Universidade de Aveiro Departamento de Biologia
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Análise funcional do domínio de ligação aos microtúbulos da proteína MAST

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Métodos Biomoleculares Avançados, realizada sob a orientação científica do Professor Doutor Claudio Sunkel da Universidade do Porto e do Professor Doutor Manuel Santos da Universidade de Aveiro.

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À Alice e ao António

o júri

presidente

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palavras-chave

Mast, Orbit, CLASP, proteínas associadas aos microtúbulos, GTPase, microtúbulos, Mitose, Drosophila

resumo

A Mast/Orbit/CLASP é uma família conservada de proteínas associadas aos microtúbulos (MAPs) essenciais para a organização e função do fuso mitótico (Inoue et al., 2000; Lemos et al., 2000; Akhmanova et al., 2001; Maiato et al., 2002; Maiato et al., 2003a; Mimori-Kiyosue et al., 2005). Estas proteínas surgem associadas aos microtúbulos, centrossomas e cinetocoros e diversos estudos sugerem que desempenham um papel importante na regulação das propriedades dinâmicas dos microtúbulos (Akhmanova et al., 2001; Maiato et al., 2002; Maiato et al., 2003a; Maiato et al., 2005). As isoformas humanas, CLASPs, fazem parte de um conjunto de proteínas (+TIPs) que exibem uma forte acumulação na ponta de crescimento (+) dos microtúbulos em polimerização (Schuyler and Pellman, 2001). Estas proteínas dissociam-se do polímero formado o que origina uma localização em forma de cometa na extremidade do microtúbulo. Neste trabalho mostramos que em *Drosophila*, a proteína Mast também é uma +TIP. Adicionalmente, definimos o domínio de ligação da proteína aos microtubulos e demonstrámos que, *in vitro,* a Mast se associa directamente com a tubulina num processo sensível a nucleótidos de guanina. O GTP favorece a ligação aos heterodímeros de tubulina, mas não influencia a ligação aos microtubulos. Contrariamente, o GDP inibe fortemente a ligação da Mast aos microtúbulos e heterodímeros de tubulina. Finalmente, provamos que a Mast liga e hidrolisa GTP, o que a torna a primeira +TIP com características de GTPase e sugere um novo mecanismo para a localização dinâmica das +TIPs. Estes resultados são consistentes com um modelo no qual a Mast-GTP copolimeriza com os heterodímeros de tubulina ou se associa directamente à extremidade (+) dos microtúbulos em crescimento. Após a associação ao microtúbulo dá-se a hidrólise do GTP e consequente formação de Mast-GDP que causará uma alteração conformacional da proteína promovendo a sua dissociação do microtúbulo. Este estudo sugere que uma proteína associada aos microtúbulos pode utilizar a actividade GTPásica na regulação da sua ligação aos microtúbulos.

keywords Mast, Orbit, CLASP, MAPs, GTPase, Microtubules, Mitosis, Drosophila

abstract

Mast/Orbit/CLASP is a conserved MAP protein family essential for the organization and function of mitotic spindle (Inoue et al., 2000; Lemos et al., 2000; Akhmanova et al., 2001; Maiato et al., 2002; Maiato et al., 2003a; Mimori-Kiyosue et al., 2005). It accumulates at centrosomes, kinetochores and microtubule plus-ends where it is thought to regulate their dynamic properties (Akhmanova et al., 2001; Maiato et al., 2002; Maiato et al., 2003a; Maiato et al., 2005; Mimori-Kiyosue et al., 2005). CLASPs, the human homologues (Akhmanova et al., 2001), are members of the microtubule plus-end tracking protein (+TIP) family (Schuyler and Pellman, 2001). +TIPs show strong accumulation at the polymerizing end of microtubules, dissociating from the polymer soon afterwards giving the appearance of a comet-like structure. Here we show that the Drosophila homologue Mast also displays +TIP behaviour. Moreover, we defined the microtubule binding domain of Mast and showed that it associates directly with tubulin in a guanine nucleotide sensitive manner. GTP favours the binding of Mast to tubulin heterodimers but does not influence binding to microtubules, while GDP strongly inhibits the binding of Mast to microtubules. More importantly, we show that Mast can bind and hydrolyse GTP demonstrating that it is the first +TIP with this feature and hence suggesting a new mechanism for +TIP behaviour. These results are fully consistent with a model in which Mast-GTP copolymerizes with tubulin heterodimers at the growing microtubule plus end. Mast is then released from the polymer due to hydrolysis of the bound GTP, causing a conformational change of the protein that promotes its release from the microtubule lattice. Our data provides evidence that a microtubule associated protein could use its GTPase activity to regulate its ability to bind microtubules.

