Dynamics and regulation at the tip : a high resolution view on microtubele assembly

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CHAPTER

1 Introduction

Fundamental processes like cell division, internal cell organization and intracellular transport rely on the proper functioning of microtubules. The microtubule cytoskeleton is a dynamic network of filaments that interact with and influence other cytoskeletal components (for example the actin network localization and organization). In turn, microtubule dynamics is highly regulated both spatially and temporally by a wide family of microtubule associated proteins. Microtubules have a complex architecture and dynamic behavior. To understand how microtubule associated proteins influence and regulate microtubule dynamics, information on the molecular mechanisms involved is required. Of special interest are a particular class of proteins that are specialized in tracking only the tips of the microtubules, the so called +TIPs. In this thesis, an in vitro approach was used in order to shed light on the molecular details of the microtubule self-assembly process itself, as well as the molecular mechanism by which selected microtubule associated proteins interact and influence microtubule dynamics.

Tubulin [1–3], the protein that forms microtubules, is a highly conserved protein. It is perhaps the most ubiquitous throughout the eukaryotic kingdom and homologs can even be found in bacteria (FtsZ [4] and bacterial tubulins [5]). Tubulin self-assembles into hollow tubes, microtubules, in the presence of GTP both *in vivo* and *in vitro* [6]. *In vivo* microtubules form a dynamic array of filaments that confer structural support and shape to the cell, and are involved in intracellular organization and fundamental processes like division and transport. In most eukaryotic interphase cells, the microtubule cytoskeleton radiates from a nucleating center near the nucleus (the centrosome) forming a filament network with various morphologies (figure 1.1 left). Before mitosis this array is rapidly disassembled and reorganized to form the mitotic spindle (figure 1.1 right). This remarkable ability to switch between different morphologies relies on the intrinsic property of microtubules: dynamic instability [7]. Dynamic instability is the microtubule behavior of switching between periods of growth and shrinkage [8]. This property makes microtubules unique among polymers. Microtubule dynamics are highly regulated within cells in order to achieve specific functions at the right time and the right place within the cell. This coordinated regulation is done by a large network of microtubule associated proteins. Regulation occurs at all levels during a microtubule lifetime: tubulin monomer folding, microtubule nucleation, growth and shrinkage, and post translational modification of the microtubule lattice.

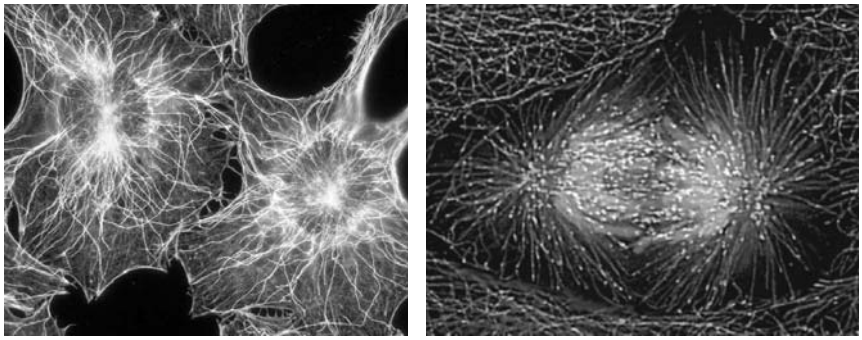

Figure 1.1: Microtubule organization *in vivo*. Left: interphase mouse fibroblasts cell displaying the aster like network of microtubules radiating from a nucleating center close to the nucleus. The fine network of actin filaments can also be observed throughout the cell. Right: a dividing kangaroo epithelial cell with the microtubules rearranged to form the mitotic spindle. The extra signal from the spindle region comes from the chromosomes. One major microtubule role in the spindle is to align the chromosomes and pull apart (this snapshot) the sister chromatids. The punctate pattern is due to the signal from EB1, an end binding protein that localizes at the growing microtubule ends. Images were acquired by Torsten Wittmann (images from http://www.ucsf.edu/sciencecafe/2007/wittmann.html).

Large effort is put into understanding the specific interactions between regulatory factors and microtubules. Malfunction in the regulation of microtubule dynamics results in erroneous mitosis and cell morphologies that are characteristic features in cancer and neurodegenerative diseases. It is established that the main players in microtubule regulation are microtubule associated proteins (MAPs) that function by targeting both soluble tubulin and microtubules [9–11]. Recent studies emphasized the importance of a special class of MAPs that are able to accumulate at and follow microtubule growing ends, the so called +TIPs [12–20]. Due to their specific localization at the microtubule tip, +TIPs have profound effects on the dynamics of the growing end. At the same time, +TIPs at microtubule ends could be delivered at specific locations in the cell or function as linkers to various cellular structures (e.g. the cell cortex or the kinetochores). Therefore, +TIPs are the main candidates to investigate when trying to understand microtubule regulation in cells. Current knowledge about +TIPs comprises important information about their function, the structure-function relationship and about the regulation of their activity [12, 21–24]. However, there is limited information about the end-tracking mechanism employed by individual +TIPs and the molecular mechanisms underlying their function. Also, +TIP influence on microtubule dynamics is usually inferred from experiments performed in living cells and in cell extracts, where it is hard to decouple the effect of other regulators. Recent *in vivo* and *in vitro* studies ([25–30] and work presented in this thesis) start to reveal the mechanisms of +TIP end-tracking and the regulation of microtubule dynamics by individual +TIPs. The advantage of an *in vitro* investigation is the minimal environment in which the influence of

various +TIPs can be dissected individually. The next logical step is to combine multiple +TIPs in order to reconstitute the *in vivo* microtubule dynamics and observe the collective effect of +TIPs.

