Coordinated Activities of Microtubule-Associated Proteins in Spatial Cytoskeleton Organisation and the Mechanisms Mediating their Microtubule Plus End-Tracking

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1 Introduction

1.1 Morphogenesis and Cell Polarity in Eukaryotes

1.1.1 Cellular Polarisation

A eukaryotic cell is a highly structured unit in which organelles and protein complexes are organised in a defined and cell-type specific way. The various components carry out specialised functions that contribute to the survival of the cell. Therefore, they need to be arranged in a specific order in the three dimensions of the cell and over time. Such spatial organisation, which includes the plasma membrane, is generally referred to as cell polarity. Cell polarity is an essential feature of all organisms, even bacteria. It is the basis for most fundamental cellular processes such as cell growth, cell division, differentiation, cell migration, cell-cell signalling or fertilisation.

To achieve a polarised organisation, cells make use of protein filaments that collectively are termed cytoskeleton. The cytoskeleton is involved for example in the positioning of organelles, the transport of material to different regions of the cell, generation of cell shape, the faithful inheritance of chromosomes in mitosis, and it also provides physical stability. There are three different forms of cytoskeletal elements, microtubules, actin and intermediate filaments, each with their own characteristics and functions. A central feature of the cytoskeleton is its ability to reorganise rapidly according to changing needs of the cell, for example in mitosis when a cell has to split its contents into two daughter cells. Cytoskeletal organisation is to a large extent determined by accessory proteins. Therefore, these proteins have a great influence on cell polarity.

1.1.2 General Principles of Polarisation

Based on work in a variety of model organisms, some general principles of cellular polarisation have emerged. To establish a polarised state, a cell either has to break an initially symmetrical structure into an asymmetric one, or it has to re-organise an already existing polarised organisation. The polarisation of cells can be described as a hierarchy of three basic steps:

1. Polarisation needs a signal that triggers the process. These signals are either localised or they activate a region that was previously defined by landmarks.
2. In response to the trigger, polarity then is established, meaning that the initial signal is amplified. This involves the activity of a number of small GTPase proteins, which are activated only at the site of the localised signal or landmark. Polarisation brings about a reorganisation of the cytoskeleton, which is achieved through a variety of GTPase effector proteins.

3. Finally, the polarisation has to be maintained. This is most likely accomplished through molecular feedback loops that mutually reinforce activation or localisation of signalling molecules, and that control cytoskeletal organisation.

1.1.3 Localised Initiation of Polarisation

Common to all polarisation processes is an initial trigger, an event that sets the cell on course towards polarisation. The trigger can be an extrinsic signal from outside the cell. Examples are sperm entry into the oocyte during fertilisation, or signalling molecules, such as growth factors. It can as well be an intrinsic signal, for example when the entry into a new cell cycle stage is triggered. The triggering signal can also provide positional information if it is locally active. However, a ubiquitous triggering signal can also lead to polarisation if only predefined regions of the cell respond to it. For example in epithelial cells, polarity depends on the cellular environment, e.g. on where a cell is attached to its neighbours.

1.1.3.1 Determination of the Site of Growth in Budding Yeast

Cellular polarisation has been most extensively studied in the budding yeast *Saccharomyces cerevisiae* (rev. in [1-4]). Budding yeast cells mostly grow asymmetrically, at the so-called bud, which defines the newly forming daughter cell. The budding site, where the bud emerges, is selected during the G1 phase of the cell cycle. In haploid cells, the bud forms adjacent to the site of the previous cell division, the bud scar, in both the mother and the daughter cell (figure 1.1). On a molecular level, the bud site is determined by a set of proteins, including Bud3, Bud4 and the integrin transmembrane receptor-like protein Bud10. These proteins localise to a patch next to the connection between the mother and daughter cells, the bud neck. The localisation of these proteins depends on the septin proteins, which form a ring structure around the bud neck, and remain there throughout the cell cycle. Bud3, 4 and 10 have been termed 'landmark' proteins, because their localisation determines the site of future growth after mother and daughter cell have divided. In vegetative growth, polarisation therefore is independent of signals from outside the cell. It is triggered by cell cycle commitment in late G1 phase and is localised by an intrinsic cue.

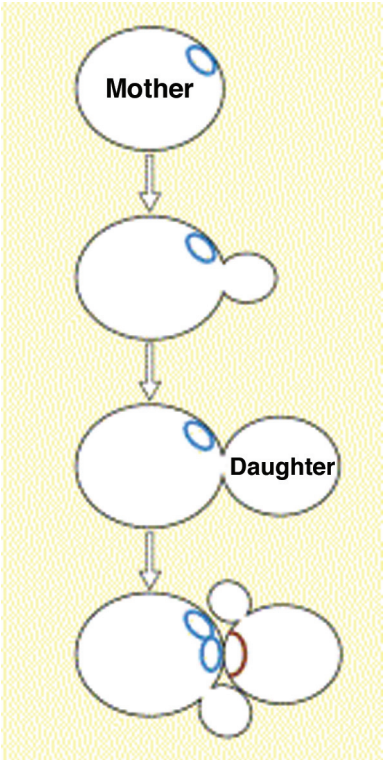

Figure 1.1 The Budding Pattern of *S. cerevisiae*
The birth scar (brown) is the point at which the daughter cell was originally attached to its mother, and the bud scar (blue) is the position of the previous bud. Both mother and daughter cell place the new bud site next to these scars. Adapted from [10].

1.1.3.2 Determining the Direction of Cell Migration

Cell migration, that is, translocation of a cell relative to its environment, is a much-studied example of cell polarity in both unicellular and multicellular organisms (rev. in [5]). It is important for the function of many cell types, for example in embryonic development, when cells have to reach their designated location in the embryo. Single cells of the slime mould *Dictyostelium* migrate either to find new places where nutrients are available, or to move toward each other to form aggregates during differentiation.

Cell migration is usually triggered by external signals coming from local sources. The signals usually are graded, meaning they are increasingly diluted with increasing distance to their source. The direction of the gradient can be sensed by the cells. Following the gradient is believed to cause directionality of migration.

The guidance signals bind to cell surface receptors, such as G-protein coupled receptors or integrins. The binding of ligands to the extracellular portion of receptors leads to their activation through conformational changes of the receptors, for example the clustering of integrins, and/or phosphorylation of intracellular domains. The activation of these receptors leads to the local induction of second messengers such as phosphoinositides, cAMP, or Ca^{2+}. The gradients of extracellular cues are usually quite shallow, and receptors are normally uniformly distributed. However, downstream of them, signal amplification takes place; consequently, the distribution of second messengers shows a much steeper gradient, allowing the cell to establish a stable direction of migration. To this end, the second messengers initiate a cascade of intracellular signals such as protein tyrosine phosphorylation, GTPase activation and changes in phospholipid biosynthesis. This induces the formation and strengthening of adhesion sites to other cells and it reorganises the cytoskeleton such that the dynamic migration process can occur.

1.1.4 Establishment of Polarity: The Role of GTPases in Signal Amplification

Once *de novo* polarisation or a change in polarisation is triggered, the subsequent polarisation process is believed to generally follow the principle of self-organisation of biological structures [6]. Such self-organisation is characterised by the interaction of robust chemical reactions in a cell or organism that aim to reach a steady state. Such a steady state does not correspond to an equilibrium. It is dynamic, characterised by continuous consumption of energy, and gain and loss of material; however, the rate of energy dissipation is minimised. It can be rapidly adjusted in response to a change in circumstances. In the case of cellular polarisation, self-organisation is based on the interplay of local activators and long-range inhibitors (see example below) [7]. Through positive feedback loops, the activators amplify as well as stabilise the initial signal, while the inhibitors suppress the formation of additional sites of polarisation.

1.1.4.1 Coordination of Polarisation Processes by Rho GTPases

A large number of different cellular mechanisms need to be regulated in a coordinated way during polarisation toward one site in the cell. How does the cell achieve this? Small guanosine triphosphate binding proteins (GTPases) of the Rho family turned out to be the key players in polarisation. They provide locally active signals that regulate, amongst other things, the reorganisation of the cytoskeleton [8, 9]. Rho GTPases are molecular switches that are conformationally regulated by the binding of a guanine nucleotide. When they are bound to GDP, they are inactive, but when the GDP is replaced by GTP, they become active. GTPases are activated by their guanine nucleotide exchange factors (GEFs), which swap the GDP with GTP, and inactivated by GTPase activating proteins (GAPs), which enhance their weak intrinsic GTPase activity to hydrolyse GTP into GDP (figure 1.2). In their active state, Rho GTPases activate a variety of downstream effector proteins.

1.1.4.2 Establishment of the Bud Site by the Activity of Rho GTPases in Budding Yeast

In *S. cerevisiae* cells, landmark proteins mark the future site of growth. Upon commitment to growth, a cascade of small GTPases establishes this location as the actual site of cellular growth. Bud1 is a ras-like GTPase that is uniformly distributed in the cell cortex. It is thought to be regulated by the GEF Bud5 and the GAP Bud2. Recruitment of Bud5 to the site of bud selection by binding to the landmark protein Bud10 locally activates Bud1 there [10]. GTP-bound Bud1 in turn binds to and activates Cdc24, which is the GEF for Cdc42, another small GTPase. This way, Cdc42 is selectively activated at the landmark.

Cdc42 is the key mediator of cell polarity, since all molecules upstream of it are not required for establishing polarity per se, but to determine its proper position; in mutants of the landmark proteins, the bud site is formed, but the localisation in the cell is random. In mating or diploid cells, other proteins mark the growth site but they also converge on Cdc42 for its establishment. Cdc42 itself is not required for growth *per se*, but to define the dimensions of a growth site. In its absence, cells still grow, but they cannot restrict growth to a defined bud site. As a consequence, the cells expand isotropically, that is, uniformly.

A particularly elegant set of experiments has further clarified this point. Thereby, budding yeast cells were arrested in G1 phase, at a time when Cdc24 is not yet available to activate Cdc42. Overexpression of a constitutively active form of Cdc42 is then sufficient to induce single polarised sites. These are marked by the accumulation of actin and Cdc42 itself. The sites are stable over time, and importantly, they are of a defined diameter. If more activated Cdc42 is expressed, the sites do not expand but instead a second or even third independent polarised zone forms. The formation of such a polarised zone depends on the formation of actin cables and motor protein-dependent transport of more Cdc42 along the cables. It is thought that Cdc42 activity leads to recruitment of actin and secretory vesicles to the region of activation, so that in turn more Cdc42 is delivered

to that region. This way, it forms a classical positive feedback loop. This experiment suggests that Cdc42 is at the centre of a self-organising circuitry, the triggering of which is sufficient to establish a defined growth zone [11].

Activated Cdc42 regulates a range of downstream effectors, which in turn regulate many aspects of polarised cell growth. Primarily, they promote the formation of a branched network of actin filaments, termed actin patches, which form a cap at the bud site. In addition, Cdc42 triggers formation of unbranched bundles of actin filaments called actin cables. The actin cables are 'spooled' out from and anchored at the cortex of the bud, and grow into the mother cell. The orientation of the actin cables then determines the position of the mitotic spindle, as the plus ends of astral microtubules are guided toward the bud along these actin cables [12]. Models for the establishment of stable cellular polarisation postulate that, in addition to the local self-amplifying activity of an activator, a global inhibitor is required that suppresses activation in the rest of the cell [13]. Much less is known about possible global inhibitors than about local activators involved in positive feedback loops, but the budding yeast GAP Bem2 is a good candidate. It negatively regulates Cdc42, is uniformly distributed over the cell cortex, and in its absence, multiple buds are formed [7].

1.1.4.3 Local Activation of Rho GTPases Coordinates Cell Migration

Also in mammalian cell migration, GTPases of the Rho family are the key players. They become locally activated as a result of extracellular signal molecules that bind to cell surface receptors, and regulate cytoskeleton organisation and cell adhesion during migration (figure 1.3) [8, 9].

At the leading edge of a migrating cell, the Rho GTPases Rac and Cdc42 become active and turn on a host of effector proteins, leading to the formation of a branched network of actin filaments

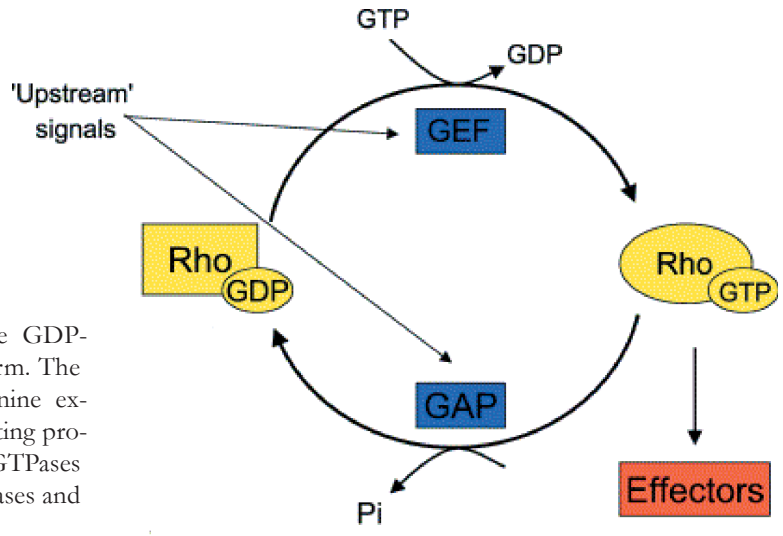

Figure 1.2 The Rho GTPase Cycle
Rho GTPases cycle between an inactive GDP-bound form and an active GTP-bound form. The cycle is tightly regulated mainly by guanine exchange factors (GEFs) and GTPase activating proteins (GAPs). In their active form, Rho GTPases can bind to effector molecules such as kinases and scaffold proteins. From [8].

below the cell cortex. The polymerisation of this network creates a pushing force against the plasma membrane, and results in the formation of broad protrusions called lamellipodia that extend in the direction of movement. Proteins downstream of Cdc42 form unbranched, bundled actin filaments at the front of the cell, which leads to the extension of thin, spike-like protrusions called filopodia. These are believed to explore the local environment.

In order to promote net cell translocation, lamellipodia have to form at the right location, meaning that the correctly localised activation of Rac is essential. Lamellipodia can form independent of Cdc42 activity. Cdc42 does, however, play a crucial role in controlling and stabilising the direction of migration, and thus in determining where the lamellipodia are formed. If Cdc42 activity is blocked, cells migrate in random, transient directions [14].

Contraction of the cell body and retraction of the plasma membrane at the rear end of the cell moves the cell toward the direction of migration. Another Rho family GTPase, Rho, directs this process by promoting the assembly of contractile actin/myosin filaments in the cell body. These are connected to adhesion sites at the leading edge. Thus, by forming protrusions and promoting contraction, actin reorganisation provides the driving force for cell translocation.

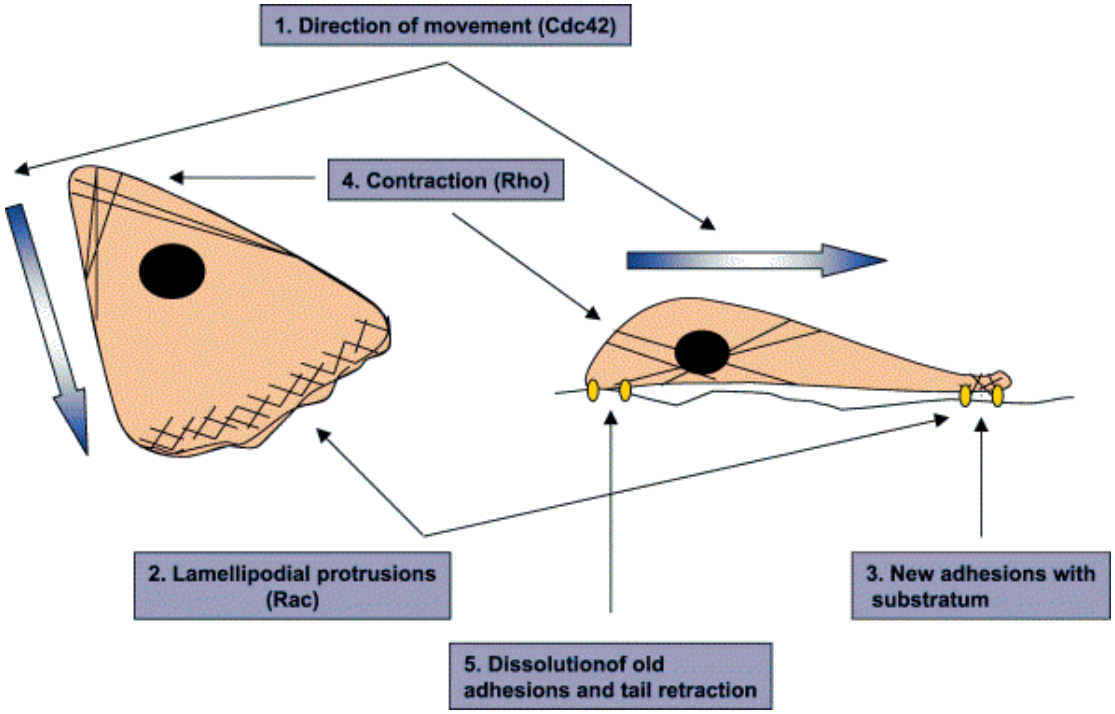

Figure 1.3 A Migrating Cell (seen from the top and side)
A migrating cell needs to perform a coordinated series of steps to move. Cdc42 regulates the direction of migration, Rac induces membrane protrusion at the front of the cell through stimulation of actin polymerisation and integrin adhesion complexes, and Rho promotes actin/myosin contraction in the cell body and at the rear. From [8].

The microtubule cytoskeleton also becomes highly polarised during migration, and its organisation is controlled by the Rho GTPases as well. Both Rac and Cdc42 can recruit microtubules to the leading edge, which reinforces cellular polarisation toward the direction of migration [15]. Cdc42 activity also leads to the repositioning of the microtubule organising centre (MTOC) in front of the nucleus [16-18]. The Golgi apparatus also becomes oriented towards the leading edge of the cell. Cdc42 activity might facilitate microtubule growth toward the leading edge, thereby promoting microtubule-mediated delivery of Golgi-derived vesicles to that site and providing the membrane components and associated proteins needed to form cell protrusions.

Rho activity also affects microtubules. It promotes the formation of extremely stable, non-dynamic microtubule plus ends [19]. These stable microtubules extend from the MTOC at the nucleus preferentially toward the leading edge in polarised cell culture cells. It is possible that these stabilised microtubules help to bias microtubule-dependent vesicle transport toward the leading edge.

1.1.4.3.1 *Formation and Turnover of Adhesion Sites in Migrating Cells*

The protrusions formed toward the direction of migration are stabilised by adhesion to the extracellular matrix or adjacent cells via integrin transmembrane receptors [5]. The formation of integrin adhesion complexes at the leading edge is controlled by Rac. These adhesions act as the "feet" of a migrating cell by serving as traction sites to generate the force for moving forward.

Disassembly of integrin adhesion sites at the rear end of the cell is also important for the cell to be able to move forward. This process is under the control of Rho GTPases as well. Microtubules are thought to play an important role also in adhesion site turnover. They were found to specifically and repeatedly target adhesion sites, possibly guided there along actin filaments. Multiple events of microtubule targeting to substrate contacts precede the release of adhesion, and addition of microtubule depolymerising drugs slows down adhesion site turnover [20].

1.1.5 Stabilisation and Maintenance of Polarity

Once polarity is established and the above mentioned 'steady state' is reached, feedback loops between signalling and cytoskeletal proteins as well as membrane lipids are operating to stabilise the desired polarised state of the cell. Specific targeting of newly synthesised proteins to the sites of polarisation further helps to consolidate it. However, because of the dynamic organisation of their components, many cells can adjust their polarity rapidly if the environment changes, for example, if guidance signals come from a different direction, through reorganisation of membranes and the cytoskeleton.

1.1.5.1 Stabilisation of the Direction of Cellular Migration

A number of self-organising mechanisms in migrating mammalian cells serve to stabilise and concentrate the localisation of active GTPase signalling through positive or negative feedback loops. GTPase-linked feedback loops help to amplify the shallow gradients of external chemoattractants

that can be sensed by the cell, so that a stable direction of movement can be achieved. In particular active Cdc42, stabilised through feedback loops, is needed to maintain a persistent, stable leading edge; when it is inhibited, only short-lived leading edges form, despite the activation of Rac [21].

Feedback loops were found to exist with many effectors of Rho GTPases. Some of them can act as GEFs or GAPs; others are activators of GEFs/GAPs.

Another example of a feedback loop involves integrin at the leading edge. Integrin adhesion sites stimulate Rac activation and its membrane targeting; Rac in turn induces recruitment and clustering of activated integrins. Yet another example involves special lipids. Lipids such as the phosphoinositide PIP_3 become polarly distributed in response to extracellular stimuli. PIP_3 accumulates at the front of a migrating cell as the result of localised accumulation and activation of PI3 kinase, which generates PIP_3. It also depends on exclusion of a PI3K antagonist, the PIP_3-phosphatase PTEN, from the leading edge [22]. PIP_3 leads to an increase in GTP-bound Rac, presumably through direct interaction of the lipid with Rac GEFs. Rac-GTP then is able to bind and stimulate PI3 kinase directly, which leads to more production of PIP_3. In this way, it forms another positive feedback loop that reinforces the gradient of activity of Rac GTPase [23, 24].

Also microtubules form a feedback loop with Rho GTPases by acting both upstream and downstream of them. As mentioned earlier, Rac can direct microtubule growth toward the leading edge of the cell. Dynamic microtubule ends, which are most abundant at the leading edge, can in turn somehow activate Rac, possibly through microtubule-associated GEFs [25]. Also Rho seems to become locally activated by increased microtubule depolymerisation, which occurs in regions outside the leading edge, and increases cell body contraction [26].

Finally the GTPases Rho and Rac can suppress each other's activity, which may help to separate their functions and keep it properly localised to distinct locations within the cell.

1.1.5.2 Differential Sorting of Proteins Stabilises Polarity

Maintenance of polarity in many cells includes the establishment of different membrane domains in different regions of the cell surface, each of which has its own characteristic protein content. One mechanism to ensure that proteins end up in specific membrane domains is differential sorting, which occurs in the pathway for vesicular transport toward the plasma membrane. Sorting is usually controlled in the trans-Golgi network but can also occur after delivery of vesicles to the plasma membrane. In this case, termed transcytosis, specific proteins are endocytosed again and subsequently transported to another membrane domain. The organisation of the cytoskeleton is essential for correct protein sorting [2]. Actin may be important for local vesicle delivery at the membrane, while microtubules provide long-range pathways for vesicle delivery. Motor proteins moving on microtubules in both directions are required for targeted vesicle transport [27].

1.1.5.2.1 *Localised Delivery of Proteins in Yeast to the Site of Growth*

For polarised growth of budding yeast cells, it is important that the delivery of proteins and lipids used for expanding the cell wall and perhaps the plasma membrane is restricted to the region of growth, and that these proteins only become exocytosed where they are needed. The polarisation of the actin cytoskeleton, with actin cables anchored with one end at the bud where Cdc42 is active, causes polarised delivery of proteins and lipids. These are transported in exocytic vesicles along the actin filaments tracks toward the bud site. Vesicle delivery along actin cables depends on the motor protein myosin (Myo2), which is the same myosin that is used for guidance of astral microtubules [28]. Also the segregation of organelles into the growing bud depends on their Myo2-dependent transport along actin cables, which remain anchored inside the bud.

In sum, cells polarise in order to arrange their components such that it allows them to fulfil a new function. This is triggered by initial signals that start the process and determine where it should happen, sometimes in combination with landmarks. Cascades of locally active signalling factors then amplify, reinforce and stabilise the signal, often through feedback loops. The cytoskeleton is the most important target of these signalling cascades, and becomes reorganised as a result of their activity. The reorganised cytoskeleton is crucial for the enforcement and the maintenance of the chosen sites. It is therefore essential to study the mechanisms regulating the spatial organisation of the cytoskeleton if we want to understand the mechanisms driving cellular polarisation.

1.2 The Cytoskeleton

The cytoskeleton provides a scaffold to structure the cytoplasm, it provides the cell with mechanical stability, it connects its different parts by serving as tracks for the transport of proteins and vesicles, and directs intracellular organisation by positioning organelles. The cytoskeleton also plays an essential role when cells rearrange their internal components as they grow, divide, or adapt to changing circumstances. These processes are accompanied and driven by a rearrangement of the cytoskeleton.

Generally, the cytoskeleton consists of protein subunits that polymerise into fibre-like structures. There are three different forms of cytoskeletal elements in eukaryotic cells: Actin microfilaments, intermediate filaments and microtubules. While actin and microtubules are both intrinsically polar because of the asymmetric conformation of their subunits, intermediate filaments are non-polar and symmetric. Bacteria seem to have a cytoskeleton as well, since they contain protein filaments that are similar in structure and function to each of the three components of the eukaryotic cytoskeleton.

1.2.1 Actin

Actin filaments are highly dynamic polymers of about 6 nm diameter that form by the polymerisation of globular G-actin monomers. The filaments consist of two intertwined helical strands that form a left-handed helix. Actin is an ATPase that binds ATP in a cleft buried deep inside the molecule, facing the minus end of the polymer. It uses the energy gained from hydrolysing ATP to rapidly polymerise and depolymerise. The two ends of the polymer show different dynamic behaviour, with the plus (or barbed) end exhibiting higher polymerisation and depolymerisation rates than the minus (or pointed) end [29, 30].

Actin is present in all cells, but is most abundant in muscle cells, where it is responsible for muscle contraction. For contraction, thick filaments containing the motor protein myosin use ATP hydrolysis to slide along thin actin fibres. In other cells, actin is essential for all kinds of motile processes that affect their morphogenesis. It is involved in membrane pushing during cell movement, vesicle and organelle transport, exo- and endocytosis, and the separation of the daughter cells at the end of mitosis through constriction of the cytokinetic ring. Myosin is present in an unpolymerised form also in nonmuscle cells, where it acts as a motor transporting cargo along actin filaments, such as vesicles, organelles or RNA. It also serves to exert contractile force by contracting actin fibres, for example in the cytokinetic ring [31].

The local formation of actin polymers directs cellular growth to specific places in the cell. Actin filaments can form different kinds of arrays in nonmuscle cells, either linear bundles where all filaments run in parallel, or three-dimensional web-like networks with branching actin filaments. These are normally concentrated at the cellular cortex below the plasma membrane [32]. The polymerisation of the actin in the network pushes against the plasma membrane, which creates the force for making broad protrusions called lamellipodia. A number of actin-interacting proteins determine which of these array types is formed, by regulating actin polymerisation dynamics [33]. At physiological actin levels, most monomeric actin would spontaneously polymerise into F-actin. This is prevented by the monomeric actin binding protein thymosin. It competes with profilin for the binding of G-actin, which helps to exchange the bound ADP to ATP and allows addition of the monomer to a growing plus end. Profilin also binds membrane phospholipids, so that actin polymerisation may be concentrated to the cellular cortex.

At the leading edge of a migrating cell, the Rho GTPases Rac and Cdc42 both become active as a result of extracellular signals [34]. They regulate WASp family proteins, which stimulate the arp2/3 complex to nucleate actin filaments. The highly conserved arp2/3 complex is responsible for the *de novo* polymerisation of actin in branched networks. The activated complex is thought to localise to the sides of existing filaments, where it nucleates a daughter branch at an angle of 70° to the mother filament [35]. This is necessary for the network to grow, since elongation of new filaments is terminated rapidly by capping proteins.

Other proteins downstream of Cdc42 form unbranched actin filament bundles at the front of the cell, which leads to the extension of thin, spike-like protrusions called filopodia that can explore the local environment. In budding yeast, it has been shown that formin proteins nucleate and stimulate the assembly of unbranched actin filament cables, independently of arp2/3 [36, 37].

Actin bundles form with the help of crosslinking proteins that cause filaments to arrange in parallel. For example, in filopodia, fimbrin causes the formation of tight actin bundles that are not contractile, with all filaments oriented in the same direction. α-actinin forms more loosely crosslinked bundles, with actin filaments oriented in both directions, that can be intercalated by myosin. This allows actin contraction, for example in stress fibres that help to contract the cell body.

Some time after addition to a polymer, the ATP bound by actin is hydrolysed to ADP, making the polymer less stable. ADP-actin filaments are disassembled through the loss of monomers from the minus end. The cycle of actin polymerisation and disassembly in cells is extremely rapid, and individual filaments have a very short lifetime. A frequently observed phenomenon in actin dynamics is treadmilling, which is important in the formation of cellular protrusions: monomers are added at the plus end and removed at the minus end at the same speed, while the overall polymer length remains stable [38]. Because *in vitro,* loss of subunits at minus ends is slower than their addition at plus ends, treadmilling depends on acceleration of actin turnover by proteins that sever older parts of existing filaments or remove monomers from the minus end (gelsolin and ADF/cofilin).

Actin fibres that extend to adhesion sites on the plasma membrane can connect to transmembrane proteins like integrins or cadherins via adaptor proteins e.g. from the ERM family. Such connections serve to transmit force or signals to or from the extracellular matrix, or other cells.

1.2.2 Intermediate Filaments

Intermediate filaments (IFs) are rope-like fibres with a diameter of around 11 nm. In contrast to the molecular structure of actin and microtubules, which are highly conserved in evolution, there are many different and divergent proteins that form intermediate filaments, and their expression varies between different cell types [39]. IF-like proteins have also been found in budding yeast [40, 41]. The subunits of intermediate filaments are themselves elongated and fibrous, they form dimers of two α-helical chains that are intertwined in a coiled-coil rod, which then associate into linear arrays [42]. Intermediate filaments can spontaneously self-assemble and do not require the energy gained from ATP or GTP hydrolysis for polymerisation. Unlike actin or microtubules, they are not intrinsically polar, because the subunits are arranged in a symmetrical way. They can form a meshwork extending across the cytoplasm, or the nucleus. Because of their high stability *in vitro*, it has long been assumed that intermediate filaments form static networks *in vivo*, providing mechanical stability and resistance to shear stress. However, there is increasing evidence that intermediate filaments are often very dynamic and motile [43]. Assembled filaments of vimentin, for example, which is the predominant type of IF in fibroblasts, turn over relatively quickly. Filaments are assembled and

disassembled constantly, and subunit exchange can even occur along the length of the filaments. In addition, vimentin filaments are moving (or treadmilling) through the cytoplasm. Vimentin particles - possibly precursors from which the long filaments are assembled - are also seen to move through the cytoplasm. The movement of both filaments and particles depends on molecular motors that transport vimentin along microtubules, and microtubules and intermediate filaments often form associated parallel arrays in the cell. It is thus thought that microtubules transport IF precursors to specific cytoplasmic regions, allowing local variation in turnover and remodelling of the intermediate filament network. The resulting motile and dynamic properties of IFs allow reorganisation of cellular structure, such as the change of cell shape that occurs during fibroblast migration.

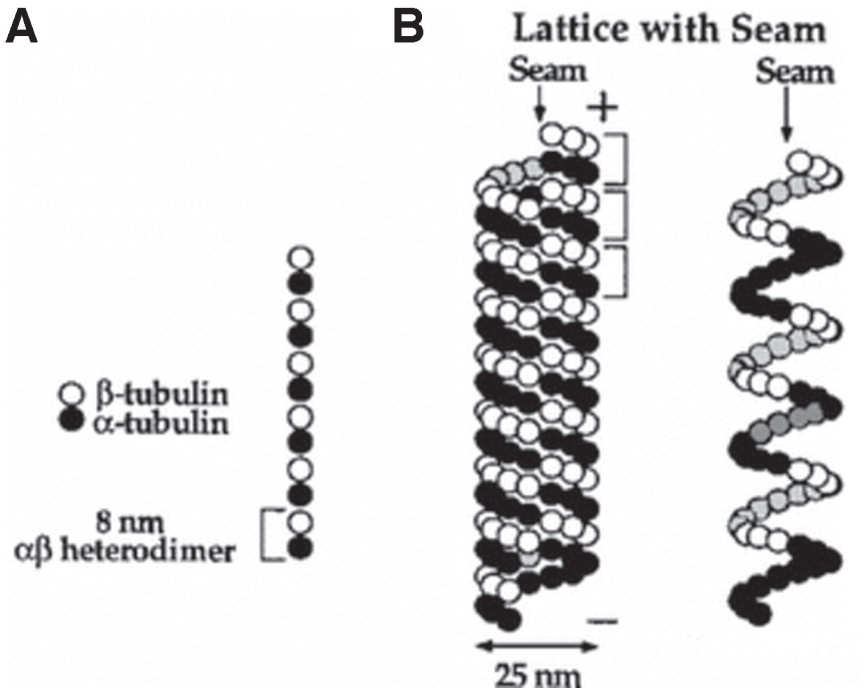

Figure 1.4 Microtubule Structure
(A) Head-to-tail interactions of alpha beta dimers form linear protofilaments. Thirteen linear protofilaments associate laterally to form 25 nm diameter hollow cylindrical microtubule polymers.
(B) A 13 protofilament microtubule with seam (left), the accepted lattice structure for microtubules. Lateral interactions between protofilaments are α to α and β to β, except at the seam. A seam is formed because one turn of a 3-start helix results in a rise of 1.5 α/β tubulin dimers (or 3 tubulin monomers). The protofilaments in a 13-protofilament microtubule are perfectly straight, whereas the protofilaments in microtubules with other protofilament numbers are helical, with a very long pitch. Plus and minus signs indicate microtubule polarity and the brackets delineate α/β dimers within the microtubule lattice. The single 3-start helix (on the right) is drawn as a visual aid (to show the lateral interactions between adjacent monomers and the helical nature of the microtubule lattice) and does not represent an assembly intermediate. Adapted from [49].

1.2.3 Microtubules

1.2.3.1 The Structure and Polarity of Tubulin and Microtubules

Microtubules are long, straight, hollow cylinders of circa 25 nm diameter that are more rigid than actin filaments. Microtubules form as the consequence of polymerisation of globular subunits into filaments. There are two types of tubulin proteins, α- and β, which have a molecular mass of about 50 kDa each. Both monomers can bind GTP, but only β-tubulin has GTP hydrolysing activity. α- and β-tubulin bind to form heterodimers. These heterodimers arrange into linear protofilaments that associate laterally to form the hollow microtubule cylinders [44]. Within a protofilament, the tubulin heterodimers associate head-to-tail (figure 1.4A). This makes microtubules intrinsically polar, resulting in structural and kinetic differences at the microtubule ends. The two different ends of a microtubule cylinder are called the plus and minus ends. The β-tubulin within the dimer is oriented toward the plus end, and the α-tubulin subunit toward the minus end. Most microtubules form from the association of 13 protofilaments into a tube (figure 1.4B).

1.2.3.2 Microtubule Nucleation Occurs at MTOCs

Microtubule nucleation, the *de novo* formation of polymers, occurs at specific structures called microtubule organising centres (MTOCs). MTOCs prevent the random formation of microtubules throughout the cell by restricting nucleation to specific locations. The microtubule minus ends are embedded within the MTOC, while the plus ends extend into either the cytoplasm or the nucleus [45, 46]. The structure of the MTOCs varies considerably between species and cell types. Work in various organisms has established that γ-tubulin, which shows some homology to α- and β-tubulin, is a universal component of MTOCs and essential for microtubule nucleation. It exists in a large complex that forms an open ring structure of 25nm diameter, called the γ-tubulin ring complex or γTuRC, which functions as a minus end capping factor for microtubule nucleation [47, 48]. The complex is also present in the cytosol but must be recruited to the MTOC to become active. This is despite the fact that it can nucleate microtubules *in vitro*. It was suggested that the γTuRC proteins form a scaffold on which 13 γ-tubulin proteins are arranged. These would then serve as adaptors for tubulin binding to form the 13 protofilaments of a microtubule. This way, the γ-tubulin complex not only nucleates microtubules but also stabilises their minus ends.

1.2.3.3 The Microtubule Dynamic Instability Model

Microtubules, once nucleated, are in mostly dynamic structures that constantly assemble and disassemble (rev. in [49]). *In vitro,* both ends show this behaviour, with the plus end growing and shrinking faster than the minus end. However, the dynamic behaviour of the minus ends might not be relevant *in vivo*, because in cells they are generally capped and thus stabilised (see last paragraph; [46]). The observation of microtubule assembly *in vivo* and the behaviour of purified tubulin *in*

vitro led to the formulation of the dynamic instability model (figure 1.5) [50]. This model states that both the phases of polymerisation and depolymerisation are persistent, with occasional transitions from one state to the other. The transition from growth to shrinkage is termed catastrophe, and from shrinkage to growth, rescue. Both catastrophes and rescues occur abruptly, infrequently and stochastically. Four parameters are used to describe microtubule behaviour: the rates of polymerisation and depolymerisation, and the frequencies with which catastrophes as well as rescues occur. *In vitro*, the growth but not the shrinkage rate is a function of the concentration of free tubulin dimers. The relationships between catastrophe/rescue frequencies and the concentration of free tubulin are more complex and not well understood: for example, catastrophe frequency seems to decrease with increasing amounts of free tubulin, because catastrophes take place less often with higher speed of polymerisation [51]. However, there are also conditions where the two parameters are uncoupled. For example, when the Mg^{2+} concentration is increased, the polymerisation rate goes up as well, but the catastrophe frequency does not change [52].

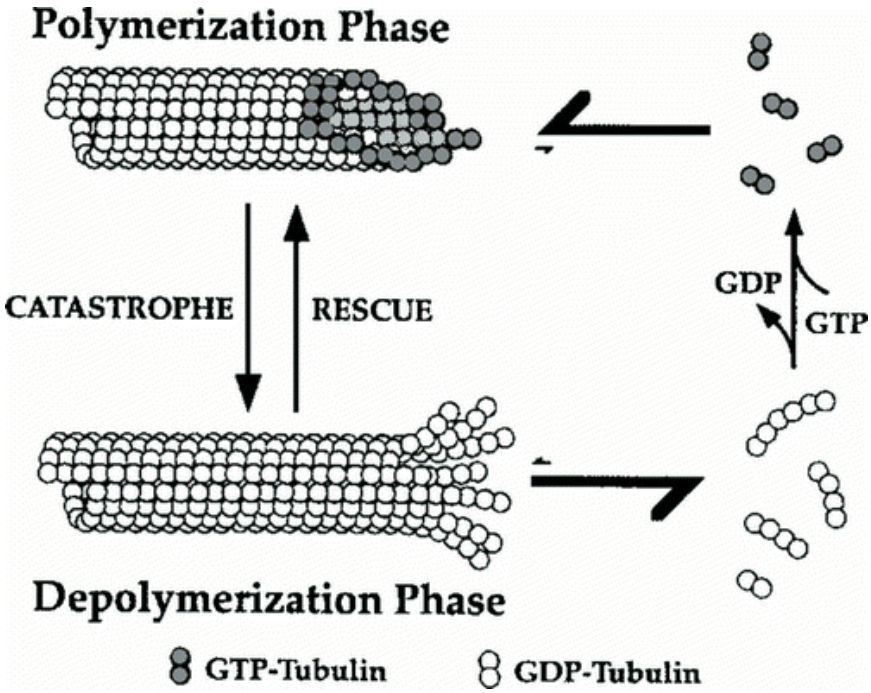

Figure 1.5 Microtubule Dynamic Instability
Dynamic instability is characterized by the coexistence of polymerising and depolymerising microtubules. GTP-tubulin is incorporated at polymerising microtubule ends, the bound GTP is hydrolysed during or soon after polymerisation, and Pi is subsequently released. Thus the microtubule lattice is predominantly composed of GDP-tubulin. Polymerising microtubules infrequently transit to the depolymerisation phase (catastrophe). Depolymerising microtubules can also occasionally transit back to the polymerisation phase (rescue). This representation incorporates the notions of a small GTP cap acting as a stabilising structure at polymerising ends and different conformational configurations at polymerising and depolymerising ends. From [49].

1.2.3.3.1 *The Mechanism Underlying Dynamic Instability*

Although a population of microtubules can maintain a stable amount of polymerised tubulin over time, individual microtubules never reach a steady state. Instead, they consume energy from GTP hydrolysis to maintain a state of dynamic instability. The GTPase activity of β-tubulin is strongly activated when a tubulin dimer is inserted into a microtubule, through direct binding of the adjacent α-tubulin to the GTP. Due to this, there might be only a single layer of dimers containing GTP, the so-called GTP cap, at the plus end, with all other dimers inside the microtubule containing GDP.

If GMPCPP, a nonhydrolysable homologue of GTP, is used for the *in vitro* polymerisation reaction, microtubules still form from the tubulin dimers. Therefore, hydrolysis is not needed for assembly; instead, it is used for the depolymerisation of microtubules. GMPCPP microtubules are indeed more stable than those formed with GTP and do not show dynamic instability [53]. This has led to the hypothesis that microtubules containing GDP-tubulin are intrinsically unstable, and that the GTP cap at the plus end is needed to stabilise microtubules. This idea was supported by the outcome of experiments where microtubules where cut in the middle, creating a new plus end without a GTP cap, upon which the microtubules depolymerised rapidly from these new plus ends [54].

