtubule dynamics in cells, EBs recruit a large family of proteins, collectively called *plus-tip interacting proteins* (+TIPs), to the growing ends of microtubules [9, 87, 109–113] (Fig. 1.4). Typically these proteins display similar, fast on and off dynamics at microtubule tips, suggesting that their plus-end localization is regulated by EB’s exchange dynamics [114–116]. The +TIP family is large, and new members are still being discovered [113]. It includes proteins which stabilize
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microtubules (i.e. CLIP-170 [115, 117, 118] and sentin [119]), microtubule depolymerases (i.e. MCAK [120]), F-actin binding proteins (i.e. CLASPs [121], MACF/Shot [122–125], and APC [126, 127]), endoplasmic-reticulum-bound proteins (i.e. STIM1 [128]), regulators of F-actin polymerization (i.e. RhoGEF2 [129]), and adaptors of motor proteins (i.e. p150\textsubscript{glued} [130, 131]).

**Figure 1.4: The +TIP family of proteins and their interactions.** The SXIP-motif containing proteins (i.e. APC, MCAK, CLASP, ACF7, RhoGEF2, melanophilin and STIM1) interact with the EBH domain. The Cap-Gly-domain containing proteins (i.e. CLIP170, p150\textsubscript{glued}) interact with EB and CLIP-170’s EEY/F acidic tails. CLASPs also have TOG-like domains with which they can directly bind to the microtubule lattice. Figure taken from [9].

Broadly speaking, the +TIP family can be broken down into three subgroups (Fig. 1.4): The first two require EBs to localize at microtubule growing ends, while a third smaller group autonomously localize at microtubule tips [9, 132]. The first group, collectively called SXIP proteins, contain the conserved sequence SXIP, embedded within a stretch of positively-charged and serine-rich amino-acids, which has been called a microtubule tip localization signal (MtLS) [107]. This MtLS is recognized by a hydrophobic cavity (the EB homology domain, EBH, Fig. 1.3 a) at the end of the coiled-coil region of EB which only forms upon EB-dimerization [133]. Many SXIP proteins contain multiple copies of the MtLS, which serves to enhance their affinity (in the nanomolar range) for EB [134]. In contrast, serine phosphorylation in the vicinity of the MtLS has been shown to inhibit the binding of SXIP-proteins to EB [107, 127, 133, 135]. The second group of +TIPs that require EB to localize at growing microtubule ends are the CAP-gly family.
of proteins. These proteins contain a hydrophobic cavity with the conserved sequence GKNDG, which specifically recognizes the acidic C-terminal EEY/F amino acids of EB (Fig. 1.3 a), α-tubulin, as well as the C-terminal zinc knuckle domain (ClipZnF) of CLIP-170 [104, 130, 136]. The third member of the +TIP family are the TOG-proteins, which autonomously localize at growing microtubule ends independent of EB. In vitro and in vivo studies have revealed that, unlike EBs, the TOG-family of proteins localize to the edge-most tubulin dimers at the microtubule tip [100, 106]. Notable members of this family are: XMAP125, ch-TOG, CLASPs, and their homologues in lower eukaryotes [137]. TOG proteins are typically elongated proteins which contain more than one copy of the TOG-domain arranged in series. This architecture allows them to act as potent microtubule polymerases. For example, XMAP215 simultaneously localizes at microtubule tips while recruiting tubulin dimers from solution [138, 139]. Furthermore, in vitro reconstitutions of microtubule polymerization with EB1 and XMAP215 have shown that these proteins act in synergy, enhancing microtubule growth speeds to near cellular levels [140]. Often, +TIPs make use of more than one domain to enhance their plus-end localization (Fig. 1.4). For instance, p150Gluexed recognizes the EB-homology domain, but also associates with CLIP-170 through its CAP-Gly domain [9]. Similarly, SLAIN2 associates both with EB via an SXIP-motif, and with and chTOG [141], and CLASPs contain both SXIP and TOG domains [142].

The variety of EB-interacting partners thus renders the microtubule plus-tip a hub where many cellular activities are regulated [87]. Not surprisingly, many interactions between microtubules and F-actin take place at this site.

1.1.3 Filamentous actin (F-actin)

Actin filaments are double-stranded helical polymers, with a diameter of \( \sim 6 - 8 \text{ nm} \), which assemble through the longitudinal addition of globular actin (G-actin) monomers (Fig. 1.5). G-actin is a \( \sim 42 \text{ kDa} \) ATP-hydrolyzing protein, whose ATP-binding cleft is located at the center of the molecule. ATP (or ADP) binding at this site is also essential to stabilize the structure of the G-actin molecule in monomeric form [143, 144]. In humans there are three main G-actin isoforms, \( \alpha \), \( \beta \) and \( \gamma \). \( \alpha \)-actin is mostly present in muscle tissue, while \( \beta \)- and \( \gamma \)-actin are the main components of cytosolic F-actin networks in non-muscle cells.

As the polymer assembles, new G-actin units are incorporated with their ATP-binding pockets facing in one direction, thus endowing the actin filament with structural polarity.
It is common to term the structurally-different ends as pointed and barbed ends, also called the minus and plus ends (Fig. 1.5). This terminology arose from electron microscopy studies of F-actin decorated with the S1 fragment of myosin motors, in which the motor heads were observed to align with an arrowhead shape in the direction of the more slowly growing actin-filament end, which was thus called the pointed end [145].

The rate-limiting step of F-actin assembly in the absence of cofactors is the formation of dimers and trimers of G-actin [147–149] (Fig. 1.5). After this slow nucleation step, F-actin polymerization proceeds from both pointed and barbed ends if the soluble G-actin concentration is sufficiently high. The rate of polymerization is markedly different at each end, and strongly depends on the concentration of divalent cations in solution [150, 151], as well as the ionic strength of the buffer [152]. In favorable conditions for F-actin polymerization in vitro (i.e. 50 mM KCl, 1 mM MgCl2, 1 mM EGTA, 50 μM CaCl2, 0.2 mM ATP, 0.5 mM DTT and 10 mM Imidazole, at 22°C [153]), it was found that the critical ATP-G-actin concentration for assembly at the pointed end \( C_{c-} = 0.6 \, \mu M \), is five-fold higher than that at the barbed end \( C_{c+} = 0.12 \, \mu M \). Thus, at steady-state, and
as a direct result of the different on- and off-rates of G-actin at barbed and pointed ends, actin filaments undergo a phenomenon called *treadmilling*, in which subunit addition at the barbed end is compensated by subunit loss at the pointed end (Fig. 1.5). This results in a flux of G-actin units along the polymer, even though the filament length remains constant [154, 155]. Concomitantly, barbed-end polymerization, pointed-end depolymerization, and their modulation by accessory proteins (i.e. profilin, capping protein, and cofilin, Fig. 1.5) [156, 157], are the main drivers of F-actin homeostasis in cells [158].