This introductory part is focused on the the system we investigated: dynamic microtubules in the presence of associated proteins (XMAP215 and the fission yeast Mal3, Tea2 and Tip1). I will present the current state of knowledge and open questions about the self-assembly process of microtubules and about the molecular mechanism used by associated proteins to interact with microtubules and influence their dynamics. The work presented in this thesis is based on *in vitro* experiments, in a minimal environment. In these experiments, growing microtubules are sometimes subject to opposing forces. At the end I will discuss the questions that fueled the research contained in this thesis and the approach taken to answer them.

1.1 Microtubules

1.1.1 Structural features

Microtubule architecture. Microtubules are hollow tubes of 25 nm in diameter, made of parallel protofilaments that comprise $\alpha\beta$-tubulin heterodimers arranged in a head-to-tail fashion [31] (figure 1.2 a). *In vivo*, microtubules are typically comprised of 13 protofilaments, but *in vitro*, the protofilament number can vary from 9 to 17, even within the same microtubule [32, 33]. Tubulin dimers are arranged in a helical pattern within the microtubule wall, with a typical pitch of 1.5 the dimer size. The pitch results in a discontinuity in the lateral interactions, called the seam, were α- and β-tubulin monomers neighbor each other (figure 1.2 a). At the seam, the lateral interactions between the adjacent protofilaments are therefore weaker and it was proposed that microtubules grow as protofilament sheets that zipper closed at the seam [34]. Recently it was also shown that the seam might have a functional role because an end-binding protein, fission yeast Mal3, specifically recognizes and binds the microtubule seam [25].

In cells, the microtubule lattice is subject to post-translational modification (PTM) that alters the surface properties [35]. The older the microtubules are, the more PTMs they accumulate. The modified lattice was shown to have an influence on the microtubule associated proteins: the plus-end-tracking protein CLIP-170 (cytoplasmic linker protein), known to stabilize microtubules by increasing rescues, is no longer properly localized on the modified microtubules [36]. Different kinesin motors are sensitive to specific PTMs and sometimes bind preferentially to the modified lattice [37], or, on the contrary, they have a lower affinity for microtubules [38].

Microtubules have polarity. Polarity is a feature of the microtubules derived from the intrinsic polarity of the tubulin heterodimer. The β-tubulin monomer is exposed at the dynamic and fast growing microtubule plus end, whereas α-tubulin is exposed at the slower and less dynamic minus end (figure 1.2 a). In cells, minus ends are usually capped (by the nucleating templates in the centrosomal matrix) and are often non-

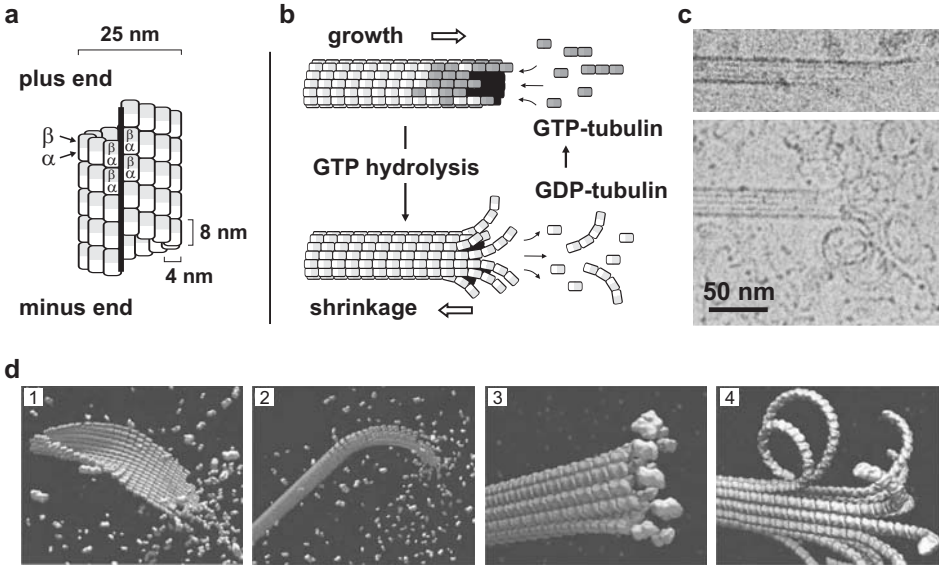

Figure 1.2: Microtubule structure and dynamic instability. **(a)** Schematic drawing of microtubule structure showing the arrangement of $\alpha\beta$-tubulin dimers within the lattice. The discontinuity marked by the thick black line represents the seam. **(b)** Schematic drawing of dynamic instability. GTP-tubulin assembles at the end of a microtubule forming a stabilizing structure that prevents microtubules from switching to a shrinking state. Upon assembly GTP is hydrolyzed. A catastrophe occurs when the stabilizing 'GTP cap' is lost (the molecular details of this process are not well understood). The cycle is completed by exchanging, upon depolymerization, the GDP of the tubulin subunits with GTP. **(c)** Electron microscopy images of what are believed to be growing (top) and shrinking (bottom) microtubules. Slightly outward curved sheet-like structures are often observed at the ends of growing microtubules. Shrinking microtubules are recognized by individual tightly-curved protofilaments peeling from the end. In addition, blunt ends are also observed and they are believed to represent an intermediate, the paused state of microtubules. Images adapted from [39]. **(d)** Artistic impression of structural intermediates of a dynamic microtubule: 1) nucleation starts by protofilament lateral association to form a sheet like structure that spontaneously closes into a cylinder. 2) A similar sheet intermediate forms at the ends of growing microtubule. 3) Loss of the GTP cap induces 4) rapid microtubule disassembly by protofilament peeling. Images adapted from [40].

dynamic. Microtubule plus ends radiate throughout the cell continuously exploring the cytoplasmic space while growing and shrinking. Microtubule polarity is exploited within the cell to create gradients by local accumulation of proteins or signaling agents: motor proteins such as kinesin and dynein follow the orientation of the microtubules and transport cargo either towards the plus end (to the cell periphery) or towards the minus end of the microtubules (to the cell interior). These motor proteins and their cargo can accumulate at the microtubule ends and be delivered at specific locations in the cell, for example when the microtubules come in contact with the cell membrane (see, for example, [41]).

