Dynamics and structural features of the microtubule plus-ends in interphase mouse fibroblasts

Zovko, S.

CHAPTER I

General introduction and scope of the thesis

General introduction

Various fundamental processes of the eukaryotic cell including intracellular vesicle transport, organelle positioning, regulation of focal adhesion dynamics (Bershadsky et al., 1996), maintenance of cell polarization , cell migration (Gundersen et al., 2004), gap junction dynamics (Shaw et al., 2007), separation of chromosomes during cell division and maintenance of cell rigidity and shape are governed by microtubules (MTs). Together with actin filaments and intermediate filaments, MTs create the cytoskeleton of the cell- a complex network of protein polymers located throughout the cell's cytoplasm. Crosstalk between the three types of filaments gives rise to an increase in the functionality and, consequently, an increase in the complexity of the cytoskeleton as a whole.

One of the important characteristics of MTs, and cytoskeletal fibers in general, is their dynamicity: the ability to rapidly rearrange into new, morphologically and functionally distinct arrays. The rapid re-arrangement is necessary for the cell to adequately respond to signals from the environment. Hence, MT-related processes require an accurate spatial and temporal regulation and coordination. The ability of MTs to participate in such a large range of processes is due to the dynamic character of MTs and their unique polarized structure, as well as due to the surplus of regulatory factors that bind to MTs and influence MT behavior within cellular context. Cellular factors associated with the MT, and in particular with its plus-end (the so-called plus-end tracking proteins or +TIPs), regulate MT plus-end dynamics and thus MT function within the cell. Malfunction of the factors influencing MT dynamics can potentially lead to perturbations of the MT cytoskeleton, which can have major consequences on the function of the cell as a whole, leading to the onset of disease. Diseases associated with the failure of proper regulation of MT dynamics include various neurodegenerative diseases (e.g. Alzheimer's disease), cardiovascular diseases (van Hagen *et al.*, 2007), pulmonary fibrosis and cancer (Aoki and Taketo, 2007).

A better understanding of the fundamental processes involved in the regulation of MT dynamics in healthy cells is essential in order to elucidate the perturbations of the MT cytoskeleton in diseased cells. Moreover, this knowledge is a prerequisite for finding potential targets among regulatory factors of MT dynamics in order to treat the diseased cells. One of the examples of such a treatment is tackling of cancer though modifications of MT-dynamics based on the targeting and stabilizing of the MTs using Taxol, an agent that binds to MTs and reduces their dynamicity, promoting mitotic arrest and cell death (Arnal and Wade, 1995).

This thesis deals with various aspects of the MT cytoskeleton, including the structure and dynamics of MTs, the role of the MT plus-end binding proteins on the function of the MT network within interphase mammalian cells and the consequences of a perturbed MT network on cell functions like cell spreading and adhesion. The various features of the MT cytoskeleton will be discussed in more detail in the following paragraphs.

Microtubules

Structural features of microtubules

MTs are hollow linear polymers ~25nm in diameter, composed of the evolutionarily highly conserved protein tubulin. Three types of tubulin are present within the eukaryotic cell: α-tubulin, β-tubulin and γ-tubulin. While α-tubulin and β-tubulin make up the MT lattice, γ-tubulin plays a role in the nucleation of the MT.

The basic structural motif of MT lattice is an α/β-tubulin heterodimer (Figure 1). Tubulin heterodimers arrange longitudinally in a head-to-tail fashion into linear arrays called protofilaments (PFs) (Figure 1), which laterally interact with each other giving rise to a hollow cylinder– a MT. Although the number of PFs (*N*) contained in a MT wall (lattice) can vary between 8-19 (Chrétien and Wade, 1991; Chretien *et al.*, 1995; Chretien *et al.*,

1999), 13 and 14 are the most frequent ones. Moreover, *in vitro* 14 PFs are favored, while *in vivo* most MTs have 13 PFs. The number of PFs within the same MT lattice can also vary, which is believed to add to the destabilization of the MT (Chretien *et al.*, 1995; Chrétien and Fuller, 2000). The PFs within a MT lattice are shifted longitudinally with regard to each other resulting in helicity of the MT. The longitudinal displacement (*S*) is defined as a total shift resulting from one full helical turn, and is given in number of monomers (*S* is in most of the cases 3). Depending on the organization of PFs within the helix, two types of lattice architectures have been described- the A and B lattice. In the A-lattice α and β-subunits alternate between the adjacent PFs (α-β). In the B-lattice, however, two α -subunits (and thus two β –subunits) of two adjacent PFs associate with each other (α-α and β-β). The 13 PFs B- lattice with *S*=3 configuration is characterized by a point of helical discontinuity or "seam"- where the α–subunits of first PF interact with the β-subunits of the 13th PF (Figure 1(Kikkawa *et al.*, 1994).