Electron micrographs show that microtubules depolymerising from their plus ends disintegrate into individual protofilaments that curl away from the cylinder, so that the ends look frayed (figure 1.6A) [55]. Hydrolysis of GTP to GDP appears to induce a structural change in tubulin so that the protofilaments have a tendency to bend. Since they cannot curl within a microtubule, this results in a mechanical strain that weakens the stability of the microtubule. This strain is released when GDP-tubulin is exposed at the plus end, leading to rapid depolymerisation [49].

EM studies have also suggested that polymerising tubulin at the plus end first forms a flat sheet that later closes into a cylinder (figure 1.6B). It has been proposed that sheet closure lags behind the site where new subunits are added, and catastrophe occurs when the closing tube manages to catch up with the polymerising end (figure 1.6C) [55]. The special structure at the plus end during polymerisation and depolymerisation probably explains why microtubule growth and shrinkage phases both persist for extended periods of time. It is not quite clear, however, what the respective roles are of the GTP cap and of the closing sheet in stabilising microtubule plus ends.

In addition to the phases of growth and shrinkage, microtubules also can remain in a pausing state without either adding or losing dimers for some time; it has been speculated that this could correspond to an intermediate state where the plus end has a fully closed tube, but no protofilament curling is occurring [56].

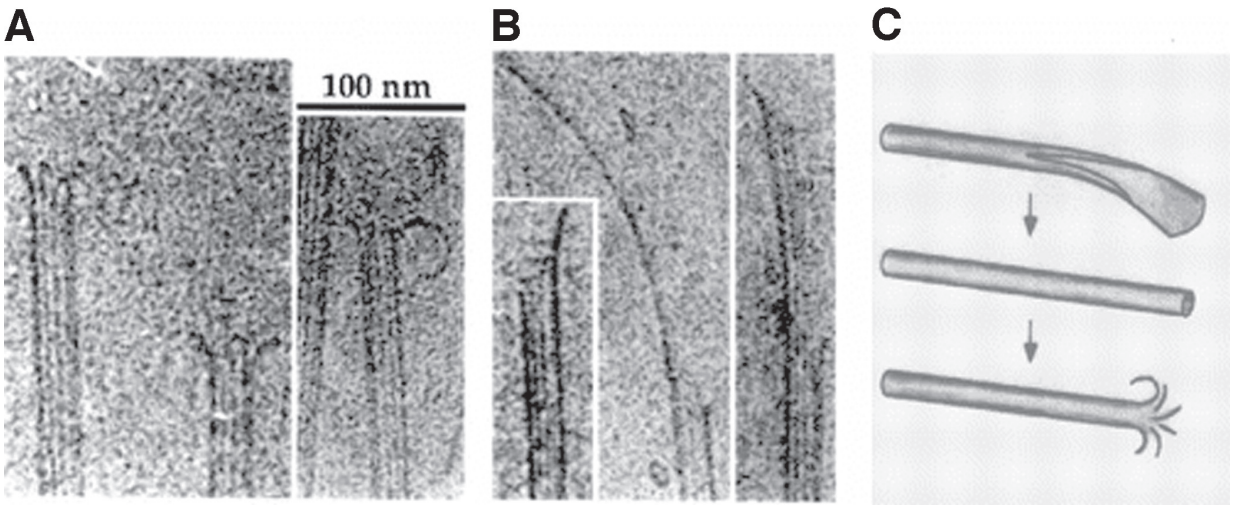

Figure 1.6 Cryoelectron Microscopy of (A) Depolymerising and (B) Polymerising Microtubule Ends
In cryo-EM images, the body of the microtubule is delineated by two thick edges. Between these thick edges, discrete lines can be seen running along the length of the microtubule, representing protofilaments. Note the curvature of protofilament oligomers at depolymerising microtubule ends in (A) and the presence of sheets at ends of polymerising microtubules in (B). The sheets tend to orient perpendicular to the surface and often appear as a single thick line.
(C) Diagrammatic representation of the structure of polymerising (top) and depolymerising (bottom) microtubule ends with a hypothetical structural mechanism for catastrophe. Catastrophe is postulated to occur as a consequence of sheet closure catching up to a microtubule end (middle). From [49].

1.3 Spatial Organisation of the Microtubule Cytoskeleton

1.3.1 Microtubule Nucleation by Centrosomes and Other MTOCs

In many animal cells, most microtubules are nucleated from a single MTOC called the centrosome, and form a radial array extending throughout the cell. Centrosomes are composed of two cylinders termed centrioles that are assembled from nine triplets of microtubules. These are embedded in a cloud of pericentriolar material that harbours the γ-tubulin complex (γTuRC) and other factors, which nucleate and anchor microtubules [57]. In budding yeast, the equivalent structure is termed the spindle pole body (SPB). It consists of three plaques. The middle plaque is embedded in the nuclear envelope, while the inner and outer plaques nucleate microtubules in the nucleus and cytoplasm, respectively [58].

In numerous cell types, however, there is no single microtubule organising centre, and microtubules are nucleated from one or several more diffuse regions of the cell. This was observed, for example, in epithelial cells. During epithelial differentiation, when distinct membrane domains are established, microtubule organisation also changes: they no longer emerge from the centrosome, but form bundles below the cortex. These bundles are usually oriented along the apicobasal axis of the cell, with the plus ends at the basal, and the minus ends at the apical side. There are in addition intertwined mats of short microtubules underneath the apical and basal membrane. This specific

orientation of microtubules allows the long-range transport of vesicles to and between specific membrane domains, and the sorting of proteins to different destinations in the plasma membrane [59]. Also plant cells do not have a discrete microtubule organising centre. In interphasic *Arabidopsis thaliana* cells, microtubule motile nucleation sites are diffusely distributed at the cell cortex and on the nucleus. γ-tubulin is distributed along the entire lengths of microtubules and on the nuclear periphery [60]. Even in cells that have a centrosome, it has been observed that there are additional peripheral microtubules, not nucleated from the centrosome, that contribute to spindle formation [61].

1.3.2 Local Regulation of Microtubule Dynamics in the Cell

Dynamic instability underlies the organisation of microtubules *in vivo*. The constant assembly and disassembly of microtubules, which is energetically costly, allows cells to rapidly reorganise their cytoplasm in response to changing requirements. It also allows microtubules to efficiently locate a specific target site by probing the space of the cell. This is followed by selective stabilisation once the target site has been contacted. This "search and capture" model has been used to explain, for example, how microtubules can attach to the kinetochores of chromosomes during mitosis [62]. Another aspect of microtubule dynamic instability is that it can be used to perform mechanical work, by generating a pushing force during polymerisation. This can be used to position organelles at a specific place in the cell. Pushing forces are used for example in fission yeast for nuclear positioning (see chapter 1.5). For these processes, the local regulation of factors that control microtubule dynamics is important.

Studies of local regulation of microtubule dynamics have been hampered by the fact that cultured cells in interphase have a very high density of microtubules around the centrosome and nucleus. For a long time, this made it difficult to follow their individual behaviour. Therefore, microtubule assembly and disassembly have been studied mainly in the cellular periphery, where the plus ends show dynamic instability. In a recent study where several new approaches where used to follow individual microtubules through their whole life cycle, also in the centre of the cell, it was observed that their dynamic behaviour differs according to where the plus end is currently located in the cell [63]. After nucleation from the centrosomes, microtubules grow persistently toward the cell margin, mostly without undergoing catastrophe. Once the microtubule tips reach the plasma membrane, they pause or immediately switch to shrinking. However, in most cases catastrophe is followed by a rescue event whilst the plus end is still in the periphery. Thus, microtubules become very dynamic and cycle between short phases of polymerisation and depolymerisation. Complete shrinkage of the microtubule back to the centrosome is relatively rare, as is the release of the minus end from the centrosome, which is followed by minus end depolymerisation. Parameters of microtubule dynamics are thus different between cell centre and cell periphery. Catastrophe and rescue rates are high at the cell margin, whereas the catastrophe but not the rescue rate is low in the interior. Growth and

shrinkage rates are essentially the same everywhere. As a consequence of this behaviour, most of the microtubule plus ends are near or at the cell margin. It is likely that the increased catastrophe frequency at the cell cortex is due to contact either directly with the plasma membrane, or with factors associated with it, such as plus end destabilising proteins. Alternatively, microtubule stabilising factors present in the interior might be excluded from the cell periphery.

In addition to the differences in microtubule regulation found between cell interior and periphery, microtubule behaviour also varies between different regions of the cortex. In migrating mammalian cells, it was shown that the catastrophe rate is considerably lower for microtubules in the leading edge as compared to the lateral edges. They also spend much more time in a growing state, resulting in net microtubule elongation toward the leading edge. Other parameters are not affected. The persistent growth of microtubules into the leading edge, which is initially devoid of microtubules, establishes a microtubular network that is essential for proper cellular locomotion [64].

A similar region-specific regulation of microtubule organisation was also observed in fission yeast cells, where catastrophes are almost entirely restricted to a certain region on the plasma membrane, at the distal cell ends (see chapter 1.5).

It should be noted that microtubule dynamics can also change globally in response to regulatory signals. For example, at the interphase-mitosis transition, microtubules become more dynamic. The opposite happens during neuronal differentiation, when microtubules become much more stable.

1.3.2.1 Regulation of Dynamic Instability by Physical Force

Microtubule dynamics might be regulated locally through the pushing force of a microtubule growing against a cellular object, which may increase the likelihood of catastrophe. This hypothesis was tested *in vitro* in a set-up where microtubules were polymerised facing a physical barrier. When the microtubules touched the barrier, they started to buckle, the growth rate slowed down, and the catastrophe rate increased about 20-fold. The increase in the number of catastrophes was probably due entirely to the reduced rate of tubulin addition. This was concluded based on the observation of free microtubules growing at reduced tubulin concentrations. Their growth speed was comparable to those of microtubules touching the barrier. These free microtubules had similarly increased catastrophe rates [65].

It is currently not known whether the effect of physical force is relevant for microtubule regulation *in vivo*. A large body of evidence has been accumulated in its place, which shows that accessory proteins are essential to regulate cellular microtubule dynamics. The *in vivo* parameters of microtubules dynamics differ considerably from those *in vitro* (at a similar tubulin concentration): polymerisation and catastrophe rates are much higher. This difference can be explained by the action of accessory proteins that modulate filament stability.

1.4 Microtubule-Associated Proteins

Among the first of these accessory proteins that were identified were the classical microtubule-associated proteins (MAPs), for example tau in neurons or MAP4 in non-neuronal cells. They suppress catastrophes as well as promote rescues, and they bind to the microtubule lattice in a nucleotide-insensitive way by electrostatic interactions. Tau and MAP4 are thought to act by crosslinking adjacent tubulin subunits, thereby preventing their dissociation and peeling away from the microtubule cylinder. Today, many additional MAPs with microtubule regulating activity are known. These use different mechanisms to localise to microtubules and to stabilise or destabilise them, respectively. Examples are the XMAP215, EB1 and Clip-170 families of proteins as well as motor proteins such as Xkcm1. It was shown that it is possible to reconstitute nearly physiological microtubule dynamics in a simple system by adding XMAP215, a microtubule-stabilising MAP, and Xkcm1, a microtubule-destabilising kinesin, to pure tubulin. In this system, XMAP215 stimulated microtubule growth, and also counteracted the catastrophe inducing activity of Xkcm1 [66].

1.4.1 Microtubule Stabilising Factors

1.4.1.1 The XMAP215/Dis1 Family of MAPs

The XMAP215/Dis1 microtubule-associated proteins are long, thin monomers that may span up to eight tubulin dimers along a protofilament [67]. At the N-terminus, these proteins have several TOG domains that are thought to mediate protein-protein interactions. The C-terminal part of several members of the family contains coiled-coil regions that are required for microtubule and MTOC localisation. XMAP215 is one of the key regulators of microtubules dynamics in *Xenopus laevis*. Also in other organisms, the respective homologues appear to play essential roles in the organisation of microtubules. XMAP215 proteins affect several different aspects of microtubule organisation, by acting both at their plus and minus ends. They act in a different way from classical MAPs, as the proteins do not simply bind along the microtubule lattice, but have enhanced binding to microtubule ends.

XMAP215 proteins might accumulate at microtubule plus ends, which has been shown for the homologues in budding yeast (Stu2) and *Dictyostelium discoideum* (DdCP224) [68, 69]. The most prominent effects of the proteins can indeed be observed at plus ends. *In vitro*, XMAP215 can bind directly to microtubules, it stimulates growth and shrinkage rates, and also reduces the rescue frequency. As a consequence, microtubules are more dynamic and have a greater steady state length [70]. In *Xenopus* egg extract, however, the main function of the protein is to stabilise microtubules by reducing the number of catastrophes. It does so by antagonising the activity of the kinesin Xkcm1, a microtubule destabilising protein [71]. The N-terminal part of XMAP215 alone is sufficient to suppress catastrophes presumably by interacting directly with Xkcm1 [72]. Together, the two proteins

appear to constitute a basic system that controls microtubule dynamics in frog extract. Also in other organisms, XMAP215 and Xkcm1 homologues have been shown to counteract each other [73]. It is possible that they constitute a conserved, basic system regulating microtubule dynamics. This basic system might then be modulated by other proteins to change microtubule dynamics in specific situations. Consistent with this view, it was shown that XMAP215 phosphorylation changes in a cell cycle-dependent way. Phosphorylation of XMAP215 *in vitro* by the cyclin-dependent kinase CDK1 changes the way it regulates microtubule dynamics, it reduces its ability to increase the polymerisation rate [74].

Microtubule stabilisation by XMAP215 proteins has also been observed in other organisms, for example in fission yeast (see chapter 1.5), *C. elegans* or human cells [75].

The most obvious localisation of XMAP215 proteins is at centrosomes / spindle pole bodies or interphase MTOCs. Microtubules are not required for their localisation as centrosomal resident proteins; instead, it is probably mediated by the TACC proteins [76, 77]. Interestingly, even in *Drosophila* meiotic cells, which form spindles lacking a centrosome, the XMAP215 homologue (Msps) localises to microtubule minus ends at the spindle poles, depending on TACC as well as a minus end directed microtubule motor (Ncd) [78]. Not so much is known about the function of XMAP215 proteins at the MTOCs, because it is obscured by their effect on microtubule dynamics at the plus ends. Msps is needed to ensure the integrity of the bipolar spindle by stabilising or bundling microtubules at the spindle poles. In *Xenopus* egg extracts as well as with pure tubulin, XMAP215 can nucleate microtubules from centrosomes and anchor the minus ends of nascent microtubules to them [79].

In budding yeast, the role of the XMAP215 homologue, Stu2, is unclear. Studies show that *in vitro*, Stu2 acts as a microtubule destabiliser by reducing the growth rate and consequently inducing more catastrophes. As a consequence the steady state length of microtubules is reduced [80]. Accordingly, depletion of Stu2 *in vivo* reduces microtubule dynamics, with fewer catastrophes or rescues [81]. These effects are the opposite of those caused by XMAP215. However, the anaphase spindle in the absence of Stu2 has a defect in elongation and eventually breaks, suggesting that in the spindle, Stu2 is required to increase microtubule length. In addition, there are conflicting results with different mutants of Stu2 about whether the protein reduces the average length of cytoplasmic microtubules or not [80, 81].

1.4.1.2 Microtubule Plus End-Tracking MAPs Regulate Microtubule Dynamics

A number of microtubule-associated proteins were shown to specifically accumulate at growing microtubule plus ends (rev. in [82, 83]). They have been termed "+TIPs", for *plus* end-*t*rack*i*ng *p*roteins [84]. Clip-170 was the first +TIP to be described [85]. Later, also other proteins were shown to localise to microtubule tips. They include EB1, tea1p, dynactin, APC/Kar9, Lis1 or the CLASPs. APC and CLASPs, however, only localise to a subset of microtubule tips, in particular those extending toward cellular growth sites, and might selectively stabilise those.

Most plus end-tracking proteins affect the dynamics of microtubules. Since in cells, microtubule dynamicity is taking place almost exclusively at the plus ends, these proteins are perfectly situated for this function. There, a few molecules are sufficient to influence microtubule behaviour, amounting to only a fraction of the number of tubulin proteins in the cell. This is unlike actin, where regulation partially occurs through the binding of every monomer in the cell by regulatory proteins.

1.4.1.2.1 *The EB1 Protein Family*

The EB1 family of microtubule-associated proteins is conserved in eukaryotes. In general, they act as microtubule stabilising proteins. Members of the EB1 family have a conserved domain structure, with a single N-terminal calponin homology domain (CH domain), followed by a less conserved, unstructured region, a short coiled-coil region and the EB1-like domain. CH domains are found in many cytoskeletal and signalling proteins [86]. Proteins with more than one CH domain were shown to be able to bind actin. The CH domain of EB1, however, binds to microtubules *in vitro*, probably through electrostatic interactions [87, 88]. Also full-length EB1 was shown to bind directly to polymerised tubulin in microtubule copelleting assays [89-91]. The C-terminus of EB1, with the coiled-coil and EB1-like domain regions, forms a four-helix bundle, and requires dimerisation of the protein for its proper structure to form (S. Honnappa, pers. comm.). This structure is responsible for the binding of several interacting proteins, for example APC or $p150^{glued}$, a component of the dynactin complex [87].

EB1 Dynamically Accumulates at Microtubule Plus Ends

In vivo, EB1 proteins are found associated with microtubules through all stages of the cell cycle. EB1 is characteristically concentrated at microtubule plus ends in almost every system where the protein was studied so far [92] (figure 1.7). It does so on interphase or astral microtubules as well as those of the spindle. EB1 also is present along the microtubule lattice, but more weakly than at plus ends. *In vitro* with purified tubulin, only the lattice, but not end binding was seen. Increasing the expression level of EB1 mainly enhances EB1 binding along the lattice [93, 94]. One exception was observed in *Xenopus* interphase extract, where EB1 localises uniformly along the microtubule lattice even at low expression levels [95]. EB1 at plus ends forms a comet-shaped structure, since the amount of protein gradually decreases with the distance from the plus end. In live imaging studies of EB1 tagged with GFP, the protein accumulates at growing microtubule plus ends, and moves together with them; it disappears from microtubule tips that undergo catastrophe and begin to shorten [95, 96].

In *Xenopus* egg extract, EB1 is thought to bind transiently along microtubules, because the signal of EB1-GFP was shown to undergo rapid recovery along the microtubule lattice following photobleaching (FRAP). Individual EB1-GFP speckles in the comets remained static over their lifetime. This argues against transport of EB1 toward the plus ends. Based on these findings, it has been suggested that EB1 binds to microtubules using two different mechanisms. It can directly associate

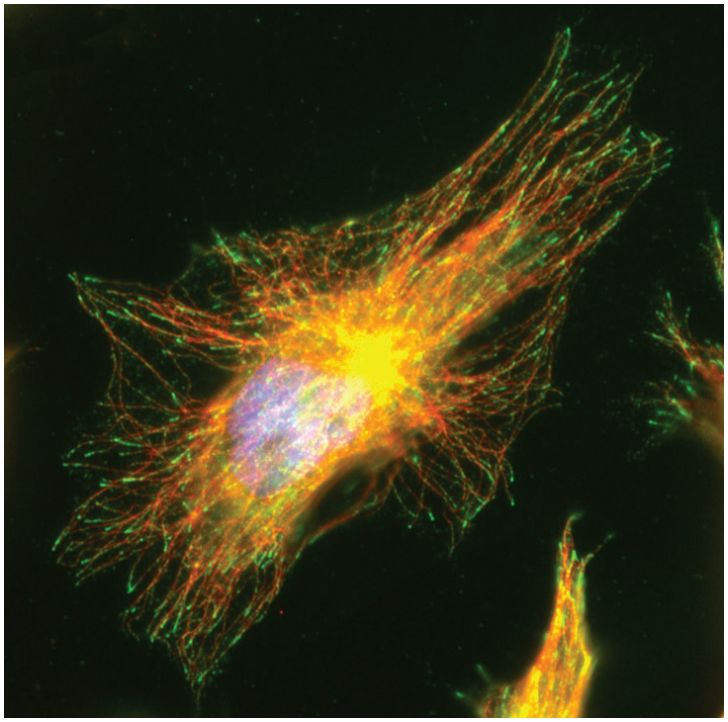

Figure 1.7 Microtubule Tip Localisation of EB1
Immunofluorescence staining of methanol-fixed HeLa cells using an anti-EB1 antibody (green) and an anti-tubulin antibody (red). DNA was counterstained with DAPI (blue). The image was kindly provided by Andrei Popov.

with low affinity to the microtubule lattice, and it shows enhanced binding to the microtubule plus ends. The plus end association might be due to copolymerisation with tubulin or by recognition of the structural or chemical properties of the plus end [95].

In addition, EB1 proteins localise to MTOCs, centrosomes and spindle pole bodies. This is most likely independent of microtubules, since depolymerisation of microtubules using drugs does not abolish localisation. In human cells, it was shown that centrosome localisation is not mediated by the CH domain, but by the C-terminal part of the protein [69, 97, 98].

EB1 Promotes Microtubule Stability and Reduces Pausing

In most organisms studied to date, EB1 proteins stabilise microtubules and increase their steady state length. This effect was not seen using only purified EB1 and tubulin [95, 99]. However, if in addition microtubule seeds are added, EB1 induces microtubule elongation and bundling. This means that EB1 can promote microtubule growth but cannot nucleate them [100].

Results from different organisms indicate that EB1 increases the steady-state length of microtubules and decreases pausing (table 1.1).

In *Xenopus* extract, depletion of EB1 from CSF (mitotic) extract causes dramatic shortening of microtubules in asters nucleated from centrosomes, although no change in microtubule length was seen in interphase extract. In both types of extract, addition of human EB1 reduces the pausing time, with microtubules neither growing nor shrinking; it also increases rescue frequency, and decreases both the catastrophe frequency and the depolymerisation rate. Together, this causes microtubules to be longer and more stable [95].

In budding yeast, the EB1 homologue, Bim1, was identified in a two-hybrid screen designed to identify α-tubulin interactors. Deletion of *bim1* results in impaired cell growth at very low as well as at very high temperatures, and confers hypersensitivity to microtubule destabilising drugs. Overexpression of the protein is lethal, and the cells arrest with undivided nuclei. Also nuclear migration to the bud neck is impaired, and spindles are short and misoriented [94]. Progression through the early phase of spindle assembly is delayed [101]. These effects are a consequence of the effect of Bim1 on microtubule dynamics, which is particularly prominent in G1 phase: the protein increases the shrinkage rate as well as the catastrophe and rescue frequencies, and strongly reduces microtubule pausing [93, 102]. Thus, in the deletion mutant, microtubules are shorter and less dynamic. As a result, the astral microtubules are less likely to capture the bud neck with their plus ends. This is essential for pulling the dividing nuclei toward the bud. The increase in catastrophe frequency caused by Bim1 is the opposite effect of that of EB1 in *Xenopus* extract. It has to be noted, however, that in the study on Bim1, catastrophe frequencies were not calculated following standard definition, and direct comparison is therefore not possible.

Knockdown of *Drosophila* EB1 resulted in the inhibition of microtubule dynamics in interphase. The catastrophe and rescue rates decreased, whereas pausing time increased. However, the overall

Table 1.1 Microtubule dynamics in functional studies of EB1 in different organisms

	Xenopus CSF Extr. 1x EB1	*Xenopus* CSF mitotic extract 8.5x EB1	*Xenopus* interphase extract 1x EB1	*Xenopus* interphase extract 2.5x EB1	*S. cerevisiae* WT G1 phase	*S.cer. bim1*Δ G1 phase	*Drosophila* S2 cells	*Drosophila* S2 cells, EB1 depleted
Polymerisation rate (μm per min)	10	10.4	6.4	14.1	2.17 ± 0.26	1.76 ± 0.63	3.8 ± 0.9	3.7 ± 1.0
Depolymerisation rate (μm per min)	9.1	-	12.5	7	3.2 ± 0.24	1.82 ± 0.23	8.7 ± 2.8	8.6 ± 3.1
Catastrophe frequency (Events per min)	0.37[2]	0[2]	0.12[2]	0.05[2]	0.498[1]	0.21[1]	1.26[1]	0.42[1]
Rescue frequency (Events per min)	0.16[2]	2.8[2]	0.39[2]	0.99[2]	0.402[1]	0.132[1]	1.74[1]	0.66[1]
Time spent polymerising %	51	96	52	78	51	8	55.2	30.0
Time spent depolymerising %	19	0	5	1	26	21	27.5	9.3
Time spent pausing %	30	3	43	21	16	66	17.3	60.6

Xenopus: from [95]
S. cerevisiae : from [93]
Drosophila: from [103]

[1] Catastrophe and rescue frequencies were defined as events per total observation time.
[2] Catastrophe and rescue frequencies were not properly defined in this study.

organisation or length of microtubules did not change, similar to the effect of EB1 depletion in *Xenopus* interphase extract. This might be explained by the fact that only one of several EB1 proteins was knocked down in this study. A more dramatic effect was seen in mitosis: Astral microtubules were short or completely absent, the spindle smaller and more compact, spindle microtubules detached from the centrosomes, and spindle poles were less focussed. Also observed were mispositioning of the spindle and defects in spindle elongation and chromosome segregation [103].

If EB1 is strongly overexpressed in cultured cells, another effect caused by the protein becomes apparent: it can induce the formation of microtubule bundles. These are long, loop around the nucleus, and are resistant to microtubule depolymerisation in the cold or by drugs [100, 104].

EB1 also localises to MTOCs. However, much less is known about possible functions of the protein there. In one study, EB1 was knocked down using RNAi. This caused a reduction of microtubules minus end anchoring at centrosomes, resulting in a less focussed array of microtubules. Also, microtubule regrowth from the centrosome after treatment with a microtubule destabilising drug was delayed [97].

EB1 Interacts with the Tumour Suppressor APC

Originally, EB1 was identified in a two-hybrid screen as a binding partner of the human tumour suppressor protein adenomatous polyposis coli (APC) [105]. APC is a large multidomain protein (300 kDa) with a variety of functions including roles in cellular signalling and the organisation of the cytoskeleton. A major role of APC in the cytoplasm is to degrade β-catenin when Wnt signalling is not activated. This function is in cooperation with Axin and the kinase GSK3β. In differentiated or confluent cells, APC mainly localises to the plasma membrane, especially to the sites of cell-cell contact, in an actin-dependent way [106]. There it may have a role in the genesis or maintenance of cell adhesion junctions, or once again in regulating the Wnt signalling pathway.

In addition, APC has an apparently separate function in the regulation of the cytoskeleton. In subconfluent or migrating cells, it clusters on a subset of microtubules close to the cortex. These are oriented toward lamellipodia or other cellular extensions. GFP-tagged APC moves specifically along these microtubules and accumulates at their growing plus ends [107]. This accumulation depends on the interaction with the plus end-directed kinesin KIF3A-KIF3B, which presumably transports the protein along microtubules [108].

One function of APC related to the cytoskeleton has been observed upon induction of migration in cultured cells (rat astrocytes). There, APC is involved in the repositioning of the MTOC, downstream of Cdc42. It has been speculated that repositioning occurs as a result of local capture of microtubules at the leading edge via APC, followed by dynein microtubule motor-dependent pulling on the microtubules [18].

The EB1-APC interaction was confirmed both *in vivo* and *in vitro* by pulldown assays. The EB1 binding site in APC is located at the C-terminal end of the protein. APC always colocalises with EB1

on the subset of microtubules with which it is associated. Whereas EB1 localisation to microtubule plus ends is independent of APC, APC probably relies on EB1 for its accumulation at microtubule tips. This assumption is based on the finding that the C-terminal part of APC with the EB1 binding domain colocalises with EB1 on growing microtubule ends all over the cell. APC also has an additional C-terminal domain that is capable of binding microtubules on its own. However, a protein fragment that contains this domain only does not accumulate at microtubule tips, but rather decorates the entire microtubule [109].

Full-length APC, and the C-terminal part of APC containing the EB1 binding site, were shown to be able to stabilise microtubules *in vitro* and *in vivo*, and a correlation was observed between APC dissociating from a microtubule end and the occurrence of catastrophe [99, 110]. One of these studies showed that the C-terminal part of APC only stabilises microtubules *in vitro* in the presence of EB1. Interestingly, the stabilising effect was maintained if EB1 was replaced by its fission yeast homologue mal3p, even though no homologue of APC is known in yeast [99]. This suggests that EB1 proteins are highly conserved with respect to their function.

The interaction with EB1 might allow the accumulation of APC at the microtubule tips, while APC would facilitate the interaction with specific membrane sites. APC could be involved in microtubule search and capture mechanisms at cellular protrusions, where it might selectively stabilise microtubules extending into the protrusions. The APC-EB1 interaction might therefore play a role in cell migration or adhesion.

A similar role for APC was predicted in targeting of microtubules to kinetochore in mitosis. APC mutant cells are defective in spindle formation and chromosome segregation, with many short spindle microtubules that are unable to connect to kinetochores. Indeed APC localises to the ends of microtubules that are embedded in kinetochores [111, 112]. It is not clear whether the interaction with EB1 is important for this function of APC, since phosphorylation of APC in mitosis by the kinase cdc2 seems to downregulate the EB1-APC interaction [109].

It is interesting to note that APC is mutated in most cases of sporadic colorectal tumours, and also in familial colon cancer, occurring in families with inherited predisposition to develop this disease. All of these mutations truncate the C-terminal part of APC, containing the EB1 binding domain. It appears that the deletion of the EB1 binding site in APC alone is not sufficient to induce tumours in mice [113]. However, the loss of the APC-EB1 interaction may lead to predisposition to cancer and could also play a role in later stages of tumour progression, for example due to resulting chromosome instability [114].

Bim1 Interacts with Kar9 in Microtubule Search and Capture

There is no homologue of APC in budding yeast. However, Bim1 binds with its C-terminus to Kar9, a protein that might have a similar function to APC. Bim1 is required for the localisation of Kar9 along microtubules and at their plus ends. Also *in vitro,* Kar9 co-pellets with microtubules only in

the presence of Bim1 [115-117]. Kar9 localises to the old spindle pole body, and in G1 phase gets transferred from there to the tips of cytoplasmic microtubules. Kar9 then binds the myosin Myo2, which translocates Kar9 and the associated microtubule along actin cables to the bud neck. Kar9 is also thought to be involved in the capture of the microtubule tip at the bud neck. The microtubule starts to shrink while remaining attached to the bud neck, so that the nucleus is pulled toward the bud. In this way, Kar9 is involved in the search and capture of the bud neck by cytoplasmic microtubules. [12, 118, 119].

Thus, Bim1 has at least two important functions in budding yeast cells: to stabilise microtubules so that they can become long enough to reach their target sites, and to localise another factor - Kar9 - to the microtubule tip, which allows it to interact with the target site at the bud neck.

Interaction of EB1 with Other MAPs

The interaction of EB1 proteins with components of the dynein/dynactin complex is well established [87, 98, 100, 120]. Dynein is a minus-end directed microtubule motor, and dynactin is a complex of proteins that binds to dynein as well as to cargo proteins and activates transport of these cargoes by dynein. The dynactin component $p150^{glued}$ was shown to colocalise with EB1 at microtubule plus ends and to punctate staining in the cytoplasm, along microtubules and at the centrosome. Furthermore, the two proteins coimmunoprecipitate and interact directly *in vitro*.

The role of the EB1-$p150^{glued}$ interaction at microtubule tips is currently not clear. Overexpression of a C-terminal fragment of EB1 disrupts $p150^{glued}$ localisation to centrosomes and causes defects in microtubule minus-end focusing and anchoring at the centrosomes. This indicates that the interaction of EB1 and dynactin may serve to anchor minus ends to the centrosome [98]. In motile cells, dynein, like APC, is required for the Cdc42-dependent repositioning of the centrosome towards the direction of migration. It is not known, however, whether interaction with EB1 plays a role in this function [17].

Recently, another link to EB1 function was established. A Rho family GTPase, Rho, was found to promote the formation of stable, detyrosinated, non-dynamic microtubule plus ends through its effector mDia, a formin that normally stimulates actin nucleation [19, 121]. These stable microtubules extend from the MTOC at the nucleus preferentially toward the leading edge in polarised, cultured cells. It is possible that these stabilised microtubules, like the reorientation of the MTOC, help to bias microtubule-dependent vesicle transport towards the leading edge. mDia was shown to directly interact with APC and with the N-terminus of EB1, suggesting that Rho/mDia act by stabilising the microtubule tip complex [122].

There is increasing evidence that also the XMAP215/Dis1 family of MAPs interacts with EB1. So far, very little is known about the putative role of this interaction. A two-hybrid screen in *Saccharomyces cerevisiae* identified Bim1 as an interactor of the XMAP215 homologue Stu2 [123]. In *Dictyostelium discoideum*, it has been shown that the two proteins co-immunoprecipitate, and that they colocalise

at the centrosome, the kinetochore and on interphase and spindle microtubules, especially on their tips. Notably, this is the case despite the fact that *Dictyostelium* interphase microtubules do not show dynamic instability. However, the functions of the proteins do not seem to be overlapping much in *Dictyostelium*, because the XMAP215 homologue DdCP224 specifically affects interphase microtubule length and microtubule interaction with the cell cortex, while DdEB1 is involved in spindle formation [69, 124].

1.4.1.2.2 *The Clip-170 Protein Family*

Clip-170 was first isolated as a microtubule-associated protein from HeLa cells. It was found to be accumulating at microtubule tips, and also localises to the mitotic spindle [85]. Clip-170 is able to directly bind taxol-stabilised microtubules.

Clip-170 contains two CAP-Gly domains at its N-terminus, each followed by a short serine-rich stretch. These domains have redundant function and are responsible for microtubule binding *in vitro* as well as *in vivo*. They are followed by heptad repeats which form a long coiled-coil domain, making up most of the protein, and two short metal binding motifs at the C-terminus [127, 128]. EM studies have shown that the protein has a highly elongated form. The central coiled-coil region mediates homodimerisation [129].

Mechanism of Microtubule Association and Plus End-Localisation of Clip-170

Using antibodies and live imaging with GFP-tagged Clip-170, the localisation of the protein on microtubules was determined more precisely. It turned out that Clip-170 specifically localises to growing microtubule plus ends. Like EB1, it forms comet-like dashes that move through the cell together with the growing microtubule end. If a microtubule slows down, the comet becomes shorter, and a shrinking microtubule filament that undergoes rescue newly acquires a comet at its tip. Upon addition of a microtubule-destabilising drug, Clip-170 comets almost immediately disappear. The N-terminal head domain of Clip-170 alone shows the same localisation and velocity as the full-length protein, suggesting that the microtubule-binding domain alone is sufficient to define the dynamic properties of Clip-170 [130-132]. Analysis of inhomogeneities of Clip-170 fluorescence intensity (speckles) within the moving comet tail show that these are immobile. Based on this observation, it was argued that the protein is not continuously transported toward the plus end by a motor-dependent mechanism [131].

In vitro, however, an N-terminal fragment of Clip-170 with the CAP-Gly and part of the coiled-coil domain does not accumulate at microtubule tips, but is found all along microtubules. It preferentially localises to microtubules that polymerised after addition of the protein, and much less so to those that were assembled before. It did not show preference for microtubule filaments polymerised with GMPCPP, a slowly hydrolysable GTP analogue, over others containing GDP-tubulin. The fragment could be crosslinked with α- and β-tubulin *in vitro*. In sedimentation velocity experiments, it

showed interaction with unpolymerised tubulin, and promoted the formation of tubulin oligomers [130].

Clip-170's interaction with polymerised tubulin is regulated by phosphorylation on serine residues. Both *in vivo* and *in vitro*, the ability of the protein to bind microtubules is inhibited by phosphorylation. The kinase that negatively modifies Clip-170 microtubule association has not been identified so far, but it is believed to be associated with microtubules as well [125]. Surprisingly, a separate Clip-170 phosphorylation activity by the kinase FRAP has been identified that positively regulates microtubule binding, indicating that the posttranslational regulation of microtubule binding of the protein is more complicated [126].

Based on these results from the *in vivo*, *in vitro*, and phosphorylation studies, it was argued that microtubule plus end-tracking of Clip-170 is not based on active transport along microtubules to the plus end, or on a higher affinity of the protein for different conformation of tubulin in the plus end GTP cap. Instead, it was suggested to bind to tubulin dimers or oligomers and to coassemble into newly polymerising microtubules. Phosphorylation by a microtubule-associated kinase would gradually release Clip-170, so that localisation of the protein is restricted to a short region at the plus end. Dephosphorylation of Clip-170 in the cytoplasm would then restore its ability to bind tubulin.

Functions of Clip-170

Clip-170 colocalises with endosomes and was shown to be involved in the binding of endocytic carrier vesicles to microtubules [127]. It is conceivable that plus end-tracking allows the protein to probe the cell for vesicles that are subsequently recruited onto microtubules for transport.

Importantly, Clip-170 also has an effect on microtubule organisation. Overexpression of Clip-170 leads to the bundling of microtubules into thick rings surrounding the nucleus. In addition, Clip-170 overexpression induces nucleation of microtubules from extracentrosomal asters if microtubules re-polymerise after removal of a microtubule-depolymerising drug [128].

Based on work in fission yeast and mammalian cells, the idea has emerged that Clip-170 is responsible for the spatial regulation of microtubule dynamics. The fission yeast Clip-170 homologue, tip1p, was the first example of a protein shown to locally affect the dynamics of microtubules. It specifically allows microtubules to continue growing when their tips touch the cell cortex in central regions of the cell, but it does not affect the microtubules growing in the cytoplasm [133] (see also chapter 1.5).

Also in a study in cultured cells where overexpression of a dominant negative construct of Clip-170, lacking the N-terminal head domain, was used to remove the endogenous protein from microtubule tips, microtubule dynamics were affected locally. Normally, microtubules in cultured cells grow persistently from the centrosome toward the periphery. There, they alternate between short growth and shrinkage phases, occasionally interrupted by pauses, before they depolymerise again

completely. This means that the microtubule tips effectively stay near the plasma membrane for extended periods of time. In the presence of the dominant negative Clip-170 construct, the microtubules no longer show their normal behaviour at the cell periphery. Instead, they immediately depolymerise all the way back to the centrosome. This means that the rescue rate is strongly reduced. Furthermore, whilst the catastrophe frequency in the cell body remains constant, catastrophes near the cell cortex are more frequent as compared to the wild type. As a result, microtubules are shorter on average, and the distribution of microtubule tips changes. These are no longer mostly found in the periphery but become symmetrically distributed along the proximal-distal axis. Localisation to microtubule tips of EB1, which is known to affect microtubule dynamics, is not abolished in these cells. Normal microtubule dynamics is restored by the additional expression of the Clip-170 head domain alone [132].

Clip-170 presumably also has a function during mitosis, because displacement of the endogenous protein results in a delay in early metaphase. Clip-170 transiently associates with prometaphase kinetochores, also on unattached chromosomes, which is mediated by its C-terminal domain [134].

Bik1, the Budding Yeast Clip-170 Protein

Bik1, the Clip-170 homologue in budding yeast, localises to the spindle pole body and on the mitotic spindle. It associates with the tips of cytoplasmic microtubules, and, more weakly, along them. In addition, Bik1 binds to kinetochores independently of microtubules. Overexpression of Bik1 causes shortening of spindle microtubules and elongation of their cytoplasmic counterparts. Bik1 deletion mutants are viable but have very short or undetectable cytoplasmic microtubules, and a shorter spindle. Translocation of the nucleus into the bud is impaired, and chromosome loss increases. Also nuclear fusion during conjugation is blocked, because the spindle pole bodies cannot fuse. The protein becomes essential in polyploid cells, where it is required to maintain kinetochore separation through the pulling force exerted by microtubules before anaphase. This function seems to be independent of the role of Bik1 in regulating microtubule dynamics [135, 136].

Clip-170 Interacting Proteins

The microtubule cytoskeleton becomes highly polarised during migration. The GTPases Rac and Cdc42, which become activated at the leading edge, are required to recruit microtubules to cortical sites of the leading edge, probably through their effector IQGAP1. This protein was shown to interact with Clip-170, and activated Rac/Cdc42, IQGAP1 and Clip-170 form a complex that may slow down removal of Clip-170 from microtubule tips a the cell cortex. This would stabilise microtubules going specifically to the leading edge, and reinforce cellular polarisation toward the direction of migration [15].

Several studies reported that Clip-170 might function to localise the dynactin complex at microtubule tips, where the proteins colocalise. Displacement of Clip-170 also removes the dynactin com-

ponent p150^{glued}, while displacement of p150^{glued} has no effect on Clip-170 localisation [132, 137]. It can be speculated that Clip-170 is involved in microtubule-based vesicle transport through recruitment of dynactin to the microtubule tips. Interestingly, Clip-170 is similar in sequence and structure to p150^{glued}, but it is not clear whether this has any functional relevance.