Similar to GTP-hydrolysis in microtubules, ATP-hydrolysis is intimately related to the polymerization dynamics of actin filaments. During filament elongation, incorporation of ATP-bound G-actin is quickly followed by ATP-hydrolysis [151], while phosphate (Pi) release proceeds more slowly. This results in the formation of a stabilizing ADP-Pi cap at the barbed end of the growing filaments [159, 160] (Fig. 1.5). Phosphate release is accompanied by destabilization of intermolecular bonds along the filament, as well as a reduction in filament bending rigidity [161, 162]. Subject of much debate, it has been recently demonstrated that phosphate release is a random process along the filament that proceeds with a half-time of ∼100 s [163]. Besides destabilizing the filament, ATP-hydrolysis also has profound effects on the association of actin-binding proteins (ABPs). For instance, the F-actin nucleator Arp2/3 [164] preferentially mediates branching from newly-polymerized F-actin regions [165], whereas the F-actin severing enzyme cofilin preferentially acts on ADP-containing portions of actin filaments [166].

In non-muscle cells, the cytoplasmic G-actin concentration is typically in the range of ∼50 – 100 μM [167]. This high monomeric pool is maintained through the activity of filament severing [156] and capping proteins [158], as well as G-actin sequestering proteins, such as profilin and thymosin-β4 [168, 169]. Both profilin and thymosin-β4 inhibit spontaneous F-actin nucleation, while profilin also participates in nucleotide exchange [170] (Fig. 1.5), and barbed-end F-actin polymerization either directly [171, 172], or through association with profilin-binding F-actin polymerases, such as the formin family of proteins [173].

Actin filaments are softer than microtubules, but stiffer than intermediate filaments (Fig. 1.1). A variety of methods have been applied to measure the bending rigidity of actin filaments, such as analysis of thermal fluctuations [162, 174, 175], and direct mechanical probing with optical tweezers [176]. Most of these studies were performed with phalloidin-stabilized actin filaments, converging on a bending rigidity in the order of ∼0.07 pN μm², while in the absence of phalloidin the bending rigidity is approximately
two-fold lower $\sim 0.04 \text{ pN \, \mu m}^2$ [162]. Such bending rigidities correspond to actin filament persistence lengths in the order of $10 - 20 \text{ \mu m}$ at room temperature (Fig. 1.1). \textit{In vitro} reconstitutions of spontaneous F-actin polymerization in the absence of cofactors have revealed that at steady state the distribution of filament lengths is exponential, in the range of $1 - 20 \text{ \mu m}$, depending on the initial G-actin concentration [177, 178]. Thus, at least \textit{in vitro}, actin filaments can be comparable to, or longer than, their persistence length, and as such are considered to be semi-flexible polymers. However, in cells actin filaments are generally much shorter. For instance, in fission yeast, the average filament length in Arp2/3-nucleated cortical patches is in the order of $50 - 100 \text{ \mu m}$ [179, 180], while in endothelial cells the average filament length is in the order of $0.5 - 3 \text{ \mu m}$ [181]. This is the result of the many accessory proteins (i.e. severing, capping, branching proteins) that participate in F-actin length regulation \textit{in vivo}. Thus, in cells, actin filaments are on average shorter than their persistence length. This is likely to have direct implications on their mechanical behavior, since at these length scales they behave closer to a stiff rod than to a semi-flexible polymer. In agreement with this, \textit{in vitro} rheological studies of F-actin networks cross-linked by filamin revealed that with decreasing average filament length (by the addition of the actin-severing protein gelsolin), the networks behaved more as a collection of stiff polymers cross-linked by flexible linkers [182], in which the elasticity of the network was dominated by the compliance of the cross-linkers, rather than by the F-actin itself.

In cells, actin filaments organize into higher-order structures with the aid of several accessory proteins [183–185] (Fig. 1.6). The list of actin-binding proteins (ABPs) is large, and includes proteins that: cross-link (fimbrin, filamin), bundle ($\alpha$-actinin, fascin), cap (gelsolin, capping protein), nucleate (formin, Arp2/3), and sever (ADF/cofilin) actin filaments, among others [186]. In different combinations, ABPs help build F-actin structures that support a variety of cellular functions, such as maintaining cellular shape during mitosis and interphase; coupling the cell interior with the extra-cellular matrix; providing protrusive and retractive forces at the front and rear of the cell; as well as acting as contractile gels that regulate the mobility of membrane-bound and cytosolic components. Broadly speaking, these F-actin superstructures can be grouped into the following categories:

- Actomyosin cortex: a thin ($\sim 190 \text{ \mu m}$ [187]) F-actin network that lines the membrane of animal cells. The cortex is primarily responsible for maintaining cellular shape both during interphase and mitosis [188–190], but is also involved in the segregation of polarity components in asymmetrical cell divisions [191, 192].
Recent work on isolated blebs of HeLa and melanoma M2 cells elucidated the proteomic content of the F-actin cortical network, revealing that it is composed of $\sim 170$ proteins. These include actin-bundling proteins, several members of the myosin-motor family, actin-filament capping proteins, membrane-anchoring proteins, and regulators of F-actin polymerization and contractility [193].

• Lamellipodium: an actively treadmilling actin filament network located beneath the plasma membrane at the leading edge of motile and growing cells [194, 195] (Fig. 1.6). The lamellipodium is a highly dynamic structure that requires a constant flux of G-actin monomers to maintain its protrusive activity. To this end, a variety of accessory proteins are required, such as capping proteins, ADF/cofilin and gelsolin (severing proteins), and profilin (monomer-sequestering proteins), among others. The lamellipodium is composed of a branched network of short actin filaments whose fast-growing barbed ends point on average in the direction of the leading edge [196, 197]. F-actin polymerization in this region is mainly driven by branching events from existing filaments through the activity of the Arp2/3 complex [164, 198, 199], which is activated by membrane-bound WAVE/Scar and N-WASP complexes [200, 201]. Membrane-anchored F-actin polymerases such as the formins and the Ena/VASP proteins [202] contribute to the lamellipodial F-actin pool as well, by generating parallel bundles of actin filaments called filopodia and microspikes. Besides aiding in the protrusive activity of the cell, these F-actin bundles are the seeds for the formation of stress fibers in the lamellum. The lamellipodium thus constitutes the main driver of membrane protrusion, since it orchestrates the force-generation of individual polymerizing actin filaments (with stall forces below 1 pN [203]), into protrusive machines that collectively generate forces in the order of hundreds of nanoNewtons [204].