Microtubules are stiff polymers. Due to their architecture microtubules are quite rigid polymers [42] conferring structural support and form to the cell. Microtubules have a persistence length l_p in the order of millimeters. l_p defines the length at which a filament displays significant bending due to thermal fluctuations. The persistence length, l_p, of a filament is related to its stiffness, κ: $l_p = \kappa / k_B T$, where k_B is Boltzmann's constant and T is the temperature ($1\ k_B T$ corresponds to 4.1 pN nm at room temperature). For microtubules $\kappa \simeq 25$ pN μm^2, although values measured in different studies vary over an order of magnitude. Recent work showed that short microtubules are easier to bend [43] and slow-growing microtubules are more stiff [44]. The length dependence was interpreted based on the complex architecture of the microtubules: shearing between adjacent protofilaments is non-negligible for short microtubules and contributes to their fluctuations [43, 45]. External factors could also affect stiffness. For example taxol-stabilized microtubules have lower stiffness [46] and it was suggested that MAPs increase microtubule rigidity [25, 46–48]. The matter, though, needs further investigation. It is clear that microtubules can not be seen as simple cylinders and that the details of the molecular lattice are important for their rigidity.

1.1.2 Dynamics

Microtubule nucleation. Microtubules are known to self-assemble from GTP-tubulin dimers. At high enough temperatures and tubulin concentrations, nucleation of new microtubules is a spontaneous process [49]. It has been proposed that nucleation starts with lateral association of short protofilaments to form a sheet, which spontaneously closes into a cylinder [50, 51] (figure 1.2 d-1). Below a critical concentration nucleation needs to be seeded by a nucleation site. In cells the soluble amount of tubulin is below critical concentration and microtubule nucleation is restricted primarily to γ-tuRC (tubulin ring complexes that include the γ-tubulin isoform and other stabilizing proteins) [52]. In *in vitro* experiments the typical nucleation sites are purified centrosomes, axonemes or short stabilized microtubules.

Microtubule dynamic instability. Dynamic microtubules switch stochastically between periods of assembly and disassembly [6]. This non-equilibrium behavior is known as dynamic instability [8]. Transition from a growing phase to rapid shortening is termed catastrophe and the reverse transition is called rescue. Figure 1.3 shows dynamic instability of a microtubule visualized with differential interference contrast (DIC) microscopy. Dynamic instability is best described by four parameters: growth speed (v_{gro}), shrinkage speed (v_{shr}) and the two transition frequencies (f_{cat} and f_{res}).

$$v_{gro} = \delta(k_{on} c_{tub} - k_{off}) \tag{1.1}$$

$$v_{shr} = -\delta^* k^*_{off} \tag{1.2}$$

$$f_{cat} = N_{cat} / t_{gro} \tag{1.3}$$

$$f_{res} = N_{res} / t_{shr} \tag{1.4}$$

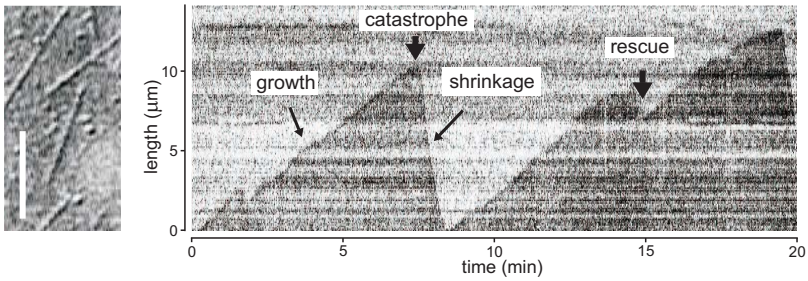

Figure 1.3: Microtubule dynamic instability. Left: Microtubules visualized by differential interference contrast (DIC) microscopy. Scale bar is 5μm. Right: A kymograph (length vs time plot) showing microtubule length changes in time. The microtubule switches back and forth between periods of growth and shrinkage. One catastrophe (transition from growth to shrinkage) and a rescue (transition from shrinkage to growth) are indicated. The kymograph was constructed from a time sequence of DIC images by measuring in each frame the intensity profile along a chosen microtubule (automated in ImageJ). The microtubule was assembled in the presence of a microtubule associated protein, Mal3.

δ is the average microtubule length increase after one tubulin subunit incorporation. Because of the helical pattern of tubulin dimers within the microtubule wall for a 13 protofilament microtubule and single dimer addition, on average, $\delta = 8/13$ nm. The growth speed depends on the soluble tubulin concentration (c_{tub}) available for polymerization and on the rate constants for GTP-tubulin association (k_{on}) and dissociation (k_{off}) during net growth. The shrinkage speed is independent of tubulin concentration and it is characterized only by the dissociation rate of GDP-tubulin from the depolymerizing end (k^*_{off}). Usually v_{shr} is an order of magnitude higher than v_{gro}. It is believed that shrinking is described by detachment of multiple subunits (oligomers) with average size δ^*. The molecular details of tubulin addition and loss is still not fully understood. The catastrophe frequency is given by the number of catastrophes observed (N_{cat}) during the total time growing microtubules were monitored (t_{gro}). Rescues are defined in a similar way. The rescue rate of dynamic microtubules is extremely low *in vitro* and cannot be reliably measured. Only an upper bound can be estimated [53].