Contents

1. Introduction

1. Introduction

1.1. Cell Cycle and Mitosis

In the nineteenth century, Theodor Schwann and Matthias Schleiden proposed that the cells were the basic unit of all living organisms and in 1855; Rudolf Virchow realized that all cells arise from pre-existing cells (reviewed by Wilson, 1925). We now know that this theory is mostly correct in that cells form the basic unit of all living organisms, whether they are plants, animals, or microorganisms. In order to reproduce, all cells go through a process of duplication and division called the cell cycle. The cell cycle of eukaryotes can be divided into two phases: interphase when cells grow and their genome is replicated (G_1, S and G_2) and cell division including Mitosis (or M phase) and Cytokinesis when the duplicated contents of the cell are equally segregated into the two daughter cells (Figure 1). G_1 is the first period of cell growth and once the cell has reached the required volume then it can progress into the next stage, which involves the replication of the genome during S phase. During G_1, depending upon the growth conditions as well as on specific factors, the cell can also exit the cell cycle temporarily and enter a resting or nondividing stage called G_0 during which metabolic activity is severely reduced. Once DNA replication is completed, the cell can now start preparing for its division. This takes place during G_2 a gap phase between S and mitosis. Once the cell is ready, it can proceed into division and enter Mitosis, a process by which the replicated chromosomes are equally distributed to the two daughter nuclei. Finally, the cell must also separate the cytoplasm and with it all the organelles of the original cell into the new formed cells. The distribution of the cytoplasm of the original cell into new cells is called Cytokinesis.

In 1879, Flemming first appreciated the significance of chromosomes during cell division and coined the term “mitosis” in 1882 (reviewed by Wilson, 1925). Mitosis is divided in five different stages: prophase, prometaphase, metaphase, anaphase and telophase (Figure 2) (Gorbsky, 1992).

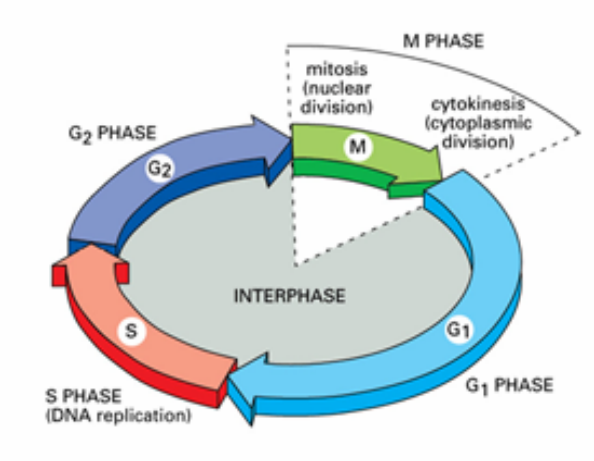

Figure 1. Schematic representation of the cell cycle. DNA replication (S phase) and Mitosis (M phase) are separated by two gap phases (G_1 and G_2). (Adapted from The Molecular Biology of the Cell, 2002)

During prophase, chromatin condenses into chromosomes and the centrosomes, the organelles responsible for the nucleation of microtubules, move into opposite sides of the cell. The end of prophase and the beginning of prometaphase is marked by nuclear envelope breakdown (NEB). At this time, microtubules growing out of the centrosomes invade the central region and begin to form the mitotic spindle. During this process, microtubules interact with chromosomes through the kinetochore, a protein-based structure located at the surface of the centromere. Initially microtubules bind only one sister chromatid and the chromosome is said to be mono-oriented, subsequently chromosomes are captured by microtubules from the opposite pole so that it becomes bi-oriented. After biorientation, the chromosome moves toward the centre of the cell and when all chromosomes align, the cell is said to have reached metaphase. Once they get there significant movement cease and the spindle became shorter and broader. Afterwards, during anaphase A, sister chromatids separate and migrate towards opposite poles of the spindle. In anaphase B, the spindle elongates and the two poles became more distant from each other. At last, the chromatids decondense and new daughter nuclei start to form. Telophase frequently includes the start of cytokinesis. In animal cell, cytokinesis starts with a cleavage furrow that divides the cell in two.