• Lamellum: localized immediately behind the lamellipodium, the lamellum is a $\sim 5 - 10$ μm-wide structure in which the newly polymerized F-actin from the lamellipodium is reorganized into contractile acto-myosin bundles that align parallel to the leading edge of the cell [205] (Fig. 1.6). Concomitantly, the lamellum is rich in actin bundling and cross-linking proteins such as $\alpha$-actinin, filamin, and myosin II [206, 207]. While eventually some of these bundles are incorporated into the stress fiber network of cells, their primary role in this region is to generate a zone of high contractility that disassembles the incoming F-actin network polymerized in the lamellipodium [208–212], thus driving retrograde actin flow [213]. What exactly triggers the transition from a lamellipodial- to a lamellar-like F-actin network is subject of debate. It has been proposed that adhesions to the
Figure 1.6: Multiplicity of F-actin structures in cells. Structured illumination microscopy image showing the diversity of actin filament structures in a crawling cell: in the lamellipodium at the leading edge of the cell, actin filaments are actively polymerized into a dense dendritic network interspersed with parallel bundles of actin filaments. These newly polymerized filaments are later on reorganized into contractile actin bundles in the lamellum. The combination of actin-filament polymerization at the lamellipodium and contractility in the lamellum drives retrograde actin flow. The contractile bundles of actin filaments created at the lamellum later on become dorsal actin arcs and actin stress fibers, which couple the cell to the extra-cellular environment. Figure taken from [220].

extra-cellular matrix might be responsible for this event, since the formation of adhesion sites co-localizes with the boundary between the two structures [195, 214]. Finally, cells with inhibited lamellipodial formation still exhibit leading-edge protrusion and migration. Thus, the individual roles of lamellipodium and lamellum in driving cell migration are not completely unraveled [215].

- Stress fibers: In the cell interior, actin filaments organize into an array of thick bundles that can at times span the whole length of the cell (Fig. 1.6). In motile cells, these bundles form through the accretion of actin filaments polymerized at the lamellipodium, and the recruitment of cross-linkers and myosin-II motors [216]. Depending on their localization and contractile properties, they can be classified into three types: Ventral stress fibers are myosin-II and α-actinin-rich anti-parallel bundles of F-actin that terminate at focal adhesion sites on each end. These bundles are under tension through myosin-contractility and are involved in focal adhesion maturation [217], and force-transduction to and from the cell exterior [218]. Dorsal stress fibers are non-contractile bundles of actin that are
anchored at a focal adhesion site on one end, and to transverse actin arcs on the other, while some are precursors of ventral stress fibers [216]. Finally, transverse arcs are contractile acto-myosin bundles localized on the dorsal surface of cells, which align parallel to the leading edge [216, 219]. Recent super-resolution live-cell microscopy studies on dorsal fiber and transverse arc dynamics revealed that myosin-powered contractility leads to actin-arc shrinkage and rearward flow [219]. This rearward flow pulls on dorsal stress fibers resulting in a flattened lamellar region [220]. In non-motile, epithelial and endothelial cells, stress fibers are also present and often terminate at cell-cell junctions [221, 222].

- Cytokinetic rings: A hallmark of dividing cells, the cytokinetic ring is a membrane-anchored contractile bundle of actin filaments, mainly polymerized by formins and cross-linked by myosin II motors, although many more key players of F-actin organization are present as well (i.e. α-actinin, septins, filamin, tropomyosin and the Arp2/3 complex [223]). The position of the cytokinetic ring defines the plane of cell division, and is tightly regulated through interactions between the actin and microtubule cytoskeletons. Although ring contraction is the main driver of cell constriction [224], actin-filament depolymerization has been shown to also contribute to this process [225].

The turnover of actin filaments is intimately related to the structures to which they belong. Studies of F-actin turnover in cells revealed that in quickly remodeling structures (i.e. lamellipodia) the actin-filament half-life is in the order of tens of seconds [226], while in more stable structures, such as stress fibers and the cytokinetic ring, the average half-life is in the order of minutes [227, 228]. The short life-times of actin filaments in actively remodeling regions in cells is the result of treadmilling combined with the activity of severing proteins such as gelsolin, which can accelerate actin-filament turnover two-fold [181].

1.1.4 Intermediate filaments (IFs)

Unlike F-actin and microtubules, intermediate filaments belong to a wide family of proteins, which in humans comprises approximately 70 different genes [229–231]. Among these, the most notable members are the cytoplasmic IFs: keratin (ubiquitous in epithelial cells), vimentin (ubiquitous in mesenchymal cells) and neurofilaments (ubiquitous in neuronal cells), and the nuclear lamins (which form a stabilizing meshwork adjacent to
the inner nuclear membrane and are present in all animal cells) [229, 232]. Intermediate filaments are expressed in most eukaryotes, with the most notable exceptions being plants and fungi. Cytoplasmic IFs are also absent from insects (with one known exception [233]), which do however express nuclear lamins.

Despite their genetic variability, the structure of intermediate-filament proteins is highly conserved. A single IF protein is an elongated molecule (∼45 nm) which contains a central α-helical rod domain flanked on each side by non-helical head and tail domains that vary widely in length, sequence and structure [234] (Fig. 1.7). These monomers assemble in two sequential steps to form a tetramer, which is the basic soluble unit of IFs. First, two monomers assemble in an anti-parallel fashion to form a dimer, by association of the central rod domains into a coiled-coil structure [235]. This step is followed by the assembly of two dimers in a partially staggered anti-parallel fashion to form the tetramer. The tetramers are thus symmetrical, which renders intermediate filaments structurally apolar (Fig. 1.7). In vitro reconstitutions of vimentin polymerization have revealed that the soluble tetramers assemble into an ∼60 nm-long structure termed the unit-length filament (ULF) [235, 236]. Through end-to-end annealing, these ULFs polymerize into elongated filaments of ∼16 nm in diameter. The final step in IF assembly is a compaction event which shrinks the filaments down to a diameter between 7-11 nm [237, 238] (Fig. 1.7).

Unlike microtubules and actin-filaments, intermediate filaments in cells are rather long-lived. In general, IF depolymerization is a triggered event [239–242], such as in mitosis, where lamin phosphorylation leads to nuclear-lamina disassembly as required for nuclear-envelope breakdown [243]. Furthermore, there is evidence that IF proteins can be incorporated laterally onto existing filaments without the need for polymer disassembly [244]. Fluorescence recovery after photo-bleaching experiments have revealed that intermediate filaments have turnover times in the order of tens of minutes, while at the whole network level, the turnover times are in the order of hours [242, 244, 245] (Fig. 1.1). In cultured cells, the majority (∼97 – 99% [246]) of intermediate filament protein is typically found in polymerized form. However, it has been recently found that this balance strongly depends on the stiffness of the substrate on which the cells are plated [246].

Intermediate filaments are the softest of the three cytoskeletal polymers, with a persistence length of ∼0.5 – 1 μm [247, 248] (Fig. 1.1). Their structural core, made up of aligned coiled-coil domains stabilized by strong hydrophobic interactions, provides IFs with remarkable mechanical properties [249]. For instance, individual IFs can be
Figure 1.7: Steps in intermediate filament assembly. First, two monomers assemble into a dimer, by association of the central rod domains into a coiled-coil structure [235, 236] (not shown). Second, two dimers associate into a tetramer in a parallel staggered fashion to form a symmetrical tetramer that renders the intermediate-filament structure apolar. Third, through lateral associations the tetramers form a unit-length filament (ULF) [235, 236], which through end-to-end annealing with other ULFs polymerizes into intermediate filaments (IFs) with a $\sim 16$ nm diameter. The final step in IF formation is a compaction event (not shown), that shrinks the IF diameter down to $\sim 7 – 11$ nm [237, 238]. ©2014 Nature Education. All rights reserved.

stretched up to 3.5-fold their original length without breaking [250]. Furthermore, rheological measurements on vimentin IF networks showed that IFs tend to become stiffer with increasing strain [251–253]. The mechanical resilience of intermediate filaments stems from their plasticity, as applied forces can induce partial unfolding and sliding of the individual subunits without triggering breakage or depolymerization [254].