The Clip-170/dynactin interaction is likely to be mediated by Lis1. Lis1, the product of a causative gene for the human brain disease lissencephaly, is a dynein/dynactin interacting protein that localises to the cell cortex and to mitotic kinetochores. Lis1 probably plays a role in microtubule-kinetochore attachment [138]. Its localisation to kinetochores is dynein-dependent. It also colocalises and directly interacts with Clip-170, and it was proposed that Lis1 recruits Clip-170 to kinetochores. Conversely, Clip-170 might target Lis1 to the tips of growing microtubules [139, 140]. Also in the budding yeast, the Clip-170 protein Bik1 interacts with the Lis1 homologue Pac1, and both proteins are required for targeting of dynein heavy chain to astral microtubule plus ends [141]. Dynein interacts with Num1, which localises to the bud neck cortex. It is then thought to move toward the minus end with the help of dynactin. This action pulls the spindle toward the bud.

A conserved family of microtubule-associated proteins that includes mammalian CLASP, *Drosophila* Orbit/Mast and yeast Stu1 has been described to interact with Clip-170 *in vivo* and directly *in vitro*. They are able to bind microtubules *in vitro*, and in cells, they colocalise with Clip-170 at the distal microtubule tips, but also on the Golgi apparatus. Removal of either protein does not affect microtubule tip association of the other [132, 142]. One recurrent observation of CLASP proteins is that they only localise to a subset of microtubule plus ends in polarised cells. This suggests that they may play a role in the polarisation of the microtubule cytoskeleton through local stabilisation, downstream of extrinsic signals. In migrating cells, the CLASPs concentrate on plus ends of microtubules that extend into the leading edge. In particular, they localise to stabilised (acetylated or detyrosinated) microtubules. CLASP overexpression induces the formation of such stable microtubules, and promotes microtubule bundling. In neurons, CLASP is required for proper axon guidance and accumulates on microtubule tips specifically in the growth cones, while Clip-170 is found on the plus ends throughout the neurons [143]. In both, the leading edge of migrating cells and the axon growth cone, Clasp localises to the microtubules that are close to the actin-rich domain at the site of cell protrusions. It is therefore possible that the protein acts downstream of signals that determine the direction of growth.

In mitosis, CLASPs localise to the plus ends of spindle microtubules, to the centrosomes, the spindle midbody and the kinetochores. Kinetochore binding is independent of microtubules and mediated by the Clip-170 interacting domain. The *Drosophila* homologue, Orbit/Mast, accumulates specifically on the microtubules of the central spindle, but not on the peripheral astral microtubules that probe the cytoplasm [144]. CLASP function is essential for spindle bipolarity and chromosome segregation. Both in *Drosophila* and cultured mammalian cells, CLASPs are required for the dynamic behaviour of spindle microtubules, in particular those that are attached to kinetochores. In its ab-

sence, the centrosomes collapse into a single aster. The kinetochores localise close to the aster's centre, attached to the plus ends of short, nondynamic microtubules [145, 146].

Also the budding yeast homologue of CLASPs, Stu1, localises to the spindle, and the spindle mid-region in anaphase. Stu1 binds β-tubulin *in vitro*. Disruption of *stu1* prevents spindle assembly, and leads to the formation of unusually long and numerous cytoplasmic microtubules [147]. The phenotype of a temperature-sensitive mutant is very similar to the loss of function of human CLASP: in an early phase of mitosis, spindle pole separation is inhibited. In a later stage, the separated spindle pole bodies collapse onto each other if Stu1 function is blocked. This effect can be suppressed by increasing the dosage of Cin8, a BimC kinesin. This suggests that, like BimC kinesins, Stu1 is involved in creating an outward force, keeping the spindle poles apart [148].

1.4.2 Microtubule Destabilising Factors

1.4.2.1 *Op18/Stathmin*

The cytosolic protein Op18/Stathmin negatively regulates microtubule dynamics, by inhibiting the formation of microtubules and / or favouring their depolymerisation. Its inhibitory effect on microtubule growth is believed to be based on its ability to sequester tubulin, which decreases the concentration of free heterodimers that are available for polymerisation. Phosphorylation inactivates the inhibitory effect of stathmin, probably by reducing its affinity for tubulin [149]. The GTPase Rac, which becomes active at the leading edge of migrating cells, inactivates stathmin. This increases the number of microtubules reaching into the leading edge [150].

1.4.2.2 *Katanins*

Another class of destabilising factors are the katanins, which sever taxol-stabilised microtubules *in vitro*. In *C. elegans* oocytes, before sperm entry, katanin is active to reduce the size of the maternal meiotic spindle, and it has to be targeted for degradation before the first mitotic cell division, when a large spindle with long microtubules is required [151].

1.4.2.3 *Kin I Kinesins*

Also the kinesins of the Kin I / MCAK family are microtubule destabilising proteins. They are unusual motor proteins (see below). They do not move along the surface of microtubule filaments. Instead, they use energy from ATP hydrolysis to bind to the ends of microtubules, remove tubulin subunits and thus trigger depolymerisation. Depletion of the Kin I kinesin Xkcm1 from *Xenopus* egg extracts suppressing catastrophes and strongly increase the size of microtubule arrays. Kin I proteins preferentially bind to curled protofilaments. It is possible that in this way they induce additional bending. At plus ends, this may weaken the association of the protofilaments with each other, perhaps removes the terminal GTP-tubulin dimer, and thus induces catastrophe [152].

1.4.3 Cytoskeleton-Associated Motor Proteins Generate Movement

A very special class of MAPs are microtubule-associated motors. Motor proteins are biological machines that are responsible for most forms of movement and intracellular trafficking in cells or organisms [153]. There are three different classes of motors: myosins, kinesins and dyneins. Whereas myosins show actin filament associated motility, dynein and the kinesins move on microtubules. All three types of motors convert energy gained from ATP hydrolysis to carry out mechanical work. being able to generate forces of 1-10 pN. The ATP hydrolysis causes a conformational change in the ATP binding site of the motor domain that is communicated and amplified to the track-binding site, leading to translocation along the cytoskeletal filament. Since both actin and microtubules are polar structures, this translocation will be polarised in a particular direction, depending on the individual properties of the motor proteins.

Microtubule motor proteins have three principal functions in the cell. They move vesicles or other cargo along microtubule filaments; they move microtubules relative to each other; and they affect microtubule dynamics. These functions are essential for intracellular transport, cell locomotion or cell division.

Dynein

Dynein is a motor protein that was first identified in cilia and flagella. A cytoplasmic form of dynein was isolated later as a minus end-directed motor. Dynein is a large protein complex with several subunits, that forms an even bigger complex with dynactin, another large protein complex. Dynactin serves as an activator that links dynein to its cargo. Dynein carries out various functions including vesicle transport, nuclear positioning, mitotic chromosome movement or spindle pole focussing [154].

Kinesin Motors

Conventional kinesin was the first described protein of this family. It was isolated from squid giant axons as a putative plus end-directed microtubule motor involved in anterograde axonal transport [155]. Subsequently, kinesins have been found in all groups of eukaryotes, with 6 members in budding yeast and over 40 in humans. Kinesins show enormous variety in composition and function; some form monomers, others are dimers, trimers or tetramers; some are processive - meaning they can translocate along microtubule filaments over many micrometers - while others dissociate after one step; some move toward the plus end, others toward the minus end. Generally, kinesins can be grouped according to where the motor domain is located within the protein [156, 157]. The kinesins with their motor domain at the N-terminus (KinN) are plus end-directed motors, while those with a C-terminal motor domain (KinC) move toward the minus end of a microtubule. Many kinesins transport cargo along microtubules for example vesicles, protein complexes, intermediate filament components, mRNA, even whole organelles [154].

1.4.3.1 Microtubule Motors Are Involved in Spindle Organisation

By forming dimers, nonprocessive kinesins can bridge microtubules, and move them relative to each other, in order to rearrange and maintain cytoskeletal organisation. This role is important in the formation and function of the bipolar mitotic spindle. Some motors are directly involved in setting up the dynamic structure of the spindle from microtubules. Plus end-directed mitotic kinesins of the BimC family can bundle microtubules that have the same orientation. At the same time they push apart microtubules that come from the two mitotic centrosomes, which is important for the formation of a bipolar spindle. The minus end directed motor dynein is involved in connecting and thus focusing microtubules at their minus ends.

Other motors are important to connect the spindle with the chromosomes. Chromokinesins such as XKid are plus end-directed motors that are associated with chromosomes. They are proposed to generate at least part of a force that moves chromosome arms away from the spindle poles, toward the microtubule plus ends in the spindle midzone. Also Xklp1, a plus end-directed motor of the Kif4 subfamily of KinN kinesins, localises to chromatin. It captures microtubules, and by moving toward their plus ends, exerts a pushing force that moves chromosomes away from the spindle poles to the spindle midzone. Kinetochores in the centromer region of chromosomes are microtubule attachment sites that are essential for sister chromatids to attach to opposite spindle poles. They are also used for chromosome separation during anaphase. Dynein localises to kinetochores and might be involved this process. Also kinetochore-associated kinesins such as MCAK might be involved, but their precise role is still unclear [158, 159].

1.4.3.2 Kinesins Can Regulate Microtubule Polymerisation Dynamics

A special group of kinesins, called the Kin I family, have a motor domain in the middle of the protein. Two members of the family, MCAK and Xkcm1, were shown to differ from other kinesins, as they do not use the energy from ATP hydrolysis to translocate along microtubules, but instead, to destabilise microtubule ends (see chapter 1.4.2).

Other kinesins do act as motors and in addition affect microtubule dynamics. For example, *S. cerevisiae* Kar3, a kinesin with a C-terminal motor domain, is a minus end-directed motor and that slows down microtubule depolymerisation rates *in vitro* [160]. *In vivo*, Kar3 is necessary to limit the number of cytoplasmic microtubules, as well as their length [161]. Xklp1 is a plus end directed motor that, as mentioned above, functions during spindle formation. In addition, it directly affects microtubule dynamics by slowing down growth and shrinkage rates [162].

1.5 Fission Yeast Morphogenesis

1.5.1 The Fission Yeast Growth Cycle

Over recent years, the unicellular fission yeast *Schizosaccharomyces pombe* has become a popular model organism to explore the mechanisms driving cellular morphogenesis. Fission yeast cells are cylindrical rods with a diameter of about 3-4 μm, and a length of 8-15 μm, depending on how far the cell has proceeded through the cell cycle (figure 1.8).

The cylindrical shape of fission yeast cells is determined by the restriction of cellular growth to one or both of the opposite cell ends. After cell division, the cells start to grow only at the old cell end, which existed already before mitosis (figure 1.9). After the cells have grown to a certain size, they switch from monopolar to bipolar growth, and start expanding also from the new cell end that was established at the site of cytokinesis. The second growth site is placed exactly opposite to the growth zone at the old cell end, resulting in a perfectly straight shape of the cell. This transition point in G2 phase is called new-end take-off (NETO).

Fission yeast cells enter mitosis after reaching a certain length. During mitosis, growth ceases at both cell ends. Cytokinesis is a fission event that takes place in the middle of the cell. In cytokinesis, actin relocalises to the cell middle and forms a cytokinetic ring, consisting of actin and myosin. As the actin ring contracts, first a septum and then two new cell walls form, which will become the new cell ends of the daughter cells (figure 1.8B).

1.5.2 The Growth Zones

In interphase, the components of the cellular growth machinery localise first to the old cell end only, and after NETO, accumulate also at the new end. Cdc42p has a central function both in promoting

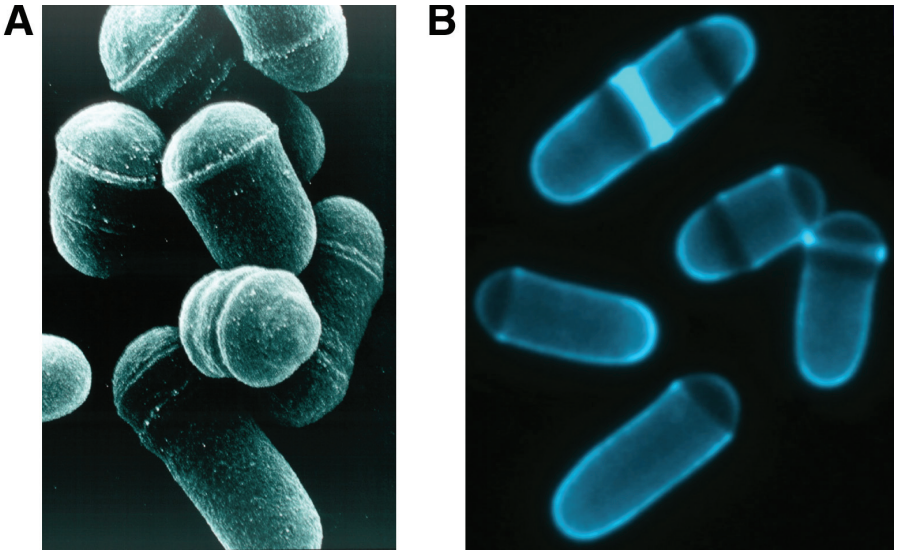

Figure 1.8 Cell Shape of *Schizosaccharomyces pombe*
(A) Scanning electron micrograph of fission yeast cells. The rings around the cells are called birth scars (from the previous attachment site to the sister cell).
(B) Staining of the fission yeast cell wall with the fluorescent dye calcofluor. The newly formed cell wall at the new cells end is stained more weakly. The bright band in the dividing cell on top corresponds to a septum. The figures were kindly provided by Damian Brunner.

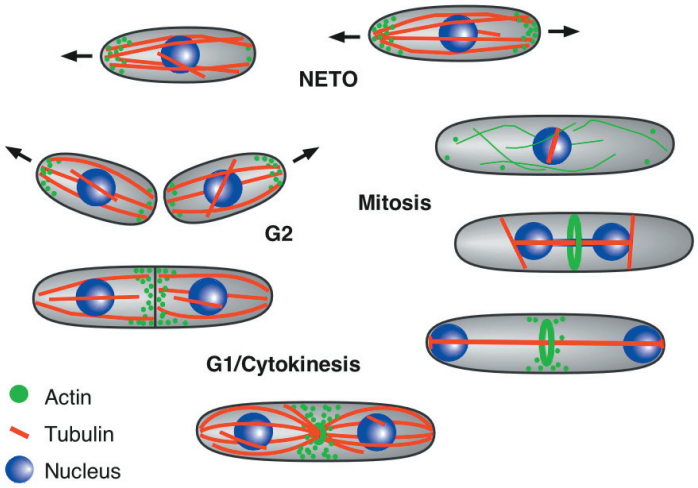

Figure 1.9 The Fission Yeast Cell Cycle
The different stages of the cell cycle are shown in clockwise order.

cellular growth and in directing it to the cell ends. The *cdc42* null phenotype is growth arrest with round and very small cells [163]. The GEF scd1p activates cdc42p. Scd1p itself is regulated by the GTPase ras1p. Ras1p is not essential for growth, but for the proper setup of polarised growth at the cell ends. It seems to localise to the growth zones, and in its absence, cells do grow but they are nearly round [164, 165]. The establishment of the growth zone is very similar to the situation in budding yeast. There, Cdc42 becomes locally activated by the scd1p homologue Cdc24, which is itself regulated by the GTPase Bud1 (see chapter 1.1).

In both fission and budding yeast, patches of actin accumulate at the growth sites, and their formation depends on the activity of another GTPase, rho1p. The patches are sites of F-actin synthesis and contain the arp2/3 complex as well as other factors involved in actin polymerisation. In addition, there are F-actin cables that extend from the cell tips throughout the cytoplasm. The patches often colocalise with or move along cables, but also are motile locally without a clear direction. Their function is not known. In budding yeast, there is increasing evidence that they mark endocytosed vesicles [166]. Both actin patches and cables depend on continuous actin polymerisation, as they disappear within a minute upon addition of an actin-depolymerising drug [167].

Fission yeast cells have a rigid cell wall covering the entire surface, which consists of crosslinked polysaccharides. (1-3)β-D glucan is the most abundant component (46-54%), followed by (1-3)α-D glucan (24-32%) and galactomannan (9-14%). To adapt to the growth of the cell, the cell wall is extended by the insertion of monomers of UDP-glucose within the cell ends. This is catalysed by the (1-3)β-D glucan synthase. This membrane-bound enzyme is activated by rho1p, which localises to the sites of growth [168].

Apart from cell wall-forming molecules and actin, it has also been shown that ergosterol-rich membrane domains seem to accumulate at the growth sites of fission yeast cells, both at the cell tips (even when growth ceases during mitosis) and the site of cytokinesis [169]. This asymmetric

distribution depends on a functional secretory pathway, but its role in cellular polarisation is not clear yet.

1.5.2.1 The Interphase Microtubule Cytoskeleton Is Involved in Cellular Morphogenesis

The overall organisation of fission yeast cells, with two exactly opposite growth sites, and the nucleus and cell division site in the cell centre, implies that some kind of long-range spatial organisation is operating in the cells. It turned out that the microtubule cytoskeleton plays an important role in the proper positioning of both the growth sites and the nucleus. Important insight into the underlying mechanisms came from the study of mutants with altered morphogenesis and abnormal cell shape. Such mutants could be easily identified by visual screening [170]. Some of these mutants are no longer able to polarise growth and develop a round cell shape; others can grow in a polar way but misplace their growth sites, resulting in bent, curved or branched cells. Often these mutants are not able to undergo NETO and grow at one end only.

The first mutants with bent or branched cell shape phenotypes were found to have mutations in the gene coding for β-tubulin [171, 172].

Further studies confirmed the role of microtubules in morphogenesis by treating cells that were blocked in G1 phase - before they undergo NETO - with the microtubule depolymerising drug TBZ [173, 174]. Such cells were shown to have a high frequency of branching after being released from the cell cycle block. This shows that microtubules are needed not for polarisation itself, but for the proper localisation of the site(s) of polarised growth. However, the induction of additional growth sites away from the cell tips also required a transient depolymerisation of the cortical actin at the growth sites [174]. This suggests that an actin-associated mechanism represents some kind of memory of the polarised growth site, which allows the cell to continue growing at or near the previously established sites in the absence of microtubules.

1.5.3 Organisation of the Microtubule Cytoskeleton in Fission Yeast

1.5.3.1 Microtubule Organisation in Mitosis

At the onset of mitosis, the mitotic spindle is formed within the nucleus. It serves to separate the sister chromatids into the two daughter cells. Shortly after the onset of spindle formation, the interphase microtubules disappear. In yeasts, the nuclear envelope does not break down during mitosis. The spindle is organised from the two spindle pole bodies (SPBs), which are the functional homologues of the centrosomes in animals. Before mitosis, in late G2 phase, the single spindle pole body, which is situated on the cytoplasmic side of the nuclear envelope, duplicates. Upon start of mitosis, the SPBs insert into the nuclear envelope probably as the duplicates [175]. Each SPB then starts to nucleate intranuclear microtubules. The unseparated SPBs initially nucleate an array of microtubules that may be involved in chromosome capture [176]. SPB separation is probably driven by interdigitation of their microtubules. The resulting spindle is made of a single bundle of microtubules,

containing more than 20 microtubules nucleated from each spindle pole body. 3-4 of them attach to each kinetochore, whilst the others interdigitate in the spindle midzone or extend to the other SPB.

As the spindle extends, the chromosomes are pulled apart and the number of microtubules decreases to about 6 per SPB. When the daughter nuclei have reached the cell ends, the spindle breaks down and disappears [176, 177].

At the onset of anaphase B, the spindle pole bodies nucleate an additional set of microtubules in the cytoplasm, the astral microtubules. These extend tangentially to the spindle axis and presumably push the spindle away from the cell cortex. In this way they align it along the long cell axis. In the absence of astral microtubules, the spindle is not properly aligned and spindle elongation is delayed [178].

During the elongation of the spindle in anaphase, an independent ring of MTOCs forms in the centre of the cell, on the actomyosin ring that positions the cell division site. It is called the equatorial MTOC or eMTOC. The eMTOC nucleates microtubules that grow toward both cell ends. They are thought to create forces that push the two daughter nuclei away from the site of cell division, so that they cannot move back to the cell centre where they would be cut apart by the newly forming septum. This process may also involve the new microtubules that start nucleating from the daughter nuclei. This transient microtubule arrangement is termed post anaphase array (PAA). Recently, it was shown that the PAA is also essential to keep the contracting actomyosin ring in the cell centre [179]. After breakdown of the spindle, the spindle pole bodies start to nucleate several cytoplasmic microtubules and eventually, interphase microtubules form again, attached to several sites on the nuclear envelope [180].

1.5.3.2 Interphase Microtubules Extend From the Nucleus Toward Both Cell Ends

Interphase microtubules in fission yeast cells form a cage-like structure [180]. There are between 2 and 6 bundles of microtubules (3 on average), which can extend along the long cell axis, often spanning the entire cell length from one cell end to the other. Each bundle in fact consists of two antiparallel microtubule bundles. It is assumed that within these bundles, microtubules are oriented with their plus ends toward the cell ends, whilst their minus ends are around the cell centre in a region where two microtubule bundles with opposite orientation overlap. These overlapping regions vary in size. The microtubule bundles in these overlapping regions are somehow attached to the nuclear envelope. Live imaging studies using α2-tubulin tagged with the Green Fluorescent Protein from *Aequorea victoria* (GFP-tubulin) show that the microtubule bundles display dynamic instability [176, 181, 182]. The tips of the bundles, usually representing a single microtubule, grow with a rate of 2-3 μm per minute from the overlap zone at the nucleus toward the cell end, either through the cytoplasm or with their tips touching the cortical membrane. When they reach the cell end, their growth rate slows down to about 1.3 μm/min, and they start to bend, following the curvature of the cell cylinder. After the microtubule tips contact the cell end for 1.5 minutes on average, catastro-

phe occurs, and the microtubules depolymerise with a rate of about 9 μm per minute (other studies reported rates of 4 or 17 μm/min) [176, 181, 182]. They shrink back to the cell centre, but do not disappear completely, in most cases the overlapping zone does not depolymerise. From there, almost immediately a new microtubule can be seen to grow out again. This whole cycle of growth and shrinkage of an entire bundle lasts around 4-6 minutes. 1-3 Secondary microtubules grow along the leading ones, so that a microtubule bundle probably consists of several microtubules with varying length. The microtubules in a bundle seem to grow and shrink independently, and also the microtubules in each of the attached antiparallel bundles show no correlation in their dynamic behaviour [176, 182].

1.5.3.3 Interphase Microtubule Organising Centres

It has been argued that the fission yeast spindle pole body nucleates microtubules only during mitosis but not in interphase, although in the latter case, one of the cytoplasmic microtubule bundles is always attached to the SPB [183].

Microtubules in interphase cells appear to be nucleated from more diffuse regions in the central part of the microtubule bundles, roughly in the region where the microtubules are attached to the nucleus. These sites are more or less the same as the zones where the antiparallel microtubule bundles overlap. They have been termed interphase microtubule organising centres (iMTOCs). If microtubules are depolymerised using a microtubule-destabilising drug, MBC, residual tubulin and proteins of the γ-tubulin complex are found in 2 to 5 dots on the nuclear envelope. Immediately after washout of the drug, microtubules are nucleated again from these dots, and grow out in both directions, confirming that the organising activity is located in several spots on the nucleus [133, 182].

Recent results suggested that upon regrowth, the iMTOCs spread out on the microtubules. Several proteins that are part of the γ-tubulin complex - rsp1p, mod20p and alp4p - were found not only on the spindle pole body, but also on iMTOCs [184, 185]. They localise to the microtubules in multiple, small satellites, which are motile and can move in both directions. These satellites are highly concentrated near the nucleus, but they also can be found further away, even on microtubule tips. Mod20p is required to nucleate microtubules from iMTOCs: In cells lacking *mod20*, there is a reduced number of interphase microtubules, and after depolymerisation by cold treatment of the cells, de novo nucleation of microtubules is strongly delayed and reduced [185].

1.5.4 Microtubule Organisation Affects the Positioning of Growth Sites and the Nucleus

1.5.4.1 Positioning of the Nucleus and the Site of Cytokinesis by Microtubules

The positioning of the septum, which splits the cell into the two daughter cells, depends on the position of the nucleus. Mutations in the two genes *mid1* and *plo1* were found to lead to random placement of the septum [186, 187]. At the onset of mitosis, the kinase plo1p phosphorylates

mid1p in the nucleus. This releases mid1p from the nucleus. It is believed that mid1p then diffuses to the nearest region of the plasma membrane, and consequently localises in a band to the plasma membrane that is closest to the nucleus. Mid1p is then thought to recruit the actomyosin ring and other components of the cellular growth machinery to the site of cell division.

It is therefore crucial that the nucleus is positioned to the cell centre, to ensure that two daughter cells of equal size are formed. Results from a number of experiments have shown that nuclear positioning is determined by the organisation of the interphase microtubules. When a microtubule plus end reaches the curvature of a cell end, it does not normally stop growing. Rather, by the continued addition of tubulin dimers, it seems to exert a pushing force. As a consequence, the region of the nuclear envelope that is associated with the respective microtubule bundle is pushed in the opposite direction. This is evident because in this situation, a deformation of the nuclear envelope occurs. If microtubule-depolymerising drugs are added, such deformations of the nucleus are no longer visible [182].

Because there are several independent bundles of microtubules, attached to multiple sites on the nuclear envelope, over time, the balance of forces pushing on the nucleus from both sides average out. The position of the nucleus is thus the result of temporal and spatial integration of many individual pushing events. In this way, even in pre-NETO cells that grow monopolar, the nucleus is always found in the centre of the cell.

This idea of microtubule pushing to centre the nucleus was confirmed by experiments where the nucleus was pushed off-centre by centrifugation in the presence of the microtubule-depolymerising drug MBC. The nucleus did move back to a central position as soon as MBC was washed out. (R. Daga and F. Chang, unpubl.).

Also in other organisms, microtubules are thought to position the nucleus in the centre of the cell [188]. In many cell types, nuclear positioning depends on the minus-end directed microtubule motor dynein. In fission yeast, however, cytoplasmic dynein only becomes essential during meiosis. There, the nucleus repeatedly moves back and forth along the cell axis in a dynein dependent manner. This movement is important for proper chromosome pairing [189].

1.5.4.2 Tea1p Links Microtubule Dynamics with Cell Polarity

Important insight into how microtubules can influence the positioning of the interphase growth sites came from studies of the *tea1* gene. *Tea1* was discovered as one of the genes that affect cellular morphogenesis when mutated. Cells lacking a functional *tea1* gene often grow bent, or they even establish completely new axis of growth, resulting in a branched cell shape. Tea1p protein contains Kelch repeats, which are also found in actin-binding proteins, and coiled-coil regions, which are generally involved in protein-protein interactions. Tea1p has weak similarity to proteins of the ERM family of proteins, which link the plasma membrane with the actin cytoskeleton. Tea1p localises to particles on microtubule tips and along microtubules, as well as to the cell ends [190]. Since it local-

ises to both cell ends already during monopolar growth, it was concluded that tea1p is a marker of cell ends. If microtubules are disrupted, tea1p is no longer at the cell ends, suggesting that microtubules deliver tea1p to the cell ends.

The mechanism of how *tea1* associates with the cell ends is not known. However, it is strongly reduced in the absence of the plasma membrane-associated protein mod5p, which accumulates at cell ends. This suggests that mod5p may serve as a tea1p anchor [191]. At the cell ends, tea1p forms a large complex containing the actin-binding protein bud6p and the formin for3p. Formins are thought to nucleate non-branched actin cables. Both bud6p and for3p depend on the presence of tea1p for their proper localisation [192]. It was suggested that tea1p might be acting downstream of the Rho GTPase cdc42p to regulate polarised cell growth at the cell ends, because it is phosphorylated by the cdc42p effector shk1p [193].

These observations led to the formulation of a model where tea1p is transported on microtubule tips to the cell ends. At the cell ends, tea1p is transferred from the microtubules to the membrane in a mod5p-dependent way. At the cell ends, it regulates bud6p and for3p in order to promote the formation of actin cables, as well as possibly other proteins that are involved in promoting polarised growth.

It has been speculated that the position of the growth sites at the cell ends could be the result of many individual events of tea1p deposition by a microtubule tip, which individually might only imperfectly determine the proper localisation of the cell end. The localisation of tea1p would become refined as a result, with a high amount of protein at the very tip of the cell and gradually decreasing amounts of both sides. Such an integrative mechanism would allow positioning the growth sites on the same axis exactly opposite of each other [194].

Tea1p itself also affects microtubular organisation: in *tea1* deleted cells, microtubules often curl around the cell ends (in 8 % of cases). Interestingly, curling was not observed in a mutant of tea1p that lacked the carboxy-terminal 200 amino acids. This truncated protein localises to microtubules but no longer to the cell ends [195].

In summary, for proper positioning of the growth sites, the nucleus and the site of cell division, it is crucial that interphase microtubules are organised such that they grow from organising centres in the middle of the cell towards the cell ends. This ensures that the cell end marker tea1p is properly deposited, and that microtubules can push against the cortex to create the force that keeps the nucleus centred. Microtubule dynamics thus must be regulated in such a way that catastrophes are restricted to the cell ends, that microtubules continue to grow there for a limited period, and that they do not grow beyond the cell end. This means that microtubule dynamics have to be locally controlled, or, in other words, there must be a mechanism that allows the microtubules to recognise the cell ends.

1.5.5 MAPs that Affect Microtubule Organisation

1.5.5.1 Tip1p Tracks Microtubule Plus Ends and Stabilises Them at the Cellular Cortex

In the screens carried out to identify morphogenesis mutants, several proteins were found that affect the organisation of microtubules.

One such protein is encoded by the *tip1* gene [133]. It is homologous to the Clip-170 protein in humans, a protein that localises to growing microtubule plus ends. The *tip1* protein (tip1p) has an N-terminal cytoskeleton-associated protein glycine-rich (CAP-Gly) domain [196], which is thought to mediate binding to microtubules, followed by a serine-rich stretch and extensive coiled coil regions. At the C-terminal end of the protein is a metal binding domain. In interphase cells, tip1p accumulates in particles at microtubule tips and along microtubules. It also concentrates at the cortex of both cell ends. In mitosis, it is found on the plus ends of astral microtubules. In cells where the *tip1* gene is deleted (*tip1*Δ), many cells are bent or branched. The frequency of abnormal shapes increases when cells recover from starvation. In *tip1*Δ cells, tea1p no longer accumulates at the cell ends, although the protein still localises along the microtubules and at their tips [133, 192]. Interphase microtubules are 30-60% shorter than in wild type cells. The ability of tip1p to stabilise microtubules resides in the N-terminal two thirds of the protein, because replacing the endogenous tip1p with a C-terminally truncated version does not affect microtubule length. However, interaction of this protein with tea1p is disturbed, and tea1p is not deposited at the cell ends. This results in cells that are bent and branched like in the *tip1*Δ strain. Following the dynamics of microtubules in *tip1*Δ cells using a GFP-tagged version of α2-tubulin revealed that tip1p has a region-specific role in regulating microtubule organisation. In the mutant, catastrophes occur almost exclusively at the cortex, like in wild type cells. However, they are no longer restricted to the cortex at the cell ends, but instead can occur in any cortical region of the cell. As a consequence, about 70 % of the microtubules no longer reach the cell ends. That means that tip1p selectively stabilises microtubules when they touch the cortex outside of the cell ends. This allows them to continue growing along the cortex, and in this way, to efficiently target the cell ends. Thus, tip1p has an essential role in restricting microtubule catastrophes to the cell ends [133].

1.5.5.2 The Plus End-Tracking Kinesin tea2p Affects Microtubule Stability

Tea2p, which shows sequence similarity to *Saccharomyces cerevisiae* Kip2, is a kinesin that has a central motor domain, followed by a short C-terminal coiled-coil region [197]. Like tea1p and tip1p, tea2p affects cell shape: *tea2* deleted (*tea2*Δ) cells are frequently bent or branched, a phenotype that is more severe upon recovery from nutrient starvation. Microtubules are shorter than in wild type cells. However, more microtubules (71%) are still able to reach the cell ends than in *tip1*Δ cells (29%). In *tea2*Δ cells or in cells carrying the temperature sensitive mutant *tea2-1*, tea1p is strongly reduced at cell ends, and the remaining protein is often associated with one cell end only. Tea1p still localises

in particles along microtubules and on their tips, and is able to move with the plus ends in *tea2*Δ cells [174, 195, 197]. This suggests that tea2p, like tip1p, plays a role in efficiently targeting tea1p to the cell ends, but that there is a mechanism independent of tea2p that allows tea1p to localise to microtubules.

Like tip1p, tea2p particles localise along the microtubules and at their tips. The protein also accumulates at the cell ends in a tea1p-dependent way. Since the protein was not found moving along microtubules like a conventional motor protein, it was speculated that it might not function as a motor, but use a different mechanism for plus end-tracking. To address this question, point mutations were introduced into the Switch II and P-loop domains of the tea2p motor domain, which abolished its ability to hydrolyse ATP and thus would inactivate any motor function. These 'rigor' mutant proteins indeed did no longer show any motility, but localised in immobile particles all along the microtubules. In these rigor mutants, tip1p and tea1p also localised in immobile particles along the microtubules and were no longer found concentrated at microtubule tips or at the cell ends [198]. ATP hydrolysis of tea2p is thus somehow involved in the plus end-tracking of tea2p, tip1p and tea1p.

1.5.5.3 The EB1 Protein mal3p Stabilises Interphase Microtubules

The microtubule-associated protein mal3p was found in a screen designed to identify genes that are required for chromosome segregation. Mal3p is the fission yeast member of the EB1 family of proteins, with 40% similarity to human EB1. Cells deleted for the *mal3* sequence are cold sensitive and often display bent or branched shapes. Microtubules in *mal3*Δ cells are much shorter than in wild type cells, the nucleus is often displaced, and the mutant cells also show hypersensitivity to the microtubule (and actin)-destabilising drug TBZ. This sensitivity can be fully suppressed by heterologous expression of human EB1. Overexpression of mal3p leads to the formation of excessively long cells, and it severely impairs spindle formation and function. Chromosome segregation to the poles is defective, and the spindles disintegrate, showing a frayed or V-shaped structure [199].

Mal3p is also required to properly localise the kinesin tea2p to microtubules. Wild-type tea2p tagged with GFP no longer accumulates at microtubule plus ends if *mal3* is deleted, although it is still found at the cell ends. However, rigor mutant variants of tea2p tagged with GFP were still found to localise to microtubules in *mal3*Δ cells in the same way as in wild type cells. This shows that in principle, tea2p can interact with microtubules independently of mal3p, but that mal3p might be required for maintaining microtubule association of tea2p with an active motor function [198].

Mal3 tagged C-terminally with GFP localises to interphase microtubules, to the spindle as well as to the post anaphase array; this corresponds to what has been found for other EB1 proteins [198, 199]. It is found along the lattice of interphase microtubules as well as concentrated at microtubule plus ends. However, mal3p tagged at the C-terminus with GFP is not completely functional, as cells are sometimes bent.

1.5.5.4 Fission Yeast Has Two XMAP215 Homologues with Partly Redundant Function

There are two fission yeast members of the XMAP215/dis1 family of microtubule-associated proteins, dis1p and alp14p/mtc1p, which have acquired partially differing functions. While the single mutants are viable, deletion of both genes is lethal and causes severe cell shape abnormalities. *Alp14*Δ cells are temperature-sensitive, while *dis1*Δ cells are cold sensitive. Interestingly, dis1p overexpression can rescue the temperature sensitivity of the *alp14*Δ mutant, but alp14p overexpression does not rescue the cold sensitivity of *dis1*Δ cells. At the restrictive temperature, *alp14*Δ cells fail to separate the spindle pole bodies or to form a bipolar spindle. Interphase microtubules become abnormally short. In *dis1*Δ cells at the restrictive temperature, a spindle forms and elongates, but the sister chromatids fail to be separated. Both mutants thus show a defect in chromosome segregation, and are presumably necessary for stable association of spindle microtubules with the kinetochores.

Dis1p localises to kinetochores in metaphase, and during anaphase it localises to the spindle, being more concentrated at the SPBs. Dis1p also localises to the astral microtubules in elongated spindles. Alp14p also localises to kinetochores in a microtubule-dependent way, and also to the spindle and to the SPBs. Both dis1p and alp14p, when tagged with GFP, are present on interphase microtubules as well. Dis1p appears to be more concentrated near or at the iMTOCs around the nucleus, while alp14p forms particles all along microtubules and at their tips [200, 201].

1.5.5.5 MTOC-Associated Proteins Are Also Involved in Microtubule Organisation

Not only MAPs affect the organisation of microtubules and consequently the shape of fission yeast cells. Also the proteins of the γ-tubulin complex do so, presumably as a consequence of a role in microtubule nucleation. Deletion of γ-tubulin is lethal, but in cells that carry a point mutation in the γ-tubulin gene, which leads to cold-sensitive growth arrest, microtubules are unusually long and curl around the cell ends at the permissive temperature. These cells frequently are bent or branched [202]. In deletion mutants of other, nonessential members of the γ-tubulin complex (mod20p, rsp1p, alp4p, alp6p and alp16p), similar cell shape and/or microtubule aberrations were observed [184, 185, 203, 204].

1.5.5.6 Most Fission Yeast Kinesins Are Nonessential for Cellular Growth

In fission yeast, there are nine kinesins. Seven of them have been characterised so far. The kip2-like kinesin tea2p affects microtubule stability as discussed above. Only one kinesin, cut7p, turned out to be essential. Cut7p, a member of the BimC kinesin family, is required for the interdigitation of the microtubules originating from the two spindle pole bodies at the onset of mitosis [205]. Cut7p localises to the spindle pole bodies and to the spindle, concentrating in its midzone during elongation [206].

Fission yeast has two members of the Kar3/Ncd family of minus-end directed kinesins, pkl1p and klp2p. Pkl1p localises to the nucleus and the mitotic spindle as well as the spindle pole bodies, but disruption of *pkl1* does not lead to any obvious growth defects. However, spindles in metaphase-arrested cells are shorter in *pkl1*Δ than in wild type cells. Furthermore, pkl1p overexpression causes the formation of abnormal V- or star-shaped spindles similar to those observed in cut7p mutants. This suggests that cut7p and pkl1p provide opposing forces on the spindle [207]. Like pkl1p, klp2p localises to the spindle, but is also present in chromosome-associated particles and is also found along interphase microtubules. Growth and cell shape are not affected in cells deleted for *klp2* (*klp2*Δ). However, the spindle appears to be affected, because it curls around the cell ends when is fully elongated. This suggests that klp2p promotes spindle microtubule disassembly at the end of mitosis [208].

Klp3p is a member of the kinesin heavy chain (KHC) family of plus end-directed motors. It localises to interphase microtubules but deletion of *klp3* does not show any effect on microtubule organisation, cell shape or organelle distribution. However, transport of membrane from the Golgi apparatus to the endoplasmatic reticulum seems to be impaired in *klp3*Δ cells. Overexpression of klp3p leads to abnormal septation, elongation and branching of cells [209], [210].

Klp5p and klp6p are the fission yeast members of the Kip3/Kin I family of kinesins that have been shown to possess microtubule-destabilising activity. Klp5p and klp6p localise along interphase microtubules. In mitosis, they are restricted to kinetochores in early stages, are subsequently found along the entire spindle, and accumulate in the spindle midzone at later stages of mitosis. In *klp5* or *6* deletion mutants, cytoplasmic microtubules curl around the cell ends and appear to be abnormally stable. This suggests that klp5p and klp6p also destabilise microtubules. While the structure of the spindle appears to be normal in *klp5*Δ *or 6*Δ cells, progression through mitosis and chromosome segregation is delayed [211-213].

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Chapter 2
The Microtubule Plus End-Tracking Proteins mal3p and tip1p Cooperate for Cell-End Targeting of Interphase Microtubules

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2.1 Abstract

Background: CLIP-170 and EB1 protein family members localise to growing microtubule tips and link spatial information with the control of microtubule dynamics. It is unknown whether these proteins operate independently or whether their actions are coordinated. In fission yeast the CLIP-170 homologue tip1p is required for targeting of microtubules to cell ends, whereas the role of the EB1 homologue mal3p in microtubule organisation has not been investigated.

Results: We show that mal3p promotes the initiation of microtubule growth and inhibits catastrophes. Premature catastrophes occur randomly throughout the cell in the absence of mal3p. mal3p decorates the entire microtubule lattice and localises to particles along the microtubules and at their growing tips. Particles move in two directions, outbound toward the cell ends or inbound toward the cell centre. At cell ends, the microtubule tip-associated mal3p particles disappear followed by a catastrophe. mal3p localises normally in *tip1*-deleted cells and disappears from microtubule tips preceding the premature catastrophes. In contrast, tip1p requires mal3p to localise at microtubule tips. mal3p and tip1p directly interact *in vitro*.