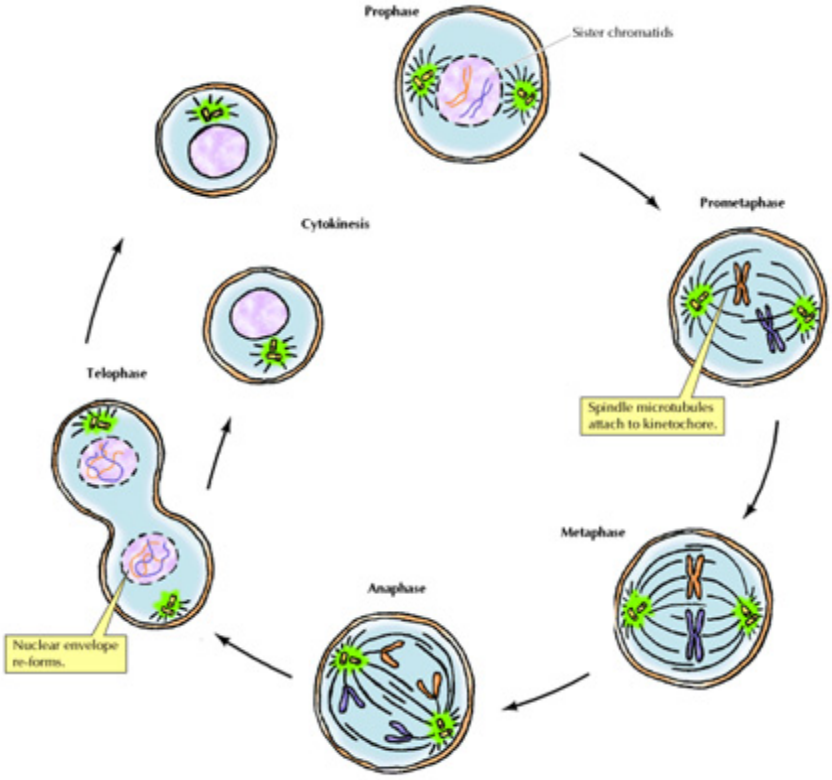

Figure 2. Stages of mitosis in an animal cell. During prophase, the chromosomes condense and centrosomes move to opposite sides of the nucleus, initiating the formation of the mitotic spindle. Breakdown of the nuclear envelope allows spindle microtubules to find and attach to the kinetochores of chromosomes. During prometaphase, the chromosomes move towards the centre of the spindle and align at metaphase plate (metaphase). At anaphase, the sister chromatids separate and move to opposite poles of the spindle. Mitosis ends with chromosome decondensation and re-formation of the two nuclei during telophase. Finally, during cell division or cytokinesis, the cytoplasm divides and two daughter cells are formed (adapted from The Cell a Molecular Approach, 2000).

The formation of the spindle, chromosome capture and chromosome alignment constitute pathways that are monitored by a cell cycle checkpoint, which prevents mitotic exit in case either of these processes does not occur properly (Cleveland et al., 2003). The checkpoint prevents exit from mitosis by inhibiting the activity of the anaphase-promoting complex (APC), an ubiquiting ligase complex that controls sister chromatids separation and exit of mitosis. The checkpoint uses a network of proteins to inhibit the activity of the APC until all chromosomes are properly aligned, at which point the checkpoint is silenced allowing the APC to promote anaphase onset (Scholey et al., 2003). The ability of the checkpoint to monitor the status of chromosome alignment is achieved by specific proteins that

localise to the kinetochore when microtubules either do not bind or are unable to exert tension across sister kinetochores.