Given their mechanical resilience, relatively slow turnover times, and the fact that IFs typically form well-connected networks of filaments and bundles that span the length of the cell [255–257] (Fig. 1.1), has led to the picture of intermediate filaments as being the mechanical integrators of cells. They are thought to provide mechanical stability against external forces, and also to integrate the much faster cytoskeletal-remodeling events carried out by microtubule and F-actin networks. Finally, as a consequence of their long life-times, IF networks can also act as scaffolds for many signaling pathways [258].
1.2 Cytoskeletal interactions

Cytoskeletal interactions between intermediate filaments, microtubules and F-actin are essential for eukaryotic cells to execute vital processes such as migration, growth, division and polarization [232, 259–262]. Historically, however, these three cytoskeletal systems have been studied independently, although the importance of their interactions has been long recognized [263, 264]. Some of the first direct evidence of microtubule-actin interactions came from in vitro reconstitutions with *Xenopus laevis* egg-extracts. In this studies, actin filaments were observed to consistently co-align and move with microtubules nucleated from demembranated *Xenopus* sperm cells [265, 266]. Conversely, microtubules were observed to inhibit cortical F-actin flows in *Xenopus* oocytes [267].

A chief obstacle in understanding how F-actin, microtubules and intermediate filaments affect each other’s organization lies in identifying when and where in the cell they interact. This is largely due to the inherent crowdedness of the cell, the density of intermediate filament networks, the diversity of F-actin structures (i.e. stress fibers, filopodia, dendritic and cortical networks), and the complicated trajectories that growing microtubules follow (Fig. 1.1). Furthermore, in cells it is often challenging to disentangle mechanical cross-talk mediated by cross-linking proteins, from biochemical interactions mediated, for instance, by the Rho family of GTPases [268, 269]. Notwithstanding, several proteins and protein complexes capable of cross-linking all three polymer systems have been identified. Some of these form passive cross-links [270, 271]; while others mediate active connections, be it through molecular motors [272–275], actin filament nucleators [276, 277], or +TIPs [125, 278–280].

In this section we focus on interactions between actin and microtubules, and mention intermediate filament networks where it is due. We propose (inspired by a previous review [260]), that the many modes of actin-microtubule cross-talk can be narrowed down into distinct functional groups: 1) steric interactions, 2) passive cross-linking, 3) microtubule plus-end capture, 4) guidance of microtubule growth, 5) actin-filament nucleation, and 6) biochemical interactions (Fig. 1.8). We relate these modes of interaction to the basic mechanical and dynamic properties of actin and microtubule filaments, in order to understand the physical basis by which they modulate the activity of actin-microtubule cross-linkers.
Figure 1.8: Cytoskeletal interaction modules. (a) Microtubule (MTs), actin filament (AFs) and intermediate filament (IFs) systems overlap in different regions of the cell. In these regions, steric interactions may play a key role. Furthermore, these systems can also interact through the activity of molecular motors, static cross-linkers, and biochemical signaling. (b) Different modes of cytoskeletal cross-talk between actin (cyan) and microtubules (red) that can be mediated by cross-linking proteins. Panel (a) was partially modified from [232].
1.2.1 Steric interactions and passive cross-linking

The simplest mode of cytoskeletal interaction would be where filaments, by nature of occupying space, sterically interfere with the mobility of neighboring polymers. These types of interactions, while oft-ignored, are likely to occur in regions where the cytoskeletal networks are densest (Fig. 1.8 a): at the cell interior (between IFs and microtubules), and in the cell periphery (between IFs and F-actin, and between microtubules and F-actin).

Steric interactions have a drastic impact on the organization and mechanical properties of cytoskeletal networks. A detailed picture of the mechanical reinforcement that steric interactions provide has come from in vitro rheological studies on composite polymer networks. For instance, the addition of a small number of microtubules to cross-linked F-actin networks promotes their non-linear stiffening [281]. This property stems from the high bending rigidity of microtubules, and it may be used by cells as a mechanism to respond to excessive deformation [281]. The addition of myosin-II molecules to these experiments revealed that contractile F-actin networks can on the one hand induce high-curvature microtubule buckling (as commonly observed in cells [282, 283]), while on the other hand mechanically reinforce the microtubules so that they can withstand higher compressive forces before breaking [284]. In a similar fashion, it has been shown that composite vimentin-actin networks have a higher stiffness than their isolated counterparts [285].

In cells, the role of steric interactions has also been probed by subjecting cells to external forces. Shearing cells by fluid flow has revealed that cells require the quick (minute time-scale) contractile response of F-actin networks in order to immobilize keratin intermediate filaments, which adjust their organization in the time-scale of hours [257, 286]. Further evidence has come from observations of F-actin-induced mobility of other cytoskeletal polymers. Both microtubules and intermediate filaments are often translocated by retrogradely-flowing and contractile F-actin networks [245, 287, 287–289]. For instance, in neuronal growth cones, translocating F-actin arcs push and compact microtubules into densely-packed bundles which are needed to stabilize the central structure of the growth cone [290, 291]. Furthermore contractile F-actin networks at the lamellum have been observed to buckle and break microtubules, a process required for proper microtubule turnover [282, 291, 292]. Conversely, during acute growth cone extension, persistent microtubule polymerization attenuates the rate of retrograde F-actin flow [293]. Similarly, intermediate-filament organization is highly dependent on the retrograde-flow transport process, as de novo filament formation often occurs at
the cell periphery, and these IF precursors need to be translocated to the cell interior to become incorporated into the cellular IF network [288]. Steric interactions also play a key role in plant cells, whose cytoskeletal cortical array is dominated by microtubules anchored to the plasma membrane [294]. These membrane-stabilized microtubules not only act as templates for further microtubule polymerization [295], but also guide F-actin polymerization [273, 296].

A likely example *in vivo* steric interactions, although passive cross-linkers could be also at play, involves the actin-driven inhibition of cytosolic flows required for the timely establishment of polarity in developing *Drosophila* oocytes. In this context, a cytosolic F-actin mesh nucleated by cappuccino and spire (*Drosophila* homologues of formins 1 and 2, and spire in humans, [297–299]), inhibits the mobility of microtubules driven by cytosolic flows generated via kinesin-1 vesicle transport [300]. In the absence of this F-actin mesh, microtubules are prematurely translocated to the cell cortex. Similar F-actin meshes are also used by large oocytes to orchestrate spindle-positioning and chromosome-condensation during meiosis [301, 302]. For instance, in mouse oocytes, a formin/spire-nucleated actin-mesh forms a cage around the spindle, and transports it towards the cell cortex through myosin-II pulling forces [303–306].