Conclusions: mal3p and tip1p form a system allowing microtubules to target cell ends. We propose that mal3p stimulates growth initiation and maintains growth by suppressing catastrophes. At cell ends, mal3p disappears from microtubule tips followed by a catastrophe. mal3p is involved in recruiting tip1p to microtubule tips. This becomes important when microtubules contact the cell cortex outside the cell ends because mal3p dissociates prematurely without tip1p, which is followed by a premature catastrophe.

2.2 Introduction

Proper spatial microtubule organisation is crucial for eukaryotic cell function. This is a highly dynamic process because microtubules constantly switch between phases of growth and shrinkage [1]. In interphase, microtubules serve as tracks for the bidirectional, long-range delivery of vesicles and molecules and for the proper positioning of cellular organelles. *In vivo*, the intrinsic dynamics of microtubules are controlled by a considerable number of microtubule-associated proteins (MAPs) [2]. These can influence different parameters such as growth and shrinkage rates or the frequency with which microtubules switch from growth to shrinkage and from shrinkage to growth, events termed catastrophe and rescue, respectively. There is increasing evidence that a subset of MAPs, which accumulate at the growing microtubule plus ends, is central to the spatial control of microtubule dynamics [3 and 4]. The prototype of such plus end-tracking proteins is CLIP-170, which links microtubules and specialized membrane domains such as endosomes and desmosomal plaques [5]. At the leading edge of migratory mammalian cells this linking action involves the binding of CLIP-170 to IQGAP1, an effecter of activated Cdc42 and Rac1 [6].

Another highly conserved family of microtubule plus end-tracking proteins is named after EB1, a factor that was identified as a binding partner to the tumour suppressor protein adenomatous polyposis coli (APC) [7]. During mitosis, EB1 proteins are involved in spindle formation and in chromosome capture [8, 9, 10 and 11], and in *Drosophila* and budding yeast the respective EB1 homologues contribute to spindle orientation by controlling the dynamics of astral microtubules [9 and 12].

EB1 proteins also affect the dynamics of interphase microtubules. Cells that are mutant for mal3p, the EB1 homologue in the fission yeast *Schizosaccharomyces pombe*, have shortened microtubules [8], and human EB1, when added to *Xenopus* egg extracts, promotes microtubule rescue and decreases catastrophe rates [13]. *In vitro*, purified human EB1 can also promote microtubule growth but only when bound to the carboxyl terminus of APC [14]. Interestingly, in this assay mal3p showed the same APC-dependent activity, although no APC homologue exists in the fission yeast genome. This suggests that the function of EB1 protein family members is highly conserved. Consistent with this, human EB1 can partially rescue the phenotype of the *mal3* deletion in fission yeast [8 and 15]. Because of their involvement in the organisation of all known microtubule structures, EB1 proteins are likely to belong to a core set of factors that control microtubule dynamics [16].

How the activities of plus end-tracking proteins are coordinated in space and time is poorly understood. In this study, we investigated the role of mal3p in organising interphase microtubules, and we explored the way in which mal3p activity is coordinated with that of the CLIP-170 homologue tip1p. Fission yeast cells possess a distinct set of interphase microtubules with a defined spatial organisation, which is important for the correct positioning of the growth zones, the nucleus, and the division site [17, 18, 19 and 20]. Interphase microtubules form three to six independent pairs

of antiparallel bundles that possess a stable overlap zone at their minus ends, termed the interphase microtubule-organising centre (iMTOC). The iMTOCs are attached to the nuclear envelope in the cell centre. From there, the microtubule plus ends grow out in opposite directions toward the two ends of the cylindrical cells, forming a cage-like structure, which can easily be visualized and resolved in three dimensions [17]. In addition, the dynamics of the microtubules can be described in great detail in wild-type as well as in mutant cells by using tubulin tagged with the green fluorescent protein (GFP) [21]. In this way it has been shown that the microtubules grow all the way to the cell ends where they then become destabilised and undergo catastrophe [22]. This ensures that the cell end-marker protein tea1p is deposited at the cell ends, thus ensuring correct positioning of the growth zones [18 and 19].

tip1p contributes to the proper spatial organisation of interphase microtubules by preventing catastrophe when microtubule tips contact the cell cortex in central regions of the cell. This allows the microtubules to continue growing below the cortex, which automatically orients them parallel to the long cell axis and allows them to efficiently target the cell ends [23]. In *tip1*-deleted cells, interphase microtubules are shortened because catastrophes can occur prematurely at the cell cortex in central regions of the cell. Shortened interphase microtubules have also been reported for cells lacking mal3p, but the cause for the shortening is not known [8 and 15]. Here we have investigated the role of mal3p in microtubule organisation. We present evidence that mal3p interacts with tip1p and that together the proteins define a system that is required for the proper organisation of interphase microtubules.

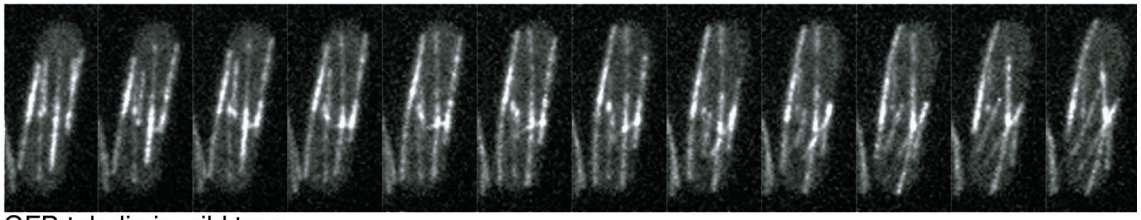
2.3 Results

2.3.1 mal3p Promotes Microtubule Growth

Cells carrying a *mal3* deletion (*mal3*Δ) possess shortened interphase microtubules [8]. To investigate how mal3p affects microtubules, we analysed their dynamics in *mal3*Δ cells that express GFP-tagged α2-tubulin to allow 3D time-lapse imaging of microtubules [21]. In these cells, microtubules grew from the usual three to six iMTOCs, reaching, on average, half the length of wild-type microtubules (Figures 2.1B and 2.1C, and Movies 2.1 and 2.2). The average growth and shrinkage rates did not change significantly, whereas the catastrophe frequency increased by more than 2-fold (Figure 2.1C).

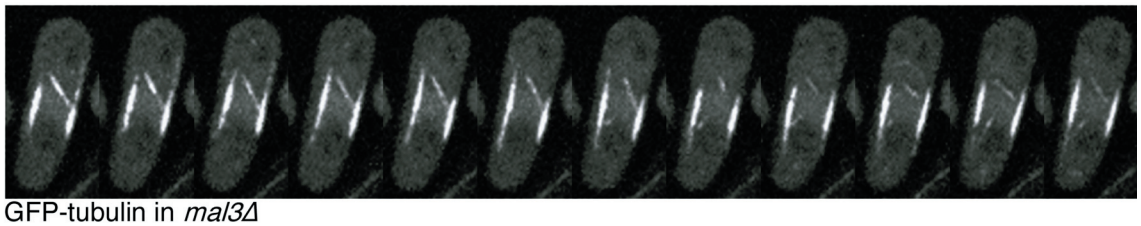
In fission yeast, events that are equivalent to rescues are largely restricted to the stable overlap zones of the iMTOCs. They can be observed only after all the microtubules in a bundle have depolymerised. We found a slight increase in the number of such events per observation time in *mal3*Δ cells (Figure 2.1C). This reflects the considerably shortened lifetimes of the microtubules and is also a function of the pausing time between completed depolymerisation and the reinitiation of

A



GFP-tubulin in wild type

B



GFP-tubulin in *mal3Δ*

C

	wild type 25ºC (32ºC)		***mal3*Δ** 25ºC (32ºC)	
Average microtubule length in µm	**7.3 ± 2.3**	(7.9 ± 1.7)	**3.6 ± 1.5**	(4.3 ± 1.5)
Average pausing time in seconds [= % of total observation time]	**28 ± 19** [12%]	(14 ± 6) [9%]	**55 ± 48** [46%]	(36 ± 25) [33%]
Catastrophe frequency	**0.33**/min	(0.45/min)	**0.81**/min	(0.81/min)
Growth re-initiation frequency	**0.24**/min	(0.38/min)	**0.38**/min	(0.49/min)
Growth rate in µm/minute	**3** ± 0.6	(3.8 ± 0.7)	**2.4** ± 1.1	(2.1 ± 0.6)
Shrinkage rate in µm/minute	**8.5** ± 2.2	(9.6 ± 3.7)	**5.6** ± 1.8	(6.7 ± 3.1)

D

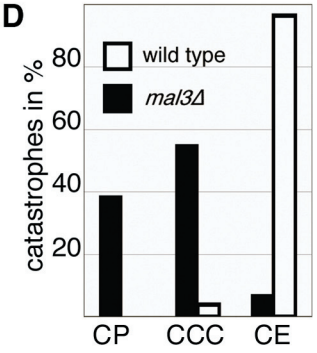


Figure 2.1 mal3p Promotes Microtubule Growth

(A and B) Movie sequences with full projections of GFP-tubulin-expressing cells showing microtubule dynamics. (A) Wild-type cells, 5.5 s/frame. (B) *Mal3*Δ cells, 11 s/frame.
(C) Quantification of microtubule dynamics in wild-type and *mal3*Δ cells.
(D) Spatial distribution of catastrophe events in wild-type (white bars, N = 187) and *mal3*Δ cells (black bars, N = 164). Abbreviations: CP, cytoplasm; CCC, central cell cortex, which is the entire cortex excluding the cell ends; and CE, cell ends.

growth of the first microtubule. Whereas in wild-type cells these pauses lasted for 28 s on average, it took 55 s on average in *mal3*Δ cells before a microtubule was seen growing out from the iMTOC (Figure 2.1C). In the absence of mal3p, the microtubules thus initiate growth with reduced efficiency. Because mal3p affects the initiation of microtubule growth and its maintenance by catastrophe inhibition, we conclude that mal3p is a general promoter of microtubule growth.

In our time-lapse experiments we recorded confocal sections covering the entire volume of the cell, which enabled us to analyse the spatial distribution of catastrophes. In wild-type cells these occurred in 96% of the cases in cell-end regions, as defined by the cell-wall curvature. In *mal3*Δ cells, however, catastrophes occurred in the cytoplasm (38%), at the cell cortex in the cell centre (55%), and in cell-end regions (7%) (Figure 2.1D). This shows that in the absence of mal3p, catastrophe events are no longer spatially restricted.

2.3.2 mal3p Localises to the Microtubule Lattice and Accumulates at Microtubule Tips

We raised a rabbit polyclonal anti-mal3p antibody, which detects two mal3p specific bands of approximately 38 kDa (Figure 2.2A). λ-phosphatase treatment eliminated the higher molecular weight band showing that it depicts phosphorylated mal3p (Figure 2.2A).

We next used the anti-mal3p antibody with an anti-tubulin antibody for immunofluorescence localisation studies. Particles of mal3p were distributed along the microtubules but were most prominent at their distal tips and on the iMTOCs (Figures 2.2B and 2.2C). Fainter staining also covered the microtubule lattices. During mitosis a similar mal3p distribution was detected on the astral spindle microtubules, and mal3p also decorated the entire mitotic spindle and both spindle poles, as well as the post-anaphase array (Supplemental Figure S2.1, Movies 2.5 and 2.6). Immunofluorescence staining of *mal3*Δ cells showed no microtubule-associated signal, and cytoplasmic fluorescence was strongly reduced as compared to wild-type, indicating that wild-type cytoplasm contains considerable levels of mal3p (Supplemental Figure S2.2).

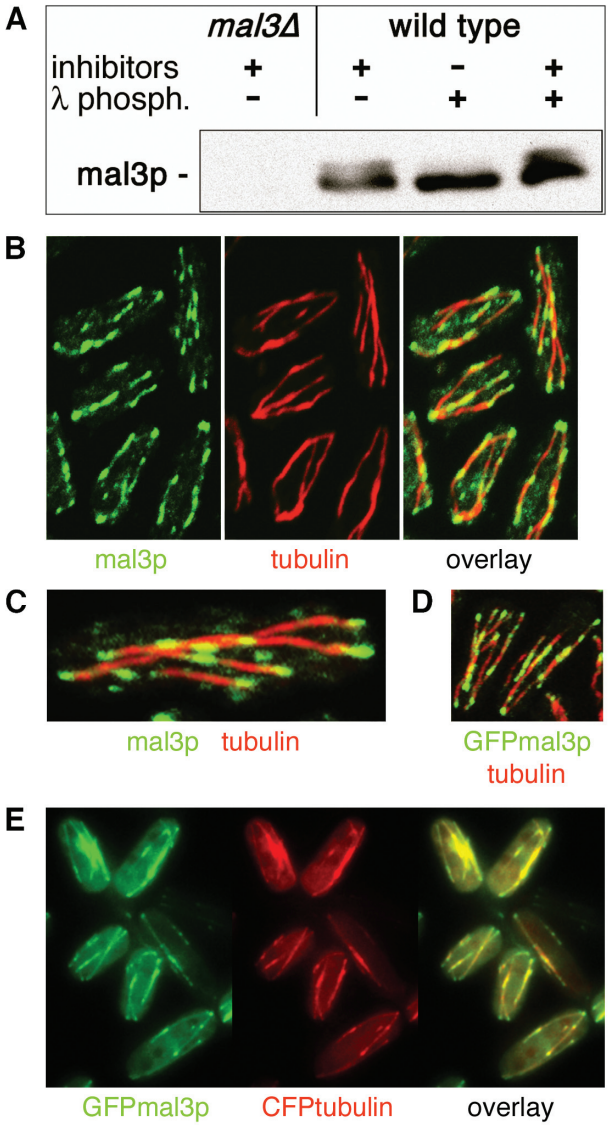
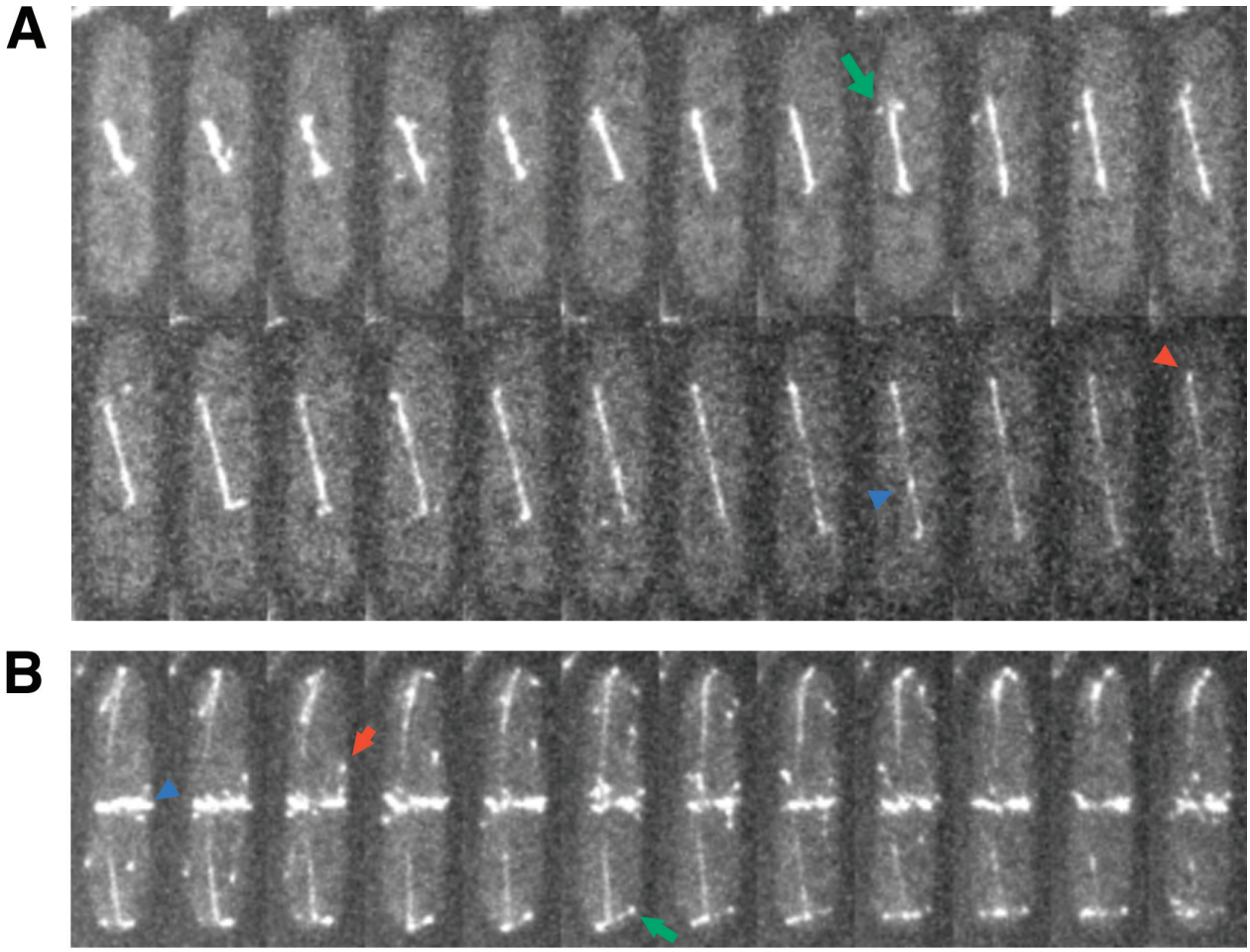


Figure 2.2 mal3p Localises along the Microtubules and Accumulates at Their Tips

(A) Western blot using the anti-mal3p antibody on total-cell extracts from wild-type and from *mal3*Δ control cells. Only the mal3p-specific bands are shown. Wild-type extracts were prepared with or without addition of phosphatase inhibitors and/or λ-phosphatase.
(B–D) Immunofluorescence staining of methanol fixed cells. Anti-tubulin staining is in red, anti-mal3p staining in green. (B) Three frames of wild-type cells showing mal3p localisation, microtubule distribution, and an overlay. (C) Enlargement of a cell from (B). (D) Cells expressing GFP-mal3p instead of wild-type mal3p.
(E) Living cells expressing GFP-mal3p and CFP-tubulin.

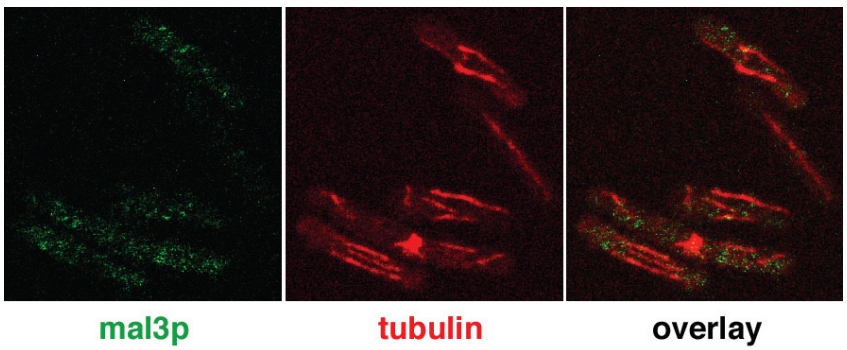
2.3.3 mal3p Particles Display Complex Movements

To investigate the dynamics of mal3p, we tagged the protein with GFP. A previously described carboxy-terminal-tagged mal3p causes altered cell morphology and microtubule appearance [8 and 15]. Therefore, we



Supplemental Figure S2.1 Cells in Mitosis Expressing GFPmal3p

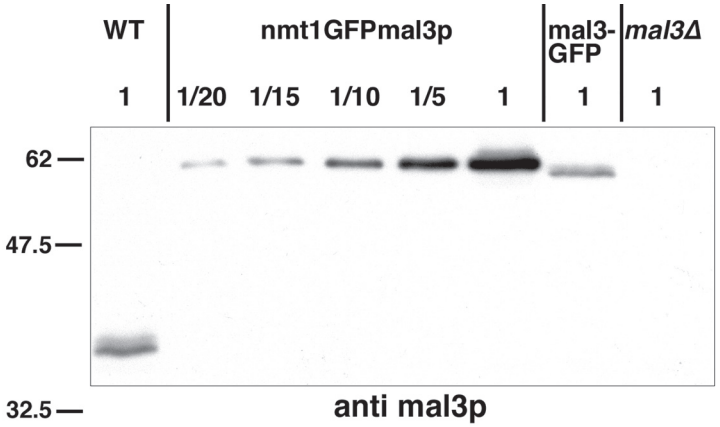
Time-lapse sequences of cells expressing GFPmal3p under control of the fully repressed nmt1 promoter. Cells are ordered from left to right and top to bottom in each sequence.
(A) Maximum projection of 15 slices covering the entire Z-axis of a cell in anaphase. Frames were taken every 38.5 seconds. GFPmal3p localises along the elongating spindle and to astral microtubules. The green arrow depicts a GFPmal3p particle at the tip of an astral, cytoplasmic microtubule. In late anaphase, mal3p presumably accumulates at kinetochores (blue arrowhead) and the spindle pole bodies (red arrowhead).
(B) Post-anaphase cell, maximum projection of 14 slices covering the entire Z-axis of the cell. Frames were taken every 20.2 seconds. GFPmal3p localises to the spindle, accumulating at its poles, to astral microtubules, accumulating at their tips (green arrow), and to a microtubule ring in the center of the cell (blue arrowhead). The ring is part of the post-anaphase microtubule array. GFPmal3p is also found on the tips of microtubules nucleated from this ring (red arrow).



Supplemental Figure S2.2 Anti-mal3p Staining in *mal3*Δ Cells

Immunofluorescence staining of methanol fixed cells. Anti-tubulin staining is in red, anti-mal3p staining in green. This control experiment shows that the anti- mal3p antibody causes only neglectable background staining.

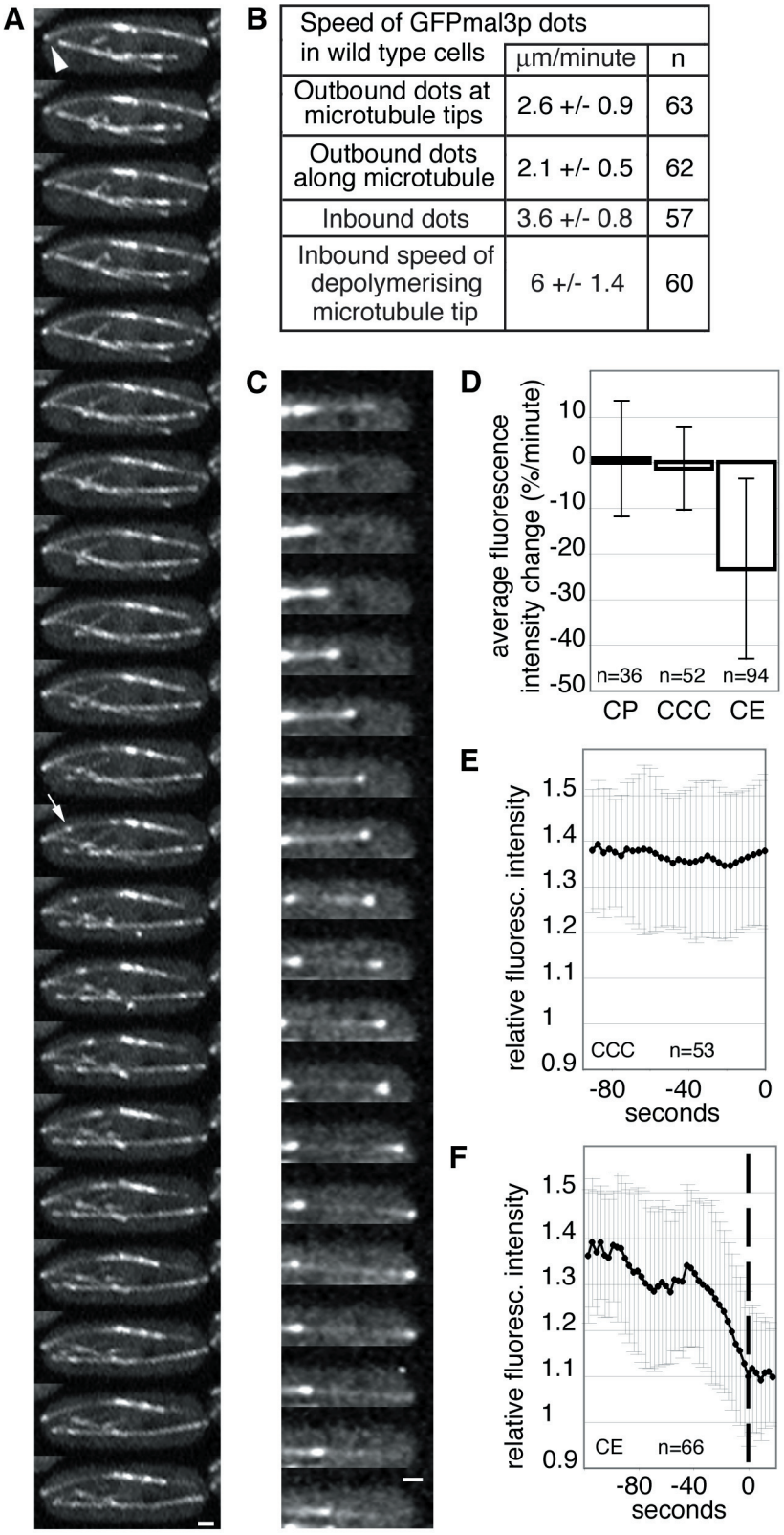
replaced endogenous mal3p with an amino-terminal-tagged mal3p (GFP-mal3p), which was expressed with the repressible nmt1 promoter. Under repressed conditions, GFP-mal3p cells were of wild-type appearance with respect to growth rates, shape, microtubule organisation (data not shown), and microtubule dynamics (catastrophe frequency is 0.3/min at 25°C, growth re-initiation frequency is 0.26/min, and average pausing time is 17 ± 14 sec). The residual promoter activity was sufficient to express GFP-mal3p at a level of approximately ten times that of the endogenous promoter (see the Experimental Procedures and Supplemental Figure S2.3). GFP-mal3p localised similarly to endogenous mal3p (Figures 2.2D and 2.2E); however, in living cells the uniform association with the microtubule lattices became more obvious. This enabled us to simultaneously follow microtubule dynamics and the behaviour of microtubule-associated GFP-mal3p particles. Time-lapse imaging revealed that the particles moved in two directions, outbound toward the cell ends and inbound toward the cell centre (Figure 2.3A and Movie 2.3). Outbound particles moved from the iMTOCs toward the cell ends, whereas inbound particles appeared de novo somewhere along the microtubules and moved toward the iMTOCs, where they fused with other particles. Inbound particle velocity was higher than outbound particle velocity but was considerably lower than the speed of depolymerising microtubule tips (Figure 2.3B).



Supplemental Figure S2.3 Expression Levels of mal3p in Strains Used for Live Imaging

Western blot analysis of total cell extracts to compare mal3p levels in wild-type cells with mal3p N-terminally tagged with GFP and expressed from the fully repressed nmt1 promoter, or C-terminally tagged with GFP and expressed from the endogenous promoter. No signal is seen in *mal3*Δ control cells. The numbers on top indicate the relative amounts of extract used.

GFP-mal3p particles were usually not associated with depolymerising, but with all growing microtubule tips (Figures 2.3A and 2.3C). From there they disappeared in cell-end regions, preceding catastrophes (Figures 2.3C–3F). Disappearance could be the consequence of reduced microtubule growth preceding catastrophes if mal3p association with the microtubule tips was dependent on microtubule polymerisation. In this scenario the amount of mal3p at microtubule tips should be a function of microtubule growth. To test this we compared microtubule growth speeds with the amount of mal3p at their tips by quantifying particle fluorescence intensities. As shown in Figure 2.4A, the amount of mal3p on growing microtubule tips and the speed of microtubule growth did not correlate. Furthermore, when microtubule tips contacted the cell ends, mal3p particles remained associated as long as the microtubule tip remained there (up to several minutes). The duration of mal3p association correlated with the time a microtubule remained at the cell end, and the particles only disappeared shortly before catastrophe occurred (Figures 2.3A and 2.3F). We conclude that



Speed of GFPmal3p dots in wild type cells	µm/minute	n
Outbound dots at microtubule tips	2.6 +/- 0.9	63
Outbound dots along microtubule	2.1 +/- 0.5	62
Inbound dots	3.6 +/- 0.8	57
Inbound speed of depolymerising microtubule tip	6 +/- 1.4	60

Figure 2.3 Microtubule-Associated mal3p Particles Are Very Dynamic

(A and C) Movie sequences of GFP-mal3p-expressing wild-type cells. The white bars in the last frame represent 1 µm. (A) 2 min, 20 s movie of an entire cell. Frames taken every 6.8 s show projections of 17 confocal sections covering the entire Z-axis of the cell. The arrow points out an inbound particle. The arrowhead depicts a microtubule that does not undergo catastrophe at the cell end and remains associated with a mal3p particle for the entire duration of the movie sequence. (C) The dynamics of a microtubule bundle are shown. The movie lasts for 1 min 14 s and covers a depolymerisation phase, a growth phase, and the beginning of a second depolymerisation phase. Note that catastrophe occurs after the fluorescence intensity of the tip-associated mal3p particle has considerably decreased.

(B) Table showing a comparison of the velocities of outbound and inbound GFP-mal3p particles and of the speed of depolymerising microtubule tips.

(D) Graph showing the average change of fluorescence intensity (percent per minute) of microtubule tip-associated, outbound GFP-mal3p particles in the cytoplasm (CP), at the cortex in central regions of the cell (CCC), and at the cell ends (CE). Error bars depict standard deviation.

(E and F) Average fluorescence intensity (particle fluorescence relative to mean cell fluorescence) including standard deviation of GFP-mal3p particles at growing microtubule tips over time in different regions of wild-type cells. (E) Fluorescence intensity of particles that are in contact with the central cell cortex (CCC). Time 0 depicts arrival at cell-end region. (F) In cell-end regions (CE), the fluorescence intensity of GFP-mal3p particles decreases before catastrophe (broken line) occurs.

mal3p localisation to microtubule plus ends is not correlated with microtubule polymerisation and that removal of mal3p from the microtubule tips may be a prerequisite for catastrophe to occur.

To exclude that GFP-mal3p localisation and particle dynamics were artefacts created by the 10-fold mal3p overexpression, we also created a carboxy terminally GFP-tagged mal3p variant. This variant showed no difference to GFP-mal3p with respect to localisation, particle velocity, and direction of movement or disappearance from microtubule tips (Supplemental Figure S2.4, Movie 2.7 and data not shown). Notably, however, the variant caused abnormal microtubule behaviour in cell-end regions, which is likely to be the cause of the previously reported shape abnormalities [8 and 15].

2.3.4 Outbound mal3p Particles Mark Additional Microtubule Tips

GFP-mal3p particles also moved outbound along depolymerising microtubules. When these particles eventually encountered the incoming microtubule tip, they often continued to move toward the cell ends, now marking the tip of a growing microtubule (Figure 2.4B). Due to resolution limitations we were unable to determine whether the outbound

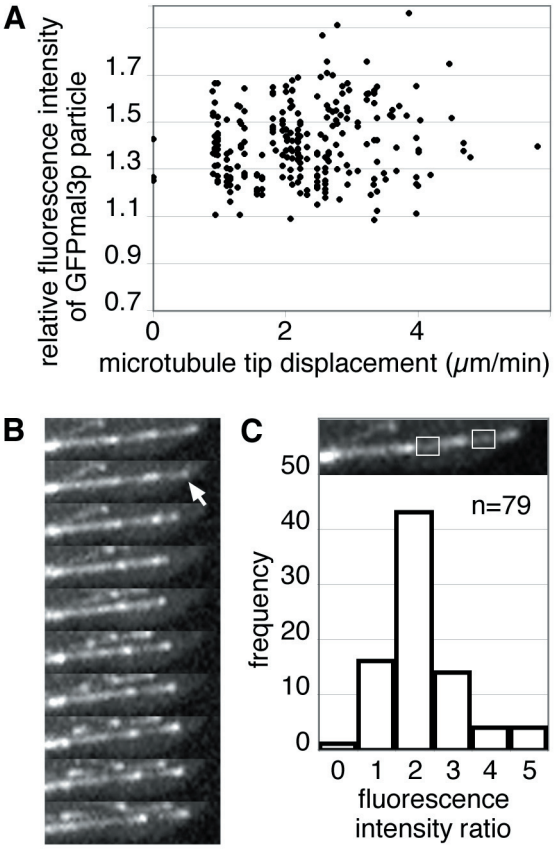
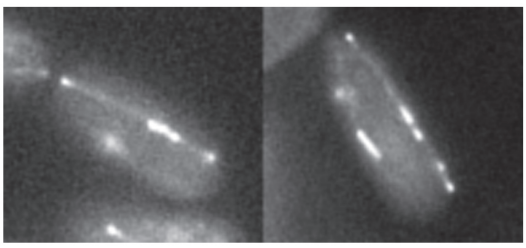


Figure 2.4 Microtubule Lattice-Associated Outbound mal3p Particles Mark the Tips of Secondary Microtubules

(A) Dot plot correlating the fluorescence intensity of microtubule tip-associated mal3p particles and preceding growth of the respective microtubules as they grow toward the cell ends.

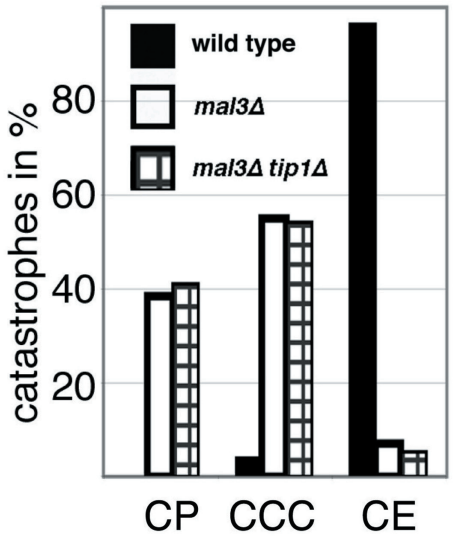
(B) Movie sequence of a GFP-mal3p-expressing wild-type cell. Frames taken every 6.8 s show projections of 17 confocal sections covering the entire Z-axis of the cell. The movie shows a depolymerising microtubule tip (arrow) encountering an outbound GFP-mal3p particle, which moves along the depolymerising microtubule and subsequently marks the tip of a growing microtubule.

(C) Histogram showing fluorescence intensity comparison of the GFP-mal3p signal in a defined section of the microtubule lattice proximal and distal of secondary GFP-mal3p particles that follow the primary particles marking the growing microtubule tips (proximal divided by distal value). The mean value is 2.2 ± 0.9. The inlay on the top shows an example. The two identical squares depict the measured and compared areas.



Supplemental Figure S2.4 Localisation of mal3pGFP

Two examples of cells expressing endogenous mal3p under the control of its own promoter and tagged with GFP at its carboxy terminus are shown. Single focal planes were recorded with a 100x NA1.4 objective on a Zeiss Axiovert 200M fluorescence microscope using Metamorph software and a Coolsnap HQ camera (2x2 binning). Mal3pGFP decorates the entire microtubule lattice and accumulates in particles/comets at distal microtubule tips and along the microtubules in the same way as shown for aminoterminally tagged mal3p.



Supplemental Figure S2.5 Spatial distribution of Catastrophe Events

Comparison of the spatial distribution of catastrophe events in wild-type (black bars, N=187), *mal3*Δ (white bars, N=164), and *mal3*Δ *tip1*Δ (chequered bars, N=56) double mutant cells. CP stands for cytoplasm, CCC, for central cell cortex (excluding the cell ends), and CE for the cell end cortex.

GFP-mal3p particles had induced a rescue or whether they marked the tips of other growing microtubules. Because there is increasing evidence that interphase microtubules are bundles of individually growing microtubules [24 and 25], we tested whether the outbound GFP-mal3p particles that move along a microtubule were marking microtubule tips. These particles moved with speeds similar to particles marking the visible distal end of a bundle (Figure 2.3B). For further analysis, we took advantage of the fact that GFP-mal3p decorates the lattice of all microtubules and that therefore two adjacent microtubules will appear brighter than a single one. We compared the GFP-mal3p signal intensity of a defined microtubule lattice stretch proximal (toward the iMTOC) and distal to the first particle behind a growing microtubule tip (Figure 2.4C and see the Experimental Procedures). The proximal signal was on average twice as bright as the distal one (Figure 2.4C), suggesting that most, if not all, outbound GFP-mal3p particles mark the tips of growing microtubules. Since we could observe up to five outbound particles in a row, there may be up to five microtubules growing in a single bundle.

Figure 2.5 mal3p Localisation to Microtubules Is Independent of tip1p

(A) Movie sequence showing microtubule dynamics in a GFP-tubulin-expressing *mal3*Δ *tip1*Δ double-deletion cell. Full projections of stacks of images taken every 16 s are shown. The phenotype is indistinguishable from that caused by the *mal3*Δ single deletion (see Figure 2.1B).

(B) Immunofluorescence of methanol fixed *tip1*Δ cells stained with the anti-tubulin (red) and anti-mal3p antibodies.

(C) Immunofluorescence of methanol-fixed GFP-mal3p (green)-expressing *tip1*Δ cells stained with the anti-tubulin (red) antibody.

(D) Movie sequence showing GFP-mal3p in full Z-axis projection of a *tip1*Δ cell. Frames taken every 9 s are shown. Inbound (green triangles) and outbound movement (red triangles) occurs. The GFP-mal3p particle disappears from microtubule tips prior to catastrophe (yellow triangles).

(E) Graph showing the average change of fluorescence intensity (percent per minute, including standard deviation), of microtubule tip-associated, outbound GFP-mal3p particles in the cytoplasm (CP) and at the cortex in central regions (CCC) of *tip1*Δ cells.

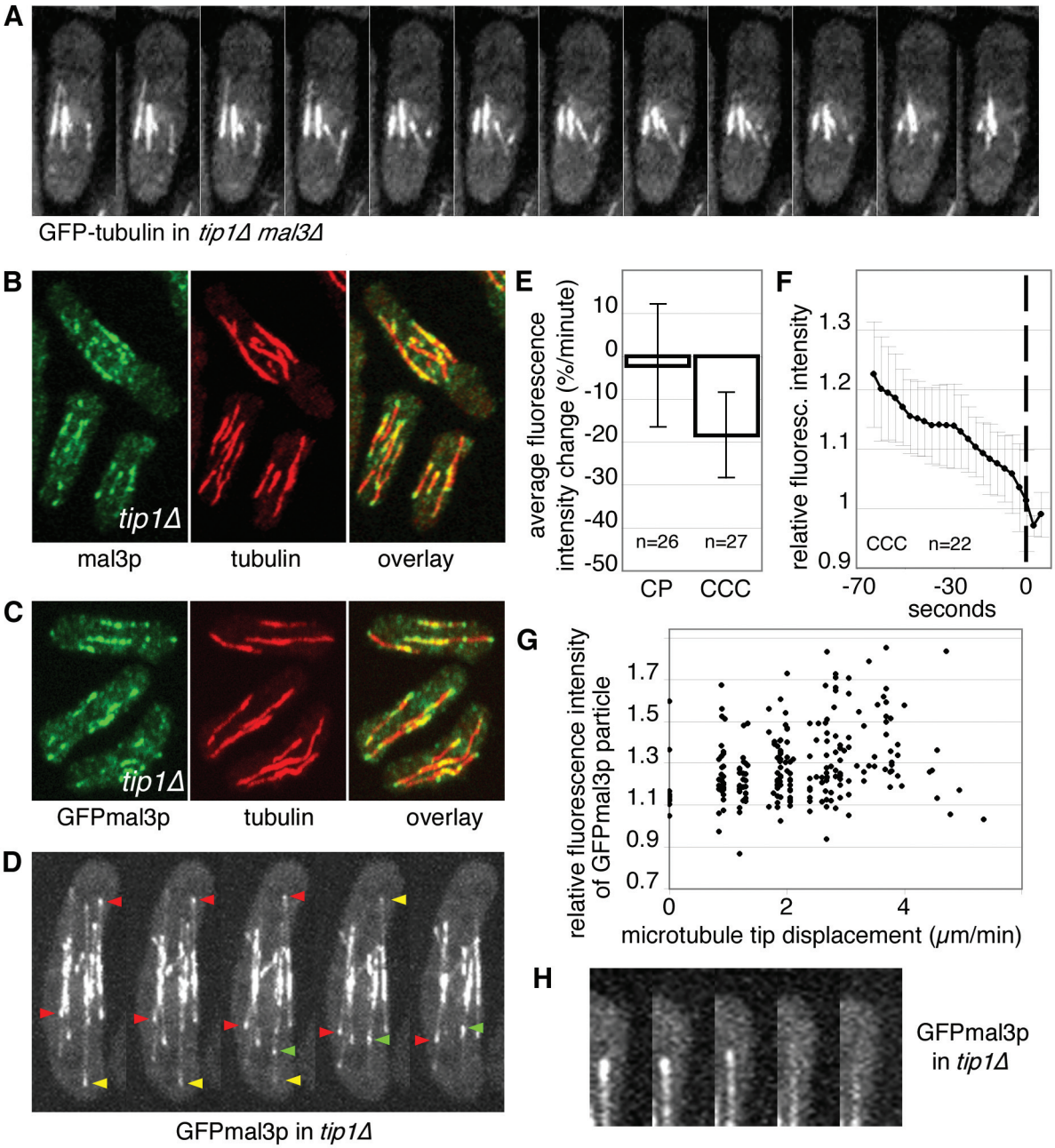
(F) Average fluorescence intensity (particle fluorescence relative to mean cell fluorescence) over time of GFP-mal3p particles at growing microtubule tips that are in contact with the central cell cortex (CCC). Time courses are aligned with respect to catastrophe (broken line), and error bars depict standard deviation.