Dynamic microtubules have been directly observed by light microscopy both *in vivo* [54, 55] and *in vitro* [56, 57]. These studies show discrepancies in the measured dynamic instability parameters. Microtubule growth speed can be up to 10 times faster *in vivo* than *in vitro* and highly dynamic microtubules are a signature of mitosis. These observations reflect the regulation of microtubule dynamics by microtubule associated proteins.

GTP hydrolysis fuels dynamics. Microtubule dynamic instability is driven by binding, hydrolysis and exchange of GTP on the β-tubulin monomer [58] (figure 1.2 b). The following paradox exists: tubulin binds and hydrolyzes GTP, though there is no energy requirement for assembly of tubulin. Only GTP binding is required for tubulin assem-

bly. GTP hydrolysis takes place after a tubulin subunit has been incorporated into the lattice, tubulin acting as its own GTPase. Studies with a slow hydrolyzable GTP analogue, GMPCPP, demonstrated the role of GTP hydrolysis: it is essential for the dynamic behavior of microtubules, the switching between growth and shrinkage. The GMPCPP-microtubules continuously assembled without transitions to a shrinkage phase [59]. The disassembly of microtubules is a fast process that does not require energy and releases GDP-tubulin back to the soluble pool of tubulin. In solution, GDP can be exchanged for GTP and the tubulin dimer is thus reconverted into a polymerization competent subunit.

Structural transitions associated with dynamics. It has been shown that GTP hydrolysis changes the conformation of the tubulin dimer from a slightly curved GTP-tubulin to a more kinked GDP-bound conformation [51]. Yet, in the microtubule lattice the GDP-tubulin dimer can not relax to its preferred curved conformation and is forced to remain straight. The free energy of the GTP hydrolysis is stored in the lattice as mechanical strain [60], which is released when the GDP-tubulin subunits are exposed at the microtubule end and provides the driving force for the rapid depolymerization phase of the dynamic instability. The straighter conformation of GTP-tubulin allows for lateral contacts between protofilaments and thus it is believed that a 'GTP cap' exists at the end of growing microtubules and stabilizes the labile microtubule lattice. Although generally accepted, the structural and biochemical details of a stabilizing cap at the microtubule growing end are still unclear. Figure 1.2 d-2 shows a possible microtubule end-structure, an open sheet of protofilaments extending while the microtubule is growing. When the GTP cap is stochastically lost (due to detachment of GTP-tubulin subunits at the end and by GTP hydrolysis catching up with addition of new subunits) (figure 1.2 d-3), the protofilaments peel outwards and the microtubule lattice collapses by rapid depolymerization (figure 1.2 d-4). This scenario is supported by the observation from electron microscopy studies that revealed the structural difference between growing and shrinking microtubule ends [34, 39] (figure 1.2 c). Growing microtubules often terminate in sheet-like structures of laterally connected protofilaments that appear to slightly bend outwards, corresponding to GTP-tubulin. At the end of shrinking microtubules individual protofilaments curl outward more strongly indicating the conformational change of GDP-tubulin. What exactly are the molecular events that trigger the switching between growing and shrinking states (catastrophes and rescues), remains poorly understood. Recent models and computer simulations based on the energetics of tubulin-tubulin bonds and the elastic properties of tubulin protofilaments are able to reproduce the growing and shrinking phases of microtubules [61–63]. They also predict a metastable intermediate state, which suggests a mechanism for rescue [61, 63] and that the GTP-cap can be composed of only a couple of GTP-tubulin subunits [62, 63].

The differences in the tubulin conformation at the growing end, shrinking end, and within the microtubule lattice could be seen as a fine-tuned mechanism of regulating

interactions with microtubule associated proteins (MAPs). MAPs could directly detect and make use of structural differences in the microtubule growing or shrinking state, a possible mechanism employed by +TIPs that recognize and follow microtubule growing ends (discussed below in section 1.2.1).

Regulation of microtubule dynamics. Many cellular functions of microtubules require that their dynamic properties are precisely controlled. The regulation is primarily done by MAPs. How could MAPs change microtubule dynamic instability parameters? If we look at equations 1.1-1.4 there are several options. Enhanced growth speed could be achieved by i) a biochemical increase in the affinity of tubulin association rate (k_{on}). The MAP could either bind first at the microtubule tip or form a tubulin-MAP complex in solution. ii) Increasing the added subunit size (δ) would also result in faster growth. This implies that MAPs would be capable of templating formation of oligomers that subsequently are incorporated at the growing microtubule end. There are experimental studies indicating that XMAP215/TOGp [64, 65] and CLIP-170 [29, 66, 67] could bind and induce formation of tubulin oligomers *in vitro*. XMAP215 and the budding yeast homologue Stu2 were also shown to destabilize microtubules [68, 69]. It is possible that a protein like XMAP215 increases both rates of tubulin association (k_{on}) and dissociation (k_{off}). Depending on the exact conditions (temperature, salt, tubulin concentration) the protein can accelerate growth or, on the contrary, enhance tubulin dissociation that could result in, for example, more frequent catastrophes. It is less obvious how MAPs could increase or reduce k^*_{off}, the dissociation rate of tubulin from a depolymerizing end. Most probably there is a correlation with the lattice stability and the interaction between protofilaments. Also the transition frequencies might depend on the stability of the lateral interactions between protofilaments and on the details of tubulin association and dissociation. We still miss information about the molecular details accompanying regulation of microtubule dynamics by MAPs. The research presented in this thesis includes new insights into the molecular mechanism of microtubule dynamics influence by two MAPs: XMAP215, known to dramatically enhance growth speed (chapter 3) and Mal3, a microtubule end-binding protein, known to have an important role in maintaining microtubule stability within cells (chapter 5).