Thus, steric interactions on the one hand enable cells to synergistically utilize the mechanical properties of the three cytoskeletal systems to increase their mechanical stability, while on the other hand they act as a driving force for cytoskeletal turnover and organization. A chief requirement for steric interactions to play a functional role is that one of the cytoskeletal systems is mechanically reinforced in order to act as an efficient obstacle for other systems. In intermediate-filament and F-actin networks, filament cross-linking serves this purpose; while in the cortical microtubule arrays of plant cells, membrane-anchoring coupled with the high microtubule bending rigidity do the job.

The addition of passive cross-linkers further enhances the effect that steric interactions have on cytoskeletal mechanical stabilization. In this context, the plakins and spectraplakins are worth noting, as they often contain in one molecule binding sites for all three cytoskeletal polymers [307–309]. For instance, the *Drosophila* spectraplakin Shot cross-links composite bundles of F-actin and microtubules in tendon cells. Since *Drosophila* lacks cytoplasmic intermediate filaments altogether, in this context the actin and microtubule cytoskeletons are organized by Shot to provide mechanical support
and resistance against pulling forces [310]. Another well-known actin-microtubule crosslinker is the protein MAP2, which can bind to and bundle actin filaments and microtubules [311]. MAP2 is required for neuronal mechanical stability, and its actin-microtubule cross-linking ability is required for neurite initiation. This probably occurs by stabilizing the F-actin protrusive machinery via the mechanical rigidity of microtubules.

1.2.2 Microtubule plus-end capture at F-actin structures

Capture and stabilization of microtubule ends is an important requirement for cells to position the mitotic spindle and to maintain cytoskeletal organization during cell-migration and apico-basal polarization of epithelial cells [312–320]. Microtubule plus-end capture has been observed in cells through the action of minus-end directed dynein motors, as well as through +TIPs (Fig. 1.8 b). The fact that end-on forces slow-down microtubule polymerization and are potent triggers of catastrophe [92, 93], suggests that for efficient microtubule capture the proteins involved must somehow stabilize microtubules against full depolymerization, be it by promoting rescues or by inhibiting catastrophes.

The best studied example of cortical microtubule plus-end capture involves the minus-end directed motor dynein [321]. This interaction allows dynein molecules to participate in spindle positioning in small cells, by capturing and pulling on astral microtubules that span the length from the spindle poles to the cortex. The position of the spindle is a key determinant of the location of the cytokinetic ring, whose proper localization ensures the segregation of cell-fate determinants in asymmetric cell divisions [320]. The mechanisms by which dynein stabilizes microtubule ends have been elucidated through in vitro reconstitutions of microtubule end-on interactions with dynein molecules. These studies revealed that dynein can inhibit microtubule growth, trigger catastrophes, and exert pulling forces on shrinking microtubules, in an ATP-dependent manner [322, 323]. This requires that dynein can bind around the circumference of the microtubule, as dynein fails to stabilize microtubules against depolymerization in a gliding-assay configuration [322]. In cells, the cortical localization of dynein is dependent on a large protein complex (NuMa/LGN/Gαi), which is highly conserved across species and whose cortical distribution is tightly regulated [320, 324, 325]. The fact that the NuMa/LGN/Gαi complex binds directly to the plasma membrane (independent of F-actin), suggests that the acto-myosin cortex may rather serve to stabilize its membrane localization [326], thus allowing cortically-anchored dyneins to exert (∼70 pN [327]), without detaching.
Cortical capture of microtubule plus-ends at the leading edge of migrating cells, and in polarizing epithelia, can also occur in the absence of molecular motors. At the core of these interactions lies the +TIP APC [126, 328–332], which participates in different microtubule-binding complexes with EB1/3 and other members of the +TIP family; notably, CLASP, CLIP-170 and MACF. APC requires F-actin for its cortical recruitment [333], where it binds IQGAP [334] and formin [335]. In addition, APC also binds to the plasma membrane associated protein AMER1 [336, 337]. A variety of in vivo studies have revealed that APC and IQGAP act in concert to form a cortical scaffold for the recruitment of CLIP1-170 [338], CLASP [135], and MACF [339]. Although these three proteins rely on EB to track growing microtubule ends, they also have microtubule-lattice binding domains (Fig. 1.4), which are likely involved in their roles as microtubule stabilizing agents.

In vivo, CLASPs have been shown to accumulate along the microtubule lattice at the leading-edge lamellum and lamellipodium. These CLASP-associated pioneer microtubules [292] grow more persistently, but twice as slow, as microtubules in the cell interior [314, 315, 340]. CLASPs have also been observed to capture microtubules in the vicinity of focal adhesions (FAs), where they are anchored to the membrane by LL5β, a PIP3-binding protein [341, 342]. Microtubule capture at FAs is required for the establishment of the kinesin-dependent delivery of FA and extra-cellular matrix (ECM) degradation factors, thus ensuring cell-detachment and migration [343, 344]. In vitro reconstitutions of microtubule growth in the presence of the S. pombe CLASP homologue Cls1p, revealed that CLASPs mediate rescues in regions of the microtubule lattice where they are locally concentrated [345]. It has been postulated that CLASPs induce rescues by delivering tubulin dimers to the microtubule tip via their TOG-domains. Consistent with this, CLASPs are required for kinetochore-fiber elongation [346], and they enhance microtubule polymerization in vitro [347]. However, whether CLASPs can indeed recruit tubulin dimers to the microtubule tip has not yet been formally demonstrated. A second, more viable alternative could be that CLASPs, by nature of binding the microtubule lattice can somehow slow-down depolymerization, increasing the chance of microtubule rescue. The fact that CLASPs can induce microtubule bundling [348] and also stably anchor microtubule minus-ends at the Golgi-apparatus [38] supports this notion. Whether, as in the case of dynein [322], CLASPs must bind around the circumference of microtubules to effectively stop them from shrinking, is yet to be elucidated.

The role of CLIP-170 in microtubule stabilization is less clear. Some observations point at CLIP-170 being a rescue factor [349], while others suggest that CLIP-170 rather
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acts as an anti-catastrophe factor \cite{115, 350}. Electron-microscopy studies showed that CLIP-170 can induce the formation of tubulin oligomers \cite{351}. It was thus proposed that CLIP-170 localizes to the microtubule tip by co-polymerizing with tubulin, and that this property allows it to enhance microtubule rescues by delivering tubulin to the microtubule tip. However, further \textit{in vitro} reconstitutions showed that CLIP-170 requires both EB and tyrosinated α-tubulin to track and quickly-exchange at microtubule tips \cite{115}. Furthermore, it was shown that CLIP-170 has a negligible effect on microtubule growth speeds (arguing against the co-polymerization model), and that it is not a potent microtubule rescue factor \cite{115, 118}. Similar observations were made for the fission-yeast CLIP-170 homologue Tip1p, although a two-fold decrease in catastrophe frequency was reported \cite{104}. These observations suggest that CLIP-170’s microtubule lattice affinity may not be high enough to trigger microtubule rescues. One likely scenario could be that through its dual association with the microtubule lattice and EB, CLIP-170 enhances the residence time of EB on the microtubule-tip, and thus reduces the catastrophe frequency. Another possible scenario is that CLIP-170’s \textit{in vivo} role as a rescue factor involves its association with CLASPs \cite{349}.