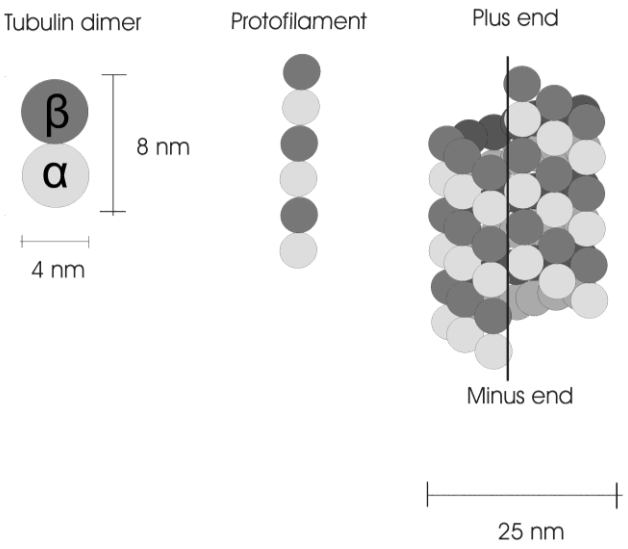

Figure 1. Microtubules (MTs) are polar tubulin polymers. Tubulin α-β-heterodimers (A) are the building blocks of microtubule. They arrange longitudinally in a head-to-tail fashion into linear arrays called protofilaments (B), which interact laterally with each other forming a hollow, tubular structure- a microtubule (C). Here (C) a scheme is depicted of a microtubule consisting of 13 protofilaments (PFs) with longitudinal displacement of 3 monomers (S=3) in which two β, and two α subunits from two adjacent protofilaments interact with each other (B-lattice). A characteristic of this MT conformation is helical discontinuity or "seam" (given by the black line between two PFs) – the point where the PFs close the tube and where thus the α–subunits of the first PF interact with the β-subunits of the 13th PF. Note that the plus end of the MT ends with β-subunits, while at the minus end α-subunits are exposed, indicating MT polarity.

Cryo-electron crystallography of zinc-induced tubulin sheets (Downing and Jontes, 1992; (Nogales *et al.*, 1995) has led to a high-resolution model of tubulin (Nogales *et al.*, 1998). Both α-and β-tubulin (each~55kDa) were found to contain three main domains: 1) the nucleotide binding domain, 2) the intermediate domain (which comprises the taxol binding site) and 3) a domain which is suggested to act as the interaction site for motor proteins (Nogales *et al.*, 1998). Although both tubulin monomers bind a guanosine 5'-

triphosphate (GTP) molecule, only the ß-tubulin is able to hydrolyze the GTP into guanosine 5'-diphosphate (GDP) (Caplow and Reid, 1985).

The intrinsic polarity of the tubulin heterodimer, in combination with its parallel orientation within the MT lattice, results in polarization of the MT. Moreover, MT polarity manifests itself by the existence of two structurally and functionally distinct ends, the “plus” and “minus” end respectively (Figure 1). At the plus-end the β-tubulin subunits are exposed, while the minus end terminates with the α-tubulin subunits. The polarity within a MT has consequences for the dynamic behavior of the two MT ends, as will be explained in the next section.

Microtubule dynamics

MTs are intrinsically dynamic structures. Purified α/β-tubulin heterodimers in solution, in the presence of GTP and proper buffer, have a property to spontaneously self assemble into MTs. MTs polymerize faster at higher tubulin concentrations. Above the so-called critical concentration of tubulin in solution, MT elongation occurs through the addition of GTP•tubulin subunits at the two MT ends. The amount of the MT polymer reaches a maximum as the system reaches a steady state in which subunit loss and addition are balanced. Dilution of the tubulin solution below the critical concentration leads to reestablishment of the critical tubulin concentration through MT shrinkage. MT shrinkage occurs through the dissociation of the GDP•tubulin subunits from the MT ends and will continue until the critical concentration of tubulin is reached again.

Both MT ends are dynamic, however, the kinetic rate constants for GTP-subunit association and GDP-subunit dissociation– $k_{on\ GTP}$ and $k_{off\ GDP}$ respectively- are greater at the plus-end than at the minus-end, due to their structural difference. Consequently, in assembly conditions, addition of GTP•tubulin is much faster at the plus-end than at the minus-end, and thus the MT elongates/shortens much faster at the plus-end then at the minus-end.

In steady state, instead of minor exchange of tubulin subunits between the MT polymer and the solution (equilibrium), the exchange is found to be rather extensive. While a majority of the MTs slowly grow, at the same time a few MTs undergo fast shrinkage and disappear, a state also termed dynamic equilibrium. This is explained by the unique behavior of the MT ends which involves continuous random abrupt alterations between growth and shrinkage phases and is termed “dynamic instability” (Figure 2). This behavior was first observed at the ends of *in vitro*-assembled MTs (Mitchison and Kirschner, 1985). Transition from growth to shrinkage is called *catastrophe*, while the reversed process, transition from shrinkage to growth is called *rescue*. Interestingly, the plus-ends of MTs undergo catastrophe more frequently, making them the more dynamic end of the MT.

The crucial feature behind dynamic instability is considered to be the difference in structure between the GTP•tubulin heterodimer and GDP•tubulin heterodimer: in the latter one monomer deviates 12 degrees with respect to the central axis of the other

monomer, resulting in a relatively *curved* conformation. In the GTP•tubulin heterodimer, however, this deviation is much smaller giving rise to a relatively *straight* conformation (Caplow and Fee, 2003; Ravelli *et al.*, 2004; Krebs *et al.*, 2005; Nogales and Wang, 2006). As the curved GDP•tubulin heterodimer in the MT lattice is forced to stay straight due to its lateral and longitudinal contacts, the energy of the curvature is stored within the lattice in the form of tension, making the MT unstable (Caplow *et al.*, 1994; Jánosi *et al.*, 2002).

Shortly after the addition of a new GTP•tubulin heterodimer at the MT end, the GTP within the previous subunit becomes hydrolyzed. Experiments with non-hydrolyzable GTP analogs showed that hydrolysis as such is not required for MT polymerization. If the addition of new subunits is faster than the hydrolysis rate inside the MT lattice, this hydrolysis "delay" will cause GTP•tubulin accumulation at the MT tip. This 'GTP-cap' is thought to stabilize the MT and promote assembly (Drechsel and Kirschner, 1994; Dimitrov *et al.*, 2008). As a consequence, the growing MT has two structurally and energetically different parts: a GDP-core in which the GDP•tubulin is trapped, not being able to switch into the energetically favorable curved conformation, and a newly polymerized part, involving the MT end itself, enriched in GTP•tubulin (GTP-cap).