(G) Dot plot correlating the fluorescence intensity of microtubule tip-associated mal3p particles and preceding growth of the respective microtubules as they grow toward the cell ends in *tip1*Δ cells.

(H) Example of a mal3p particle disappearing from the microtubule tip while the microtubule continues growth in a *tip1*Δ cell.

2.3.5 mal3p Is Epistatic to tip1p and Its Localisation Is tip1p Independent

Like mal3p, the CLIP-170 homologue tip1p localises to growing microtubule tips and suppresses catastrophes. To investigate the functional relationship between mal3p and tip1p, we analysed a *mal3*Δ*tip1*Δ double-deletion strain. This strain displayed a *mal3*Δ mutant phenotype. The microtubules were of similar length (3.2 ± 1.2 µm, N=198 at 25°C, compared to 3.6 µm for *mal3*Δ) and catastrophes were not spatially restricted like in *tip1*Δ cells (Figure 2.5A and Supplemental Figure S2.5). *mal3* is thus epistatic to *tip1*.



We next investigated whether mal3p localisation is tip1p dependent. Immunofluorescence studies showed that mal3p localised normally to the shorter microtubules of *tip1*-deleted cells (*tip1*Δ) (Figure 2.5B). In living *tip1*Δ cells, the GFP-mal3p also localised similarly to the wild-type (Figure 2.5C), and the dynamic behaviour of GFP-mal3p particles was largely unchanged (Figure 2.5D, Movie 2.4 and Table 3.1). The binding of mal3p to microtubules and the dynamics of mal3p particles are therefore independent of tip1p.

The main difference between wild-type and *tip1*Δ cells is that in the latter the microtubule tips rarely reach the cell ends because premature catastrophes occur when they encounter the cortex in central regions of the cell. The disappearance of the GFP-mal3p particles from the microtubule tips also preceded these mislocated catastrophes (Figures 2.5E and 2.5F). Similar to wild-type cells, we could not find a correlation between microtubule growth rate and the amounts of mal3p at the microtubule tips (Figure 2.5G). Furthermore, we could observe that the microtubules continued to grow for short periods of time even while the mal3p signal disappeared from their tips, which excludes that mal3p disappearance is caused by reduced microtubule polymerisation (Figure 2.5H).

Our results suggest that the localisation of mal3p to interphase microtubules is independent of tip1p but that tip1p is needed to restrict the dissociation of microtubule tip associated mal3p particles to the cell-end regions.

2.3.7 tip1p Localisation to Microtubules Is mal3p Dependent

tip1p, like its mammalian homologues, directly binds to purified microtubules *in vitro* [23]. This suggests that the localisation of tip1p to microtubules occurs independently of mal3p. To confirm this, we examined tip1p localisation in *mal3*-deleted cells (*mal3*Δ). In wild-type cells, tip1p particles localise to growing microtubule tips, along the microtubules, and to the cortex at cell ends (Figure 2.6A) [23]. In *mal3*Δ cells we could only observe very faint tip1p particles, which were dispersed

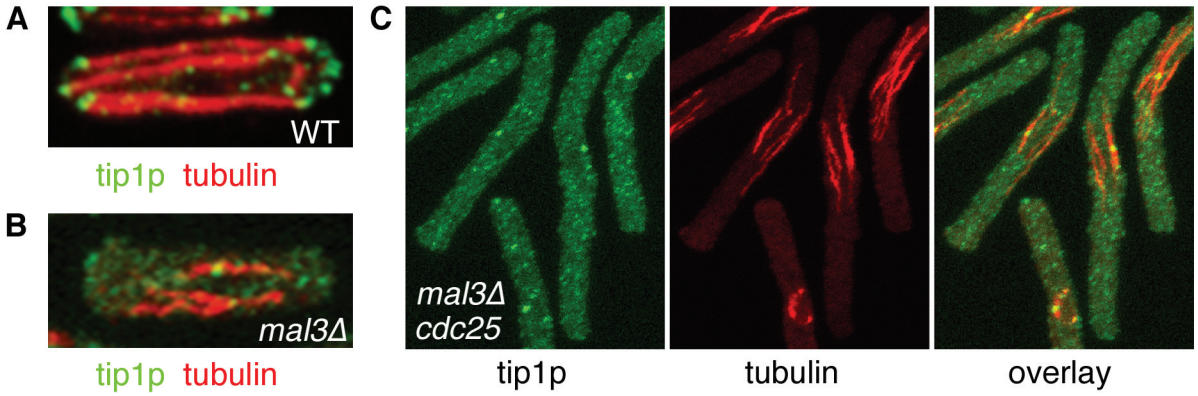


Figure 2.6 Microtubule Tip Association of tip1p Requires mal3p

(A–C) Immunofluorescence staining of fixed cells using the anti-tip1p antibody (green) and the anti-tubulin antibody (red). (A) Wild-type cell. (B) *Mal3*Δ cell. (C) *Mal3*Δ *cdc25ts* cells grown for 4 hr at the restrictive temperature.

throughout the cell (Figure 2.6B). Because the particles were present at a relatively high density, we were unable to unambiguously determine whether they were associated with the short microtubules of *mal3*Δ cells. We therefore repeated the experiment in *mal3*Δ cells carrying a temperature-sensitive *cdc25* allele (*mal3*Δ*cdc25ts*). At the restrictive temperature these cells are blocked in G2, become elongated, and possess longer microtubules than *mal3*Δ cells [26]. No tip1p was associated with these microtubules except for single tip1p particles that occasionally localised to the iMTOCs (Figure 2.6C). Colocalisation studies using a sad1p-DsRed construct showed that some tip1p particles colocalised with the interphase spindle pole body (data not shown). Hence, in the absence of mal3p, tip1p only appears to be able to associate with microtubules at the iMTOCs, but not along the microtubules or at their tips.

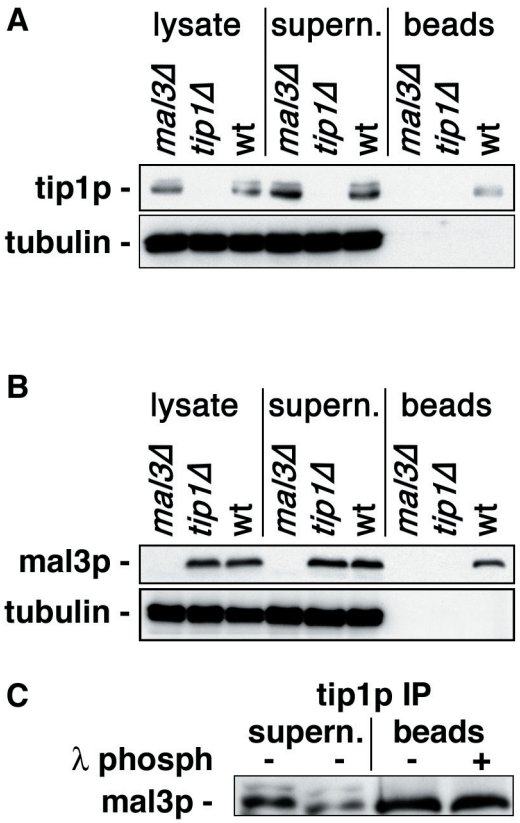


Figure 2.7 tip1p and mal3p Coimmunoprecipitate

(A–C) Western blots of coimmunoprecipitation experiments done by using yeast extracts and antibodies recognizing tip1p, mal3p, and α-tubulin. The first three lanes in (A) and (B) show total cell lysate. "Supern." (supernatant) refers to the unbound and "beads" to the bound fractions. (A) mal3p immunoprecipitation done with the anti mal3p antibody. *Mal3*Δ and *tip1*Δ cells are used as controls. The Western blot shows coimmunoprecipitation of tip1p, but not α-tubulin. (B) tip1p immunoprecipitation done with the anti tip1p antibody. *mal3*Δ and *tip1*Δ cells are used as controls. The western blot shows co-immunoprecipitation of mal3p but not α-tubulin. (C) Two tip1p immunoprecipitation experiments are shown. One of the immunoprecipitates was treated with λ-phosphatase showing that the coimmunoprecipitating mal3p band represents the dephosphorylated form of mal3p.

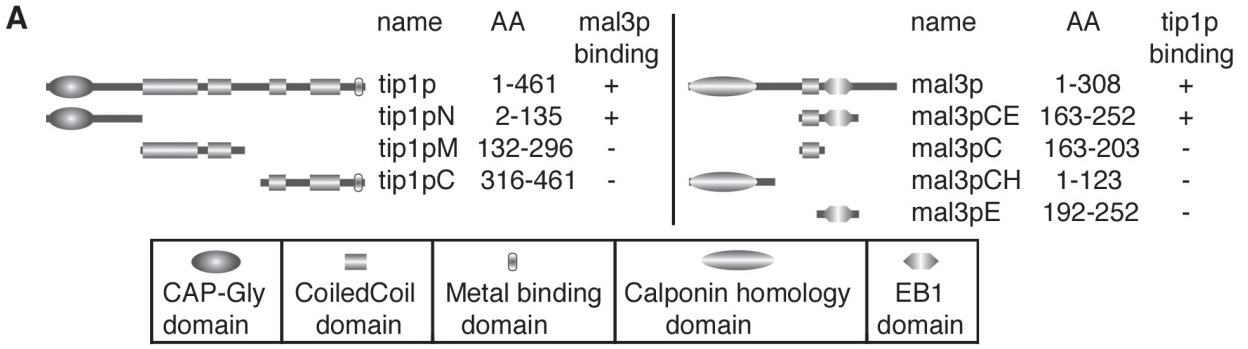
2.3.8 mal3p and tip1p Interact Directly

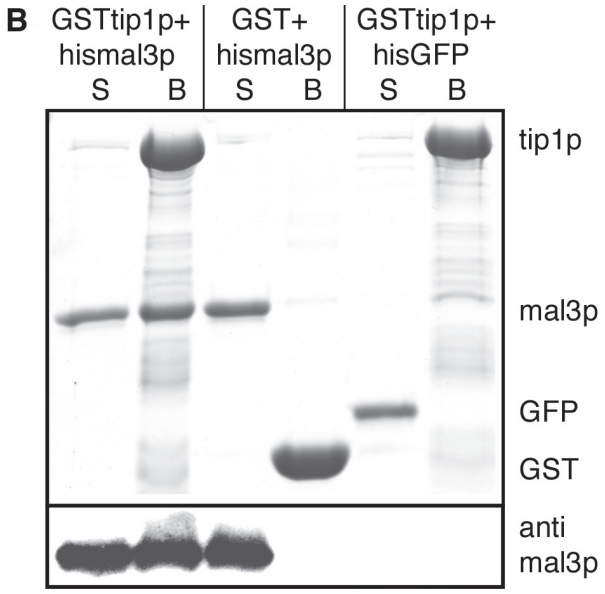
We have found that tip1p localisation to growing microtubule tips is mal3p dependent and that tip1p is required to maintain mal3p association with the growing microtubule tips at the central cell cortex. To test whether these interactions could be direct, we performed coimmunoprecipitation experiments and protein binding assays. We isolated tip1p and mal3p from cell extracts with the respective antibodies and found that a significant fraction of tip1p or the non-phosphorylated mal3p coimmunoprecipitated with mal3p and tip1p, respectively (Figures 2.7A–7C). Notably, we could only coimmunoprecipitate mal3p or tip1p when performing the standard washing steps within a short time period, indicating that the tip1p/mal3p interaction is unstable. In both immunoprecipitates we could not detect any traces of tubulin (Figures 2.7A and 2.7B).

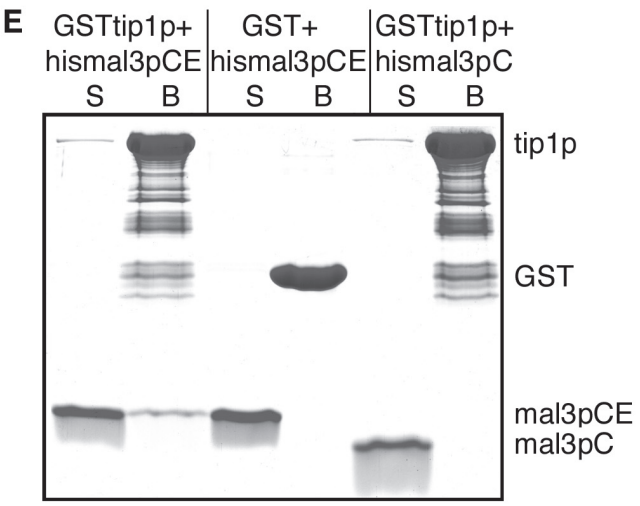
To test for direct association of tip1p and mal3p, we performed protein-binding assays with bacterially purified proteins. In our experiments, GST-tagged tip1p was able to bind to His-tagged mal3p,

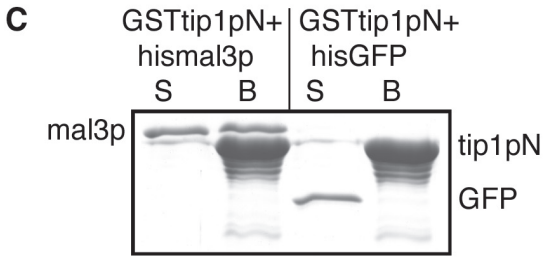
but not the His-tagged GFP control (Figure 2.8B). His-tagged mal3p was unable to bind GST alone. Hence, tip1p can interact directly with nonphosphorylated mal3p.

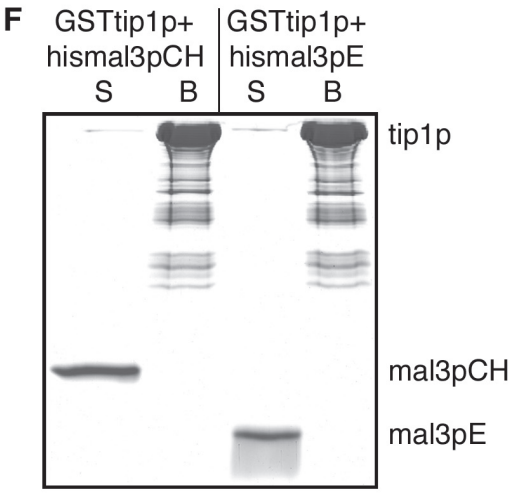
We went on to map the interacting regions of tip1p and mal3p. mal3p contains a conserved amino-terminal calponin homology domain, which mediates microtubule binding in EB1. Like most EB1

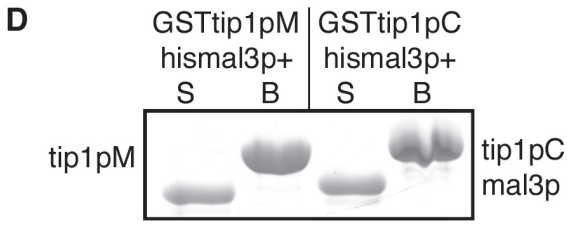












family members, mal3p also contains a short coiled-coil stretch followed by the conserved EB1 domain at the carboxyl terminus. tip1p has a conserved CAP-Gly domain at its amino terminus, followed by a serine-rich amino acid stretch, several coiled-coil regions, and a metal binding domain. We tested multiple bacterially purified mal3p and tip1p fragments for their ability to bind to full-length tip1p and mal3p respectively (Figure 2.8A). Only the amino-terminal, CAP-Gly domain containing part of tip1p bound to mal3p (Figures 2.8C and 2.8D), whereas the region of mal3p containing the coiled coil and the EB1 domain mediated binding to tip1p (Figures 2.8E and 2.8F).

2.4 Discussion

We have investigated how the microtubule plus end-tracking protein mal3p contributes to the organisation of interphase microtubules and how its activity is related to that of another microtubule plus end-tracking protein, tip1p. We found that mal3p is a general promoter of microtubule growth. In its absence the microtubules are less efficient in initiating growth from the iMTOCs, and they can maintain growth only for short periods of time before a catastrophe occurs. This results in the formation of shortened microtubules as previously described [8 and 15]. Similar to the other EB1 proteins, it is not clear how mal3p affects microtubule dynamics at the molecular level. This could be through direct interaction with the microtubules or by recruiting other growth promoting proteins such as tip1p.

Shortened microtubules have also been observed in *tip1*Δ cells. This is because catastrophes are no longer restricted to cell ends but are triggered prematurely when microtubules come in contact with the cell cortex outside the cell-end regions [23]. tip1p function is therefore required only in cortical regions of the cell. This is different from the *mal3*Δ cells where catastrophes frequently occur in the cytoplasm even before growing microtubule tips have reached the cell cortex. This suggests that mal3p is required for microtubule growth throughout the cell and that mal3p may

Figure 2.8 tip1p and mal3p Interact Directly

(A) Domain organisation of tip1p and mal3p and fragments used for interaction domain mapping.
(B–F) The figures show Coomassie-stained gels loaded with supernatants (S column) or bead eluates (B column) of binding assays with recombinant His-tagged mal3p and GST-tagged tip1p or with fragments of the two proteins and with GST- or His-tagged GFP. In all experiments the GST-tagged protein was bound to glutathione sepharose beads and was subsequently incubated with the His-tagged protein. For each experiment 1/15 volume of the supernatants and 2/3 volume of the eluates were loaded. (B) The first two lanes show binding of Hismal3p to GSTtip1p. Hismal3p cannot bind to the GST control protein (middle lanes), and no binding to GSTtip1p occurs with the His-tagged GFP control protein (last lanes). The frame at the bottom shows an anti mal3p immunoblot of the same experiment. (C) Hismal3p (first lanes), but not the HisGFP control protein (last lanes), binds to the amino-terminal fragment of tip1p containing the CAP-Gly domain. (D) Hismal3p cannot bind to central and carboxy-terminal fragments of tip1p. (E) A mal3p fragment containing the coiled coil and the EB1 domain binds to tip1p (first lanes), but not to GST alone (middle lanes). The mal3p coiled-coil domain is unable to bind to tip1p (last lanes). (F) Neither the amino terminus with the calponin homology domain (first lanes) nor the EB1 domain (last lanes) is on its own sufficient for binding to tip1p.

be more fundamental to microtubule growth than tip1p. Several substitution experiments between mal3p and human EB1 have suggested that their function is highly conserved [8, 14 and 15]. This is further supported by experiments performed in *Xenopus* egg extracts, where human EB1 was shown to promote microtubule growth by reducing catastrophe rates similar to mal3p [13]. However, EB1 homologues in *Drosophila* and budding yeast were reported to increase catastrophe rates [9 and 12]. At this point it is not clear what this difference means. It is possible that not all EB1 homologues have identical roles in controlling microtubule dynamics. In *Drosophila*, only one of several EB1 family members has been thus far eliminated by RNA interference. It is possible that removal of other family members will produce effects similar to *mal3* deletion. It should be interesting to test other EB1 family members besides the human homologue for their ability to substitute for mal3p activity.

In addition to its role in controlling microtubule dynamics, mal3p is also required for the localisation of tip1p to growing microtubule tips. This is surprising because recombinant tip1p, like CLIP-170, can bind to pure microtubules [23 and 27]. The conserved CAP-Gly domain is thought to mediate this interaction. This is based on the finding that a fragment of human CLIP-170, containing the CAP-Gly domain and an adjacent serine-rich amino acid stretch, can bind to the lattice of purified microtubules [28 and 29]. Furthermore, *in vivo*, the same protein fragment is necessary and sufficient for microtubule plus-end binding [28 and 29]. Intriguingly, the equivalent CAP-Gly domain fragment from tip1p directly binds to mal3p. This raises the possibility that in fission yeast cells, tip1p is recruited to the growing microtubule tips by mal3p. However, we cannot exclude that the requirement of mal3p is indirect, for example, if binding of mal3p to the CAP-Gly region modifies its affinity to microtubule tips. Alternatively, mal3p could somehow modify the structure of the microtubule tip and in this way facilitate tip1p binding independent of a direct interaction.

Our results raise another intriguing possibility, namely that mal3p inhibits microtubule catastrophes by recruiting the catastrophe inhibitor tip1p. However, we show that unlike tip1p, mal3p is already required for catastrophe inhibition when microtubules grow in the cytoplasm. In this situation mal3p would therefore be acting through another catastrophe inhibitor.

To get more mechanistic insight into how tip1p and mal3p affect microtubule dynamics and how they localise to growing microtubule tips, it will be important to study their interaction with the kinesin tea2p. Also the localisation of tea2p to microtubule tips is mal3p dependent [15]. However, it seems that mal3p does not directly mediate microtubule binding of tea2p because tea2p rigor mutants, which have blocked motor activity, localise to microtubules even in the absence of mal3p. *Tea2*Δ cells possess shortened microtubules and abnormal morphology similar to *mal3*Δ and *tip1*Δ cells [30]. Although the effects of tea2p on microtubule dynamics have not been investigated in detail, it is likely that it closely interacts with tip1p and mal3p.

The finding that tip1p localisation is mal3p dependent is consistent with the view that mal3p has a more basic function than tip1p. Also consistent is that in turn, mal3p localisation to the microtu-

bules is not tip1p dependent. This is similar to what was observed with mammalian EB1. In hamster ovary cells, overexpression of a dominant-negative CLIP-170 protein removed endogenous CLIP-170 from the microtubule tips but did not affect EB1 localisation [31]. Also the various aspects of mal3p localisation to interphase microtubules, namely its association with the microtubule lattice and its accumulation at the iMTOCs and the growing plus ends, are similar to what has been observed with other EB1 family members [11, 32, 33 and 34].

It is not clear which aspect of mal3p localisation is critical for the control of microtubule dynamics. However, we noticed a correlation between the presence of mal3p at microtubule tips and the occurrence of catastrophes suggesting that mal3p dissociation may be a prerequisite for catastrophe to occur. After microtubules had reached the cell ends, catastrophes did not immediately take place but could be delayed for up to several minutes. During this time the microtubule tips remained relatively immobile and were associated with a mal3p particle. Particle disappearance was only triggered shortly before catastrophe occurred. In principle mal3p disappearance could be the consequence of reduced microtubule growth if the recruitment of mal3p to microtubule tips requires microtubule polymerisation. However, we could not find a correlation between microtubule growth rates and the amount of mal3p at microtubule tips. This was the same in *tip1*Δ cells, where mal3p particles also disappeared from microtubule tips preceding catastrophe. In these cells we could observe that during and immediately after mal3p particle disappearance the microtubules were still able to grow for short periods of time. In these situations mal3p was therefore no longer recruited to, or retained at the growing tips. This suggests that either the properties of the growing microtubule tips or of mal3p itself have changed. It will now be important to identify the nature of these changes since they may be the key for understanding how microtubule dynamics can be locally controlled in a cell.

2.4.1 Conclusions

Our results show how two microtubule plus end-tracking proteins act in synergy to locally regulate microtubule dynamics in a cell. Both tip1p and mal3p are required to promote the growth of cytoplasmic microtubules. Whereas mal3p is important for microtubule growth throughout the cell, tip1p is required to maintain growth only when microtubule tips come in contact with cortical regions in the cell centre. mal3p therefore appears to have a more basic function in the control of microtubule dynamics than tip1p. Consistent with this view, mal3p is also essential for the microtubule plus-end association of tip1p. It is possible that this involves direct binding of mal3p at or near the CAP-Gly domain of tip1p. In turn, mal3p localisation is not dependent on tip1p, but its behaviour is influenced by tip1p. In wild-type cells mal3p disappears from microtubule tips, followed by microtubule catastrophe, only when microtubules have reached the cell ends. In *tip1*Δ cells, however, mal3p disappears already when microtubule tips contact the cortex in any region of the cell, which is followed by premature catastrophe events. tip1p and mal3p therefore define a system that is capable of sensing the cortical organisation of the fission yeast cell.

2.5 Experimental Procedures

2.5.1 *S. pombe* Methods

Standard methods were used for growth, transformation, and genetic manipulation of *S. pombe*. Cells were generally grown and imaged at 25°C unless otherwise stated.

2.5.2 Antibody Production

Full-length *mal3* cDNA was PCR amplified from an S. *pombe* cDNA library (constructed by B. Edgar and C. Norbury) by using the primers AATTGGATCCAGATGTCTGAATCTCGGCAAGAGC and ATTCTCGAGTTAAAACGTGATATTCTCATCG. The product was cloned as a BamHI-XhoI fragment into pGEX-5X-1 (Amersham, Little Chalfont, UK) to create an amino-terminal GST fusion protein with *mal3*. The protein was expressed in *E. coli*, purified by using a GSTrap and a Resource Q column (Amersham, Little Chalfont, UK), and then used to inoculate rabbits. On Western blots from wild-type cell extracts the resulting antibody detected two mal3p specific bands at 38 kDa, which were absent in extracts from *mal3*Δ cells, and after long exposure times, an additional, unspecific band of about 35 kDa.

2.5.3 Immunofluorescence Techniques

Cells were methanol fixed and processed through standard methods [18]. Images were taken on a TCS SP2 laser-scanning confocal microscope (Leica, Wetzlar, D) with a Plan-Apochromat 100× oil objective (Leica; NA 1.4), equipped with an Argon laser (100 mW) and a green 1 mW He/Ne laser. The 488 and 543 nm laser lines were used. Images were processed with Adobe Photoshop. Primary antibodies used were rabbit anti-mal3p (this study), rabbit anti-tip1p [23], and anti-α2-tubulin (clone B-5-1-2, Sigma). Secondary antibodies used were anti-rabbit-Alexa 488 and anti-rabbit-Alexa 546 (Molecular Probes, Eugene, OR).

2.5.4 Construction and Growth of the GFP-mal3p Strain

The homologous recombination-based method described in [35] was used to tag endogenous mal3p with GFP at its amino terminus and place it under the control of the full-strength nmt1 promoter. The primers used were: CTAATTGAGAGATAGTTATTTTAATGTTTGCAACGAATATTCCG GAATACATGTAAATTTTCAGTTATAATACCCATTAAGAATTCGAGCTCGTTTAAAC and GATAATTTTTTCAATCTAACTAAGATATTAAAGCGTACTTGGTTGATCCAAGCTAAG AGCTCTTGCCGAGATTCAGACATTTTGTATAGTTCATCCATGC. GFP-mal3p cells were grown with fully repressed promoter, which was leaky enough to express GFP-mal3p at a level ten times higher than the endogenous promoter as judged by Western blots of dilution series of total-cell extracts.

The same method was used to insert a GFP at the c-terminus of the endogenous mal3p using primers CGTTCCCTCTGCACCAGATTTCGTACATGCTAGGCTACAAAGTTTAGAGGTTG ATGACGATGAGAATATCACGTTTatccttggagctccttcagg and GTATACAGAATGCTATATGTTAAGGAAAAAGAACGAAATTAAATGAATTTGGAGG TCATGAGGCACGCAACATTCGATCAgaattcgagctcgtttaaac, where capital letters denote *mal3*-specific sequence. GFP was PCR-amplified from a modified plasmid pFA6A-GFP(S65T)-kanMX6 (B. Hülsmann, unpublished), creating a ILGAPSGGGATAGAGGAGGPAGLI linker sequence between the *mal3* and GFP ORFs.

2.5.5 Live Imaging and Image Analysis

Cells expressing GFP-mal3p or GFP-tubulin under the control of the full-strength nmt1 promoter [21] or the 81nmt1 promoter (only in the *mal3*Δ *tip1*Δ strain under fully derepressed conditions) were grown to midlog phase and transferred to glass bottom microwell dishes (MatTek, Ashland, MA) coated with 1 μl of 2 mg/ml Lectin BS-1 (Sigma, St. Louis, MO). After 15 min, unattached cells were washed away, and 2.5 ml of fresh medium was added.

To image GFP-mal3p and CFP-tubulin, GFP-mal3-expressing cells were transformed with the plasmid pRL72 [36]. Images were taken with a Coolsnap HQ camera (Roper Scientific, Tucson, AZ) on an Axiovert 200 M microscope (Carl Zeiss, Göttingen, Germany) with a Plan-Apochromat 100× NA 1.4 objective (Zeiss). A stack of eight slices (0.5 μm distance between planes) was maximum projected with ImageJ. To avoid bleed-through from the CFP-tubulin signal when imaging GFP-mal3p, a YFP-specific filter set (AHF Analysentechnik, Tuebingen, Germany) was used. The CFP-specific filter set used for CFP-tubulin imaging allows some bleed-through of the GFP-mal3p signal.

Other live images were recorded on an UltraView LCI spinning-disc confocal microscope system (Perkin Elmer, Wellesley, MA) by using a 488 nm Argon Krypton laser (50 mW; Melles Griot, Carlsbad, CA) and a Nikon Eclipse TE200 microscope with a 100× oil objective (Plan Fluor, NA 1.3, DIC, Nikon, Kawasaki, JP). Pixel size was 136 nm (binning 2). Z stacks were taken every 7–9 s, with 17–18 planes per stack (0.3 μm distance between planes). The Z stacks were maximum projected with custom routines, written by Timo Zimmermann, EMBL, and analyzed with ImageJ. Growth reinitiation frequencies were calculated as events per observation time. Growth rates, shrinkage rates, and catastrophe frequencies were calculated as described in [37], and the relevant measurements were done by following the position change of MT plus ends with the crosshair tool of ImageJ. For measurement of fluorescence intensity of GFP-mal3p particles over time, the mean intensity of particles in the maximum projection was measured and tracked using the region of interest (ROI) function of ImageJ. To correct against photobleaching, the intensities were divided by the mean intensity of the whole cell. To average fluorescence intensity measurements, curve data of individual experiments were linearly interpolated to obtain the values at standardized time points. If the dura-

tion of measurements varied, they were standardized with respect to a defined event (i.e., arrival at cell end, catastrophe). The correlation between fluorescence intensities of mal3p particles and the speed of microtubule plus-end growth was analysed by plotting the speed of displacement of microtubule plus ends between two time points of a movie with the fluorescence intensity (mean grey value of a region of fixed size encompassing the mal3p particle) of the associated mal3p particle in the second time point. Only microtubules growing outside the cell-end region were considered. The mean signal intensity of GFP-mal3p bound to the microtubule lattice proximal and distal to GFP-mal3p particle was compared by measuring the intensities of particles free microtubule segments of identical size on either side of the GFP-mal3p particles excluding the comet tail and subtracting the mean intensity of a microtubule free cellular region of the same size. Up to three intensity measurements per particles were averaged. Image series were processed with Adobe ImageReady. Normalization and plotting of numerical data was done with Microsoft Excel.

2.5.6 Coimmunoprecipitation

Fission yeast native extracts were prepared as previously described [38]. Cells were broken by using glass beads (Sigma, St. Louis, MO). Immunoprecipitations were performed with anti-tip1p and anti-mal3p antibody bound to protein A sepharose beads (Amersham, Little Chalfont, UK) in Hepes buffer (25 mM Hepes [pH 7.2], 50 mM potassium acetate, 1 mM $MgCl_2$, 1 mM EDTA, 1% Triton X-100, 1 mM DTT, 40 μg/ml Aprotinin, 20 μg/ml Leupeptin, 1 mM PMSF, 2 mM Benzamidine, 1 μg/ml Pepstatin A, Roche EDTA-free protease inhibitor mix, 0.1 mM Sodium Vanadate, and 15 mM p-Nitrophenylphosphate). After incubation of beads with extract, three consecutive and rapid washing steps (<5 min) were performed for every tube separately. Coimmunoprecipitation of mal3p, tip1p, and tubulin was detected by using the respective antibodies (see Immunofluorescence section) on Western blots. For the mal3p immunoprecipitations anti-*mal3* antibody was crosslinked to the protein A beads as described in [39] to eliminate the IgG bands, which interfered with Western blot detection of tip1p. In this case proteins were eluted three times from the beads with 150 μl 0.1 μM glycine (pH 2.5), pooled, and neutralized with 200 μl 0.5 M Hepes (pH 7.6), and precipitated with TCA [40].

2.5.7 Phosphatase Treatment

Extracts were incubated for 45 min to 1 hr at 30°C, with or without 5 μl λ-protein phosphatase (NEB, Beverly, MA)/75 μl extract, and 25 mM sodium fluoride/5 mM sodium orthovanadate phosphatase inhibitors were added in the controls.

2.5.8 *In Vitro* Binding Assay

Full-length *mal3* cDNA was cloned into pET28c(+) (Novagen, EMD Biosciences, Madison, WI) by using the primers and restriction sites described for antibody production. cDNA fragments of tip1p

and mal3p were generated by PCR by using the plasmids with the corresponding full-length cDNA inserts and the following primers: for tip1p full-length, ATATGGATCCTTTCCTCTTGGCAGT GTC (forward) and AATTCTCGAGTTAAGCTTCGTCTGTGCTG (reverse); for tip1pN 2-135, ATATGGATCCTTTCCTCTTGGCAGTGTC and AATTCTCGAGTTATTCGGTTGGCGTTA AGG; for tip1pM 132-296, ATTAGGATCCACGCCAACCGAAAAAATTCTTC and AATTCTC GAGTTAGGGTGAACTGCTTTCCATA; for tip1pC 316-461, ATATGGATCCCCTGAAAACC ATCCTCAAC and AATTCTCGAGTTAAGCTTCGTCTGTGCTG; for mal3pCH 1-123, AATT GGATCCAGATGTCTGAATCTCGGCAAGAGC and AATTCTCGAGTTAAGCAGGTCCTC TATTCCC; for mal3pCE 163-252, ATTAGGATCCAATCTTCGACTGCAAATACTGCGG and ATTCTCGAGTTAGGGTTGATCAGGTGGTAACTCA; for mal3pC 163-203, ATTAGGATCC AATCTTCGACTGCAAATACTGCGG and AATTCTCGAGTTAATCACGTTCTCTCTCCAA ACCAAAC; and for mal3pE 192-252, ATTAGGATCCCTAATGAGACGATGTTTGGTTTGG and ATTCTCGAGTTAGGGTTGATCAGGTGGTAACTCA.

The products were cloned by using BamHI-XhoI, the tip1p fragments into pGEX-6P-1 (Amersham, Little Chalfont, UK), and the mal3p fragments into pET28c(+), creating amino-terminal GST or His fusions. The GST-tip1p protein fragments and the GST vector alone were expressed in *E. coli* as described in [23], affinity purified with glutathione sepharose (Amersham), and dialysed in PBS. His-mal3p fragments and His-GFP were expressed in *E. coli*, affinity purified using Ni-NTA agarose and dialysed in PBS. For the binding assays, 40 μl glutathione sepharose beads were washed three times with PBS + 0.1 % Triton X-100, then incubated in PBS/Triton for 4 hr at 4°C with 20 μg each of the recombinant proteins. As controls, purified GST or His-GFP [41] were used. The supernatant was isolated and the beads were subsequently washed three times with PBS/Triton before boiling to elute the bound protein. 1/10 of total supernatants and 2/3 of total eluates were loaded on 10% (full length and long fragments) or 18% (short fragments) SDS-gels and stained with G-Coomassie. Half amounts of each sample were used for mal3p detection on Western blots.

Supplemental Movies

Movie 2.1. Wild type cells expressing GFP-tubulin
Each of the 43 frames is a maximum projection of 15 slices covering the entire Z-axis of the cell. Frames were taken every 5.6 seconds (shown 58x accelerated). The majority of the microtubule bundles grow until they contact the cell ends where they undergo catastrophe. After full depolymerisation, the microtubules almost immediately re-initiate growth.

Movie 2.2. *Mal3*Δ cells expressing GFP tubulin
Each of the 33 frames is a maximum projection of 17 slices covering the entire Z-axis of the cell. Frames were taken every 7.5 seconds (shown 39x accelerated). Microtubule growth is strongly reduced. Microtubules rapidly undergo catastrophe after initiating growth. After depolymerisation, the microtubules have relatively long pausing times before re-initiating growth. The long cell on the right of the image centre is in the last stage of spindle elongation and has initiated cytokinesis as depicted by the microtubules originating from the equatorial MTOC in the cell centre.

Movie 2.3. Wild type cell expressing GFP-mal3p under the control of the fully repressed nmt1 promoter, resulting in about 10 fold higher expression levels than endogenous promoter. Each of the 54 frames is a maximum projection of 17 slices covering the entire Z-axis of the cell. Frames were taken every 6.8 seconds (shown 35x accelerated). GFP-mal3p particles move outbound from the iMTOCs toward the cell ends with the growing microtubule tips. Particles moving outbound along pre-existing microtubules most likely mark the tips of secondary microtubules. GFP-mal3p particles also move inbound towards the iMTOCs along the microtubules. These particles show increasing fluorescence intensities as they approach the iMTOCs.

Movie 2.4. *Tip1*Δ cells expressing GFP-mal3p
Each of the 49 frames is a maximum projection of 17 slices covering the entire Z-axis of the cell. Frames were taken every 9 seconds (shown 47x accelerated). GFP-mal3p particles show the same dynamics of movement in *tip1*Δ as in the wild type cells.

Movies 2.5 and 2.6. Wild type mitotic cells expressing GFPmal3p under the control of the fully repressed nmt1 promoter
Movie 2.5, showing a cell in anaphase. Each of the 117 frames is a maximum projection of 15 slices covering the entire Z-axis of the cell. Frames were taken every 7.7 seconds (shown 98x accelerated). GFPmal3p localises along the spindle and to cytoplasmic astral microtubules, where it accumulates at their tips. In late anaphase, mal3p weakly localises to the spindle pole bodies and to two particles on the spindle in a location where the kinetochores would be expected to be.
Movie 2.6, showing a post-anaphase cell. Each of the 105 frames is a maximum projection of 14 slices covering the entire Z-axis of the cell. Frames were taken every 6.7 seconds (shown 53x accelerated). GFPmal3p localises to the spindle, with decreasing intensity in the midzone. It accumulates at the spindle poles. It also localises to the tips of the astral microtubules nucleated from the cytoplasmic side of the SPBs. In addition, it is found also on a ring-like structure in the middle of the cell, which is part of the post-anaphase microtubule array. GFPmal3p localises to the tips of microtubules nucleated from this ring.

Movie 2.7. Wild type interphase cell expressing carboxy-terminally GFP-tagged mal3p from the endogenous promoter
Single focal planes were recorded with a 100x NA 1.4 objective on a Zeiss Axiovert epifluorescence microscope using a Coolsnap HQ camera (2x2 binning). Frames were taken every 1.1 seconds (shown 5.5x accelerated). A mal3-GFP particle can be seen moving inbound along a microtubule bundle toward the cell centre.

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Chapter 3
Tea2p Kinesin Is Involved in Spatial Microtubule Organization by Transporting Tip1p on Microtubules

Karl Emanuel Busch, Jacky Hayles, Paul Nurse and Damian Brunner

3.1 Abstract

The positioning of growth sites in fission yeast cells is mediated by spatially controlled microtubule dynamics brought about by tip1p, a CLIP-170-like protein, which is localised at the microtubule tips and guides them to the cell ends. The kinesin tea2p is also located at microtubule tips and affects microtubule dynamics. Here we show that tea2p interacts with tip1p and that the two proteins move with high velocity along the microtubules toward their growing tips. There, tea2p and tip1p accumulate in larger particles. Particle formation requires the EB1 homologue, mal3p. Our results suggest a model in which kinesins regulate microtubule growth by transporting regulatory factors such as tip1p to the growing microtubule tips.

3.2 Introduction

In many polarised cells, the interphase microtubule network is oriented within the cell to allow the delivery of cellular components to specific locations. Although a number of proteins affecting microtubule dynamics have been identified, little is known about how they are networked to control the spatial organisation of cytoplasmic microtubules. In the cylindrical cells of the fission yeast, *Schizosaccharomyces pombe*, the distribution of cytoplasmic interphase microtubules is important for the precise positioning of growth sites (Sawin and Nurse, 1998). Approximately three to six pairs of antiparallel microtubule bundles nucleate from several locations in the vicinity of the nuclear envelope at the cell centre termed interphase microtubule organising centres (iMTOC) (Hagan and Hyams, 1988). The minus end of each bundle appears to be tethered within the antiparallel region while the plus ends of both bundles grow toward the opposing cell ends (Drummond and Cross, 2000). Interphase microtubules go through repetitive phases of growth and shrinkage, a process termed dynamic instability (Drummond and Cross 2000 and Mitchison and Kirschner 1984). They grow until they reach the cell ends, where they then undergo catastrophe and depolymerise. This local restriction of catastrophe events shows that microtubule dynamics are spatially regulated (Brunner and Nurse, 2000b). The well-defined microtubule organisation in fission yeast allows the delivery to the cell ends of marker proteins like tea1p and pom1p, which control the correct positioning of the two antipodal growth zones (Bähler and Pringle 1998 and Mata and Nurse 1997). If microtubules are shorter and fail to reach the cell ends, then growth sites are displaced and cells become bent or branched (Hiraoka et al. 1984; Toda et al. 1983 and Umesono et al. 1983).