1.2. Mitotic Spindle

Chromosome segregation is mediated by a complex protein superstructure called the mitotic spindle. It was first described by Boveri at the turn of the century as a system of astral rays extending between the centrosomes of a diving cell (Nurse, 2000). The spindle is composed mostly of microtubules that are arranged in two antiparallel arrays with their fast growing plus ends at the equator and their slow growing or depolymerising minus ends at the centrosomes (Karsenti and Vernos, 2001). The three classes of microtubules within the spindle are defined by the position of their plus ends (Compton, 2000). Astral microtubules are nucleated from the spindle poles (centrosomes) and extend away from the cell centre toward the cortex. This interaction with the cell cortex plays an important role in positioning and orientating the spindle within the cell and determining the localization of the cleavage furrow during cytokinesis. A second class of microtubules, interpolar microtubules, grows from each pole into the central spindle and interacts with each other in an antiparallel fashion. They appear to confer stability to the spindle and are capable of moving spindle poles relative to one another. The third class of microtubules are the kinetochore microtubules, that extend from the spindle pole to chromosomes, where they contact kinetochores. In most eukaryotes, kinetochores interact with a number of microtubules forming a kinetochore bundle also referred to as a kinetochore fiber (Karsenti and Vernos, 2001; Wittmann et al., 2001).

There are significant changes in the behaviour and organization of microtubules as the cell progresses from interphase to mitosis. In general, spindle assembly appears to depend on two modifications in microtubule behaviour: a shift from one to two microtubule organizing centres and the stable interphase microtubule population is replaced by a highly dynamic and unstable microtubule population (Compton, 2000). In addition, spindle associated molecular motors and microtubule associated proteins (MAPs) like Dynein, NuMA, Kin C and bim C

family, alter their behaviour so as to contribute towards changes in microtubule dynamics that underlay the formation of the microtubule bipolar array (Sharp et al., 2000; Wittmann et al., 2001). Current models suggest that the activities of microtubules and their associated motors are responsible for the carefully orchestrated sequence of movements that underlie mitosis.

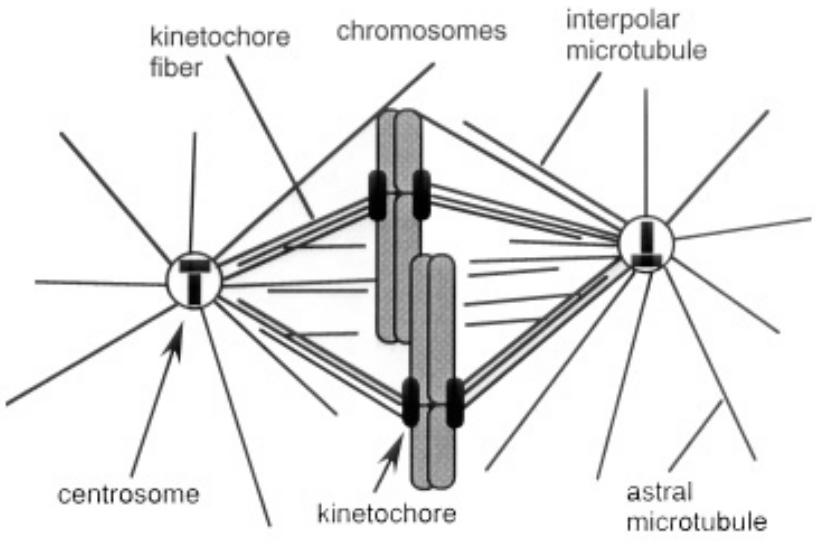

Figure 3. Organization of the mitotic spindle during metaphase. The diagram show the organization of the cell at metaphase including the centrosomes, chromosomes, kinetochores, astral microtubules, interpolar microtubules and kinetochore microtubules (adapted from Compton, 2000).

1.3. Microtubules

Microtubules are dynamic polar polymers of α and β tubulin heterodimers subunits that normally organize in thirteen linear protofilaments to form a 25 nm diameter cylindrical structure (Figure 4) (Wade and Hyman, 1997). Tubulin was first purified by is affinity for colchicine, a natural drug that binds tubulin and arrests cells in mitosis because it prevents polymer growth (Borisy and Taylor, 1967; Weisenberg et al., 1968). Much later, it was shown that β tubulin binds and also hydrolysis GTP (Weisenberg et al., 1976), a nucleotide that is required for the dynamic behaviour of microtubules.