The moment the GTP-hydrolysis catches up with the growth (or through stochastic dissociation of GTP-tubulin), the GTP-cap is lost and the PFs can adopt a curved conformation, which results in weakening of the lateral interactions between the PFs. Eventually, peeling off of the curved PFs and rapid collapse of MT occurs (Caplow *et al.*, 1994; Jánosi *et al.*, 2002; Nogales and Wang, 2006).
Dynamic behavior of MTs characterized by the addition of tubulin subunits at the plus-end and simultaneous loss of subunits at the minus-end is termed "treadmilling", or flux. When the velocities of subunit addition and subunit loss are in balance, at a certain concentration of free tubulin, the steady-state of the polymer is reached, resulting in a constant net length of the treadmilling polymer. This MT behavior can be observed in cells lacking MTOCs, thus with both plus end minus ends free in the cytoplasm.
Both MT treadmilling and dynamic instability have been observed as two distinct phenomena *in vivo* (Margolis and Wilson, 1998; Howard and Hyman, 2003).

Microtubules in vivo
The MT array of many animal cells is characterized by a radial architecture (Howard and Hyman, 2003) as a consequence of the embedment of the minus-ends of MTs in a microtubule organizing center (MTOC) and the "search and capture" behavior of the plus-ends throughout the cytoplasm (Kirschner and Mitchison, 1986) (Figure 3). MT "search-and-capture" behavior manifests itself in the exploration of the cytoplasm by the MTs and is accompanied by the growth and shrinkage of the plus-ends of MTs. This dynamic behavior of the MT plus-end is essential for its (random) targeting towards and its capture at specific sites in the cell cortex, like the focal adhesion sites (Krylyshkina *et al.*, 2003) and the cortical actin meshwork (Fukata *et al.*, 2002). Through the temporally and spatially regulated capturing of individual MTs at the cell cortex, the stabilization of the usually dynamic MTs takes place. By this local stabilization of a subset of MTs, the

asymmetry within the cell, cell polarization, is promoted, providing the basis for the cell morphogenesis and cell migration.

In the cell interior MTs grow persistently; catastrophes are rare and rescue is rapid. However, in the area close to the cell edge the MT plus-ends are highly dynamic, exhibiting dynamic instability. In some cases peripheral MTs undergo a period of shrinkage long enough to depolymerize to their nucleation point: the MTOC. The persistent MT growth in the cell interior is thought to be a result of a combination of relatively high elongation velocity and low catastrophe frequency (Komarova *et al.*, 2002b). Recently it has been found that EB1 and EB3 act as catastrophe suppressors thereby promoting persistent MT growth in cells. Experiments *in silico*, however, suggest that the increase of free tubulin concentration, resulting from the boundary-induced catastrophes, could also explain the existence of this phenomenon (Gregoretti *et al.*, 2006). Nonetheless, asymmetric dynamic instability of MTs results in accumulation of the plus-ends at the cell periphery.

The growing MTs that hit the barrier at the cell cortex, if not rescued, rapidly depolymerize. Catastrophe in the cell periphery can be prevented if the plus-end is stabilized by attachment to another cellular factor or structure (e.g. adhesion structures) present in the periphery. In this way a dynamic MT becomes a long-lived MT, also called 'stable' MT. Stable MTs comprise only a fraction of the total population of cellular MTs. However, this fraction is very high in neurons (Bulinski and Gundersen, 1991; Bulinski, 2007). The stabilization of MTs is thought to be necessary for the polarization of the MT network. The formation of a polarized network in specific locations in cells is essential for proper functioning of the cell and in generation of cellular asymmetries.

One of the consequences of the extended capture and/or stabilization of MTs is that α-tubulin within the lattice of the captured MTs becomes a subject of post-translational modifications (PTMs) including acetylation (addition of acetyl group) and detyrosination (removal of C-terminal tyrosine). This makes stable MTs biochemically and functionally distinct from the dynamic MTs (Bulinski, 2007). Processes occurring on the 'long-lived' MTs are found to be time-dependent: the "older" the stable MTs are ($t_{1/2}$ can vary between ~1h to even 16h) the more enriched in acetylated and/or detyrosinated α-tubulin they become (Khawaja *et al.*, 1988). Detyrosinated and acetylated MTs are also termed nocodazole-resistant MTs as they are less susceptible to nocodazole-induced depolymerization (Bre *et al.*, 1987; Gundersen *et al.*, 1994).

α-tubulin within the MT is detyrosinated by a tubulin-specific carboxypeptidase, while the reverse reaction, tyrosination, is performed on cytosolic (detyrosinated) α-tubulin by tubulin tyrosine ligase (TTL), an enzyme involved in tumor progression. TTL-mediated tyrosination of α-tubulin is important for the binding of those +TIPs, that comprise a cytoskeleton-associated protein glycine-rich (CAP-Gly) MT binding domain, to the MT plus-end *in vitro* and *in vivo* (Peris *et al.*, 2006).

The enzyme responsible for acetylation of α-tubulin is alpha-tubulin acetyltransferase. MTs enriched in acetylated α-tubulin will further in the text be labeled as *acetyl*-MTs, those enriched in detyrosinated α-tubulin will be labeled as *glu*-MTs (glu for the newly exposed C-terminal glutamate residue), and those without these PTMs, the dynamic ones, or the Tyr-MTs.