The microtubules are oriented parallel to the long axis of the cell by a guidance mechanism (Brunner and Nurse, 2000a). If microtubules grow off this axis, they first contact the cell cortex in central regions of the cell where they are deflected into the correct orientation. This microtubular cortical guidance mechanism requires tip1p, a member of the CLIP-170 protein family. Tip1p localises to the tips of growing microtubules where it protects them from catastrophe if they encounter the cortex in central regions of the cell. In *tip1*-deleted cells (*tip1*Δ), the microtubules undergo catastrophe when they contact the cell cortex regardless of the subcellular region and so they are generally shorter than wild-type microtubules. The consequence of this is that the cell end marker proteins are not deposited at the cell ends, resulting in misplaced growth sites.

The microtubule tip localisation of tip1p is dependent on mal3p, a member of the EB1 family of microtubule plus end tracking proteins (Beinhauer et al. 1997 and Busch and Brunner 2004). Mal3p localises to microtubules and functions as a general promoter of microtubule growth. This function is independent of tip1p as long as the microtubules grow within the cytoplasm. However, when microtubules contact the cell cortex in central regions of the cell in the absence of tip1p, mal3p is prematurely removed from microtubule tips, which is followed by a catastrophe.

The localisation of the kinesin-like motor protein, tea2p, to growing microtubule tips also requires mal3p (Browning et al., 2003). Furthermore, cells deleted for *tea2* (*tea2*Δ) possess misplaced growth sites and shorter microtubules similar to cells deleted for *tip1* or *mal3* (Browning et al., 2000). Several kinesins have been reported that affect microtubule dynamics although their mode of action is not understood (Cottingham and Hoyt 1997; Huyett et al. 1998 and Walczak et al. 1996). Our results suggest that tea2p and tip1p act in a common pathway to organise cytoplasmic microtubules. We propose that tea2p localises tip1p to the growing microtubule tips where tip1p regulates microtubule dynamics. For this, tea2p forms a complex with tip1p and moves the protein along the microtubules toward their growing tips, where dissociation of the two proteins is prevented by a mal3p-mediated anchoring mechanism.

3.3 Results

3.3.1 Tip1p Particles Move with Growing Microtubule Tips

Tip1p is known to localise to cell ends and to particles at microtubule tips and along the microtubules in fixed cells. However, the dynamics of tip1p localisation have so far not been described. We therefore performed time-lapse image analysis on living fission yeast cells expressing endogenous tip1p tagged with yellow fluorescent protein (tip1pYFP). In these cells, there was a constant flux of tip1pYFP particles from the cell centre toward the cell ends (Figure 3.1B; Supplemental Movie 3.1). The particles always started off near the nucleus in the cell centre where they usually appeared de novo or emerged from an already existing particle. These centrally located tip1pYFP particles sometimes showed a bidirectional oscillatory movement before moving away from the cell centre in one direction. In many cases, tip1pYFP particles followed one another through the cytoplasm on what appeared to be defined tracks. Also, the oscillatory movement of the central particles always followed the direction of these tracks. To see if the tracks corresponded to microtubules, we followed the particles in a strain expressing CFP-tagged tubulin (CFP-tubulin) (Glynn et al., 2001). The tip1pYFP particles moved with the growing microtubule tips and along the microtubules (Figure 3.1C; Supplemental Movie 3.2). The average speed of tip1pYFP particle movements was 3.5 μm/min (ranging from 1.6–6.8 μm/min) (Figure 3.1D). This is similar to the rate of microtubule growth in fission yeast (Drummond and Cross, 2000). The movement of some tip1pYFP particles stalled for short periods before continuing toward the cell end (arrows in Figure 3.1D), and occasionally particles disappeared before reaching the cell ends. When arriving at the cell end, tip1pYFP particles often seemed to fuse with each other. However, the overall signal at the cell ends appeared to remain relatively constant, suggesting that the protein may be continually turned over. Occasionally we could observe tip1p particles moving away from the cell ends. Mostly, these particles rapidly lost fluorescence intensity and vanished between cell end and cell centre (Supplemental Movies 3.1 and 3.3).

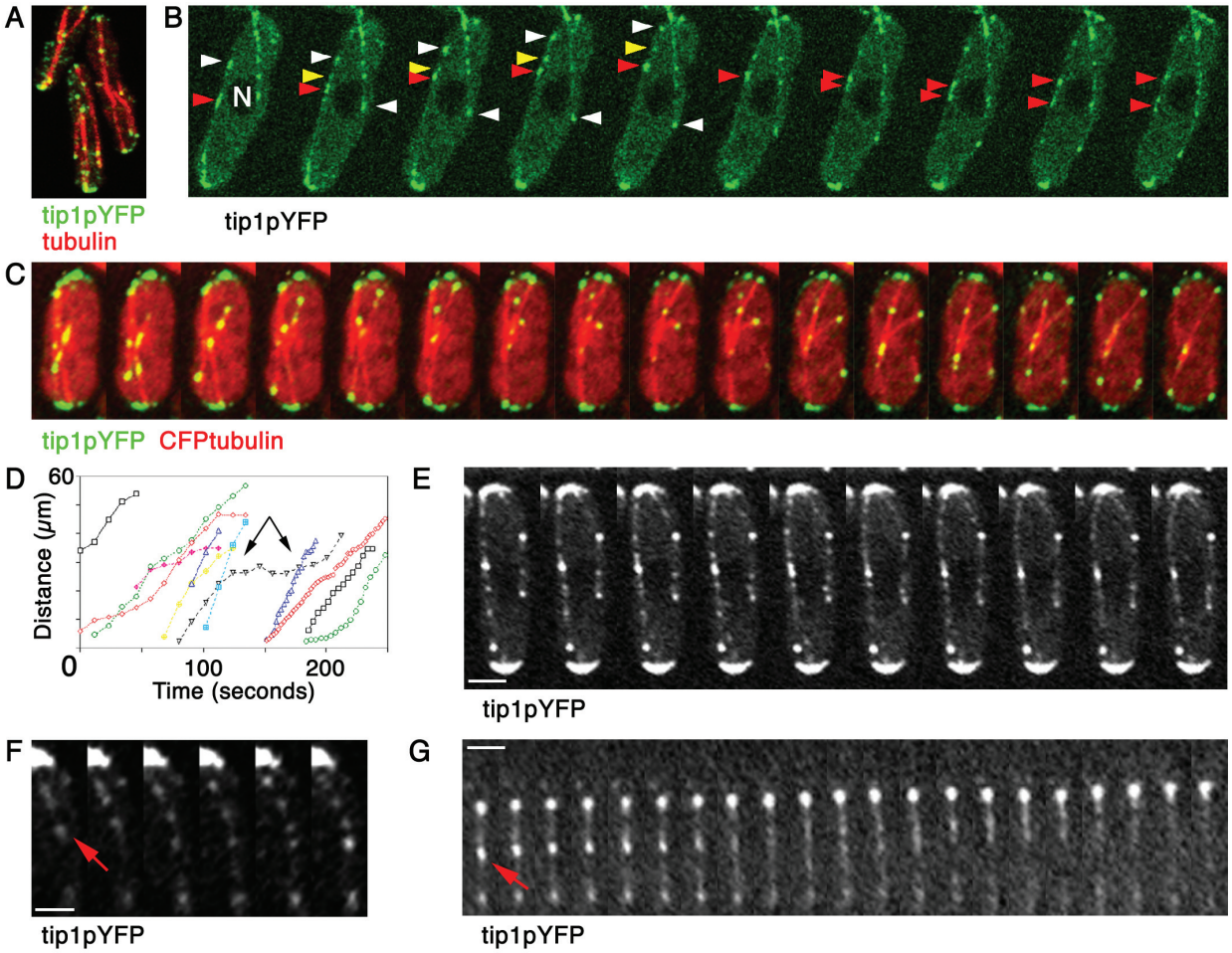


Figure 3.1 Movement of Tip1p on the Microtubules

(A) Immunofluorescence staining of methanol-fixed interphase cells expressing tip1pYFP. Tip1pYFP localisation and microtubules were detected using the respective antibodies. Tip1pYFP localises to particles along the microtubules and at their tips as previously described for the wild-type protein.
(B) Image series showing movement of tip1pYFP particles in a central slice of the cell (0.5 μm thickness). Pictures were imaged every 10.5 s. Arrowheads mark outbound moving tip1p particles. Yellow arrowheads highlight a particle following another particle, and red arrowheads indicate an oscillating and budding particle. "N" depicts the nucleus.
(C) Image series showing tip1pYFP particles (green) moving with growing microtubule tips in CFP-tubulin-expressing cells. Projections of confocal stacks covering approximately 80% of the cell volume were imaged every 10.4 s.
(D) Graph showing velocity of several tip1pYFP particles moving from the cell centre toward the cell ends.
(E–G) Fast time-lapse image series (1 frame/s) showing moving and stalling tip1pYFP speckles in single confocal planes of wild-type cells. Note that the brighter tip1pYFP particles do not move much during this short time period. (E) The white bar represents 2 μm. (F) Same as (E) but enlargement of different cell. The arrow highlights an outbound moving speckle. The white bar represents 1 μm. (G) Same as (E) but enlargement of a different cell. The arrow highlights a tip1pYFP particle that follows a leading particle but then appears to dissolve into speckles, which catch up with the leading particle. The white bar represents 1 μm.

When performing faster time-lapse imaging of single planes only, we noticed, in addition to the tip1p particles, much fainter and smaller, linearly moving tip1p speckles. Tip1pYFP produced a relatively weak signal, which did not allow us to follow these speckles over sufficiently long time periods and with appropriate time resolution to unambiguously describe their dynamics. We therefore used a strain carrying a second tip1pYFP gene copy, which enhanced the signal but did not change

the overall behaviour of tip1p (Experimental Procedures). These cells expressed approximately 1.5 times more tip1p than wild-type cells, but were of wild-type appearance, and when cells were fixed, tip1pYFP localisation was indistinguishable from that of native tip1p, indicating that the YFP tag and moderate tip1pYFP overexpression do not interfere with tip1p function (Figure 3.1A). In these cells, we found tip1pYFP speckles distributed along the tracks outlined by tip1p particle movement (Figures 3.1E and 3.1F; Supplemental Movie 3.3). The speckles moved irregularly, mostly outbound toward the microtubule tips but sometimes also inbound toward the cell centre, particularly in central regions of the cell. Their velocity was extremely variable, reaching from 8.6 up to 29.5 μm/min (on average 16.5±5.7 μm/min, N = 41). Speckles sometimes stalled movement and they appeared and disappeared randomly. Some speckles were only visible in a single frame. Because our maximal time resolution for imaging was 1 frame/0.5 s, we could not discriminate whether such speckles were moving with very high velocity or whether they disappeared by dissociating from the microtubules. Occasionally we could observe that the larger tip1p particles seemed to dissolve into multiple faster-moving speckles (Figure 3.1G; Supplemental Movie 3.4).

These experiments establish that microtubule-associated tip1p moves in two ways in cells. The protein forms discrete particles in the region of the microtubule nucleation sites in the cell centre, which subsequently move with the speed of growing microtubule tips toward the cell periphery, along preexisting microtubules and at their growing tips. In addition, tip1p speckles move with higher velocity along the microtubules.

3.3.2 Tip1p and Tea2p Colocalise and Act in the Same Process

A *tea2* deletion (*tea2*Δ) causes the same mutant phenotype as *tip1*Δ (Browning et al., 2000) (Figure 3.2A), and, like tip1p, tea2p localises to cell ends and forms particles along the microtubules and at their tips (Browning et al., 2000). Tea2p particles have also been shown to move along the microtubules and with growing tips, in a similar manner to tip1p (Browning et al., 2003). To investigate whether tip1p and tea2p function in the same pathway, we constructed a double mutant *tip1*Δ*tea2*Δ. The phenotype was indistinguishable from either of the single mutants: it was viable with short microtubules (Figure 3.2A), and with bent or branched cells being formed at the same frequency as in the *tip1*Δ or *tea2*Δ single mutants (Figure 3.2B; data not shown). This suggests that tip1p and tea2p act in the same process. We next tested colocalisation between tip1p and tea2p. Particles of both tip1p and tea2p are found at microtubule tips, along the microtubules, and at the cell ends. To simultaneously monitor tip1p, tea2p, and microtubules, we performed immunostaining on cells expressing myc-tagged tea2p and GFP-tagged tubulin. Tea2p-myc and tip1p showed a mostly overlapping staining pattern (Figure 3.2C). Only a few tip1p and tea2p-myc particles did not colocalise, and this was mostly on the microtubules in the cell centre (arrows Figure 3.2C). There was a clear overlap of tip1p and tea2p-myc particles at the microtubule tips and along the microtubules, while at the cell ends we only found a partial overlap, mostly coinciding with the end of a microtubule. We also analysed living cells ex-

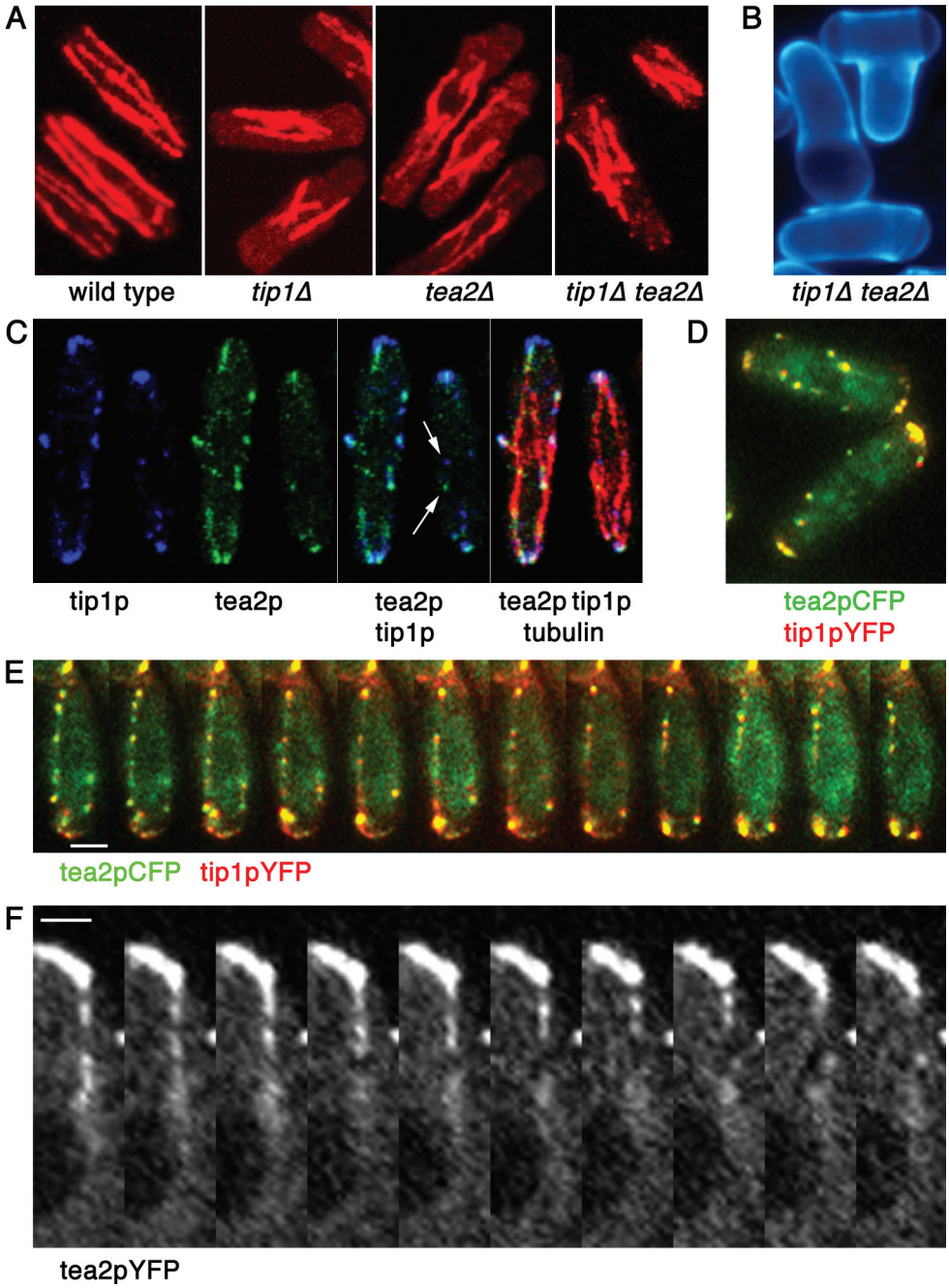


Figure 3.2 Comparison of Tip1p and Tea2p Localisation and Deletion Phenotypes

(A) Anti-tubulin immunofluorescence staining of wild-type, *tip1*Δ, *tea2*Δ, and *tip1*Δ*tea2*Δ cells.

(B) Cell walls of exponentially growing *tip1*Δ*tea2*Δ cells stained with the fluorescent dye calcofluor.

(C) Immunofluorescence staining with anti-tip1p and anti-myc antibody of wild-type cells expressing GFP-tagged α2-tubulin and myc-tagged tea2p. Tip1p localisation is shown in blue, tea2p localisation in green, and GFP-tubulin in red. Arrows mark particles that may not colocalise.

(D and E) Wild-type cells expressing endogenous tip1p and tea2p, tagged with YFP (red) and CFP (green), respectively. (D) Maximal projection of focal planes covering the entire volume of the cells. Note that the particle signals are not fully overlapping, which is possibly caused by the time delay that occurs when switching filters. (E) Time-lapse sequence showing a single focal plane. Images were taken every 4.7 s. The white bar represents 2 μm.

(F) Time-lapse sequence of a partial cell showing tea2pYFP speckles. Frames are 1.5 s apart and represent single confocal sections. Cell end is on top (bright signal). The dark region in the bottom half is the nucleus. The white bar represents 1 μm.

pressing tip1pYFP and CFP-tagged tea2p (tea2pCFP). In projections of focal planes covering the entire cell volume, tip1pYFP and tea2pCFP mostly colocalised also at the cell ends, which is different from the fixed cells (Figure 3.2D). Particles were often not fully overlapping, which is probably due to the time delay caused by filter switching (about a second). We also produced time-lapse movies of the tip1pYFP- and tea2pCFP-expressing cells. In these movies, the proteins colocalised at cell ends and to particles that moved along defined tracks. Also here, the position of the two particles was usually slightly shifted, mostly along the long cell axis, as would be expected if the two proteins comigrated but were sequentially imaged (Figure 3.2E). In the cell centre, the particles usually formed simultaneously. These results suggest that tip1p and tea2p colocalise to the microtubule-associated particles and at cell ends.

We next performed fast time-lapse imaging of cells expressing endogenous tea2p tagged with YFP (tea2pYFP) to see if, similarly to tip1p, we could detect tea2p speckles in addition to the previously reported particles. We could indeed observe tea2pYFP speckles that moved mainly outbound, but also inbound along the tracks defined by tea2pYFP particles (Figure 3.2F; Supplemental Movie 3.5). Tea2pYFP speckles behaved similarly to the tip1p speckles, moving with variable speed (range 8.7–31.9 μm/min, average 16.3±5.5 μm/min, N = 37) and occasionally stalling.

These results establish that the behaviour of microtubule-associated tip1p and tea2p is similar and suggest that localisation and function of tea2p and tip1p may be closely linked.

3.3.3 Tea2p Interacts with Tip1p and Mal3p

In a yeast two-hybrid screen performed to identify tea2p interacting proteins, 12 of the 54 isolated cDNAs encoded the *tip1* gene. One of these cDNAs was truncated at the 5' end, deleting the first 179 amino acids of the protein, and thus lacked the CAP-Gly microtubule binding domain, the serine-rich domain, and part of the first coiled-coil domain. Yet this truncated protein was able to interact with tea2p almost as efficiently as wild-type tip1p (data not shown). We further investigated this tip1p/tea2p interaction by immunoprecipitating tip1p from wild-type cell extracts and testing for tea2p coimmunoprecipitation. In these experiments, most of the tea2p protein was present in the immunoprecipitate, showing that tea2p was efficiently complexed with tip1p (Figure 3.3A). In a second experiment, GFP- or myc-tagged tea2p (tea2pGFP; tea2p-myc) was immunoprecipitated using GFP- or myc-specific antibodies, respectively. Again we found efficient coimmunoprecipitation of tip1p and tea2p (Figure 3.3B, data not shown), demonstrating that tip1p and tea2p interact in cells.

It has been shown that tip1p binds directly to another microtubule plus end tracking protein, mal3p, and that the two proteins coimmunoprecipitate (Busch and Brunner, 2004). We could also detect mal3p in the tea2p immunoprecipitates, suggesting that tea2p also interacts with mal3p (Figure 3.3C). The interaction of tip1p and mal3p is partly independent of the presence of tea2p, as the proteins do co-immunoprecipitate in *tea2*Δ cells, but to a lesser amount than in wild type cells (Supplemental Figure S3.1).

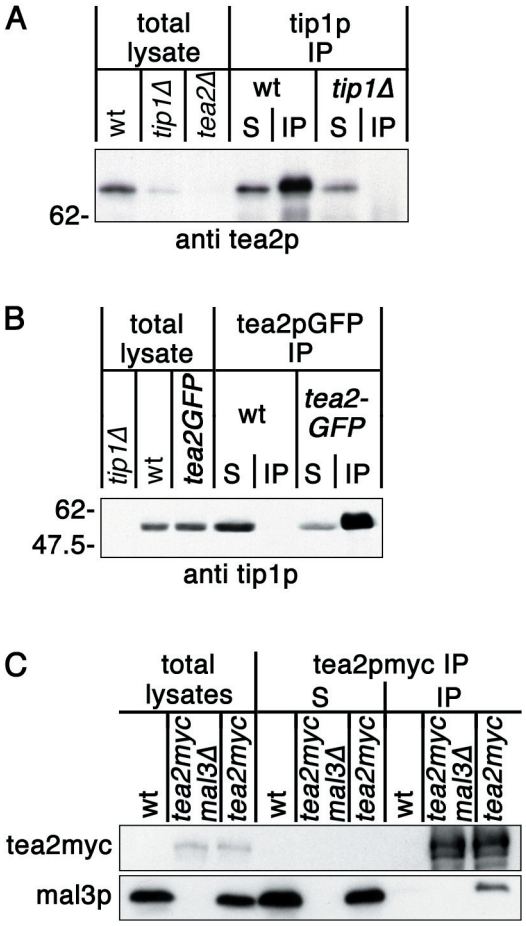


Figure 3.3 Tea2p/Tip1p/Mal3p Coimmunoprecipitations

(A–C) Western blots of coimmunoprecipitation experiments. "S" marks lanes loaded with the supernatant after immunoprecipitation, "IP" marks lanes loaded with immunoprecipitate. Three times more precipitate than supernatant was loaded. (A) Anti-tea2p Western blot showing the amount of tea2p present in total lysate, supernatant, and, in the "IP" lane, the amount of tea2p coimmunoprecipitating with tip1p from wild-type (wt) cell extracts. *tip1*Δ and *tea2*Δ cells served as negative controls. Note that the amount of tea2p is reduced in total lysates of *tip1*Δ cells. (B) Tea2pGFP immunoprecipitation from tea2pGFP-expressing cells and wild-type control cells using an anti-GFP antibody. The amount of tip1p present in total lysate and supernatant and, in the "IP" lanes, the amount of coimmunoprecipitating tip1p are shown. The total lysate from *tip1*Δ cells served as a control for the specificity of the anti-tip1p antibody and the immunoprecipitation from wild-type cells as a control for the specificity of the anti-GFP antibody. (C) Western blot of a tea2pmyc immunoprecipitation experiment with an anti-myc antibody. Tea2pmyc-expressing and wild-type control cells were used. The top panel shows the efficiency of tea2pmyc immunoprecipitation. The bottom panel shows the amount of mal3p present in total lysate and supernatant and, in the "IP" lanes, the coimmunoprecipitating mal3p.

Tip1pΔ299, a protein form lacking the carboxy-terminal 162 amino acids, is able to rescue the shortened microtubule defect but not the aberrant tea1p localisation or the morphological defects associated with *tip1*Δ cells (Brunner and Nurse, 2000a). Tip1pΔ299 localisation to microtubules is indistinguishable from that of wild-type tip1p, but the protein does not accumulate at cell ends, suggesting that this localisation is mediated via the tip1p carboxy terminus. Tea2p was found to coimmunoprecipitate also with tip1pΔ299, although with reduced efficiency compared to the two wild-type proteins (data not shown). The tip1p/tea2p interaction therefore does not absolutely require the tip1p carboxy terminus and does not need tip1p attachment to cell ends. Taken together with the finding that in the two-hybrid system the tip1p CAP-Gly domain was dispensable for interaction with tea2p, this indicates that tip1p is likely to interact with tea2p via the coiled-coil region.

3.3.4 Tip1p Microtubule Localisation Is Much Reduced in *tea2Δ* Cells

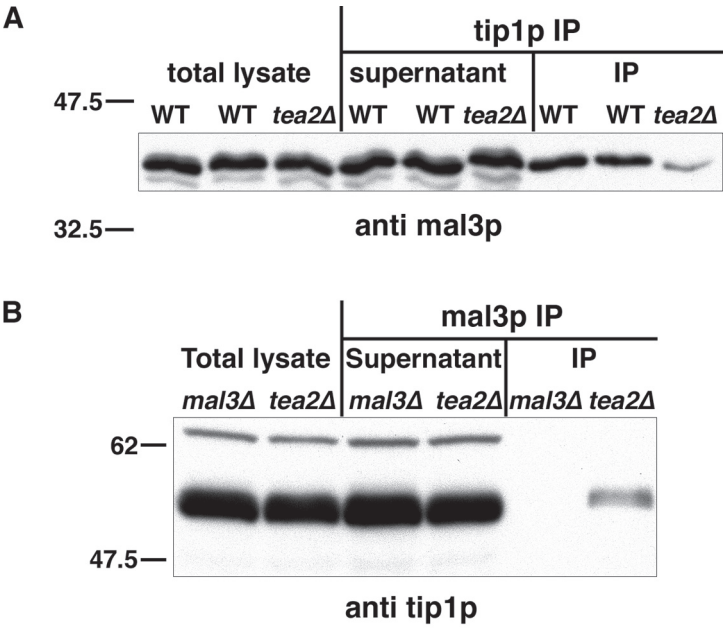
To test the localisation dependency between tip1p and tea2p, we performed immunofluorescence staining on fixed cells that expressed a temperature-sensitive *tea2* mutant (*tea2-1*) (Verde et al., 1995). Whereas in wild-type cells tip1p localises to cell ends and to larger particles at microtubule tips, tip1p was absent from cell ends and microtubule tips in *tea2-1* cells grown at the restrictive temperature (Figures 3.4A and 3.4B). Only few larger tip1p particles were present. These colocalised with microtu-

bules mostly at the iMTOCs in the vicinity of the nucleus but not at the microtubule tips or at the cell ends.

We also followed tip1pYFP localisation in living *tea2*Δ cells. Western blots demonstrated that the level of tip1p protein was not affected in *tea2*Δ cells (Figure 3.4C). Immunostaining in fixed *tea2*Δ cells showed that the tip1pYFP localisation pattern was similar to that of tip1p in *tea2-1* cells (Figure 3.4D). In living *tea2*Δ cells, the detectable tip1pYFP signal was very much reduced compared to the wild-type (Figures 3.4E–4H). We could detect few tip1pYFP particles concentrated in the cell centre, which mainly showed oscillatory movements along defined tracks (Figure 3.4H; Supplemental Movie 3.6). We could also occasionally detect faint speckles moving toward the cell ends. These "speckles" were different from those seen moving along microtubules in wild-type cells; they were much fainter and they moved with 2.6±0.6 μm per min (n = 39) on average (Figure 3.4H; Supplemental Movie 3.6). This is similar to the velocity of growing microtubule tips but considerably slower than that of the speckles in wild-type cells. We could not detect any of the fast-moving tip1pYFP speckles present in wild-type cells.

We conclude that normal tip1p localisation to microtubules requires tea2p activity. However, some residual tip1p association with the microtubules occurs in the absence of tea2p, mostly in the vicinity of the nucleus.

We also investigated tip1pYFP speckle behaviour in cells expressing different tea2p rigor mutant proteins (P loop and switch II, which cannot bind or hydrolyse ATP) in place of endogenous tea2p (Browning et al., 2003). The behaviour of the larger, slow-moving tea2p and tip1p particles in such cells was described previously (Browning et al., 2003). The tea2p rigor mutant protein was shown to be enriched at the iMTOCs and to decorate the entire length of the microtubules. No motile particles were detected. This was also the case for tip1p, which was reported to

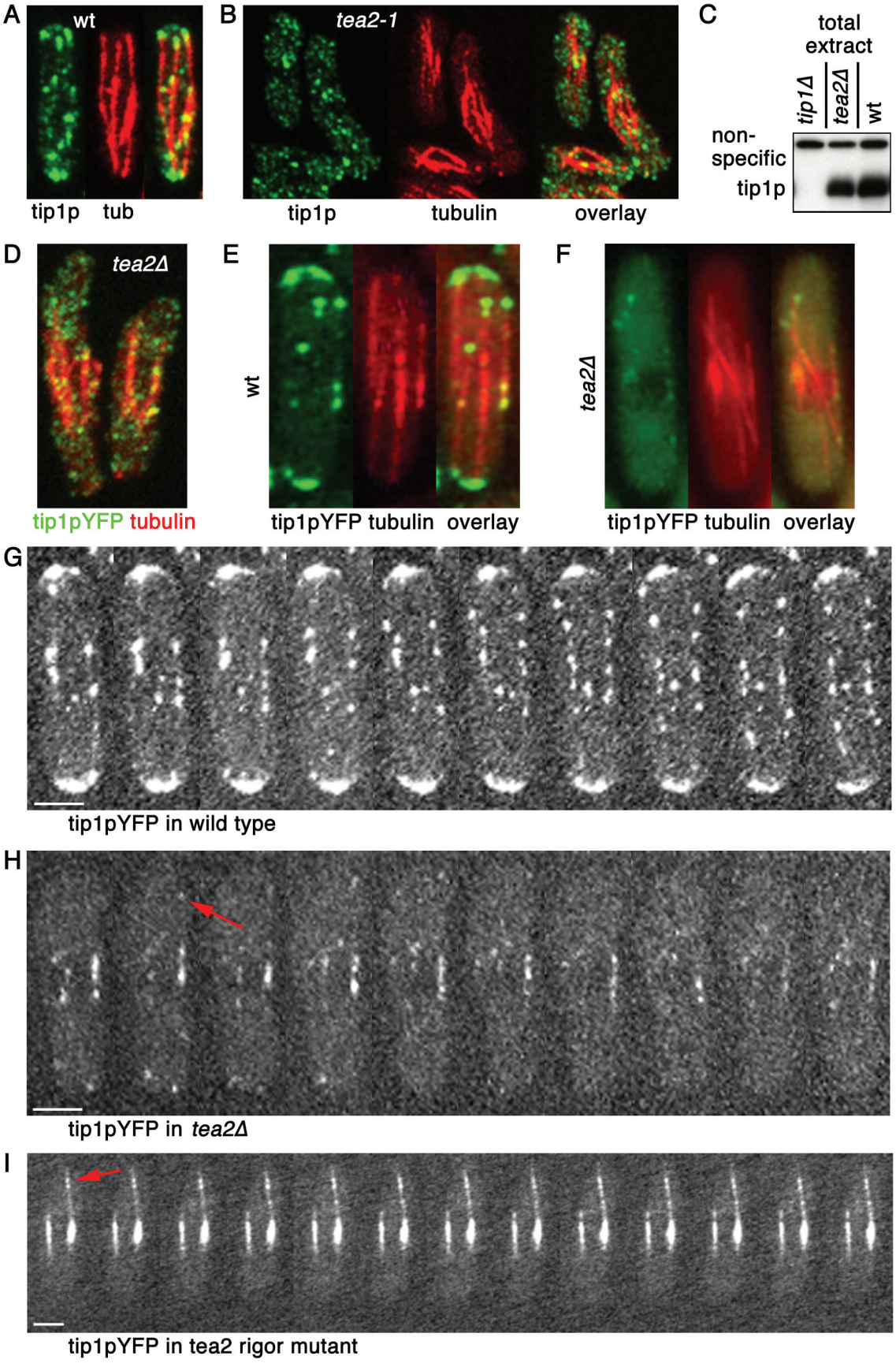


Supplemental Figure S3.1 Tip1p/mal3p Coimmunoprecipitation in the Absence of Tea2p

Western blots of co-immunoprecipitation experiments carried out in cell extracts with antibodies recognizing tip1p and mal3p. The first lanes show total cell lysate, 'Supernatant' shows the unbound and 'IP' the bound fractions.

(A) tip1p immunoprecipitation with the anti-tip1p antibody. Compared with the wild type, less mal3p is co-precipitated in *tea2*Δ cells. The lowest band is unspecific.

(B) mal3p immunoprecipitation with the anti-mal3p antibody. Tip1p co-immunoprecipitates in *tea2*Δ, but is not present in the immunoprecipitate of *mal3*Δ control cells. The band above 62 kDa is unspecific.


A
wt
tip1p tub
B
tea2-1
tip1p tubulin overlay
C
total extract
tip1Δ
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wt
non-specific
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D
tea2Δ
tip1pYFP tubulin
E
wt
tip1pYFP tubulin overlay
F
tea2Δ
tip1pYFP tubulin overlay
G
tip1pYFP in wild type
H
tip1pYFP in tea2Δ
I
tip1pYFP in tea2 rigor mutant

colocalise with the rigor mutant protein. Performing fast time-lapse imaging of cells coexpressing tip1pYFP and four different tea2p rigor mutant protein forms, we could not detect any tip1pYFP speckle movement (Figure 3.4I; Supplemental Movie 3.7; and data not shown). Tip1p speckle movement therefore is dependent on tea2p ATP hydrolysis.

3.3.5 Tea2p Is Reduced in *tip1*Δ Cells but Can Still Associate with Microtubules

To investigate the interdependency of tip1p and tea2p localisation, we monitored tea2pGFP in the absence of tip1p. In fixed wild-type cells where the microtubules were fluorescently labelled with an anti-tubulin antibody, tea2pGFP was present in particles along microtubules, at their tips, and also at the cell ends (Figure 3.5A). In contrast, in *tip1*Δ cells no GFP fluorescent signal was detectable, but using a GFP-specific antibody to enhance the signal, faint tea2pGFP speckles were observed mainly in the region of the short microtubules present in *tip1*Δ cells (Figure 3.5B). Some of the speckles appeared to align along the microtubules, but tea2pGFP did not accumulate at the microtubule tips and was not found at cell ends. Western blots comparing tea2p protein levels in wild-type and *tip1*Δ cells showed that the reduced levels of GFP signal were due to a reduction of tea2p protein in *tip1*Δ cells (Figure 3.5C). Tip1p thus is required to maintain normal tea2p protein levels.

To follow the behaviour of tea2p in the absence of tip1p, we expressed tea2pGFP in place of the endogenous tea2p from a repressible nmt1 promoter in a *tip1*Δ strain. Under fully repressed conditions, the residual promoter activity was sufficient to overexpress tea2pGFP about 7-fold (Figure 3.5D). Tea2p overexpression did not rescue the shortening of microtubules in *tip1*Δ cells (average length 4.8±1.7 μm, N = 180 versus 4.7±1.9 μm, N = 180 without overexpression). In immunofluorescence experiments with anti-tubulin and GFP antibodies, we detected tea2pGFP speckles decorating the entire length of the microtubules whereas larger particles were absent (Figure 3.5E). We also detected a strong, diffuse cytoplasmic signal, which appeared more concentrated toward the cell ends. In living *tip1*Δ cells, we could observe tea2pGFP signal forming lines of speckles that appeared to move toward the cell ends (Figure 3.5F; Supplemental Movie 3.8). Due to their high density, it was not possible to unambiguously follow single speckles. At the tips of the lines defined by the speck-

Figure 3.4 Tip1p Localisation in *tea2* Mutant Cells

(A, B, and D) Immunofluorescence staining experiments with anti-tip1p and anti-tubulin antibodies. Tip1p localisation is shown in green and the microtubules are shown in red. (A) Wild-type cells. (B) *Tea2-1* mutant cells grown at the restrictive temperature (36°C). (D) Tip1pYFP-expressing *tea2*Δ cells.

(C) Western blot showing tip1p present in total extract of wild-type and *tea2*Δ cells. The *tip1*Δ lysate identifies a non-specific band that served as loading control.

(E and F) Living wild-type and *tea2*Δ cells expressing tip1pYFP (green) and CFP-tubulin (red).

(G and H) Movie sequences showing maximal projections of confocal slices covering the entire Z-axis of tip1pYFP-(white) expressing cells. Imaging conditions were identical to allow direct comparison. The time interval between frames is 8 s. The white bars represent 2 μm. (G) Wild-type cells. (H) *Tea2*Δ cells. The arrow depicts an outbound moving tip1pYFP dot.

(I) Movie sequence showing a single focal plane of a cell coexpressing tip1pYFP (white) and a tea2p Switch II (E336A) rigor mutant protein. The time interval between frames is 1 s. The white bar represents 2 μm. The red arrow depicts an immobile tip1pYFP speckle.

les, we sometimes observed a slight accumulation of tea2pGFP signal but not to the extent of the larger particles seen in the wild-type. We could not detect any enrichment of tea2pGFP at cell ends. We conclude that in the absence of tip1p, tea2p is able to bind and move along the microtubules

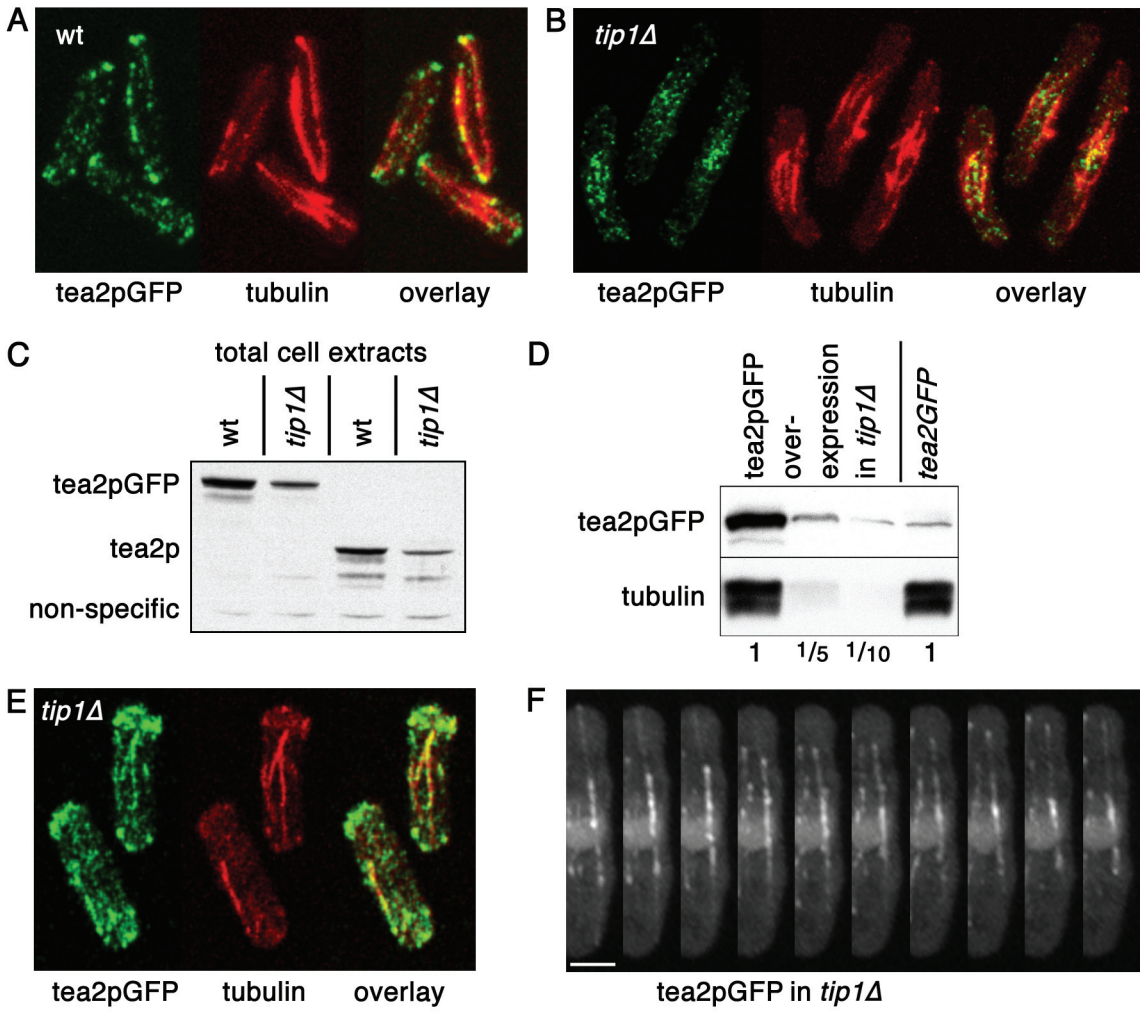

Figure 3.5 Tea2p Localisation in *tip1*Δ Cells

(A and B) Immunofluorescence stainings with anti-GFP (green) and anti-tubulin (red) antibodies. (A) Wild-type cells expressing endogenous tea2p tagged with GFP. (B) *Tip1*Δ cells expressing endogenous tea2p tagged with GFP.