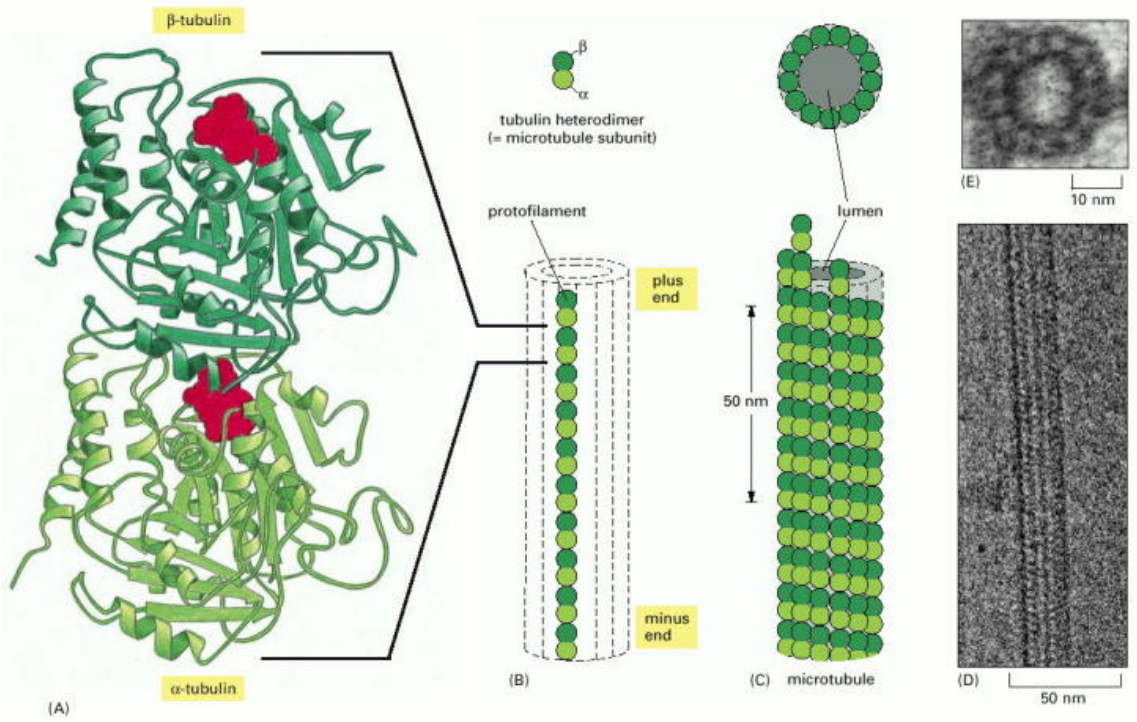

Figure 4. The structure of a microtubule and its subunit. (A) The subunit of each protofilament is a tubulin heterodimer formed from a very tightly linked pair of α and β tubulin monomers. The GTP molecule in the α tubulin monomer is so tightly bound that it can be considered an integral part of the protein. The GTP molecule in the β tubulin monomer, however, is less tightly bound and has an important role in filament dynamics. Both nucleotides are shown in *red*. (B) One tubulin subunit (α-β heterodimer) and one protofilament are shown schematically. Each protofilament consists of many adjacent subunits with the same orientation. (C) The microtubule is a stiff hollow tube formed from 13 protofilaments aligned in parallel. (D) A short segment of a microtubule viewed in an electron microscope. (E) Electron micrograph of a cross section of a microtubule showing a ring of 13 distinct protofilaments. (D) Electron micrograph of a microtubule (adapted from Molecular Biology of the Cell, 2002)

Microtubules are highly dynamic and can switch stochastically between phases of grow or shrinkage, both *in vivo* and *in vitro* (Nogales, 2000). This non-equilibrium behaviour is based on the binding and hydrolysis of GTP by tubulin heterodimers. Each tubulin monomer binds one molecule of GTP. The nucleotide binding site on α tubulin is non-exchangeable and is referred to as the N site, whereas the binding to β tubulin at the E site is exchangeable (Spiegelman et al., 1977). Only dimers with GTP in their E site can polymerize into a protofilament, but after polymerization, this nucleotide is rapidly hydrolysed and becomes non-exchangeable. In the late 1970s, *in vitro* observations of continuous incorporation

of tubulin into microtubules at steady state (when polymer level is constant) led to the concept of treadmilling (Margolis and Wilson, 1978; Rodionov and Borisy, 1997). At steady state, a treadmilling polymer has constant assembly of α-β heterodimers at one end, with a balanced loss of α-β heterodimers at the opposite end. However, when microtubules were first grown out of centrosomes it was observed that microtubules never really reach a constant length, they display a behaviour named dynamic instability (Mitchison and Kirschner, 1984) (Figure 5). According to this model, a single microtubule never reaches a steady state length but persists in prolonged states of polymerization or depolymerization that interconvert infrequently (Desai and Mitchison, 1997). In sumary, GTP-tubulin is preferentially incorporated at the fast growing or polymerizing end (plus end) but soon after the dimer is incorporated the bound GTP is hydrolysed and Pi is subsequently released. The transition from polymerization to depolymerization is referred as a catastrophe and is characterized by the rapid loss of GDP-tubulin subunits and oligomeres from the microtubule end (Walker et al., 1988). Depolymerising microtubules can also infrequently transit back to the polymerization phase, which is termed as rescue (Walker et al., 1988; Desai and Mitchison, 1997).