In motile cells, glu-MTs and acetyl-MTs are found to be preferentially localized towards the leading edge (Bulinski and Gundersen, 1991). It has been shown that only acetylated MTs support kinesin transport in neurons (Reed *et al.*, 2006; Bulinski, 2007; Verhey and Gaertig, 2007). Moreover, MT detyrosination negatively affects the MT disassembly and inhibits the activity of the neuronal depolymerizing motor KIF2A (Peris *et al.*, 2009).

At the onset of mitosis the relatively stable interphase array transforms into a mitotic array with shorter, more numerous and more dynamic MTs (Gregoretti *et al.*, 2006). The drastic reorganization of the MT array results in the formation of a new structure- the mitotic spindle. The MTs now search and capture the chromosomes with their plus-ends. After the capture of the MT by the kinetochore, and subsequent to the spindle assembly checkpoint, MTs pull the chromosomes away from each other, towards their centriole-embedded minus-ends. This process, in which MT plus-ends function as molecular machines pulling the cargo while shrinking, is thought to be mediated by the Dam1 ring (Westermann *et al.*, 2006) and/or recently described fibrils (McIntosh *et al.*, 2008).

Factors involved in cortical capturing of the microtubule

Cortical capture of the MTs is thought to take place at the plus-end itself. +TIP cytoplasmic linker protein (CLIP-170), strategically positioned at the plus-end of the MT, was shown to directly interact with cortical actin binding protein IQGAP at the leading edge of migrating Vero cells (Fukata *et al.*, 2002). Additionally, IQGAP acts as an activator of two Rho GTPases, the signaling proteins that regulate cell morphology and migration, Rac1 and Cdc42. Increased Rac1 activity has been shown to correlate with enhanced MT re-growth after depolymerization induced by a MT depolymerizing agent-nocodazole (Waterman-Storer *et al.*, 1999).

CLIP-associated protein 2 (CLASP2) has also been demonstrated to specifically localize to the plus-ends directed towards the leading edge (Akhmanova *et al.*, 2001). Moreover, Drabek et al, showed, using scratch wounding assay that CLASP2, but not CLIP-170, is required for the MT interaction with the cortex at the leading edge and for persistent cell motility. Interestingly, CLASP2 localization at the cell edge was found to be regulated by actin-MT crosslinking factor ACF7 (MACF1,macrofilin1, microtubule-actin crosslinking factor 1) (Drabek *et al.*, 2006). In contrast to these findings, Watson and Stephens (Watson and Stephens, 2006) reported that siRNA-mediated depletion of CLIP-170 in HeLa cells caused defects in the ability of cells to polarize in response to scratch wounding. They speculate that decreased recruitment of dynactin component, $p150^{Glued}$, to the MT plus-ends in CLIP-170 depleted cells, could partly be responsible for the observed effects on polarization. Combined data indicate that CLIP-170 might be required for cell migration in a cell-type dependent fashion.

Microtubule plus-end

The minus ends of MTs are often associated with the gamma tubulin-containing nucleation point, the MTOC, which dictates their position in the cell and orientation. Release of MTs from the MTOC results in MTs with both plus and minus ends free in the cytoplasm. These 'free' MTs can also arise by breakage of the centrosomal MTs. In cells free MTs persist only when their minus end is stabilized or capped (epithelial cells and neurons). The stabilizing factors are not known yet, but might involve gamma tubulin (O'Toole *et al.*, 2003). In fibroblasts free MT minus-ends are not capped and are highly prone to rapid disassembly, while only the MTOC-associated MTs persist.

In fibroblasts MTs are arranged in a radial array, growing from the MTOC towards the cell periphery. Plus-ends of these MTs represent the essential sites where the MT dynamics is exhibited and regulated. Alterations of the dynamics of the MT plus-end lead to the alterations of the dynamics of the MT array. Therefore, it is of major importance to unravel the mechanisms of plus-end dynamics and its regulation, as well as how these affect the MT array as a whole and thereby cell behavior.

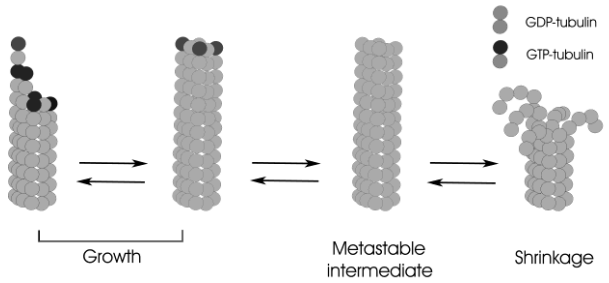

Figure 2. Proposed model of dynamic instability of the microtubule. MT exhibits dynamic fluctuations in length at its plus-end. The plus-end fluctuates between the phases of growth (A and B), pause (C) and shrinkage (D). Transition from growth to shrinkage is called catastrophe, while the reversed process, transition from shrinkage to growth or pause is called rescue. Growing plus-end is characterized by a sheet of PFs extending from the tube or by a blunt end which forms when the sheet closes (A and B respectively). The elongation and sheet closure occurs by addition of GTP-tubulin dimers and oligomers at the plus-end. The GTP-dimers at the tip of growing plus-ends provide stabilization via lateral PF-interactions. At the same time GTP-hydrolysis of GTP-tubulin occurs within the lattice (GTP-> GDP). When all the GTP-tubulin in the lattice is hydrolyzed the metastable intermediate state is reached (C). MT can now keep on growing (A) or start shrinking (D). Shrinkage is characterized by peeling off of the GDP-tubulin PFs generating frayed' plus-end conformation (D). A shrinking plus-end (D) can be rescued, temporarily stabilized (C) which would allow for subsequent addition of new tubulin units (A).