1.1.3 Force generation

Several studies show that microtubules can produce both pushing and pulling forces [70, 71]. On one hand, during microtubule polymerization, the free energy gain associated with addition of tubulin subunits is sufficient to generate a force of several tens of piconewton. On the other hand, the mechanical strain stored in the GDP-lattice can fuel pulling forces on a target coupled to shrinking microtubule ends. Microtubules can thus perform mechanical work [72, 73] and drive intra-cellular movement of organelles. A well known example is chromosome separation during anaphase in dividing cells, which is partly powered by depolymerization of kinetochore-attached microtubules [74]. Also, microtubule growth generates forces that help position organelles

and microtubule organizing centers within the cell [75]. The biophysical aspects of microtubule force generation have been studied in experimental [70, 76, 77] and theoretical [78–80] work by several groups.

The simplest mechanistic way to think about force generation by growing microtubules is provided by the Brownian ratchet model [81]. In this model, thermal fluctuations of a confining barrier allow for occasional addition of new subunits to a growing filament. The filament can grow even in the presence of an opposing external force. Since the amplitude of the fluctuations reduces as the force increases, the rate at which new subunits can insert slows down. The force at which no net growth occurs anymore and subunits only occasionally detach is defined as the stall force. The Brownian ratchet predicts an exponential dependence of the filament growth rate on the applied force (F):

$$v_{\mathrm{gro}}(F) = \delta(k_{\mathrm{on}} c_{\mathrm{tub}} e^{-F\delta/k_{\mathrm{B}}T} - k_{\mathrm{off}}) \tag{1.5}$$

For microtubules, more elaborate models accounting for multiple filaments predict a slightly different force-velocity curve, whose functional details depend on the assumed structure of the growing microtubule end [78–80].

Microtubule growth is influenced by force in the crowded intracellular environment. In the cell, microtubules often grow against obstacles like organelles, membranes and the cell cortex. In mitosis, microtubules push and pull chromosomes in order to align and separate them. There is still a lack of quantitative knowledge about the magnitude of these forces. A recent study analyzed the shapes of bent microtubules in the cell and concluded that microtubules can withstand much higher loads than expected from *in vitro* studies [82]. The authors explain this discrepancy by microtubule mechanical reinforcement from the surrounding elastic cytoskeleton [82, 83]. Although not studied from this perspective, the microtubule end-binding proteins that decorate the tips of growing microtubules could also influence microtubule stability under force. There is still an open question: do MAPs regulate the force generation of microtubules and if so, what is the molecular mechanism underlying this regulation?

1.2 Microtubule plus-end tracking proteins, +TIPs

In vivo, the regulation of microtubule dynamics is largely the responsibility of microtubule associated proteins (MAPs) [9–11]. As their name says, the shared feature is the affinity for tubulin and microtubules. Their activity covers a wide range of regulation. There are MAPs that control microtubule nucleation and microtubule bundle organization. MAPs, like Op18/stathmin, have been proposed to sequester soluble tubulin [84, 85]. Classical MAPs such as tau and MAP2 bind to the microtubule lattice and stabilize it, but other lattice binding MAPs have the opposite effect (e.g. the severing protein katanin [86]). Microtubule associated motors are a class of MAPs that have the ability to walk along the microtubules (kinesins and dyneins). Motor proteins transport

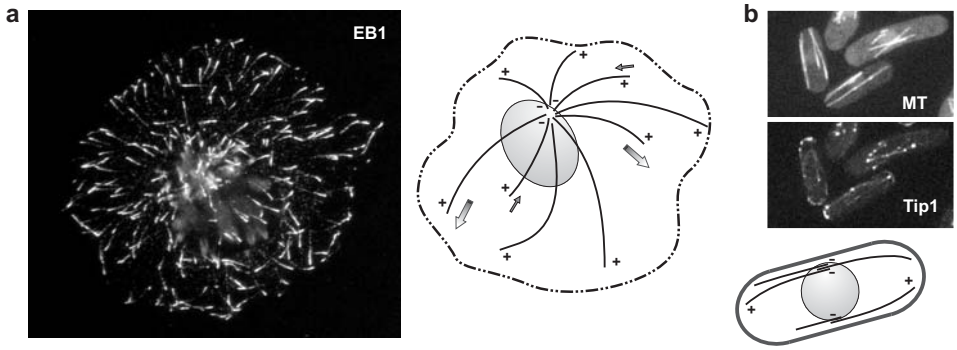

Figure 1.4: Plus-end tracking proteins, +TIPs. **(a)** Fluorescent comets of EB1, a mammalian plus end-binding protein, at the growing microtubule ends in an interphase COS-7 cell. The cell shows immunofluorescence staining of endogenous EB1. Image courtesy of Anna Akhmanova. Right: Schematic drawing of microtubule organization in a similar fibroblast cell in interphase. Dynamic microtubule plus ends radiate towards cell periphery. +TIPs, like EB1, are able to specifically localize at the growing plus ends. **(b)** Fluorescence images of microtubules (top image) and Tip1-GPF decorating the microtubule plus ends (lower image) in fission yeast cells. Tubulin was tagged with mCherry. Images courtesy of Christian Tischer. Below: Schematic drawing of microtubule organization in an interphase fission yeast cell. Microtubules are organized in parallel bundles and grow with the plus ends towards the cell poles.

and deliver material throughout the cell [87,88], they are involved in maintaining the integrity and function of microtubule bundles, for example, in the mitotic spindle [89] or in the beating flagella. Motors can anchor microtubules at the cell cortex [90] or to kinetochores [91], and by themselves can influence the stability of microtubules [92–95].