(C) Western blot analysis of wild-type and *tip1*Δ cells using anti-tea2p antibody. Cells in the first two lanes express endogenous tea2p tagged with GFP. The unspecific band at the bottom serves as loading control.

(D) Western blot analysis of total cell extracts to compare tea2pGFP levels in wild-type cells expressing tea2pGFP from the endogenous promoter (*tea2GFP*) and in *tip1*Δ cells expressing tea2pGFP from the fully repressed nmt1 promoter. The numbers at the bottom indicate the relative amounts of extract used. Tubulin protein levels served as a loading control.

(E) Immunofluorescence staining with anti-GFP (green) and anti-tubulin (red) antibodies of the *tip1*Δ cells expressing tea2pGFP from the fully repressed nmt1 promoter. A single confocal section is shown.

(F) Movie sequence showing a maximal projection of confocal stacks covering the entire Z-axis of a partial *tip1*Δ cell expressing tea2pGFP from the fully repressed nmt1 promoter. The time interval between frames is 6.8 s. The white bar represents 2 μm.

but tip1p is required to attain wild-type tea2p protein levels, for the efficient accumulation of tea2p particles at growing microtubule tips, and for its association with cell ends.

3.3.6 Mal3p Localisation Is Tea2p Independent

It has been shown that mal3p is required for the localisation of tip1p and tea2p particles along the microtubules and at their growing tips and that mal3p microtubule localisation is tip1p independent (Browning et al. 2003 and Busch and Brunner 2004). We tested whether mal3p localisation is affected in *tea2*Δ cells using a GFPmal3p-expressing yeast strain (Busch and Brunner, 2004). In fixed *tea2*Δ cells, GFPmal3p localised normally, decorating the microtubule lattice and forming particles along the microtubules and at their tips (Figures 3.6A and 6B). In living wild-type cells, GFPmal3p parti-

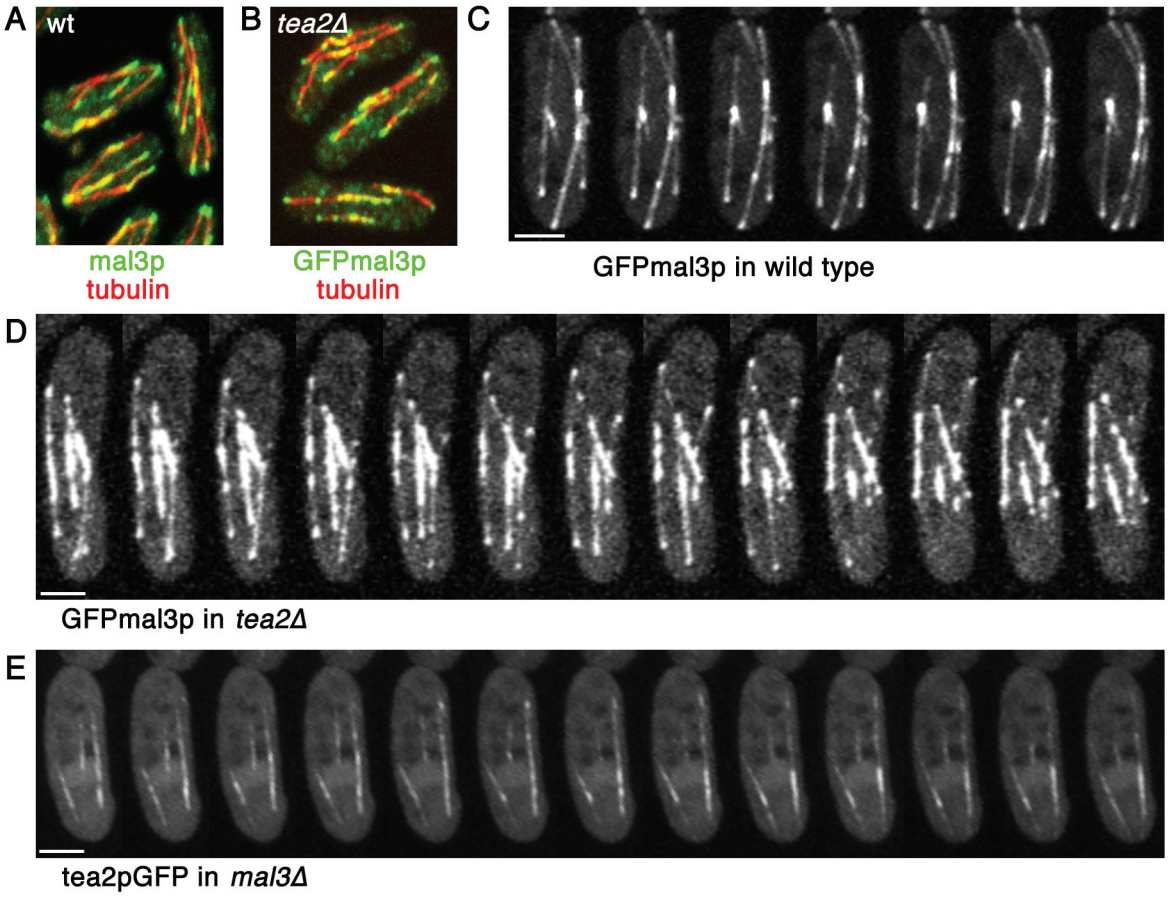

Figure 3.6 Partially Independent Localisation of Tea2p and Mal3p

(A) Immunofluorescence staining of wild-type cells stained with a mal3p- (green) and a tubulin-specific antibody (red).

(B) Immunofluorescence staining of GFPmal3p- (green) expressing *tea2*Δ cells stained with a tubulin-specific antibody (red).

(C–E) Movie sequences showing maximal projections of confocal stacks covering the entire volume of the cell. The white bars represent 2 μm. (C) GFPmal3p-expressing wild-type cell. Frames imaged every 6.8 s are shown. (D) GFPmal3p-expressing *tea2*Δ cell. Frames are 8.5 s apart. (E) *Mal3*Δ cell expressing tea2pGFP from the fully repressed nmt1 promoter. The time interval between frames is 6.8 s.

cles display bidirectional movements along the microtubules and they move with the growing microtubule tips (Figure 3.6C). Time-lapse imaging of *tea2*Δ cells showed that the pattern of GFPmal3p particle movement was mostly unchanged (Figure 3.6D; Supplemental Movie 3.9 and Supplemental Table 3.1). We conclude that mal3p localisation to the microtubules is tea2p independent.

	Wild type	*tip1*Δ		*tea2*Δ	
	µm / min	µm / min	N	µm / min	N
Outbound particles	2.6	2.7 ± 0.6	54	2.5 ± 0.7	16
Inbound particles	3.6	4.8 ± 1.6	51	4.8 ± 1.5	11
Inbound speed of depolymerising microtubule tip	6.0	8.4 ± 2.0	54	9.6 ± 2.6	18

Supplemental Table 3.1 Velocity of GFPmal3p Particles in *tip1*Δ and *tea2*Δ Cells at 25°C

3.3.7 Tea2p Speckles Move Independently of Mal3p

Previous studies have suggested that the microtubule localisation of tip1p and tea2p requires mal3p. This was based on the observation that tip1p and tea2p particles were no longer detectable in *mal3* deleted (*mal3*Δ) cells (Browning et al. 2003 and Busch and Brunner 2004). Because tip1p and tea2p speckles had previously not been described, we reexamined the dependence of tip1pYFP and tea2pGFP microtubule localisation on mal3p, this time focusing on the speckles in *mal3*Δ cells. Tip1p and tea2p protein levels are not affected in *mal3*Δ cells (Browning et al. 2003 and Busch and Brunner 2004). In both tip1pYFP- and tea2pGFP-expressing *mal3*Δ cells, we observed a strong cytoplasmic background signal, which was too strong to allow the detection of putative speckles. To increase a potential signal of microtubule-associated tea2p, we overexpressed tea2pGFP in *mal3*Δ cells. We could now detect tea2pGFP speckles, which fairly evenly decorated what appeared to be the short microtubules present in *mal3*Δ cells (Figure 3.6E; Supplemental Movie 3.10). These speckles were motile, but because of their density and the short microtubule stretches it was not possible to unambiguously track single speckles over long enough time periods. None of the larger tea2pGFP particles were detectable. This suggests that tea2p can associate with and move along microtubules in the absence of mal3p, but is no longer able to accumulate into the larger tea2p particles found at growing microtubule tips, along the microtubules, and at the iMTOCs.

3.3.8 Tip1p and Tea2p Associate More Stably with Microtubules Than Mal3p

The finding that mal3p is essential for tea2p/tip1p accumulation at microtubule tips raises the possibility that mal3p is important for the maintenance of tea2p and tip1p at growing microtubule plus ends. To further investigate this possibility, we looked at the rates with which the three proteins

turn over at microtubule tips. We performed photobleaching experiments using the tea2GFP-, the tip1pYFP-, and the GFPmal3p-expressing strains, respectively. Using an inverted FRAP technique (iFRAP), we bleached the entire cell except for single particles or parts of a single microtubule bundle (Experimental Procedures) (Dundr et al., 2002). We then followed the unbleached signal over time. Control experiments showed that this did not interfere with microtubule dynamics during the experiment (Experimental Procedures). After photobleaching, the remaining signal of the GFPmal3p particle at microtubule tips rapidly disappeared, with a half-life of approximately 1 s and a constant of fluorescence loss k of $0.8s^{-1}$ (Figures 3.7A and 3.7B, Supplemental Movie 3.11). We also performed iFRAP on the GFPmal3p that had been shown to decorate the microtubule lattice and on the particles moving inbound along the microtubules (Busch and Brunner, 2004). The half-lives after photobleaching were again approximately 1 s each, and the respective constants of fluorescence loss k were $0.8s^{-1}$ and $0.9s^{-1}$ (Figure 3.7B). In contrast to the GFPmal3p particles, the

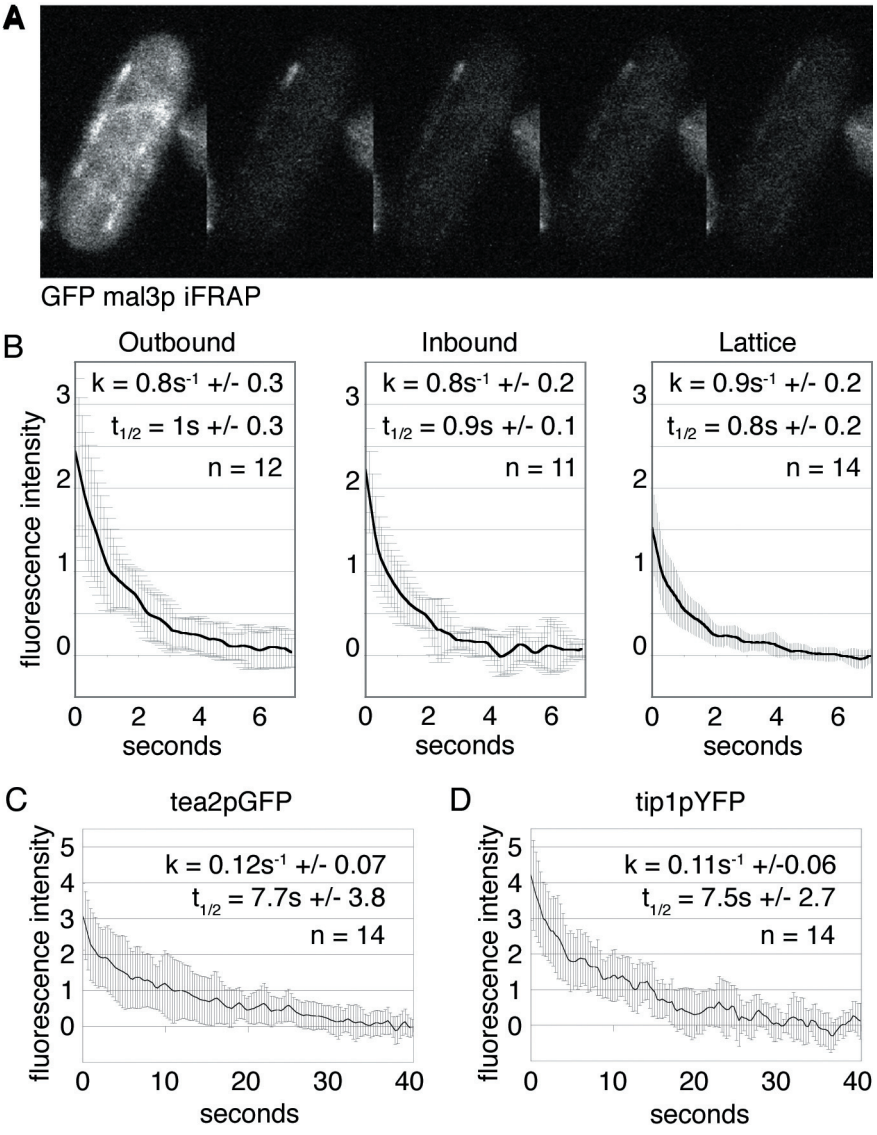

Figure 3.7 Mal3p/Tip1p/Tea2p Turnover on Microtubules

(A) Movie sequence showing a single confocal section of a cell in a typical iFRAP experiment. Frames imaged every 0.8 s are shown.

(B–D) Graphs showing the kinetics of fluorescence loss of unbleached GFPmal3p, tea2pGFP, and tip1pYFP signal in iFRAP experiments. The curves represent the averaged decays of n individual experiments, which were linearly interpolated at standardized time points. Error bars depict the standard deviation. k and $t_{1/2}$ values were calculated from 11–14 measurements.

tea2pGFP and tip1pYFP particles at growing microtubule tips disappeared more slowly, with a half-life of approximately 7.7 and 7.5 s, respectively. The constants of fluorescence loss k were $0.12s^{-1}$ and $0.11s^{-1}$ (Figure 3.7C). Tip1p and tea2p thus have similar turnover rates.

Our experiments show that tea2p, tip1p, and mal3p bind transiently to microtubules and suggest that the interaction of tea2p and tip1p with the microtubules is more stable than that of mal3p.

3.4 Discussion

Proteins localising to the plus ends of growing cytoplasmic microtubules are involved in the control of microtubule dynamics, spindle orientation, vesicle trafficking, and signal transduction (Schuyler and Pellman, 2001). Here we have studied the mechanism that leads to the accumulation of tip1p at the growing microtubule tips. We show that although tip1p has some affinity for microtubules, efficient accumulation at their growing tips requires the kinesin tea2p. It has been postulated that tea2p may act as a conventional motor protein based on the finding that tea2p rigor mutants that are no longer able to bind or hydrolyse ATP, formed immobile tea2p particles on the microtubules (Browning et al., 2003). Given that the reported velocity of particles formed by wild-type tea2p is similar to that of growing microtubule tips, it is possible that tea2p associates and/or moves specifically with growing microtubule tips rather than moving along the microtubules.

The results presented here support the view that tea2p can act as a kinesin motor that moves along microtubules and suggest that a central function for tea2p is to transport tip1p along the microtubules toward their growing tips, where the proteins accumulate by a mal3p-mediated mechanism. Tea2p's function as a motor protein is supported mainly by the presence of small, fast-moving tea2p speckles, which we observed in addition to the previously reported larger tea2p particles. These speckles were present all along the length of the microtubules, and their velocity was higher than that of the larger particles or the rate with which microtubules grow. This excludes the possibility that these speckles are associated with growing microtubule tips and suggests that their movement is motor based.

Consistent with the idea that tip1p is a tea2p cargo, we detected small, fast-moving tip1p speckles, which were similar in behaviour and velocity to the tea2p speckles. Tip1p speckle motility depended on tea2p ATP hydrolysis and fast-moving speckles were absent in *tea2*Δ cells. Only few residual "speckles" were moving in these cells. However, their velocity was similar to that of growing microtubule tips, suggesting that low amounts of tip1p directly interact with microtubule tips. Importantly, this residual tip1p is not sufficient to fulfil normal tip1p function.

Conversely, tea2p was still able to associate with the microtubule lattice in the absence of tip1p. This was detectable, however, only when tea2p was overexpressed because tea2p is either not efficiently expressed or is more efficiently degraded in the absence of its putative cargo tip1p. Strong

support for a close interaction between the two proteins comes also from the findings that tip1p and tea2p coimmunoprecipitated from cell extracts and interacted in two-hybrid analysis. Furthermore, comparison of *tip1*Δ and *tea2*Δ cells with a *tip1*Δ *tea2*Δ double mutant indicates that the two proteins act in the same process.

Interestingly, we also observed speckles and particles of tea2p and tip1p, which moved inbound, toward the cell centre. At this stage, we do not understand the nature of these movements. It is possible that minus end-directed motor activity is associated with tea2p/tip1p. In the regions of the iMTOCs where microtubules form antiparallel overlaps, this could also be explained by hopping of tea2p/tip1p between microtubules. Alternatively, it is possible that microtubules with opposite polarity are present in a bundle or that minus end-directed sliding of microtubules within a bundle cause backward movement of a growing plus end.

Tip1p and tea2p colocalise to the previously described particles at growing microtubule tips and along the microtubules. Because the latter particles also move with the speed of growing microtubule tips, they probably depict the tips of microtubules growing within the bundle, which was also suggested to be the case for the respective mal3p particles (Busch and Brunner, 2004). Although we cannot exclude the possibility that tip1p and tea2p particles and speckles are completely independent entities, it is likely that multiple speckles that have arrived at the growing microtubule tips form the particles. Consistent with this idea, we observed tip1p and tea2p particles moving along a microtubule bundle that dissolved into multiple speckles, which then moved with much higher velocity toward the bundle tip.

A model where outbound moving tea2p and tip1p speckles form particles by accumulating at growing microtubule tips requires some docking mechanism, which prevents tea2p/tip1p from falling off the microtubule ends. It is possible that tip1p, via its CAP-Gly domain, fulfils such a docking function. The CAP-Gly domain-containing regions of human CLIP-170 have previously been shown to specifically bind microtubule plus ends (Diamantopoulos et al. 1999 and Perez et al. 1999). However, more recently it was shown that in fission yeast, the EB1 homologue mal3p is essential for the accumulation of tip1p and tea2p at growing microtubule tips (Browning et al. 2003 and Busch and Brunner 2004). Because tip1p binds directly to mal3p via the CAP-Gly domain-containing region, this suggests that mal3p may be responsible for tip1p/tea2p tethering. Consistent with this, we show that in *mal3*Δ cells tea2p can no longer accumulate at microtubule tips even when overexpressed, although in such cells motile tea2p speckles are associated with the microtubules. Furthermore, mal3p is present in tea2p immunoprecipitates, suggesting that the two proteins closely interact. It is therefore possible that tea2p/tip1p become tethered at growing microtubule tips by a direct interaction with mal3p. Our finding that mal3p turns over much faster at microtubule tips than tea2p or tip1p appears to contradict this model. However, turnover rates need not be similar if the protein interactions are not stochiometric. It is conceivable, for example, that consecutive interaction with multiple mal3p molecules retains a given tip1p or tea2p molecule at microtubule

tips. Alternatively, tea2p/tip1p may exist in multimeric complexes that can be retained by few mal3p interactions. Since the microtubule tips are constantly displaced by the addition of new tubulin subunits, a constant interplay of tea2p/tip1p capture and release by mal3p would allow intermediate motor steps to occur, which would enable tea2p/tip1p to follow the growing microtubule tips. In this scenario, mal3p would thus affect the processivity of tea2p, particularly at the microtubule tips, by slowing down tip1p/tea2p and thus preventing their dissociation. This would automatically result in tea2p/tip1p accumulation and thus explain particle formation and at the same time particle movement.

Because some mal3p is present along the entire microtubule lattice, it is possible that mal3p also promotes the association of tea2p/tip1p speckles with the microtubules. Consistent with this, we were not able to detect tea2p or tip1p speckles in *mal3*Δ cells expressing wild-type tea2p/tip1p levels. In these cells, however, speckle detection may have been hampered by the increased cytoplasmic background fluorescence, which reduced the imaging contrast. This is likely to be the consequence of increased amounts of free tea2p/tip1p, which are no longer concentrated into particles.

Our model, implicating the tea2p kinesin and mal3p in tip1p localisation and movement at the microtubule tips, is different from that explaining the similar localisation of its human homologue, CLIP-170. For CLIP-170 a "treadmilling" mechanism has been proposed, whereby the protein is either directly picked up from the cytoplasm by the growing microtubule plus ends or is recruited there after binding to free tubulin dimers, which are then added on to the microtubule plus end (Diamantopoulos et al. 1999 and Perez et al. 1999). It is possible that different ways of localising homologous microtubule tip proteins are operative, but we should not exclude the possibility that efficient CLIP-170 localisation in human cells also involves kinesin activity. Conversely, we cannot exclude that a treadmilling mechanism contributes to tip1p localisation. This may account for the residual tip1p that is present at growing microtubule tips in *tea2*Δ cells. This could also be explained by the existence of another kinesin possessing overlapping function with tea2p. Alternatively, it is possible that some tip1p is recruited to the microtubule tips by direct binding to the mal3p particles that are still present in *tea2*Δ cells. Direct binding to mal3p would also explain the presence of oscillating tip1p particles on the microtubules around the iMTOCs, where oscillating mal3p particles are highly abundant (Busch and Brunner, 2004). This may point toward a mechanism by which tip1p, and possibly also tea2p, becomes efficiently loaded onto the microtubules at the iMTOCs, which would explain why tip1p and tea2p particles do not form in these regions in *mal3*Δ cells.

Kinesins have long been known to affect microtubule dynamics; both stabilising and destabilising kinesins have been described (Cottingham and Hoyt 1997; Huyett et al. 1998 and Walczak et al. 1996), but only the microtubule destabilising kinesins XKCM1 and XKIF2 have been analyzed in detail (Desai et al., 1999). XKCM1/XKIF2 action apparently does not involve conventional motor activity. Our findings suggest a different mode of action for tea2p. We propose that tea2p kinesin regulates microtubule dynamics indirectly by acting as a motor that transports tip1p along micro-

tubules, first toward and then with their growing tips. In this way, tea2p ensures that there is always sufficient tip1p present at the growing microtubule tips where tip1p influences microtubule dynamics according to where the tip is located within the cell. As a consequence, proper spatially organised interphase microtubules are brought about within the polarised fission yeast cell. Dissociation of tip1p/tea2p from the growing microtubule plus ends is prevented by a mal3p-mediated docking function. Our results offer a new explanation for how some microtubule stabilising factors are organised once they are associated with the microtubules.

3.5 Experimental Procedures

3.5.1 *S. pombe* Methods

Standard methods were used for growth, transformation, and genetic manipulation of *S. pombe* cells (Moreno et al., 1991). Most cells were grown at 32°C (if not mentioned otherwise) on YE medium with supplements. Cells carrying plasmids were grown on minimal EMM2 medium with appropriate supplements, and cells used for time-lapse imaging were grown and imaged at 25°C.

3.5.2 Immunofluorescence Techniques

Cells were fixed with methanol and were processed using standard methods (Mata and Nurse 1997 and Sawin and Nurse 1998). Antibodies used were: Tat1 monoclonal antibody for tubulin detection (Mata and Nurse 1997 and Woods et al. 1989), rabbit anti-tip1p antibody for tip1p detection (Brunner and Nurse, 2000a), anti-GFP (gift from Ken Sawin), anti-mal3p (Busch and Brunner, 2004), and 9E10 monoclonal antibody for tea2p-myc detection (Chan et al., 1987). Secondary antibodies were Alexa 546 and Alexa 488 (Molecular Probes) and CY5 (Amersham). Images were taken with a Zeiss LSM 510 laser scanning confocal microscope.

3.5.3 Extract Preparation and Immunoprecipitation Experiments

Fission yeast native extracts were prepared as previously described (Moreno et al., 1991). Immunoprecipitations were performed as described in Busch and Brunner (2004) with anti-tip1p (Brunner and Nurse, 2000a), anti-GFP (gift from Ken Sawin), and 9E10 or 9B11 anti-myc antibodies (Chan et al., 1987) (NEB). Antibodies recognizing tea2p (Browning et al., 2000), tip1p (Brunner and Nurse, 2000a), and mal3p (Busch and Brunner, 2004) were used for detection of tea2p, tip1p, and mal3p. For tip1p detection in the immunoprecipitate, the IgG bands were masked by incubating the Western blot after the blocking step, overnight, with goat anti-IgG followed by thorough washing (12 hr, including six changes of washing solution).

3.5.4 Two-Hybrid Screen

This was carried out as described in the ClonTech Matchmaker two-hybrid system 2 handbook, using full-length *tea2* in pAS2 as the bait and an *S. pombe* cDNA library in pACT constructed and provided by Steve Elledge (Baylor, USA). 10^7 clones were screened and 54 cDNAs were selected for further analysis.

3.5.5 Strains Constructed for Time-Lapse Imaging

Tea2pCFP and tea2pYFP were constructed as described in Browning et al. (2003). The tip1pYFP strain was a gift of A. Decottignies. It expresses the endogenous gene carboxy-terminally tagged with *YFP*. The tip1pYFP overexpression strain in addition expressed *tip1YFP* from a randomly integrated gene copy controlled by the partially repressed nmt1 promoter (0.2 μM thiamine). The growth rate of these cells did not differ from that of wild-type cells. *Tea2GFP* was expressed from the endogenous promoter (Browning et al., 2000), or from the fully repressed nmt1 promoter, which was inserted in front of the *tea2GFP* gene after elimination of the kanamycin marker gene with ura^+. Both were done by the homologous recombination-based method described in Bähler et al. (1998). Protein expression levels in overexpressing strains were estimated from Western blots of dilution series of total cell extracts.

3.5.6 Time-Lapse Imaging and Image Analysis

Time-lapse imaging and movie sequence processing/analysis were done as described in Busch and Brunner (2004); we used a spinning disc confocal microscope equipped with an additional 442 nm HeCd-laser (92 mW) to produce time-lapse movies of single confocal planes, of confocal stacks covering the entire cell volume, and for tip1pYFP/CFP-tubulin imaging. For the fast acquisition of the single focal planes used for speckle velocity measurements, and for tea2pCFP/tip1pYFP dual colour imaging, an epi-fluorescence microscope (Zeiss) was used.

3.5.7 Inverted Photobleaching and Analysis

Inverted photobleaching (iFRAP) experiments were performed on a Zeiss LSM 510 confocal microscope, with a setup as described in Daigle et al. (2001). The objective was a Plan-Apochromat 40x NA 1.0 oil objective (Carl Zeiss). To bleach and detect the whole depth of the cell, the pinhole was opened to approximately the equivalent of 4x the Airy disk diameter. The entire cell was bleached except for a small region containing a single particle of interest, or a microtubule segment free of mal3p particles. Single confocal planes containing the unbleached GFPmal3p signal were subsequently imaged. Bleach masks were defined using the region of interest (ROI) definition of the LSM 2.8 software. A ROI was bleached by a single exposure with 100% laser transmission. Three images were recorded before and 200 images after bleaching, all at 0.2%–2% laser transmission

every 250–500 ms. GFP bleaching was 60%–90% complete, such that the movement of bleached GFPmal3p dots could be followed, showing that the behaviour of mal3p and microtubule growth was not affected by the bleaching procedure. The mean fluorescence intensity of the unbleached region was measured in ImageJ. The fluorescence intensity decay was fitted with an exponential decay curve, using the function $f(t) = (I_{unbl}^{0} - b / I_{bl}^{0} - b) (e^{-kt}) + (I_{unbl}\infty - b / I_{bl}\infty - b)$, where I_{unbl}^{0} is the initial mean fluorescence intensity of the unbleached structure, I_{bl}^{0} is the initial intensity of a comparable, bleached structure, or of the whole cell, b is the background outside the cell, and the I∞ values are the mean intensities of the structures after completed fluorescence redistribution. The time dependence of the bleached signal is described by k, which is the fluorescence decay constant, calculated from a best-fit exponential curve through the individual data points in Microsoft Excel. The decay half-life $t_{1/2}$ was calculated as ln(2)/k.

Supplemental Movies

Movie 3.1. Tip1p particles in wild type cells
Tip1pYFP particle movements occurring in wild type cells are shown (a selection is marked by arrows and coloured in green). Frames are maximal projections of confocal slices covering the entire cell volume. Stacks were imaged every 8 seconds. Total movie duration in real time was 3 minutes 52 seconds (shown 40x accelerated).

Movie 3.2. Tip1p particles in wild type cells
Tip1pYFP particles (green) moving with growing microtubule tips in CFP-tubulin (red) expressing wild type cells are shown. Maximal projections were made of confocal stacks covering approximately 80% of the cell volume. Stacks were imaged every 10.4 seconds. Total duration of movie was 1 minute 44 seconds (shown 47x accelerated).

Movie 3.3. Tip1pYFP speckles in wild type cells
Movie of a single, 1 μm confocal section of a wild type cell expressing endogenous tip1p tagged with YFP. Frames were taken every second. Total movie duration was 29 seconds (shown 5x accelerated). A selection of outbound speckles is marked with red arrows and a much slower particle is highlighted in green. In the bottom half of the cell, towards the end of the movie, a particle (marked in blue) can be seen moving away from the cell end towards the cell centre before vanishing.

Movie 3.4. Dissolving tip1pYFP particle
Movie of a single, 1μm confocal section of a wild type cell expressing endogenous tip1p tagged with YFP. Frames were taken every 0.5 seconds. Total movie duration was 14.5 seconds (shown 3.5x accelerated). A particle (marked by green arrow) is shown, which dissolves into a number of outbound moving speckles.

Movie 3.5. Tea2pYFP speckles in wild type cells
Movie of a single, 1 μm confocal section of a wild type cell expressing endogenous tea2p tagged with YFP. Frames were taken every 1.4 seconds. Total movie duration is 42 seconds (shown 7x accelerated). A selection of outbound speckles is marked with red arrows and a much slower particle is highlighted in green.

Movie 3.6. Tip1pYFP in *tea2*Δ cells
Movie shows tip1pYFP motility in *tea2*Δ cells overexpressing tip1pYFP approximately 1.5 fold. Maximal projections were made of confocal stacks covering the entire cell volume. Stacks were imaged every 8 seconds. Total duration of movie is 2 minutes 32 seconds (shown 40x accelerated). Only very faint dots are detectable. These are usually concentrated in the nuclear regions (example marked with a white circle) where they display bi-directional movement. Sometimes an outbound dot can be followed over several frames (i.e. green arrow). Note that this is a similar time resolution as used for the much brighter tip1pYFP particles present in wild type cells.

Movie 3.7. Tea2pYFP in *tea2* rigor mutant
Movie of a single, 1μm confocal section of a cell co-expressing tea2p Switch II (E336A) rigor mutant protein in place of the endogenous tea2p and endogenous tip1p tagged with YFP. Frames were taken every second. Total movie duration was 29 seconds (shown 5x accelerated). The bright signal in the centre is the tip1pYFP accumulating at the iMTOCs in the cell centre. The alignment of speckles in the top left hardly changes over time.

Movie 3.8. Tea2pGFP in *tip1*Δ cells
Movie shows tea2pGFP in *tip1*Δ cells overexpressing tea2pGFP approximately 6 fold. Maximal projections were made of confocal stacks covering the entire cell volume. Stacks were imaged every 6.8 seconds. Total duration of movie is 3 minutes 17 seconds (shown 34x accelerated). Tea2pGFP decorates the microtubule lattices. Speckles are present but are too dense to be followed over more than two frames also in movies with higher time resolution (data not shown). The larger tea2pGFP particles that are present at growing microtubule tips in wild type cells are absent in *tip1*Δ cells although transient tea2pGFP accumulation may still occur.

Movie 3.9. GFPmal3p in *tea2*Δ cells
GFPmal3p particle movements in *tea2*Δ cells are shown. Maximal projections were made of confocal stacks covering the entire cell volume. Stacks were imaged every 8.5 seconds. Total duration of movie is 4 minutes 6 seconds (shown 61x accelerated). Mal3p localises normally to the shortened microtubules present in *tea2*Δ cells.

Movie 3.10. Tea2pGFP in *mal3*Δ cells
Movie shows tea2pGFP motility in *mal3*Δ cells overexpressing tea2pGFP approximately 6 fold. Maximal projections were made of confocal stacks covering the entire cell volume. Stacks were imaged every 6.8 seconds. Total duration of movie was 3 minutes 17 seconds (shown 49x accelerated). Similar to the *tip1*Δ cells (movie 7), tea2pGFP decorates the microtubule lattices. Speckles are present but are too dense to be followed over more than two frames also in movies with higher time resolution (data not shown). The larger tea2pGFP particles that are present at growing microtubule tips in wild type cells are absent in *mal3*Δ cells.

Movie 3.11. iFRAP experiment with wild type cells expressing GFP-mal3p
Each of the 90 frames is a single slice covering the entire Z-axis (about 4 μm) of the cell. Frames were taken every 0.4 seconds except for the first bleached frame which was taken with an 0.7 seconds interval. The unbleached GFP-mal3p particle at the microtubule tip rapidly disappears showing that there is fast protein turnover on the microtubules.

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Chapter 4
Additional Results
Mal3p Modulates Microtubule Stability and Moves on Microtubules in Both Directions Independent of Microtubule Motors

4.1 Levels of mal3 Protein Modulate the Length of Interphase Microtubules

Microtubules in *mal3*Δ cells are shorter than in wild type cells (chapter 2). Mal3p accumulates at growing microtubule tips and remains associated with these tips at the cell end. The loss of mal3p from the tips correlates with catastrophe occurring. This raises the possibility that the relative amount of mal3p localising to microtubule tips controls the stability of microtubule filaments. In this case, changing the levels of mal3p in a cell should either increase or decrease the stability and consequently the length of microtubules. To investigate this possibility, we created cells expressing mal3p under control of different regulable promoters that result in different expression levels. We used the strong promoter of the fission yeast nmt1 gene [1]. Two weaker derivatives, designated 41nmt1 (medium strength) and 81nmt1 (low strength), contain mutations that attenuate both repressed and induced levels of expression [2]. Each version of the promoter allows expression at a relatively low level (with thiamine in the medium) or a relatively high level (without thiamine in the medium), thus allowing a wide range of protein expression in the transformed cells. Because of the small dimensions of a normal fission yeast cell, where interphase microtubules vary considerably in their individual length, differences in microtubule length as a result of different protein expression levels might not be easily detectable. Therefore we used cells with a mutation in the cdc25 gene (cdc25-22) that at the restrictive temperature blocks the cells at the G2/Mitosis transition and causes cell elongation. Cells are shifted to 36˚C for 4.5-5 hours and keep growing in G2 until they are about three times as long as normal cells (Figure 4.1A). In such cells expressing wild type levels of mal3p, most microtubules were able to reach the cell ends. *mal3* deleted cells had considerably shortened microtubules. Also expression from the weakest promoter resulted in shortened microtubules (Figure 4.1A). In our experiments, cells were shifted to the restrictive temperature, and samples were taken after 4.5-5 hours both for cell extract preparation and subsequent western blotting of mal3p, and for immunofluorescence studies with anti-tubulin antibody. Decreased mal3p levels correlated with a decrease in average microtubule length (Figure 4.1B). Microtubules were shortest in cells completely lacking mal3p. Overexpression of mal3p using the strong nmt1 promoter, which

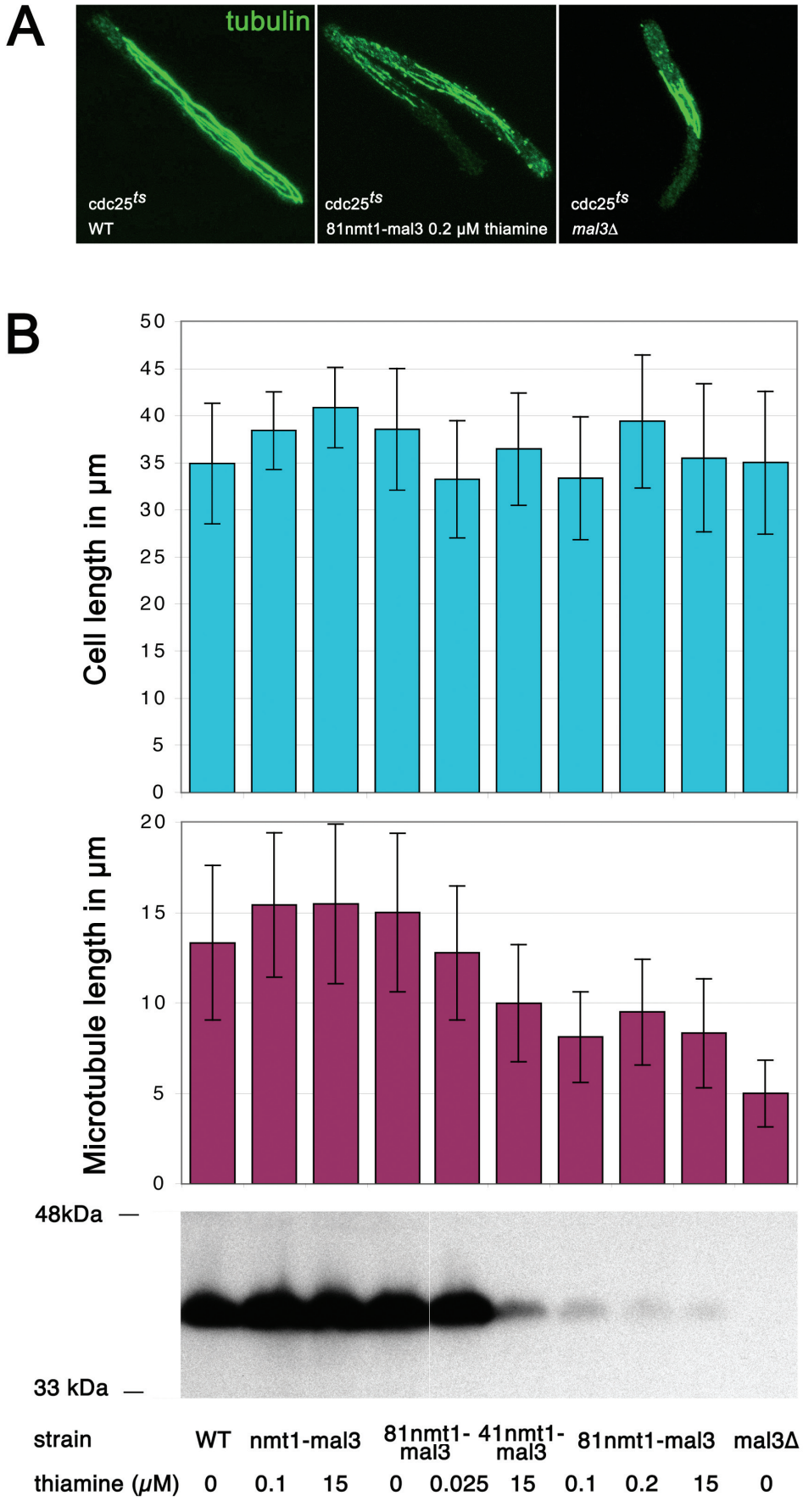


Figure 4.1 Mal3p Levels Determine Interphase Microtubule Length

(A) Immunofluorescence staining of fixed cdc25^{ts} cells using the anti-tubulin antibody. Cells grown at the restrictive temperature for 5 hr are shown, expressing wild type levels of mal3p, reduced levels or no mal3p.

(B) Comparison of cell length, microtubule length and mal3p levels in cdc25^{ts} cells grown for 4.5-5 hr at the restrictive temperature. Mal3p was expressed at wild type levels, from regulated promoters with different concentrations of thiamine, or was completely absent. Cell lengths and microtubule lengths were measured in fixed cells stained for tubulin. Error bars depict standard deviation. Samples of the same cells were taken to monitor protein levels. Mal3p levels in total-cell extracts of the respective cells are shown by western blotting with the anti-mal3p antibody (bottom panel).