Plus-end dynamics and conformations in vitro

Electron microscopy (EM) studies on *in vitro*-assembled negative-stained and frozen-hydrated MTs demonstrated that the plus-ends exhibit various structural features depending on the particular condition that either favors MT polymerization or depolymerization (Mandelkow and Mandelkow, 1985; Simon and Salmon, 1990; Wade and Chrétien, 1993; Tran *et al.*, 1997a). These studies showed that specific morphological features of the plus-end are related to phases of shrinkage, pausing and

growth. The morphology of the plus-end was determined by considering the length and curvature of the PFs at the tip of MTs observed in 2D electron micrographs. The three main plus-end conformations described were: 1) frayed (*coiled, "ram's horn*"), 2) blunt and 3) sheet-like (*straight, tapered, extended*) (Mandelkow *et al.*, 1991; Chretien *et al.*, 1995; Arnal *et al.*, 2000).

In frayed end MTs the PFs curve inside out away from the MT axis, while in the blunt end MTs the PFs terminate simultaneously (Figure 2, blunt end (B and C), frayed end (D)). The sheet-like end appears like a blunt end with a number of PFs extending beyond the rest, resulting in a two-dimensional "sheet" (Figure 2A). The length of the extensions was reported to be dependent on the growth rate (which is dependent on the tubulin concentration) reaching 2µm under certain conditions (Chretien *et al.*, 1995). The minus-end was found to display similar features as the plus-end (Mandelkow *et al.*, 1991).

From the previous kinetic studies in combination with cryo-EM studies, a dynamic instability model has evolved in which the elongation phase, as the GTP•tubulin dimers are added at the plus-end, gives rise to a gently curved two-dimensional sheet, which closes in a zipper-like fashion at the seam (the point where the sheet is folded) (Wang and Nogales, 2005; Kerssemakers *et al.*, 2006) (Figure 1). As the sheet gradually straightens, due to the lateral interactions between the PFs, it closes forming a hollow cylinder, a tube thereby adopting the *blunt* end conformation (Chretien *et al.*, 1995; Arnal *et al.*, 2000), which was suggested to be a metastable transition state between growth and shrinkage (Tran *et al.*, 1997b; Arnal *et al.*, 2000; Jánosi *et al.*, 2002). The loss of the GTP-cap destabilizes the lateral interactions between the PFs and initiates curving of the PFs which then peel off away from the MT axis (*frayed* end).

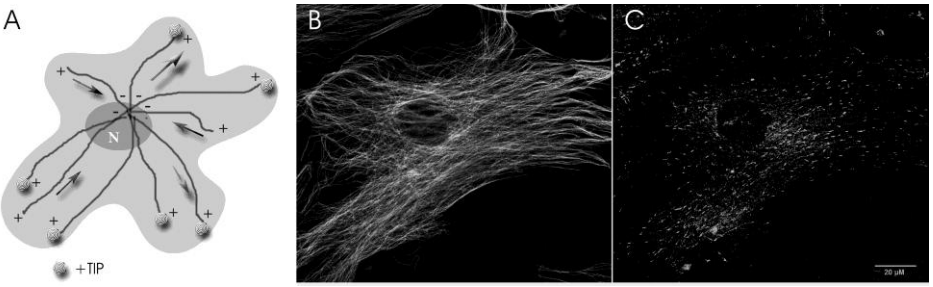

Figure 3. Radial architecture of MT array in interphase mammalian cells. (A) Scheme of a mammalian cell in interphase showing MTs (black lines) with their minus ends embedded in microtubule organizing center near nucleus (N) oriented with their plus-ends towards the cell edge. The plus-ends continuously grow and shrink (arrow-marked MTs). The MTs probe the cell periphery with their plus-ends, exhibiting "search and capture" behavior. Growing plus-ends are decorated with +TIPs. (B and C) Fluorescence microscopy images showing an interphase MT array (B) of an embryonic mouse fibroblast and (C) dot like pattern of +TIP protein, CLIP-170, in the same fibroblast. Magnification in B and C is the same (Bar in C, 20µm).

Plus-end dynamics and conformations in vivo

O'Toole and colleagues have used electron microscopy (EM) and electron tomography (ET) to describe the morphology of the chromosome-associated MT plus-ends and spindle pole body-associated minus-ends in *C.elegans in situ* (O'Toole *et al.*, 1999; O'Toole *et al.*, 2003). Two main distinct morphologies of the minus-ends, open and closed, were observed, while the plus-ends adopted an open conformation. The open conformation was subdivided into 1) flared and 2) blunt conformations. The minus end was often found to be capped by a structure suggested to comprise γ-tubulin (O'Toole *et al.*, 1999; O'Toole *et al.*, 2003)

EM/ET study of the plus-end structure in mitotic plant cells (*Arabidopsis)* revealed five different plus-end morphologies: 1) blunt (open), 2) extended (sheet-like), 3) horned (frayed, 'ram's horns'), 4) hybrid (horned/extended) and 5) flared. The lengths of the sheets in the extended ends were reported to range between 50-300nm (Austin *et al.*, 2005). VandenBeldt et al described two classes of plus-end conformations of the kinetochore-associated MTs of *Drosophila* and Ptk1 cells: straight and curved. The straight class consisted of: 1) blunt, 2) extended (sheet-like), 3) capped and 4) flared plus-end conformations. The curved class included: 1) typical frayed ends and 2) frayed-like ends which PFs were curving just like in frayed ends, but were much shorter than in frayed ends (VandenBeldt *et al.*, 2006). Taxol, a MT stabilizing agent that modifies lateral interactions between PFs (Nogales *et al.*, 1999; Perez *et al.*, 1999) gave rise to a higher frequency of MT plus-ends with straight conformation. On the other hand, nocodazole, an agent that binds to cytoplasmic tubulin and thereby causes MT depolymerization, resulted in a significant increase of curved plus-ends.