An important class of MAPs are the end-binding proteins, generally termed +TIPs [12–20], including both microtubule associated motors and non-motor proteins. Their distinguishing feature is the microtubule end localization (figure 1.4). When marked with a fluorescent tag, the +TIPs appear as comets at microtubule tips moving throughout the cell as the microtubules are growing. Some +TIPs also have the ability to follow the shrinking microtubule ends.

The +TIPs most obvious function is the regulation of microtubule plus-end dynamics, where tubulin is incorporated and forms the stabilizing cap. 'Traveling' on microtubule ends provides a mechanism for cargo delivery at specific locations in the cell. An emerging role for +TIPs is in cell motility and polarized cell growth where signals are delivered to the cortex to control actin assembly, organization and contractility (one example can be found in fission yeast [96]). A scenario for protein delivery at the cell periphery would require the combined effect of several +TIPs: stabilization of the growing end such that the microtubules reach the target and continuous presence of the cargo at the plus end. As a consequence the +TIPs form a highly interactive protein network where several +TIPs act together to achieve one function [12, 21, 24]. Some +TIPs can

directly interact with membrane proteins, actin associated proteins and kinetochore proteins and thus provide coupling between microtubules and other cellular components [14, 24]. For example, EB1, a microtubule plus-end-tracking protein, interacts with a transmembrane protein STIM1, which is localized to the endoplasmic reticulum (ER) (membranous network within cells). EB1-STIM1 interaction mediates ER remodeling by coupling the dynamic microtubule plus-ends to the ER membrane [97]. Some of the coupling +TIPs have been proposed to transduce the pulling forces generated by the microtubule disassembly during chromosome segregation. A peculiar example of force coupling through a +TIP is the alignment and positioning of the mitotic spindle in the budding yeast [98]. In this example the +TIP is dynein, a minus end directed motor. Here, the force is produced by both the +TIP motor tracking the shrinking microtubule ends and by disassembly of microtubules. The force generated results in spindle motion through coupling of dynein to the cell cortex.

1.2.1 End-tracking mechanisms

Structural and cellular studies together with recent *in vitro* experiments (including part of the work presented in this thesis) start to unravel the molecular mechanisms of plus-end localization. Possible mechanisms are described below.

Copolymerization implies that a +TIP is incorporated together with tubulin at the growing microtubule end (figure 1.5-1). There are some requirements in order to get tip tracking through copolymerization. The +TIP should have high affinity for soluble tubulin to form relatively stable complexes with tubulin in solution. +TIP affinity for the microtubule lattice should be low. Upon end incorporation the +TIP releases from the older parts of the filament by stochastic detaching or induced by a conformational (straightening of protofilaments) and/or biochemical (GTP hydrolysis) change. Modification of +TIP affinity for the microtubule by other proteins is also possible. In the copolymerization scenario the +TIP would appear stationary on the microtubule while the end elongates. After a delay, the +TIP would then release its fixed point on the microtubule. As defined above, the copolymerizing +TIPs would only follow growing microtubules.

Among +TIPs, CLIP-170 was shown to bind tubulin *in vitro* and copolymerization was suggested to be a likely mechanism of CLIP-170 end-tracking [29, 66, 67]. This conclusion was recently challenged in a study showing rapid turnover of the protein at microtubule ends *in vivo* [30]. The underlying mechanism of CLIP-170 end-tracking was suggested to be, therefore, a tip recognition mechanism.

Tip recognition requires the existence of a specific structure at the growing microtubule end that is recognized by +TIPs (figure 1.5-2). Cryo-EM studies show the existence of slightly curved protofilaments at the ends of growing microtubules. Further, GTP-tubulin is assembled at the tip, while GDP-tubulin represents the majority within the microtubule lattice. The structural and biochemical details of the microtubule end-structure remain to be clarified. Assuming the existence of such a struc-

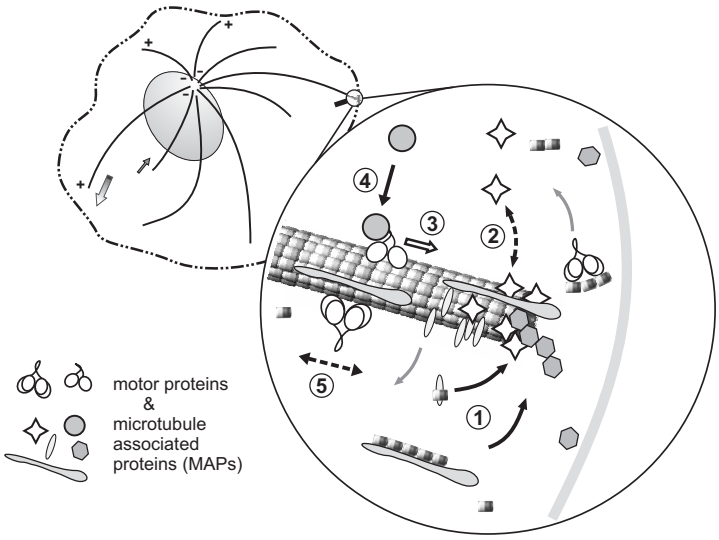

Figure 1.5: Microtubule end-tracking mechanisms. Cartoon of a cell with dynamic microtubules radiating from the centrosome. The enlarged image gives an artistic impression of the complex interactions at the growing microtubule plus-end. Specialized proteins, so called +TIPs, can bind and track dynamic microtubule ends, where they control microtubule dynamics, deliver cargo or mediate interactions with the cell cortex. Possible end-tracking mechanism of +TIPs include: (1) copolymerization with tubulin and later detachment from the microtubule lattice, (2) specific end-structure recognition, (3) direct movement of kinesins and dyneins, (4) hitchhiking on other motor or non-motor +TIPs already present at the tip, and (5) 1-D diffusion on the lattice to facilitate end-finding.