Another important issue is that overall the microtubule maintains a defined polarity, which is a consequence of the different polymerization rates of the two ends of the microtubule. The faster growing end is the plus end and the slower the minus end (Allen and Borisy, 1974). In addition, the fact that α-β heterodimers are arranged head-to-tail in the microtubule lattice also contribute to the polar lattice. Later on, a consensus has been reached on the orientation of the α-β heterodimer relative to the polarity of the microtubule lattice. Henceforth, β tubulin is exposed at the plus end and α tubulin is exposed at the minus end of the microtubule (Mitchison, 1993). In the context of a diving cell, all microtubules growth out of the Microtubule Organizing Centre (MTOCs), the centrosome in animal cells, and therefore have their minus ends anchored there while the plus ends reach the cortex or the cell centre.

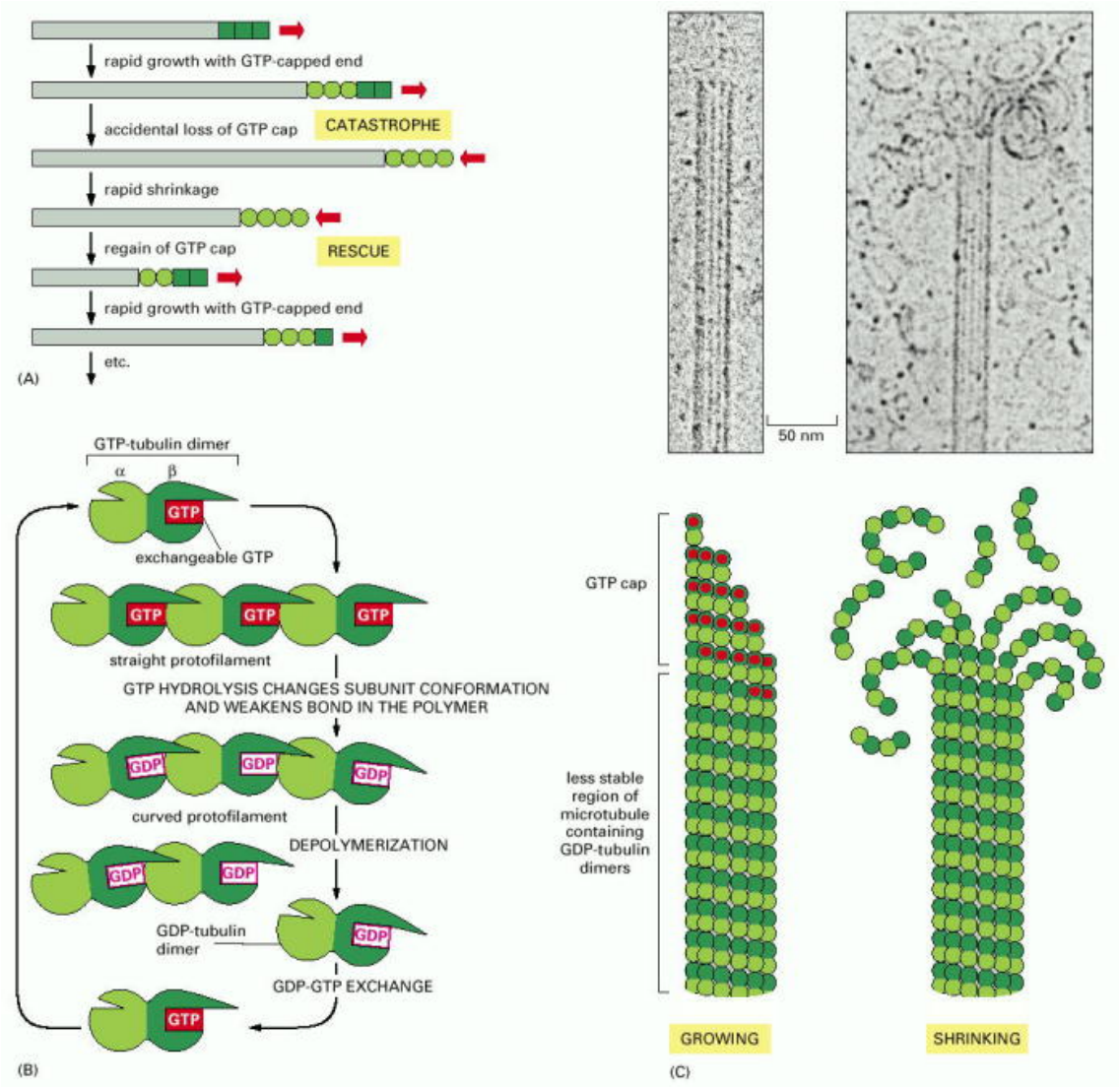

Figure 5. Dynamic instability of microtubules. (A) If the free tubulin concentration is between the critical values, a single microtubule end may undergo transitions between a growing state and a shrinking state. A growing microtubule has GTP-containing subunits at its end, forming a GTP cap. If nucleotide hydrolysis proceeds more rapidly than subunit addition, the cap is lost and the microtubule begins to shrink, an event called a "catastrophe." However, GTP-containing subunits may still add to the shrinking end, and if enough add to form a new cap, then microtubule growth resumes, an event called "rescue." (B) Model for the structural consequences of GTP hydrolysis in the microtubule lattice. The addition of GTP-tubulin subunits to the end of a protofilament causes the end to grow in a linear conformation. Hydrolysis of GTP after assembly changes the conformation of the subunits and tends to force the protofilament into a curved shape. (C) In an intact microtubule, protofilaments made from GDP-tubulin subunits are forced into a linear conformation given a stable cap of GTP-containing subunits. Loss of the GTP cap, however, allows the GDP protofilaments to relax into their more curved conformation. This leads to a progressive disruption of the microtubule. Above the drawings of a growing and a shrinking microtubule, electron micrographs show actual microtubules in each of these two states. Note particularly the curling, disintegrating GDP-containing protofilaments at the end of the shrinking microtubule (adapted from Molecular Biology of the Cell, 2002).