MACF (also called ACF7 in mammals \cite{279}, and Shot in \textit{Drosophila}) has also been implicated in microtubule capture at the leading edge of motile cells and in polarized epithelial cells \cite{339}. Besides its APC-mediated cortical targeting, MACF also contains an N-terminal F-actin-binding domain which is required for its localization at F-actin-rich regions in the cell edge and at adherens junctions \cite{123, 352}. The C-terminus of MACF contains a microtubule binding domain (MTBD) encompassing a GAR-domain and an SXIP-containing MtLS. C-terminal fragments of MACF have been observed to bind and stabilize microtubules against depolymerization \textit{in vivo} \cite{123, 125, 353, 354}. \textit{In vitro} reconstitutions with MACF are notably lacking. However, the observation that MACF-localization in actin-rich regions correlates with elongated EB1 plus-end intensity profiles \cite{123, 125} suggests that these proteins could act in concert to stabilize microtubules, possibly by creating an extended zone of binding to the microtubule lattice.

Finally, IQGAP and APC participate in yet another microtubule-capture and stabilization complex required for polarized cell migration \cite{313}. This complex involves both EB and the F-actin polymerase and Rho-effector formin mDia1 \cite{355–357}. IQGAP is required for the cortical localization of Rho-activated mDia1 \cite{358}, which in turn recruits EB and APC in a microtubule-dependent manner \cite{335}. \textit{In vitro} work has revealed that mDia1 and mDia2 directly bind microtubules through their FH1-FH2 domains, and in doing so can slow-down both microtubule growth and shrinkage.
This type of stabilization mechanism is potent and long-lived, as formin-associated microtubules accumulate post-translational modifications indicative of long-term stabilization [360]. The same microtubule-capture mechanism is present in budding yeast, where homologues of APC, EB and formin anchor astral microtubules at the daughter-cell cortex to position the spindle [280, 361–363]. In this context, cortically-anchored dynein is not required for microtubule plus-end capture, but rather to reel-in the spindle to the daughter cell after astral microtubules have been captured [363].

The multiplicity of microtubule-capturing complexes at F-actin structures, many of which are to some extent orchestrated by the APC/IQGAP scaffold, suggests that perhaps not one mechanism is sufficient, but rather that cells utilize them in concert. A mechanistic understanding of the ways by which these microtubule binding +TIPs differentially participate in microtubule capture and stabilization at F-actin structures is still lacking.

1.2.3 Guidance of microtubule growth by F-actin structures

Contrary to the effects that retrogradely-flowing and contractile F-actin networks have on microtubule organization in cells, which can both translocate and induce microtubule breakage [282, 292], bundles of F-actin seem to act as positive guides for microtubule growth (Fig. 1.8 b). From a mechanical standpoint, there are three requirements for successful microtubule guidance: first, capture and stabilization of the microtubule tip, second, mechanical deflection of the stiff microtubule lattice by the F-actin bundle, and third, that the microtubule and F-actin bundle remain associated. The best studied examples of microtubule guidance occur in crawling cells and growing neurons, where microtubule growth is targeted along actin-stress fibers in the cell interior, and along actin bundles inside filopodia at the growth-cone periphery. In mammalian cells this guidance is mediated by the +TIP MACF, whose homolog in Drosophila is called Shot [279, 354].

As explained above, guidance of microtubule growth by stress fibers is required for microtubules to be captured in the vicinity of FAs by CLASPs, where they become tracks for the delivery of FA and ECM degradation factors to ensure cell detachment and motion [279, 342–344, 364–367]. A key aspect of this process remains unanswered: namely, what the mechanical constraints are for successful capture and redirection of microtubule growth. Studies of cells plated on adhesive micro-patterns have revealed that microtubules can undergo sharp deformations upon encounters with a stress fiber,
and that they remain attached to the fiber as they continue to grow [368]. This suggests that cross-linking proteins must be present not only at the microtubule tips, but all along the actin-microtubule interface, in order to maintain their tight association. However, MACF is not particularly enriched along stress fibers, but rather has been observed to localize at microtubule growing ends and in actin-rich regions in the cell edge and near FAs [279]. Two scenarios are thus possible: one, that the subset of microtubules that are captured is at least already partially co-aligned with the stress fiber; or two, that MACF’s interaction with the stress fiber is dependent on the presence of an overlapping microtubule. The second scenario is supported by recent evidence that Shot can adopt an auto-inhibited conformation in which only its MtLS is exposed. This observation suggests that encounters with F-actin structures may release the auto-inhibition, thus exposing Shot’s actin-binding and microtubule-binding domains [369].

In neuronal growth cones, guidance of microtubule growth by filopodia is required for axonal extension [124, 291, 354]. In this context, microtubules have been implicated in dictating the direction of growth, as local microtubule stabilization by taxol induces growth-cone turning in the direction of drug application, while local depolymerization with nocodazole has the converse effect [370]. However, the precise ways by which invading microtubules aid the actin protrusive machinery are not yet clear. Since growth-cone advance is inversely proportional to the rate of actin retrograde flow [213], one possibility is that invading microtubules act as mechanical struts against retrograde flow. Microtubules that co-align with filopodia often remain attached to, and are translocated rearwards by, the polymerizing F-actin bundle, resulting in short-wavelength microtubule buckling [291]. Similar observations of microtubule buckling in cells have revealed that the elastic environment provided by the surrounding F-actin networks can aid microtubules to withstand compressive forces in the order of tens to hundreds of picoNewtons [283, 371]. This range of forces may be sufficient to at least locally slow-down retrograde actin flow [372], and thus promote forward motion. Yet another lingering question in this context regards the ways by which microtubules selectively invade the peripheral zone of the growth cone along filopodia, since in conditions where filopodia are removed without disturbing the surrounding dendritic F-actin network, microtubules are observed to better-invade the peripheral zone [373]. Recent observations suggest that the +TIP drebrin, which localizes at the filopodial-base, may act as a gate to guide microtubules along bundles [270].

In the above-mentioned examples, microtubule guidance occurs by F-actin bundles that are anchored to their surrounding environment: in the case of stress fibers through adhesions to the ECM; and in the case of filopodia by the neighboring lamellipodial
actin meshwork. However, instances of microtubule guidance exist in which the F-actin bundle is not mechanically anchored to its surroundings. The best-studied example of this occurs in budding yeast, where microtubule growth is guided through the action of the +TIP Kar9 (the yeast homolog of APC), which binds microtubule ends via Bim1 (the yeast homolog of EB1), and which is also associated with the myosin V myo2, which guides the microtubule tip along actin cables polymerized at the cell cortex by the formin bni1 [272, 280, 361, 363, 374, 375]. Given that Bim1, like EB1, quickly exchanges at microtubule tips poses the problem of microtubules losing the connection with the actin cables. Recent in vitro reconstitutions of microtubule guidance by template microtubules through plus-end tracking kinesins could shed some light on this process [376, 377]. In these experiments, the locally enhanced concentration of motors at microtubule tips generated by EB1 was sufficient for microtubules to remain attached and even sustain deflections as they polymerized along template microtubules. This occurred up to a point, since at encounter angles above 90° the ability to guide microtubule growth strongly depended on the force required to bend the growing microtubule. This mechanical constraint may play a lesser role in microtubule guidance by actin cables in yeast, which are free to fluctuate, thus allowing the growing microtubule to force the F-actin bundle to move as the microtubule follows.