ture, +TIPs could recognize either the presence of GTP-tubulin, specific protofilament arrangement, protofilament curvature and/or might bind to exposed tubulin sites otherwise hidden in the rest of the tube. Also, the +TIPs should not prevent the progressive conversion of the end structure into a regular lattice conformation (a closed tube). The GTP-cap was predicted to be quite small (< 0.5 μm) [56, 99–101], while in cells +TIP comets are in general covering a more extended area (0.5 -2 μm). However, biochemical recognition of the tip cannot be excluded as the GTP-cap size prediction is based on limited experimental evidence. A structural recognition of the growing end by EB proteins is supported by a recent electron microscopy study of Mal3 [25]. Mal3, the fission yeast EB homologue, showed specific binding at the microtubule seam. At the seam, the lateral interactions between adjacent protofilaments are different than elsewhere in the lattice. It is possible that the EB binding sites of tubulin are only available at the seam. A similar picture can be envisioned at the growing end. An open structure of protofilaments could provide exposed EB binding sites. Experimental results presented in chapter 4 and chapter 5 bring new information about the molecular mechanism of Mal3 end-tracking.

Some +TIPs are able to follow shrinking microtubules, commonly known as backtracking. In this case, the proteins would recognize the highly curved protofilaments observed at the ends of disassembling microtubules. The budding yeast Bik1 (CLIP-170 homologue) and kinesin Kip2 were observed to follow shrinking microtubules [102]. The backtracking of Bik1 was dependent on the kinesin Kip2 that seems to transport Bik1 to the plus end. It is possible that the two yeast +TIPs together or independently recognize the curved disassembling protofilaments. Future experimental investigations are needed to fully understand the mechanism of Bik1 and Kip2 backtracking.

Directed motion is a potential end-tracking mechanism used by motors, provided that the motors walk faster than the microtubules grow (figure 1.5-3). A simple explanation for motor accumulation at the tip could be that newly formed parts of the microtubule 'receive' motors both from solution and from the older lattice. The extent of the end accumulation would depend on the run length of individual motors. Some of the kinesins, like the fission yeast Tea2 [103], are known to end-track growing microtubules. It is not yet established if their directed movement is solely responsible for their end-tracking behavior. It is possible that once reaching the ends, these motors are retained due to specific affinity for the end-structure or by interaction with other +TIPs already present there. A motor dependent end-tracking mechanism does not exclude tracking of shrinking ends. The protein directed motion would be sufficient to produce accumulation. The Kip2 kinesin shows backtracking in budding yeast, but the molecular mechanism has not yet been investigated.

Hitchhiking by association with motors and other microtubule end-binding proteins seems to be common for many +TIPs [17] (figure 1.5-4). This type of mechanism involves an intermediate protein at the microtubule tip, implying that hitchhikers do not efficiently interact with tubulin or microtubules. Several +TIPs were proposed to be transported as cargo to the growing microtubule ends. For example, the yeast homologs from the CLIP family, Bik1 and Tip1, are moved processively by the Kip2 and Tea2 kinesis, respectively [102, 103]. STIM1 is another example of hitchhiker that is involved in linking the ER membrane to microtubule tips by direct binding to EB1 [97].

1-D diffusion on microtubules is common for nonprocessive motors or proteins that have weak affinity for the microtubule lattice. Affinity for the microtubule will target these MAPs to the lattice where they diffuse for a short period exploring the 1-D space (figure 1.5-5). If they land close to the tip they might accumulate at the microtubule end but only through another mechanism (tip recognition or interaction with other +TIPs). 1-D diffusion is therefore not per se an end-tracking mechanism. It can rather be seen as a microtubule-end targeting mechanism employed by proteins that do not use active motion. The kinesin-13 MCAK was shown to target microtubule ends by lattice diffusion [26], together with recognition of the protofilament curvature at the microtubule tips.

One common feature of the end-tracking mechanisms is that +TIPs are transiently present at the microtubule end. Interaction with the tip is dynamic and new proteins

from solution are constantly repopulating the tip. The majority of +TIPs have low affinity for tubulin in solution and most +TIPs are dimers (EBs, CLIPs, tip tracking kinesins) or have multiple binding sites for tubulin indicating that cooperativity might be necessary for end-tracking [21]. Some interactions within the +TIP network seem to be based on similar amino acid motifs as some +TIP-tubulin interactions [104, 105]. The competition raised by this similarity could prevent overcrowding at the microtubule tip and, more importantly, could induce sequential recruitment of +TIPs at the microtubule end.

1.2.2 Regulation of microtubule dynamics

+TIPs in general promote microtubule growth and/or enhance the dynamic behavior of microtubules. The first +TIP discovered, CLIP-170, was found to stabilize microtubule growth. CLIP-170 functions as a rescue factor [106] and the fission yeast homologue, Tip1, suppresses microtubule catastrophes [107, 108]. The mechanism of rescuing shrinking microtubules remains puzzling as CLIP-170 does not seem to bind the depolymerizing ends. The EB (end binding) family members are also known as stabilizers. EB proteins were reported to suppress catastrophes in fission yeast [109] and in *Xenopus* egg extracts [110]. In extract experiments, stabilization of microtubules was achieved also by EB1 increasing the rescue rate. *In vitro* experiments supported the idea that the EBs promote polymerization [25, 111, 112], but might dependent on the presence of other +TIPs [111]. On the other hand, in budding yeast [113] and *Drosophila* [114], EB1 enhances both the catastrophe and the rescue rate. However, it remains unclear if EBs can modulate microtubule dynamics on their own and if the *in vivo* effects can be attributed solely to the EB proteins. Among the destabilizers are kinesin-8 (Kip3 [115, 116]), kinesin-13 (MCAK [117], KLP10A [118]) and kinesin-14 (Kar3 [119]) family members that are known to catalyze microtubule depolymerization. One possible mechanism is based on structural changes at the microtubule tip where these kinesins can force protofilaments into a more bent conformation.