The polarity of microtubules has very important repercussions for the GTP cap model (Carlier et al., 1984; Mitchison and Kirschner, 1984). In this model, the microtubule lattice, made of GDP tubulin, is unstable, and the microtubule structure is stabilized by a layer of tubulin heterodimers at the plus end that still retain their GTP, the GTP cap (Nogales, 2000). When this cap is lost, which could occur by hydrolysis of GTP to GDP in the E site, the microtubule rapidly depolymerize and the dynamic instability increase (Davis et al., 1994). A GTP cap is generated when the rate of heterodimers addition is faster than that of GTP hydrolysis. However for a long time scientists speculate if the stabilizing cap at microtubules ends could be composed of either GTP-tubulin or GDP-Pi-tubulin (Desai and Mitchison, 1997), but later studies showed that GDP-Pi-tubulin do not stabilize microtubules (Caplow and Shanks, 1998; Caplow and Fee, 2003). Studies of tubulin polymerization in the presence of nonhydrolyzable GTP analogues GMPPNP and GMPPCP led to the conclusion that polymeryzation does not require GTP hydrolysis (Caplow, 1992; Hyman et al., 1992). Attempts to measure the size of the GTP cap required to stabilize a microtubule have established that only a few GTP heterodimers are sufficient to stabilize a growing microtubule (Drechsel and Kirschner, 1994; Caplow and Shanks, 1996).

Since Cryo-EM was used to study microtubules polymerization, a relationship between microtubule dynamics and the conformation of shrinking and growing plus ends comes into sight. Growing microtubules have relatively straight ends in an open sheet structure, whereas depolymerising ends contain highly curved protofilaments oligomers (Figure 5) (Mandelkow et al., 1991).

Nowadays, the current models of microtubule dynamics generally accept that treadmilling and dynamic instability are two processes that are likely to coexist in cells, and may account for the execution of different processes such as kinetochore capture during early mitosis and passive transport of associated organelles, including chromosomes (Waterman-Storer and Salmon, 1997; Margolis and Wilson, 1998). On the other hand, it has been shown that many proteins that bind microtubules (MAPs) appear to regulate microtubule dynamics and therefore modulate the polymerization dynamics of microtubules.