All the mentioned studies on the plus-end geometries were performed on kinetochore – associated MTs. The first study describing the plus-end morphologies of interphase MTs was published in 2007 (Höög *et al.*, 2007). Using ET, authors showed the existence of five distinct plus-end morphologies in the periphery of the fission yeast cell: 1) capped, 2) blunt, 3) flared, 4) extended (sheet-like) and 5) curled (termed 'frayed' in other studies). In this study it was suggested that flared ends were the growing ones, as they were found at the tips of the longest MTs.

Microtubule plus-end tracking proteins (+TIPs)

The dynamic behavior of the MT plus-end, an intrinsic property of MTs, is spatially and temporally regulated by a group of proteins that are associated to the MTs, the so-called MT associated proteins or MAPs. Among them are also proteins that specifically bind to the MT plus-ends, i.e. plus-end tracking proteins (+TIPs). Plus-end tracking was for the first time observed in live cells expressing GFP-tagged CLIP-170, where the MT tips appeared as comet-tails moving throughout the cell (Diamantopoulos *et al.*, 1999; Perez *et al.*, 1999).

+TIPs comprise a) non-motor proteins and b) motor proteins. Among the non-motor +TIPs are cytoplasmic linker proteins (CLIP family), end binding proteins (EB family) , CLIP-associated proteins (CLASP family), adenomatous polyposis coli tumor suppressor

protein (APC), the dynactin subunit p150^{glued}, dynein regulatory factor LIS1, the spectraplakin ACF7, and navigator proteins (Perez *et al.*, 1999; Carvalho *et al.*, 2003; Kodama *et al.*, 2003; Akhmanova and Hoogenraad, 2005; Martínez-López *et al.*, 2005; van Haren *et al.*, 2009). The +TIP motors include the minus-end directed motor dynein and the plus-end directed motor kinesin-13, or mitotic centromere-associated kinesin (MCAK), in mammalian cells. In yeast Tea2/Kip2 displays plus-end tracking behavior (Akhmanova and Steinmetz, 2008)

EB proteins exhibit anti-catastrophe activity. They also recruit other +TIPs, like CLIPs, to the MT ends (Bieling *et al.*, 2007; Bieling *et al.*, 2008; Dixit *et al.*, 2009). CLIP-170 in mammalian cells and Tip1p, its homologue in yeast, have been found to promote rescues and act as anti-catastrophe factors, respectively (Brunner and Nurse, 2000; Komarova *et al.*, 2002a). CLIP-170 is also involved in targeting of dynactin to the plus-ends. CLASPs enhance MT polymerization and link the MTs to the cell cortex, kinetochores and Golgi. APC is an anti-catastrophe +TIP, also involved in linking of MTs to the cell cortex. The motor protein MCAK on the other hand promotes MT disassembly and induces catastrophes by curving the dimers within PFs of the MT lattice. Kinesin-14 promotes depolymerization of the MTs, and is , together with dynein, involved in transport via MT tracks. Dynein, whose processivity (i.e ATP-driven movement along a microtubule) is regulated by the dynactin subunit p150^{Glued}, is involved in pulling of the MTs at the cell cortex and in transport of organelles over MTs.
Most of the +TIPs have the ability to bind to one or more +TIPs members thereby influencing each others behavior and thus MT dynamics. EB proteins seem to be in the center of the large network of interactions. At their N-terminus the MT binding (MTB) domain of EB proteins contains a calponin homology (CH) domain. This CH domain is necessary for tracking of MT plus-ends. CLIP-170 and p150^{Glued} share a conserved cytoskeleton-associated protein glycine-rich (CAP-Gly) domain involved in binding to the MT and to EB proteins (Carvalho *et al.*, 2003; Mimori-Kiyosue and Tsukita, 2003; Akhmanova *et al.*, 2005; van Haren *et al.*, 2009). These two proteins, together with CLIP-115, also share short basic and Ser-rich domains implicated in MT binding. The N-terminus of CLIP-115 is structurally similar to that of CLIP-170. EB proteins, CLIP-170 and p150^{Glued}, also contain coiled-coil domains, which mediate parallel homodimerization of protein monomers (note: recent data have shown that EB-family members can also heterodimerize). Homodimerization is found to be required for the anti-catastrophe activity of EB proteins (Komarova *et al.*, 2009). At their C-terminus, EB proteins and CLIP-170 share the EEY/F sequence motif. Interestingly, this sequence motif is also present at the C-terminus of alpha-tubulin and it is this short motif that is recognized by the CAP_GLY domain. The MTB domains in APC, ACF7, CLASP and navigators are represented by extensive regions enriched in basic and serine residues. Tracking of the growing plus-ends by kinesins and dynein is mediated by domains outside the microtubule-interacting motor domains and relies on their association with other +TIPs.

Mechanisms of plus-end targeting by mammalian +TIPs

In the past, three mechanisms by which + TIPs were thought to accumulate at the MT plus-ends have been proposed, i.e. motor driven transport, hitchhiking and treadmilling.

Recently, a new model has been established for the tracking of the plus-ends by mammalian proteins, including CLIP-170, EB3 and CLASP. In this model an excess of binding sites, present at the polymerizing plus-end, bind and release +TIPs a number of times before disappearing. The disappearance of these binding sites is exponential, suggesting the control of these binding sites by the specific conformation of the plus-end (Dragestein *et al.*, 2008).