1.3 This thesis

The work presented in this thesis concerns the regulation of microtubule dynamics by microtubule associated proteins. Our effort was directed towards understanding the molecular mechanisms underlying this regulation. We chose to investigate a number of representative MAPs, individually, *in vitro*. The advantage of an *in vitro* minimal system is the possibility it offers to uncouple the regulation of different MAPs. If desired, the level of complexity can be increased by introducing a combination of MAPs.

In order to achieve molecular resolution on the microtubule assembly process, we developed a high resolution technique based on optical tweezers. Chapter 2 contains a detailed description of the technique. Dynamic microtubules could be followed with unprecedented resolution of a few nanometers and allowed us to zoom in on micro-

tubule assembly process. In chapter 3, molecular details of microtubule growth are discussed and how this process is altered, on a molecular scale, by the microtubule associated protein XMAP215. XMAP215, a *Xenopus* MAP, attracted out attention due to its effect on microtubule assembly, XMAP215 being probably the most potent microtubule growth enhancer known [120]. Previous *in vitro* experiments showed that XMAP215 sped up microtubule growth up to 10-fold [121, 122], similar with its behavior *in vivo*. Using our high-resolution technique we observed fast length changes during microtubule growth, that correspond to the size of the XMAP215 protein itself. The implications of this new finding are discussed. Triggered by the observations at high-resolution, we further attempted to investigate XMAP215-tubulin interactions in solution and the possibility that XMAP215 templates formation of tubulin oligomers (XMAP215 could bind multiple tubulin dimers along its length [64, 123]). Our observations using two independent techniques, fluorescence speckle microscopy and fluorescence correlation spectroscopy, are presented and discussed.

Chapters 4 and 5 present experiments related to a set of end-tracking proteins, namely three fission yeast +TIPs: the EB1 homologue Mal3, the kinesin Tea2 and the CLIP-170 homologue Tip1. These +TIPs were shown to have an important role in regulating microtubule dynamics *in vivo*. However, due to lack of *in vitro* investigations, it is not clear if EBs, for example, can regulate microtubule dynamics independently or if they only modulate the influence of other +TIPs. First *in vitro* reconstitution of microtubule plus-end tracking by the three fission yeast +TIP system is presented in chapter 4. We found experimental evidence for the molecular mechanisms utilized by the three proteins, individually and together, to specifically end-track microtubule growing ends [124]. Among the three +TIPs only Mal3 was able to autonomously end-track microtubule growing ends. Tea2 and Tip1 needed each other and Mal3 to end-track. As Mal3 is able to localize at microtubule growing ends on its own, it is possible that the EB1 homologue has also an effect on microtubule dynamics. Chapter 5 contains our results on Mal3 regulation of microtubule dynamics *in vitro*. The experimental work includes quantification of microtubule dynamic instability parameters by light microscopy, quantification of Mal3 localization on microtubules by fluorescence microscopy, and investigation with the optical tweezers based technique of the modifications induced by Mal3 at the microtubule dynamic tip. The sole presence of Mal3 induced a surprising and complex effect on microtubule dynamics. Combining results from the three independent techniques, we propose a mechanism of Mal3-regulation.

Chapter 6 focuses on microtubule catastrophes, the transitions from a growing to a shrinkage phase. Knowledge about a mechanism for catastrophes has been limited to models based on structural details of the microtubule ends imaged with cryo electron-microscopy. Information about how MAPs act on a molecular level to regulate catastrophes is even less extensive. Using our optical tweezers based technique we followed dynamic microtubule ends with near molecular resolution. When zooming in at the events just before microtubule fast disassembly, we observed a slow decrease in micro-

tubule length of several tens of nanometers. Our observations suggest that catastrophes are represented by loss of the stabilizing end-structure or a conformational change at the microtubule tip. The influence of XMAP215 and Mal3 on the molecular details preceding fast depolymerization were also investigated. First observations are presented.

Chapter 7 includes a general discussion of the results comprised in this thesis and future directions of investigation:

(i) From the *in vitro* end-tracking experiment we found that Tea2 and Tip1 need Mal3 to localize at the plus end. It is possible that the presence of Tea2 and Tip1, individually or in combination, at the microtubule tip affect Mal3-induced regulation. Preliminary experiments were done to assess the interplay between the two +TIPs (Tea2 and Tip1) and Mal3 and their combined regulation of microtubule dynamics.

(ii) Mal3 is the fission yeast homologue of mammalian EB proteins. The high sequence homology between Mal3 and EB1/EB3 would suggest a similar behavior of the three proteins on microtubules, but *in vivo* studies indicated that EB1 effect on microtubule dynamics might depend on the presence of another +TIP, the dynactin component $p150^{Glued}$ [125]. First *in vitro* reconstitution of the EB3 end-tracking is presented. Fluorescently tagged EB3 autonomously end-tracked growing microtubules, similarly with Mal3, but also showed an intriguing interaction with the microtubule lattice at high concentrations.

(iii) An important aspect of microtubule dynamics is regulation by force. This effect has relevance for the intracellular crowded environment where microtubules often encounter barriers. The presence of MAPs might affect the behavior of microtubules growing against force. Our high-resolution technique involves optical tweezers allowing for quantification of the force generation by growing microtubules. The first observations of microtubule force generation in the presence of XMAP215 and Mal3 are presented.