Finally, guidance of microtubule growth has also been observed by non-bundled F-actin structures. For example, microtubules often enter actin-rich neuronal protrusions such as dendritic spines and axonal branches [378]. The fact that in dendritic shafts and axons microtubules are tightly packed and bundled, raises the question of how they turn and enter these protrusions in the first place. In this context, septins seem to serve the role of microtubule stabilizers and guides. Septins are GTP-binding proteins, which assemble into filamentous structures that are often associated with F-actin structures near the plasma membrane [379]. Septin7, which accumulates at the base of axonal branches [380], is involved in redirecting microtubule growth into these branches [381]. Similarly, in polarizing epithelia, microtubule growing ends follow, and are captured by, septin filaments made up of the Septin2-6-7 hetero-hexameric complex [382]. To date, however, the mechanisms by which septins associate with microtubules, and how this association can result in microtubule stabilization and guidance, are not yet clear.
1.2.4 Positioning sites of F-actin polymerization by microtubules

Besides physically interacting with F-actin, microtubules are also involved in positioning sites for F-actin nucleation and polymerization. For instance, growing microtubules can trigger the formation of intrapodia, which are traveling waves of actin polymerization at the leading edge of motile cells, structurally similar to the actin comet-tails used by many pathogens to power their motion in the cell interior [383, 384].

The best studied examples of microtubule-mediated F-actin nucleation occur in budding and fission yeast, where microtubules that contact the cell-cortex help recruit the protein bud6, a binding-partner and activator of formin (bni1 and for3, in budding and fission yeast respectively), which results in actin-cable formation [375, 385–387]. This process is required for maintenance of cell polarity and shape, as formin-nucleated actin cables serve as tracks for the delivery of cellular growth factors [385, 388, 389]. Although bud6 directly interacts with the APC-homologues kar9 and tea1 (in budding and fission yeast, respectively) [390], recent work suggests that this interaction is not required in this context, as direct interactions between bud6/tea1 and the yeast EB1 homologues (Bim1p and Mal3 in budding and fission yeast, respectively) are sufficient to capture microtubules at the cortex [391–394].

EB1, APC and mDia1 have also been implicated in the polymerization of F-actin bundles that emanate from the cell cortex towards the cell interior [335]. Similar to their yeast counterparts, it is not clear whether their role in enabling microtubule capture at the cell cortex collaborates with, or inhibits the polymerization of F-actin by mDia1 [359]. mDia1 binds both microtubules and F-actin via the formin-homology domain, FH2 [357, 395, 396], and recent work indicates that microtubule-binding by mDia1 (and mDia2) inhibits their ability to polymerize F-actin [395]. Furthermore, in vitro reconstitutions of F-actin polymerization with mDia and APC revealed that APC enhances mDia-dependent actin polymerization, but that these proteins eventually detach as APC remains associated with the pointed end of the filament, while mDia moves with the polymerizing barbed end [397, 398]. Further in vitro studies will thus be required to elucidate how interactions between EB1, APC and the different formins are orchestrated to induce on the one hand microtubule capture and stabilization at the cell cortex, and on the other, F-actin polymerization [399].
1.2.5 Interactions through biochemical signaling

Last but not least, microtubules and F-actin profoundly affect each other’s organization through biochemical signaling pathways. In this context, chief players are the RhoA, Rac1 and Cdc42 members of the Rho-family of GTPases [269, 400, 401], and the protein kinase GSK3β, all of which have been implicated in regulating actin and microtubule organization and dynamics in cells. Rho GTPases are active when bound to GTP and localized at the plasma membrane, while induction of GTP-hydrolysis through GTPase-activating proteins (GAPs) renders them inactive. Conversely, Rho GTPase re-activation occurs via guanine-nucleotide exchange factors (GEFs). Inactive Rho GTPases can be also sequestered in the cytosol by guanine nucleotide dissociation inhibitors (GDIs). The main actin-related functions of RhoA, Rac1 and Cdc42 can be generally grouped into three categories.

RhoA and acto-myosin-based contractility: RhoA achieves myosin-II activation in two ways. First, through activation of myosin light-chain kinase (MLCK), which phosphorylates the myosin light-chain (MLC), leading to myosin-II activation and contractility. Second, through activation of Rho-kinase (ROCK) which, by phosphorylating myosin light-chain phosphatase, inhibits MLC dephosphorylation. RhoA-induced myosin contractility in turn triggers stress fiber and cytokinetic ring assembly. Additionally, RhoA can bind to, and activate, the formin mDia1, leading to filopodial formation [402–405].

Rac1 and lamellipodial formation: Rac1 binds to and activates the SCAR/Wave complex, which in turn activates the Arp2/3 complex, thus triggering the formation of lamellipodial F-actin networks near the plasma membrane [406].

Cdc42 and filopodial and lamellipodial formation: Cdc42 binds to, and activates, some formins (such as mDia2), resulting in the formation of filopodial extensions. In addition, Cdc42 activates both Rac1 and the Arp2/3 activator N-WASP, and is thereby also involved in the formation of lamellipodia [201, 407].

Coupled to their roles as orchestrators of F-actin architecture, RhoA, Rac1 and Cdc42 are also involved in mediating actin-microtubule coordination; for instance, in order to establish and maintain a polarized cytoskeleton during cell migration [261, 269, 401, 408].

The first evidence of Rho GTPase-mediated cytoskeletal coupling came from the observation that nocodazole-induced microtubule depolymerization leads to enhanced stress fiber networks, as a result of RhoA activation [366]. Microtubules modulate RhoA activity by sequestering RhoA GEFs along their lattice, which are released upon
microtubule depolymerization [129, 409]. Conversely, RhoA binds to, and activates, the formin mDia1, which together with APC is required to capture and stabilize microtubules that reach the cell cortex [355, 387, 410, 411]. In neuronal growth cones it has been shown that Rho kinase activation by RhoA is required to generate contractile actin bundles, which in turn create a compact bundle of microtubules that stabilizes the growth cone [293]. Yet another example of RhoA mediated cross-talk occurs during mitosis, where microtubules help determine the position of the cytokinetic ring by defining a region of locally concentrated active RhoA at the cell cortex [412].

Rac1 and Cdc42 have also been implicated in microtubule stabilization [413]. Active Rac1 and Cdc42 are required to localize IQGAP at the leading edge of motile cells, where it participates in the recruitment of several microtubule-capturing proteins such as APC, CLIP-170 and CLASP [334, 335, 338]. Conversely, microtubules at the leading-edge are required for Rac1 activation [268], and it has been further shown that APC stimulates the activity of a Rac1-specific GEF, Asef [414]. Conversely, Rac1-mediated lamellipodial activity is also involved in microtubule turnover, which stems from enhanced microtubule breakage at the contractile lamellum [413].