The +TIP comet-like structure fades away the moment a MT plus-end encounters the barrier of the cell edge and starts shrinking. A similar behavior is observed upon treatment of the cells with a shrinkage-inducing agent like nocodazole. The disassociation of the +TIPs from a shrinking MT is thought to be a consequence of the loss of the GTP-cap or the sheet-like structure at the plus-end.

It has been shown that the MT-stabilizing agent taxol causes displacement of CLIP-170 and EB1 from the plus-ends when applied in macromolar concentrations (Perez *et al.*, 1999). Interestingly, at low concentrations of taxol, GFP-tagged CLIP-170 and EB3 still decorate the plus-end, of not only the growing, but also of shrinking and pausing MT plus-ends. Low (nanomolar) concentration of taxol has been shown to enhance MT dynamics, rather than suppressing it (Pasquier *et al.*, 2005). These observations suggest that +TIPs do not recognize the dynamic state (growth, shrinkage, and pause) as such, but rather a structural/biochemical feature at the plus-end. Moreover, this feature can be modified by taxol in certain conditions. In the presence of low concentration of taxol, the +TIP-decoration pattern of the plus-end changes, i.e. the comet-like plus-end accumulation of these proteins becomes dot-like. Previous studies have shown that taxol binding to the MT leads to the modification of the lateral interactions between PFs (Nogales *et al.*, 1999) and prevents the GDP subunit from adopting a kinked configuration after GTP hydrolysis (Arnal and Wade, 1995; Elie-Caille *et al.*, 2007). The above described observations suggest that taxol treatment leads to the exposure of the binding sites for +TIPs, like CLIP-170, EB3 and CLASP.
Since in this thesis a large part of work involving CLIPs is presented (Chapter 5), in the next paragraph their function as +TIPs will be discussed more thoroughly.

Cytoplasmic linker proteins (CLIPs)
Structure of CLIPs. The cytoplasmic linker protein of 170 kD, CLIP-170, is characterized by two conserved cytoskeleton-associated protein glycine-rich (CAP-GLY) domains situated in the N-terminal region. These domains target the acidic C-terminal region present in both α-tubulin and the +TIP end-binding 1 (EB1) protein, which indicates that both proteins compete for the same binding site (Honnappa *et al.*, 2006; Mishima *et al.*, 2007; Slep and Vale, 2007). The two CAP-Gly domains (Cap-Gly1 and Cap-Gly2) have been proposed to interact with α-tubulin from two tubulin dimers (Slep and Vale, 2007). Interestingly, only tyrosinated α-tubulin is able to bind CLIP-170; detyrosination of α-tubulin abolishes this interaction (Peris *et al.*, 2006).

At the C-terminal region of CLIP-170 a metal binding domain, or Zn-finger domain, is localized, necessary for targeting of two other +TIPs, namely LIS1 and the dynactin

subunit, p150^{Glued} to MT ends. The Zn-finger domain is also able to interact with the CAP-GLY domain of the same molecule, resulting in intramolecular folding leading to auto-inhibition of CLIP-170 (Lansbergen *et al.*, 2004). The N- and C-terminal regions are separated by a coiled-coil region which facilitates homodimerization of CLIP-170. This region is also responsible for binding another +TIP, namely CLIP-associated protein or CLASP.

In contrast to CLIP-170, its homologues in budding and fission yeast, Bik1 and Tip1p respectively, comprise only one CAP-Gly domain and one Zn-finger motif per monomer.

The closest mammalian homologue of CLIP-170 is CLIP-115 (De Zeeuw *et al.*, 1997). All domains present in CLIP-170 are also present in CLIP-115, except for 1) C-terminal Zn-finger domain, indicating lack of ability of CLIP-115 for auto-inhibition (Coquelle *et al.*, 2002) and 2) C-terminal EEY/F domain, involved in mediation of interaction with proteins containing CAP-Gly domain.

Function of CLIPs. In mammalian cells, the removal of CLIP-170 and CLIP-115 inhibits the rescue of MTs near the cell cortex (Komarova *et al.*, 2002a; Arnal *et al.*, 2004). Instead of having frequent fluctuations of growth and shrinkage near the cell edge, the MTs in these cells display persistent growth and persistent shortening (Komarova *et al.*, 2002a). CLIP-170 recruitment to the plus-ends was found to be enhanced in CLIP-115 knock-out fibroblasts, suggesting a compensatory role of CLIP-170 (Hoogenraad *et al.*, 2002). Elimination of Tip1p, the CLIP-170 homologue in fission yeast, resulted in impaired MT growth, leading to abnormal cell morphology (Brunner and Nurse, 2000). While in mammalian cells CLIP-170 acts as a rescue factor (converting shrinking MT plus-ends into growing ones), the role of Tip1 in fission yeast is that of an anti-catastrophe factor (inhibiting conversion from growth to shrinkage) (Brunner and Nurse, 2000).

In mammalian cells CLIP-170 has been implicated in the regulation of cell polarity (Fukata *et al.*, 2002). CLIP-170 is responsible for regulation of accumulation of CLASPs (unpublished data) and the dynactin subunit, p150^{Glued} at the MT plus-end (Watson and Stephens, 2006).