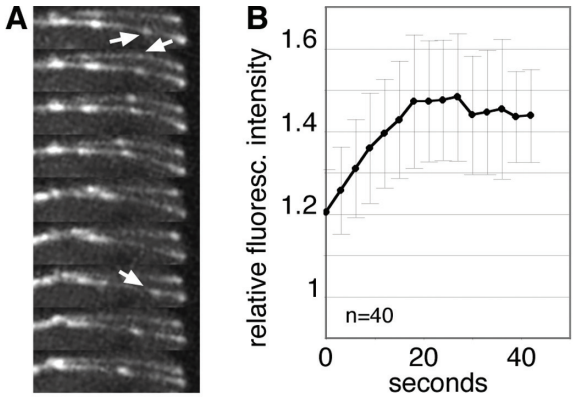
results in protein levels higher than in wild type cells, did not increase microtubule length (Figure 4.1B, lane 2). However, this is not surprising because at wild type mal3p levels, microtubules were already able to reach the cell end, so the potential to increase their length is obviously limited by the boundaries of the cell. Alternatively, since microtubules have the potential to continue growing by curling around the cell end, the availability of other microtubule stabilising factors that interact with mal3p might be limiting.

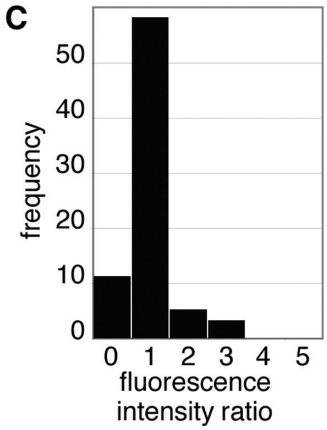
We conclude that there is a correlation between mal3p levels and microtubule length.

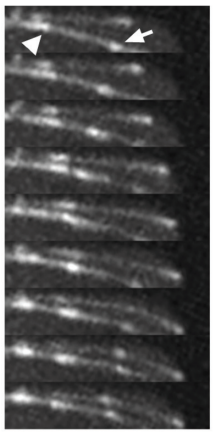
4.2 Inbound GFPmal3p Movement

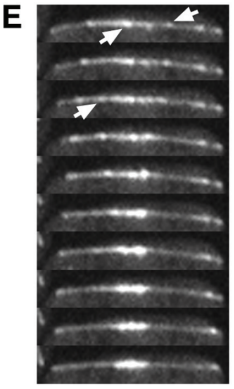
An interesting observation from our live imaging studies with GFPmal3p was that GFPmal3p particles not only moved outbound toward the cell ends with growing microtubule tips, but also inbound along the microtubules toward the interphase microtubule organising centres (iMTOCs; Figure 2.3A, Movie 2.3). The particles were never seen to spontaneously switch their direction of movement. Inbound moving GFPmal3p appeared mainly on fully elongated microtubules that had been in contact with the cell end regions (Figure 4.2G). They were first detectable as weak dots that appeared at random locations along the microtubules (Figure 4.2A). The particles rapidly gained intensity as they moved towards the iMTOCs (Figure 4.2B). The brighter particles displayed a comet-like shape, with the tail facing away from the iMTOCs, which is the opposite orientation of outbound moving comets (Figure 4.2A). Inbound particles moved with an average speed of 3.6±0.8 μm/min (Figure 2.3B). This is faster than outbound moving particles, but considerably slower than the speed of a depolymerising microtubule tip (6±1.4 μm/min). Interestingly, in *tip1*Δ or *tea2*Δ mutants, the inbound movement of GFPmal3p particles is about a third faster than in wild type cells (Table 3.1). Together with the finding that the depolymerising tip of a bundle is not associated with a mal3p particle, this suggests that inbound particles are not associated with depolymerising microtubule tips (Figure 2.3B). To further investigate this, we measured the fluorescence intensity of the GFPmal3p signal on the microtubule lattice, proximal and distal to inbound particles. If inbound particles should highlight depolymerising microtubule tips, then the signal intensity of mal3p decorating the microtubule lattice distal to the particle should be reduced compared to the proximal signal. However, both signals were similar, further strengthening the view that inbound particles are unlikely to highlight depolymerising microtubule tips (Figure 4.2C).

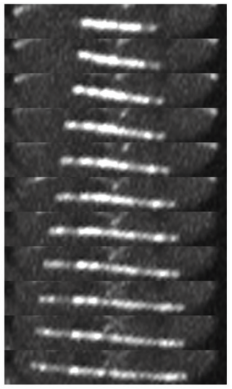
We also observed situations where inbound particles encountered an outbound particle. In this case the outbound particle was often absorbed, a process during which the inbound particle slowed down (Figure 4.2D). In some cases the colliding particles seemed to ignore each other. Due to resolution limitations we could not be unambiguously sure that such particles were indeed in direct contact with each other.











G

Inbound particle appears: Position of MT tip	%
Growing	16
At cell end	58
At cell end, then depolymerising	13.5
Depolymerising	12.5

Figure 4.2 Inbound Moving mal3p
(A and D-F) Movie sequences of subregions of GFPmal3p expressing wild type cells. Frames taken every 7 seconds show projections of 17 confocal sections covering the entire z-axis of the cells.
(A) GFPmal3p particles (arrows) move inbound along the microtubule bundles.
(B) Average fluorescence intensity (particle fluorescence relative to mean cell fluorescence) of inbound GFPmal3p particles over time, including standard deviation.
(C) Histogram showing fluorescence intensity comparison of the GFPmal3p signal on the microtubule lattice proximal and distal of 77 inbound GFPmal3p particles (proximal divided by distal value). The mean value is 1.0 +/- 0.6.
(D) Inbound GFPmal3p particle (arrow) absorbing an outbound particle (triangle).
(E) Central pool of GFPmal3p gaining intensity as inbound particles (arrows) reach the iMTOC.
(F) Central GFPmal3p pool at the iMTOC "exploding" into multiple outbound particles, co-incident with new microtubule outgrowth.
(G) Table showing the position or dynamic state of the tip of a microtubule bundle at the time when an inbound particle appears on that microtubule. 71.5% of particles appear on microtubules whose tip is at the cell end. Most microtubules remain stable while the inbound particle is moving toward the cell centre, some of them undergo catastrophe during that time.

When reaching the iMTOCs, inbound particles performed oscillating movements, sometimes fusing with each other or immediately leaving the iMTOCs on the antiparallel microtubule bundle with reduced speed (Figure 4.2E). In few cases they disappeared near the iMTOC. The amount of GFPmal3p at the iMTOCs strongly fluctuated but this central GFPmal3p pool was the source of all outbound particles. Microtubules were not seen to initiate growth without a considerable amount of GFPmal3p at their growing tips. Occasionally we observed that the entire central pool suddenly fragmented into several particles, which simultaneously moved outbound in opposite directions (Figure 4.2F). This kind of event was usually linked with the outgrowth of two new antiparallel microtubule bundles.

To confirm that the observed inbound movement of GFPmal3p was neither due to the fact that we slightly overexpressed GFPmal3p nor the consequence of amino-terminal tagging of mal3p, we performed live imaging on a yeast strain expressing mal3p tagged with GFP at its carboxy-terminus.

In those cells, we were also able to observe mal3pGFP particles that moved inbound toward the nucleus (Movie 2.5).

Our observations suggest that mal3p particles move inbound, and that inbound movement contributes to the accumulation of GFPmal3p at the iMTOCs.

4.3 Mal3p Particle Movement in Microtubule Motor Mutants

We showed that accumulation of tip1p at growing microtubule tips depends on its delivery by the kinesin tea2p. Mal3p, on the other hand, does not rely on tea2p for plus end accumulation (see chapter 3). It might, however, be transported there by other microtubule motors. Also, the observed inbound movement of mal3p could be motor-driven. To test this hypothesis, we performed live imaging to study the dynamic behaviour of GFPmal3p in deletion mutants of fission yeast microtubule motor proteins. There are ten such proteins known, nine kinesins and dynein, the heavy chain of which is encoded by the *dhc1* gene [3]. So far, only seven of the kinesins have been characterised; one of them, cut7p, is an essential chromokinesin and was not studied here. There are two putative minus-end directed kinesins, homologues of *S. cerevisiae* Kar3 (pkl1p and klp2p); two Kip3 family kinesins that are thought to facilitate microtubule depolymerisation (klp5p and klp6p); and one KHC family kinesin, klp3p, whose function is largely unclear.

The results from these observations are shown in Figure 4.3 and 4.4. In all mutants, GFPmal3p localisation dynamics appeared normal. GFPmal3p localised along the microtubule lattice. It formed particles at the microtubule tips and along microtubules. These displayed both inbound and outbound movement (arrows, Figures 4.3 and 4.4). Mal3p remained associated with microtubule tips at the cell ends for extended periods of time (arrowheads), and disappeared when catastrophe occurred (Figures 4.3 and 4.4). Mutations of *klp5* and *klp6* were shown to lead to excessively stable microtubules that can curl around cell ends [4]. We could also see such curling of GFPmal3p-associated microtubules in the *klp5*Δ *klp6*Δ double mutant (arrowheads, Figure 4.4A). A GFPmal3p particle was localised also at the tips of such curling microtubules until catastrophe took place. This indicates that klp5p or klp6p could be involved in a process that removes GFPmal3p from microtubule tips and prevents them from curling around the cell ends.

Our results show that neither pkl1p, klp2p, klp3p and dynein alone nor klp5p and klp6p together affect GFPmal3p dynamic localisation and are not involved in the observed inbound movement of particles.

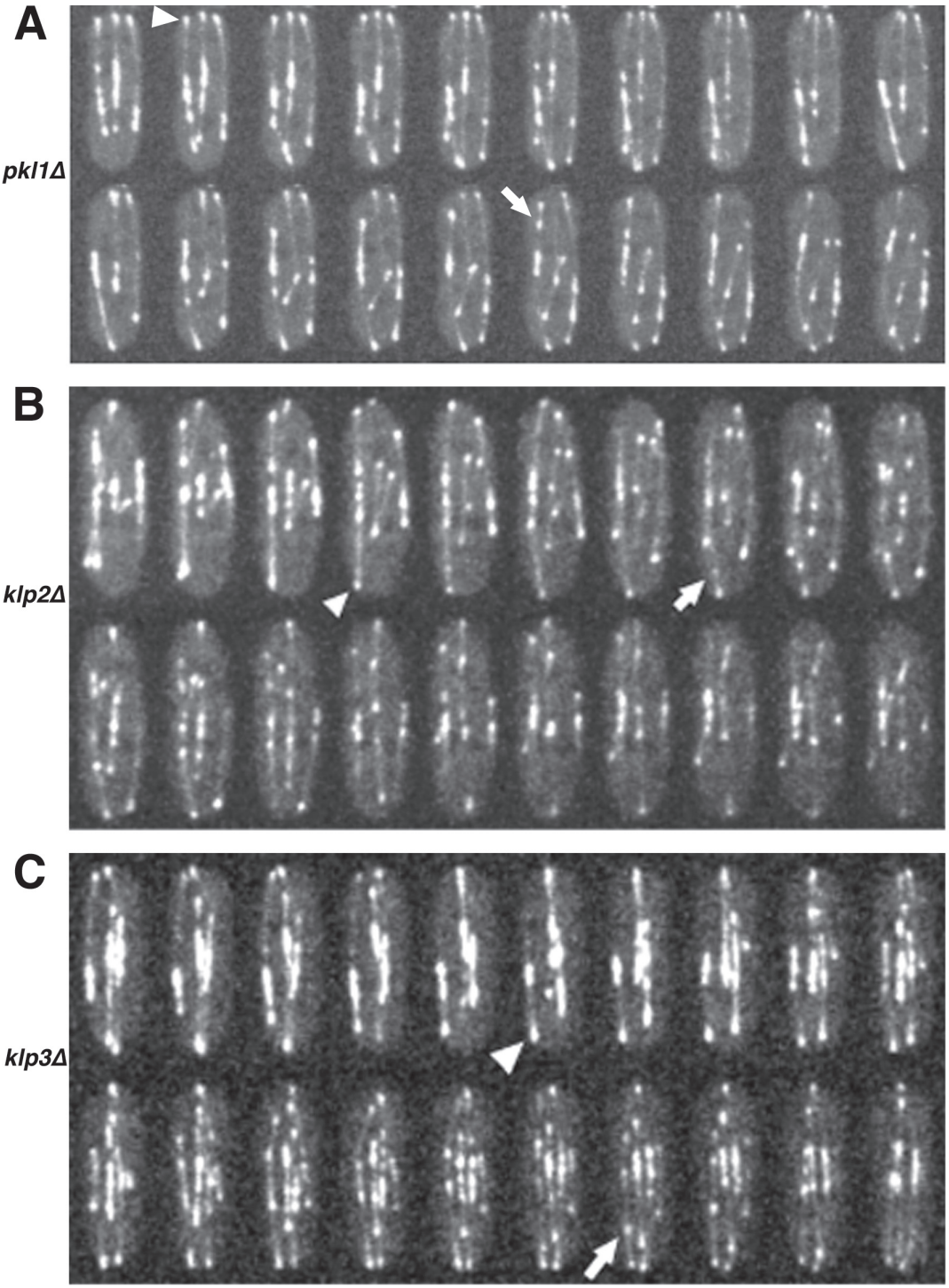


Figure 4.3 Localisation of GFPmal3p in Kinesin Deletion Mutants
(A-C) Time-lapse sequences showing full z axis projections of GFPmal3p-expressing cells. Cells are ordered from left to right and top to bottom in each sequence. Both outbound movement and inbound particles movement (arrows) can be observed. Arrowheads depict microtubules that have reached the cell end and do not immediately undergo catastrophe, but instead remain associated with a GFPmal3p particle.
(A) *pkl1* deleted cell, frames taken every 9 s.
(B) *klp2* deleted cell, frames taken every 13.4 s.
(C) *klp3* deleted cell, frames taken every 10.7 s.

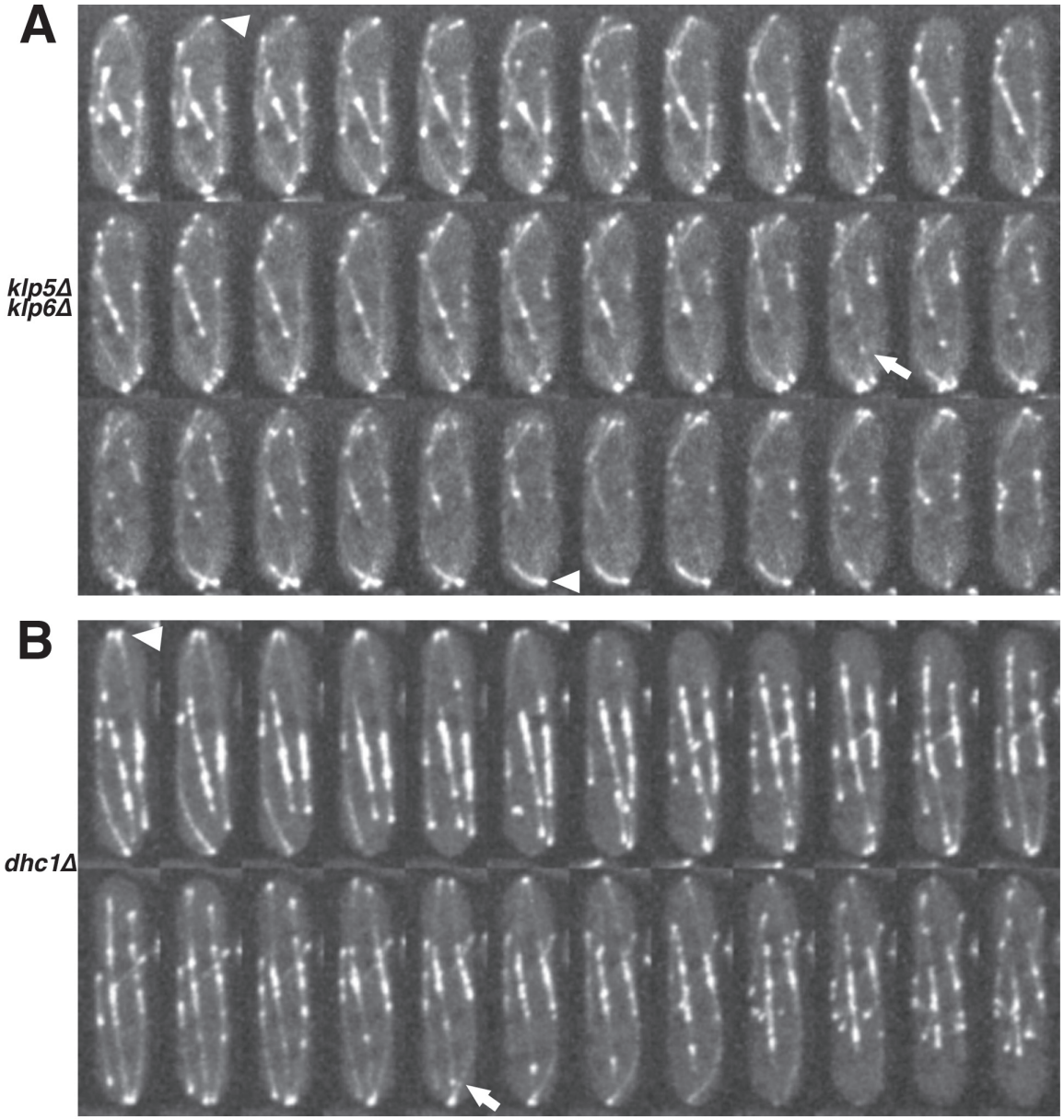


Figure 4.4 Localisation of GFPmal3p in Deletion Mutants of Kip3-family Kinesins and Dynein

(A-B) Time-lapse sequences showing full z axis projections of GFPmal3p-expressing cells. Cells are ordered from left to right and top to bottom in each sequence. Both outbound movement and inbound particles movement (arrows) can be observed.

(A) *klp5 klp6* double deleted cell, frames taken every 8.3 s. The arrowheads point to microtubule plus ends that curl around the cell ends, while remaining associated with GFPmal3p particles. The arrow highlights an inbound particle.

(B) *dhc1* deleted cell, frames taken every 12.5 s. The arrowhead depicts a microtubule that has reached the cell end and remains associated with a GFPmal3p particle until shortly before catastrophe occurs.

Supplemental Movies

Movies 4.1-5. Motor mutant cells expressing GFPmal3p under control of the fully repressed nmt1 promoter

Movie 4.1. *pkl1* deleted cells expressing GFPmal3p. Each of the 44 frames is a maximum projection of 17 slices covering the entire Z-axis of the cell. Frames were taken every 9 seconds (shown 55x accelerated). GFP-mal3p particles localise along the microtubules and at their tips. Also inbound particles are seen, one of which is marked in yellow. At the cell ends, GFPmal3p remains associated with microtubule tips for extended periods of time during which no catastrophe occurs. It also localises along the microtubule lattice. The protein thus shows the same localisation dynamics as in wild type cells.

Movie 4.2. *klp2* deleted cells expressing GFPmal3p. Each of the 47 frames is a maximum projection of 16 slices covering the entire Z-axis of the cell. Frames were taken every 6.7 seconds (shown 43x accelerated). Localisation dynamics of GFPmal3p particles are similar to those in wild type cells. Also inbound moving particles can be seen (one is marked in yellow).

Movie 4.3. *klp3* deleted cells expressing GFPmal3p. Each of the 44 frames is a maximum projection of 17 slices covering the entire Z-axis of the cell. Frames were taken every 10.7 seconds (shown 65x accelerated). GFP-mal3p particles show a dynamic localisation in *klp3*Δ that is similar to that in wild type cells. Inbound movement of particles occurs (one of them being highlighted in yellow).

Movie 4.4. *klp5 klp6* double deleted cells expressing GFPmal3p. Each of the 45 frames is a maximum projection of 16 slices covering the entire Z-axis of the cell. Frames were taken every 8.3 seconds (shown 51x accelerated). Dynamics of localisation of GFP-mal3p particles closely resembles the wild type situation, except that microtubules regularly curl around the cell ends. GFPmal3p remains accumulated at these curling microtubule tips until catastrophe occurs. An example of an inbound particle is marked with yellow.

Movie 4.5. *dhc1* deleted cells expressing GFPmal3p. Each of the 36 frames is a maximum projection of 18 slices covering the entire Z-axis of the cell. Frames were taken every 12.5 seconds (shown 84x accelerated). GFP-mal3p particles show similar dynamic movement in the absence of dynein activity as in wild type cells. An example of an inbound particle is highlighted in yellow.

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Chapter 5
Conclusions and Perspectives

In fission yeast, microtubules play an important role in morphogenesis. They are involved in the positioning of the interphase growth zones and of the nucleus, which in turn positions the cell division site. In order to do this, microtubule dynamics must be regulated in the intracellular space such that microtubules grow with their plus ends from the nucleus to the cell tips, and that catastrophes are restricted to the cell ends. In this work, we have investigated how a subset of microtubule-associated proteins contributes to the proper organisation of the microtubule cytoskeleton. In particular, we were interested in how the different activities of these proteins are coordinated in space and time.

5.1 The Plus End-Tracking Mechanism(s) of Clip-170 Proteins

The Clip-170 protein, as well as its fission yeast homologue, tip1p, is thought to link specific membrane structures with microtubules, an important process in cellular morphogenesis. For this role of Clip-170 it is essential that the protein localises to growing microtubule plus ends. The current "treadmilling" model that explains how Clip-170 localises to microtubule tips is mostly based on *in vitro* studies, as well as on live imaging studies in cultured cells (see chapter 1.4). It states that Clip-170 binds to tubulin dimers directly through its CAP-Gly domain and that it co-polymerises with the dimers into the microtubule filaments. Shortly after incorporation, it is actively removed from the microtubule lattice through phosphorylation by a microtubule-associated kinase. Based on the fact that speckles of the protein near the plus end are not dynamic, it was concluded that transport along microtubules toward the plus ends plays no role in Clip-170 plus end-tracking [1].

We now show that previously undetected speckles of both tip1p and tea2p move with high speed toward the microtubule tips, where the proteins accumulate. In the absence of the kinesin tea2p, tip1p no longer forms speckles, and accumulation at microtubule tips is strongly reduced. The two proteins co-immunoprecipitate, and turnover of both proteins on microtubule tips is relatively high.

Recent results from budding yeast are highly consistent with our results. The mechanism of plus end targeting of Bik1 - the budding yeast Clip-170 protein - was investigated [2]. Bik1 also accumulates at the spindle pole bodies (SPBs), but this pool of protein is not necessary for plus end targeting. Instead, Bik1 seems to be recruited onto microtubule tips from the cytoplasm. Like with tip1p, speckles of Bik1 were observed to be highly dynamic and to move along microtubules, mostly toward their plus ends. Speckle movement depends on the kinesin Kip2, which forms a complex with

Bik1. Speckles of Kip2 and Bik1 comigrate along the microtubules, and both proteins accumulate at their plus ends. Deletion of *kip2* practically abolishes Bik1 plus end association. Overexpression of Kip2 increases microtubule stability and length by recruiting almost all Bik1 in the cell to microtubule tips; Kip2 levels vary over the cell cycle, and high levels in mitosis correlate with an increased amount of Bik1 on microtubule tips and with more stable microtubules.

However, unlike Clip-170 or tip1p, Bik1 not only accumulates at the tips of growing, but also of shrinking or pausing microtubules, and it still targets microtubule plus ends in a β-tubulin mutant where microtubule dynamics are strongly reduced. In FRAP experiments, Bik1 proteins at microtubule tips turned over with a half-life of about 20 seconds, even on microtubules that are not dynamic. Thus Bik1 is targeted to microtubule plus ends independently of their dynamicity.

In conclusion, both studies show that tea2p and Kip2 interact with and transport tip1p and Bik1, respectively, along microtubules toward their tips. There, tip1p and Bik1 act to stabilise microtubules. The kinesins regulate microtubule organisation indirectly through the yeast Clip-170 protein family members.

Together, these results strongly challenge the postulated treadmilling mechanism used to explain plus end association of Clip-170 proteins. It is though possible that the mechanisms for plus end localisation of yeast and human Clip-170 proteins are different, with motors being involved in plus end targeting in one case, but not the other. However, motor-dependent transport of human Clip-170 *in vivo* was ruled out largely relying on a single result. This is that speckles of the protein lying within the comet-shaped tail that forms at the microtubule tip were not seen to be motile. We were only able to visualise tip1p speckles with a very sensitive microscope setup that allowed rapid imaging of fission yeast cells. Therefore, one can easily imagine that such motile speckles exist also with Clip-170, but that they have so far eluded detection, because they were too faint or too fast to be seen. In the published results on Clip-170 live imaging, there was an interval of 2.8 seconds between the recorded frames, which might be too slow for detection of speckles moving with high velocity [1]. Several examples of non-motile speckles are shown in these recordings; however, in our study, we also see many cases of speckles that are stalling over several frames. For these reasons, I think it would be premature to exclude motor-dependent transport as a mechanism for plus end targeting of human Clip-170.

We show that tip1p accumulation is not due to transport alone, but that there is an additional, but weaker, tea2p-independent mechanism for microtubule plus end accumulation. Co-immunoprecipitation experiments in *tea2*Δ cells showed that mal3p and tip1p are able to interact independently of tea2p, and we have shown that the recombinant proteins bind each other directly. Therefore, it is likely that this second mechanism of tip1p plus end-tracking relies on direct binding to mal3p. Also human Clip-170 might rely on more than one mechanism for efficient plus end targeting.

One might argue that the plus end-tracking mechanisms of Clip-170 and of its yeast homologues differ because the proteins are different in amino acid sequence, and because Bik1 not only localises

to growing microtubule tips, but can also associate with the tips of pausing and even depolymerising microtubules. However, a chimeric protein, in which the Clip-170 microtubule binding head domain replaced the corresponding region of Bik1 shows the same localisation as wild type Bik1 [2]. The behaviour of Bik1 is consequently not due to species-specific differences in the structure of the microtubule binding domain.

Our results, and those of a related study [3], show that tip1p and tea2p depend on mal3p to accumulate at microtubule tips. Intriguingly, deletion of *bim1*, the budding yeast homologue of mal3p, does not abolish Kip2 - and most likely, Bik1 - accumulation at microtubule tips [2]. This might mean that the role of mal3p in plus end targeting of tip1p and tea2p is not a conserved mechanism. An alternative possibility is that the mechanism is conserved in evolution but has been lost specifically in budding yeast. Bik1 and Kip2 are unusual plus end-tracking proteins in the sense that they accumulate not only at growing, but also at shrinking ends. Since EB1 proteins do generally not target shrinking plus ends, Bik1/Kip2 might have evolved a different mechanism for plus end targeting, independently of Bim1, that allows them to track microtubule tips in all their dynamic states.

It is not clear how mal3p "docks" tip1p and tea2p at microtubule plus ends. Given that mal3p turnover on microtubule tips is higher than that of tip1/tea2p, it is possible that they bind to several mal3p molecules at the same time, so the dissociation from one mal3p does not cause their unbinding. Alternatively, there might be an excess of mal3p at microtubule tips such that dissociation of tip1p/tea2p from one mal3p molecule is immediately followed by binding to another. An intriguing possibility is that this mechanism would slow down tip1 and tea2p when they arrive at the microtubule tip, so that they do not "fall off" the microtubule. It would allow the two proteins to continue moving with the growing microtubule tips. To shed light on this process, localisation and function of tip1p, tea2p and mal3p on microtubules *in vitro* should be studied, especially to answer the question whether changes occur when several of the proteins are added simultaneously.

5.2 The Mechanism of mal3p Plus End-Tracking

Mal3p seems to target to microtubule plus ends with a mechanism which is different from that of tip1p. We show that the protein dynamically localises to microtubule tips, and co-migrates with the growing plus ends. However, individual protein molecules most likely do not move, as turnover of the proteins at the growing microtubule tips is very high, with a half-life in the range of one second. Also, none of the microtubule motors tested so far, including tea2p, is essential for mal3p plus end targeting. Overexpression of the protein increases mainly its localisation along the microtubule lattice. Together, these results suggest that mal3p plus end-tracking is not due to transport along the microtubules toward their tips. Instead it could be the result of a higher binding affinity of mal3p for the GTP cap, or the sheet structure at the growing microtubule plus end, or another protein re-

siding there as compared to the rest of the microtubule. A high protein turnover, most likely caused by high dissociation rates, might be necessary for such a mechanism of plus-end tracking, because otherwise the protein would accumulate in long stretches at the microtubule plus ends.

Another possible mechanism is that the protein copolymerises with tubulin into the polymer and is later somehow removed from the microtubule lattice. However, unpolymerised tubulin dimers do not co-immunoprecipitate with mal3p. Furthermore, such a mechanism cannot easily explain why mal3p localises uniformly to the entire microtubule lattice, in addition to its accumulation at their tips. The high turnover of mal3p along the microtubule lattice means that the protein most likely binds the lattice directly and that the signal is not a remnant of the plus end-associated mal3p. In other words, there would have to be different mechanisms of mal3p that co-polymerised with tubulin onto microtubule tips and of mal3p binding to the lattice, and only the former would be subject to the postulated removal mechanism. We found that turnover of mal3p is basically the same at the microtubule tips and on the lattice, which is similar to observations made with EB1 in *Xenopus* extracts [4]. This suggests that the dissociation mechanisms at the tip and the lattice are similar. Together, these results argue against a co-polymerisation model operating for microtubule plus end-tracking of mal3p.

Finally, we cannot fully exclude that one of the so far uncharacterised kinesins are able to transport mal3p along microtubules toward their tips, or that several kinesins and/or dynein have redundant functions. However, given the high turnover of mal3p both along the microtubule and at the tip, movement along the filament seems unlikely. The protein would dissociate from the microtubule before it could move any significant distance, unless it moves with extremely high speed. If we assume that mal3p molecules move with 30 μm per minute, the maximum speed that we observed for tip1p and tea2p speckles, the half-life of 0.8 seconds for lattice-bound mal3p would mean that half of the protein has turned over after having moved 0.4 μm. This is only 5% of the average length of a microtubule bundle!

Our model of mal3p plus end-tracking is essentially in agreement with the model proposed for mammalian EB1 plus end targeting. This is mainly based on results with human EB1 in *Xenopus* egg extract. It is thought that the protein recognises a specific property of the polymerising plus end. Consequently, it shows higher affinity binding to polymerising microtubule tips and lower affinity binding along the lattice, with continuous dissociation [4]. In that respect, it is interesting to note that human EB1 can at least partially replace mal3p function in fission yeast cells [3, 6].

To unravel the mechanism of how mal3p specifically recognises microtubule plus ends, structural studies on the binding of mal3p to polymerised tubulin *in vitro* will be helpful, especially if the binding to the microtubule wall can be compared to plus end binding.

5.3 Inbound Movement of mal3p Particles

A still unsolved problem is the inbound movement of mal3p particles toward the cell centre. For now, it is unclear how this movement comes about, what purpose it serves, and what other factors are involved.

One possible explanation is that inbound mal3p highlights plus ends of microtubules that are nucleated far away from the nucleus and grow antiparallel to the existing microtubule bundle. Consistent with this, recent results in fission yeast show that proteins of the γ-tubulin complex, which nucleate microtubules and most likely form the iMTOCs, localise not only to the SPB or to the region of the interphase microtubules that is attached to the nucleus, but also form "satellites" (or speckles) that are distributed along the microtubules, and can even localise near microtubule tips [7, 8]. If these satellites are able to nucleate microtubules, it is easy to imagine that they do so also at peripheral locations. In an extension of this model, it is furthermore possible that microtubule motor proteins play a role in this process by sliding antiparallel microtubules along each other Such sliding could move a microtubule "seed" that has been newly nucleated at a random position along existing microtubules, to the place where it is supposed to be, that is, to the overlap zone of the antiparallel microtubule bundles at the nucleus.

Some of our results argue against a model where inbound moving particles mark the tips of growing microtubules. The average velocity of these particles is more than a third higher than that of outbound particles at the tip of growing microtubules. However, this speed increase could be explained if, in addition to the polymerisation-based dislocation, the postulated inbound sliding would occur. Should inbound moving particles mark growing microtubule tips, one would also expect that the microtubule lattice staining increases distal to an inbound particle because an additional microtubule is created. We found no such increase, not even in cases where up to four particles moved inbound on the same microtubule. Several experiments can be carried out to investigate this problem. If nucleation of microtubules is required for this phenomenon to occur, it will be informative to study GFPmal3p dynamics in mutants of γ-tubulin complex proteins. In those mutants, interphase MTOCs are not active anymore, and no microtubules are nucleated except those associated with the SPB. Double imaging with a marker for the γ-tubulin complex will also be important to study the relationship between movement of the nucleating complex and of mal3p particles. Using electron microscopy and subsequent three-dimensional reconstruction of the microtubule cytoskeleton in fission yeast cells, it should be possible to detect microtubules that have their minus ends far away from the nucleus. One might, however, need to look at many cells to find a suitable example, which would require a major effort. By photobleaching stretches of GFP-marked microtubule bundles, it should also be possible to detect microtubule polymerisation directed toward the cell centre.

Alternatively, with a new microscopy technique called second harmonic imaging microscopy, it is in principle possible to distinguish antiparallel from parallel microtubule bundles. The method can detect highly ordered, polarisable, asymmetric structures in cells, such as microtubules. These emit light at half the excitation wavelength, but the signal is quenched if the structures are arranged in an antiparallel way (for details on the physics see [9]). The feasibility of such an experiment in fission yeast cells is an open question.

Another possible explanation of inbound mal3p particle movement is that these mark the growing plus end of a spontaneously nucleated, γ-tubulin independent microtubule with a non-capped minus end that is not stable and depolymerises shortly behind. This would result in a short stretch of a treadmilling microtubule. With photobleaching of GFP-tubulin stretches, it should also be possible to detect such events.

Yet another possibility is that Mal3p directly binds to a minus end directed motor protein or that it may highlight an entity such as a vesicle or a protein complex that is transported inbound by microtubule motors. To investigate the question whether motors are involved in the process, we have studied candidate motor proteins. So far, however, none of them seem to affect inbound movement of mal3p. It is possible that the kinesins klp8p or klp1p (SPBC15D4.01c), the only fission yeast kinesins that we have not tested so far, are responsible for the observed particle movements. More likely, however, is that several kinesins, and possibly dynein, have redundant functions in this process. Therefore, GFPmal3p dynamics needs to be analysed in cells where multiple microtubule motors, including klp8p/klp1p, are deleted.

Also other proteins might be part of these inbound movements. Although particles of tip1p and tea2p are seen to move toward the nucleus, this appears to be more rare than is the case for mal3p. Instead, the proteins seem to be deposited at the cell ends in a tea1p-dependent way. Preliminary results suggest that particles of the kinesin klp2p, and the XMAP215 homologue alp14p, might not only localise to microtubule tips, but also show inbound-directed movement. Therefore, the proposed studies on mal3p inbound movement should be extended to include these proteins.

5.4 Mal3p at Microtubule Minus Ends

EB1 proteins, including mal3p, generally do not only accumulate at microtubule plus ends, but also at the various forms of nucleation sites (e.g. centrosomes, iMTOCs or spindle pole bodies). Very little is known about the functional significance of EB1 minus-end / MTOC association. Colocalisation studies of mal3p with γ-tubulin complex proteins, which like mal3p seem to move bidirectionally, might reveal which (if any) of the mal3p locations on microtubules correspond to iMTOCs. The microtubule-binding CH domain at the N-terminus of EB1 seems to be dispensable for MTOC localisation [10]. Deletion of the corresponding part of mal3p should show whether this is also

the case for the fission yeast homologue. If the C-terminus of mal3p does indeed confer MTOC localisation, it might become a useful tool to study the function of the protein when associated with this location.

5.5 The Regulation of mal3p Localisation and Function

Another unsolved question is whether mal3p plus-end localisation is involved in stabilisation of microtubule tips at the cell end. As described in chapter 1.5, it is necessary that microtubules do not undergo catastrophe before their tips reach the cell end, but before they start curling around the cell ends. Only in this way they can exert a pushing force on the nucleus.

We observed a correlation between removal of mal3p from microtubule tips and subsequent catastrophe at the cell cortex in wild type and *tip1*Δ cells. We also showed that microtubules are considerably less stable in *mal3*Δ cells and have increased catastrophe rates. In addition, variation of the amount of mal3p in cells roughly correlates with the average microtubule length. Based on these results, we speculate that stabilisation of microtubule growth, induction of microtubule catastrophe at cell ends, and possibly also microtubule nucleation are regulated through modulation of the amount of mal3p at the microtubule tip. It is still unclear how this might work in mechanistic terms. In order to shed light on this question, it will be useful to try to identify mal3p-interacting proteins that might remove the protein from the microtubule tips. One intriguing possibility is that posttranslational regulation of mal3p plays a role in this process. Phosphorylation in particular is an obvious candidate, because we found that mal3p is a phosphoprotein and that only its unphosphorylated form interacts with tip1p. For several microtubule-associated proteins, it is assumed that phosphorylation controls their association with microtubules. However, nothing is known so far about the possible role of phosphorylation in the regulation of EB1 proteins.

5.6 Effect of mal3p, tip1p and tea2p on Microtubule Organisation

Currently we know little about the mechanism underlying mal3p activity. Mal3p clearly has a microtubule stabilising function that is independent of its role in retaining tip1p and tea2p at the plus ends. *In vitro*, EB1 strongly induces microtubule polymerisation if seeds are added that allow the nucleation of microtubules [11]. It is likely that mal3p has the same ability to directly affect microtubule plus end stability *in vitro*, but this has to be tested.

Our results suggest that tip1p, in contrast to mal3p, affects microtubule growth only indirectly, probably by modulating mal3p activity. Mal3p does not require tip1p for plus end-tracking. However, tip1p ensures that mal3p is retained at the growing microtubule tips when these touch the cell cortex

in central regions of the cell. Because at the same time tip1p localisation to microtubule plus ends depends on mal3p, this means that mal3p brings along its own regulator. This allows mal3p to exert its stabilising activity also at the cortex. At the cell ends, tip1p becomes deposited on the membrane and is removed from the microtubule tip. As a consequence, mal3p might no longer be able to bind and stabilise the plus end, which results in catastrophe. The mechanism of this process is unclear, and much additional work will be required for clarification. The identification of new proteins that interact with tip1p or mal3p will be an important first step. In particular, membrane-associated factors interacting with tip1p are likely to play a key role.

Our results show that also the kinesin tea2p influences microtubule dynamics indirectly, by transporting tip1p toward the microtubule plus ends. This is supported by the fact that overexpression of tea2p cannot rescue normal microtubule length if *tip1* is deleted. The same is true for its budding yeast homologue, Kip2, which cannot stabilise microtubules in *bik1*Δ cells [2]. In the absence of tea2p, a residual amount of tip1p localises to microtubule tips, independent of the kinesin. Therefore, if tip1p is the stabilising agent of the two proteins, one would expect microtubules to be slightly more stable in *tea2*Δ cells compared to *tip1*Δ cells, which is indeed the case [3, 12].

An intriguing observation is that tip1p particles occasionally dissolve into speckles that move with increased speed toward to the cell end. We interpret this as a process where, at the microtubule plus end of a secondary microtubule, which is growing along a longer primary microtubule, tea2p and with it tip1p "switches tracks" and jumps onto the adjacent microtubule. This could lead to destabilisation of the secondary microtubule which has lost its tea2p / tip1p cap. Such a model would explain why secondary microtubules within a bundle often do not reach the cell ends.

5.7 Interaction of mal3p / tip1p / tea2p with Other Microtubule-Associated Proteins

While we have been able to unravel some of the mechanisms underlying the actions and localisations of mal3p/EB1, tip1p/Clip-70 and the tea2p kinesin, very little is known about how these proteins interact with other microtubule-associated factors. Of particular interest is the possible interaction with members of the XMAP215 protein family. In two species (*Dictyostelium* and *S. cerevisiae*), an interaction has been reported between EB1 and XMAP215 proteins, but nothing is known about the possible roles of this interaction. There are two XMAP215 homologues in *S. pombe*, alp14p and dis1p. It has been shown that deletion of *alp14* leads to shortened interphase microtubules, but the parameters of microtubule dynamics have not been measured. In the case of dis1p, no effect on interphase microtubules has been reported. Future plans include co-immunoprecipitation experiments in order to test whether the proteins interact. Also the localisation dynamics of alp14p/dis1p in living cells deserve further investigation.

Several studies have suggested that XMAP215 and the kinesin Xkcm1 may constitute a basic system of microtubule regulation that allows physiological microtubule dynamics. This is because the two proteins have antagonistic activities: one stabilises, the other destabilises microtubules (see chapter 1.4). Also in fission yeast, it has been proposed that alp14p/dis1p form a system of microtubule regulation with the microtubule destabilising kinesins klp5p/klp6p [13]. It has been speculated that other proteins might modulate one or both of these components to locally adapt microtubule organisation within certain regions of the cell. It is possible that mal3p and tip1p might be such factors that regulate the activities of the fission yeast XMAP215/Xkcm1 homologues. However, it is also conceivable that mal3p/tea2p/tip1p activity is completely independent of other MAPs. Therefore, it will be interesting to investigate whether mal3p/tip1p and alp14p/disp1p (or klp5p/klp6p) depend on each other for their localisation or function.

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