Finally, one emerging player in the field of actin-microtubule cross-talk, particularly during cell migration, is the protein kinase GSK3β (glycogen synthase kinase 3/β) [415]. GSK3β has been shown to phosphorylate a variety of microtubule +TIPs that are normally required for cortical microtubule capture at the leading-edge of motile cells, and thus ensure cell polarity and persistent motility. Phosphorylation by GSK3β typically inhibits their ability to bind EB. However as many SXIP-motif containing +TIPs contain multiple phosphorylation sites, the effect of GSK3β is often cumulative [107]. Notable GSK3β targets are: APC [330, 416], CLASP [135, 315, 417], and MACF [339, 418]. Finally, GSK3β is inhibited at the leading edge of motile cells through Cdc42 activation of the Par6-atypical protein kinase [419].

Thus, biochemical regulation by members of the Rho-family of GTPases and GSK3β plays a defining role in the establishment and maintenance of cytoskeletal polarity during cell migration. One open question is how these different cytoskeletal regulators are spatially controlled to effect their functions at the correct cellular sites [400]. Recent work has revealed that, while RhoA is activated at the leading edge of motile cells, Rac1 and Cdc42 become active a few micrometers behind, suggesting that their activation is spatio-temporally regulated during cell migration [420].
1.3 Multiple roles for the cytoskeletal coordination toolbox

The same tools that cells use to polarize their cytoskeleton during migration are often put to use in entirely different contexts, thus revealing novel functions. For instance, during mitosis, EB, APC, CLASP and CLIP-170 are all required for the maintenance of kinetochore-microtubule attachments [102, 346, 421–426]. In polarized epithelial cells, APC, EB1 and MACF have also been implicated in the maintenance of polarized actin and microtubule cytoskeletons. In this context, APC and EB1 anchor and organize the basal microtubule network, where they do not act as +TIPs but rather localize to the microtubule lattice [427]. Similarly, EB1 and MACF are required for the formation of apico-basal composite bundles of F-actin and microtubules [428], and in the establishment of parallel arrays of microtubules anchored by APC in Drosophila tendon cells [310]. Moreover, cells often use a combination of interaction modules to properly organize their cytoskeleton. For instance, astral microtubule capture by cortically-anchored dynein is a requirement for proper spindle positioning [320], while acto-myosin cortical flows aid in this process by dragging the cortex-anchored dyneins to the sides of the cell, thus helping in centrosome separation [429].

1.4 Motivation and thesis outline

The multiplicity of actin-microtubule cross-linkers found in cells, their often overlapping localization, and the fact that they seem to behave differently depending on the cellular context is, to say the least, puzzling. Reconstituted model systems using purified proteins are ideally suited to unravel these issues, as they can separately address biochemical and mechanical interactions, and identify the minimum requirements to achieve a certain functional organization. Not surprisingly, in vitro reconstitutions have played a fundamental role in our current understanding of the cellular cytoskeleton [430–432]. However, despite the wealth of evidence that actin-microtubule crosstalk is vital for cellular function, to date, in vitro reconstitutions of actin-microtubule interactions are still rare. Notable exceptions include studies on the mechanical properties of composite actin-microtubule networks [281, 284], molecular motor motility at actin-microtubule cross-roads [433, 434], and observations of actin-microtubule co-alignment both with Xenopus egg-extracts [265, 266], and purified proteins [435]. However, in vitro studies
of the interaction between dynamic microtubules and F-actin are notably lacking. This is unfortunate, given that actin-microtubule coordination is to a very large extent mediated at the growing microtubule end, where EB acts as the organizing hub.

This thesis thus describes the development and characterization of a simplified in vitro model to study interactions between dynamic microtubules and F-actin. To this end, we engineered a cross-linking molecule, which we called TipAct, that can bind to F-actin directly and also localize at growing microtubule ends via EB. We subjected microtubule growth to different F-actin organizations, with and without TipAct. This was done in order to elucidate the separate contributions of steric and cross-linker-mediated interactions in dictating actin-microtubule coordination, and also to find out the ways by which the mechanical properties of pre-existing cytoskeletal architectures modulate the activity of actin-microtubule cross-linkers. The thesis is thus organized as follows:

In Chapter 2, we describe the general experimental methods applicable to all the in vitro assays described in this thesis. In order to work with dynamic microtubules and actin filament structures, cross-linked via fluorescently-labeled EB and TipAct molecules in solution, we developed a variety of surface assays amenable for total internal reflection fluorescence (TIRF) microscopy and quantitative image analysis.

In Chapter 3, we describe the design, purification and characterization of TipAct. To this end, we measure its actin binding affinity, and monitor its localization in cultured cells. We show that both in cells and in vitro, TipAct binds actin filaments, and localizes at growing microtubule ends in an EB-dependent manner. Furthermore, we show that even though TipAct has a low affinity for F-actin, when locally concentrated at microtubule tips it can efficiently link microtubules to actin filaments. Finally, we demonstrate that TipAct’s actin-binding domain is insensitive to actin filament polarity.

In Chapter 4, we study the effects that actin bundle architectures can have on microtubule organization. We find that microtubule growth can be guided by F-actin bundles via two distinct mechanisms: steric deflection, when TipAct is absent, or through a capture and zippering mechanism, when TipAct is present. We show that despite its low affinity for single actin filaments, TipAct stably binds F-actin bundles and also recruits EB to the bundles. Through fluorescence recovery after photo-bleaching (FRAP) experiments, we confirm that the off-rates of TipAct and EB at actin-microtubule overlaps are reduced. These observations explain the efficiency of the capture and zippering mechanism (via TipAct), in guiding microtubule growth. Finally, we demonstrate that TipAct enables parallel arrays of F-actin bundles to globally dictate microtubule organization.
In Chapter 5, we describe the effects that growing microtubules can have on F-actin organization. To this end, we reconstitute interactions between dynamic microtubules and mobile actin filaments and bundles. We show that cross-linking via EB and TipAct can lead to both F-actin bundle and microtubule deformation as they interact. Further, we find that TipAct allows growing microtubules to transport freely-diffusing actin filaments, and to pull on actin filaments partially tethered to a glass surface. In addition, we show that when microtubules interact with two or more actin filaments, they can create F-actin bundles which subsequently recruit TipAct and act as guides for microtubule growth. Finally, we demonstrate that a radial array of microtubules (nucleated from a centrosome) can drastically reorganize an otherwise isotropic solution of actin filaments.

In Chapter 6, we study the TipAct-mediated transport of soluble actin filaments by growing microtubules. To this end, we reconstitute interactions between dynamic microtubules and single actin filaments in solution. Concomitantly, we develop a model of biased diffusion of actin filaments at microtubule tips, in order to explain the transport phenomenon. We further compare the model predictions to experimental data on actin filament transport for variable EB, TipAct and tubulin concentrations, and find a close agreement between simulations and data. Finally, we find that the biased-diffusion mechanism of actin filament transport, potentially generates forces in the picoNewton range, which are comparable to those generated by molecular motors in cells.

In Chapter 7, we give a brief conclusion and an outlook for future work.