Molecular interactions of plus-end tracking CLIPs. CLIP-170 recognizes and binds the C-terminal acidic tail of α-tubulin, which is exposed at the surface of tubulin dimers/oligomers but also within the MT lattice, and thus in all cases accessible for CLIP-170 (Mishima *et al.*, 2007). Moreover, a recent study has shown that tyrosination of α-tubulin subunits is essential for the recruitment of CLIP-170 to the plus-end (Peris *et al.*, 2006). Even though CLIP-170 is capable of binding directly to α-tubulin, thus probably also α-tubulin within the MT lattice, another study has shown that the +TIP EB1 is required for recruitment of CLIP-170 at the plus-end of the MT in mammalian cells and for the homologues in yeast (Busch and Brunner, 2004; Watson and Stephens, 2006; Bieling *et al.*, 2008). In contrast, a study using *in vitro*-assembled MTs by Ligon and colleagues demonstrated the opposite mechanism - EB1 targeted the MT plus-ends

specifically only in presence of CLIP-170 (Ligon *et al.*, 2006). The association of Bik1 and Tip1 (CLIP-170 homologues in budding yeast and in fission yeast, respectively) to the plus-ends was found to be via motor-driven transport by motor protein kinesin (Busch *et al.*, 2004; Carvalho *et al.*, 2004). In fission yeast, even though the targeting of the plus-end by Tip1p is kinesin-mediated, it still requires Mal3, which is a homologue of EB1 (Busch *et al.*, 2004; Carvalho *et al.*, 2004; Bieling *et al.*, 2008). However, in budding yeast, plus-end tracking by Bik1 is not dependant on Bim1 (EB1 homologue in budding yeast) (Badin-Larcon *et al.*, 2004). It is possible that different systems, in spite of a great degree of homology among the +TIPs, engage different mechanisms of plus-end tracking. Recently Dragestein and colleagues (Dragestein *et al.*, 2008) proposed a plus-end tracking model in which an excess of binding sites present at the polymerizing plus-end bind and release +TIPs a number of times before disappearing. The disappearance of these binding sites is exponential suggesting the control of these binding sites by the conformation of the plus-end itself. The association/dissociation of the CLIP-170-GFP molecules, as well as EB3-GFP molecules, with the MT plus-ends in this system, was found to be diffusion-limited. Interestingly, these authors found that at low concentration of the MT-stabilizer taxol, instead of CLIP-170-GFP dissociation from the plus-end observed at high taxol concentrations, these molecules remained bound to the plus-end. Moreover, in the presence of low concentration of taxol, instead of a comet-like structure at the plus-end, a dot like pattern of GFP-CLIP-170 was observed on the plus-ends, also of some shrinking and pausing MTs.

Scope of the thesis

The aim of this thesis was to a) investigate and describe the relationship of the MT plus-end morphologies and dynamics, and b) investigate the effect of CLIP170/CLIP115,-deficiency on the formation of a particular MT plus-end morphology in interphase mouse fibroblasts. Moreover, we set out to investigate the effect of CLIP170/115-deficiency on the formation of MT network and on the cellular morphology of mouse interphase fibroblasts in general.

In order to visualize and investigate the structural features of the MT plus-ends we have used an electron microscopy approach combined with a relatively new technique: electron tomography. Chapter 2 of this thesis concerns a functional description of these techniques and discusses their application in the study of MT plus-ends. Also the advantages and disadvantages of these techniques, regarding visualization of the MT plus-end conformations (morphologies), are discussed.

Chapter 3 describes a study of the various plus-end conformations present in the periphery of interphase mouse fibroblasts (3T3 cells). We show nine major interchangeable plus-end conformations, one of which was not previously reported in the literature. Investigating the plus-end structures in cells treated with the MT depolymerizing agent nocodazole, and thus shifting the dynamic instability of the MTs in favor of disassembly, enabled us to follow the chronology of events occurring at the plus-

end just before, and at the very onset, of MT shrinkage. Using kinetic analysis of the nocodazole-induced shifts, we were able to link a particular plus-end conformation to the corresponding dynamic state. Concluding, we propose a pathway of transitions of one plus-end conformation into the other conformation, corresponding to the change from one dynamic state into the other. Additionally, we show here that the various plus-end conformations are non-randomly distributed over the cell periphery, but rather that particular conformations are stabilized by factors present in the cell periphery.

Chapter 4 concerns a cryo-electron microscopy and cryo-electron tomography (cryo-ET) study of the periphery of whole-vitrified primary interphase mouse embryonic fibroblasts (MEFs). We show that whole-mount cryo-ET applied on extremely thin cells like MEFs enables high resolution study of impeccably preserved structural features of various cellular structures including the MTs and MT plus-ends. Furthermore, we show that MEFs contain densities inside their MTs, as was shown recently for MTs in neurons (Garvalov *et al.*, 2006).

Chapter 5 includes a study describing the effects of CLIP170/115-deficiency on various aspects of MTs investigated in interphase MEFs at three related levels. First, we investigated these effects on the level of structural features characterizing the MT plus-end using electron microscopy and electron tomography. By comparing plus-end conformations in CLIPs-deficient MEFs with those in wild type MEFs, we were able to identify conformations which are likely to be stabilized and/or rescued by CLIPs. Furthermore, we used fluorescence microscopy in order to investigate the effects of a CLIP170/115-deficiency on the level of MT network. We focused on both dynamic (tyrosinated) MTs and stable MTs (acetylated and detyrosinated MTs). Our findings indicate a role of CLIPs in MT distribution in interphase MEFs which can be ascribed to perturbed dynamic instability caused by loss of CLIPs as rescue factors. Moreover, our findings indicate that CLIPs are involved in the formation of nocodazole-resistant MTs suggesting a stabilizing mechanism governed by CLIPs. Finally, we studied the effects of CLIPs-deficiency on cell processes like cell spreading and adhesion. We found that CLIPs play a role in cell spreading by targeting and signaling to focal adhesion sites.

Chapter 6 integrates our findings with the information obtained from the literature on the subject of MT plus-end structure and dynamics and discusses diverse aspects of this and similar research. Chapter 6 includes Summary (English) and is followed by Samenvatting (Nederlands).

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