1.4. MAPs and Molecular Motors

Microtubule associated proteins (MAPs), known to modulate the behaviour of microtubules dynamics, were originally identified as proteins that copurified with tubulin through repeated cycles of microtubule polymerization and depolymerization (Olmsted, 1986; Maiato et al., 2004). Some promote microtubule polymerization and stability while others induce depolymerization. MAPs are divided in two major groups: the nonmotors MAPs and the molecular motors.

MAP4 was the first nonmotor MAP, initially described as a stabilizing factor (Bulinski and Borisy, 1980), is evolutionarily conserved from *Drosophila* (DMAP205) (Goldstein et al., 1986) to human. MAP4 promotes microtubule assembly *in vitro* by strongly enhancing the rescue frequency without decreasing the catastrophe frequency (Ookata et al., 1995). Another group of classical MAPs included MAP1, MAP2 and tau (Mandelkow and Mandelkow, 1995). These proteins are present in neurons and bind to, stabilize and promote microtubule polymerization. More recently, the Dis1-TOG family of MAPs has been intensively studied because of their ability to either stabilize or destabilize the microtubule polymer, promoting microtubules turnover. XMAP215 was initially described in *Xenopus* (Gard and Kirschner, 1987) as a protein required to promote microtubule growth. Later several others homologues have been identified, including the human ch-TOG (Charrasse et al., 1995; Charrasse et al., 1998); Stu2p in *S.cerevisiae* (Wang and Huffaker, 1997) and Msps in *Drosophila* (Cullen et al., 1999). All Dis1-TOG family members localize to interphase microtubules and are found throughout the spindle during mitosis.

Molecular motors are also MAPs but with the special capability to convert chemical energy form ATP into force and/or movement (Barton and Goldstein, 1996). Microtubule based motors proteins form two distinct families of ATP-dependent force-generating enzymes, the kinesins and the dyneins (Sharp et al., 2000). These two families differ mostly in their size and direction of movement along microtubules. Kinesins were first found in neural tissue, where they appear to generate plus end-directed movement needed for axonal transport. There are three different classes of kinesins: N-terminal kinesins, C-terminal kinesins and

bipolar kinesins. The bipolar kinesins like the conserved Bim C family (Bim C/Eg5/KLP61F9) move toward the plus end of microtubules and are required for bipolar spindle formation (Hagan and Yanagida, 1990; Sawin et al., 1992; Heck et al., 1993). On the other hand, C-terminal kinesins that include the protein Ncd/Kar3p are minus end directed motors and exert forces that antagonise the activity of bipolar kinesins during mitosis (Endow et al., 1990; Walker et al., 1990; Hoyt et al., 1993).

Dynein was first discovered in cilia and flagella, where it powers microtubule sliding in the axoneme by generating minus end-directed microtubule movement and plays a role in the assembly of mitotic spindle (Gibbons, 1988). Cytoplasmatic dynein works in close cooperation with dynactin an activating multisubunit complex (Karki and Holzbaur, 1999).

In addition, an important class of MAPs includes microtubule destabilizing factors. Such factors destabilize microtubules, reducing net assembly and increasing catastrophe rate. The first microtubule-destabilizing factor to be discovered was katanin, which is an ATPase that utilizes energy from nucleotide hydrolysis to mediate microtubule depolymerization (Vale, 1991; McNally and Vale, 1993). Katanin localize to centrosomes throughout the cell cycle and was suggested as a candidate to mediate disassembly of microtubule minus ends during poleward flux of tubulin (McNally et al., 1996). Op18/stathmin was first purified as a microtubule destabilizing factor (Belmont et al., 1996; Belmont and Mitchison, 1996; Cassimeris, 2002). Op18/stathmin was shown both to serve as a tubulin-sequestering enzyme and to induce tubulin GTPase activity (Howell et al., 1999; Larsson et al., 1999). Immunodepletion of Op18/stathmin caused a large increase in the amount of polymerized tubulin in mitotic asters, affecting both the length and density of microtubules (Belmont and Mitchison, 1996). A third class of proteins implicated in microtubule destabilization is the Kin I members of the kinesin superfamily (Desai et al., 1999). Some examples are *Xenopus* XKCM1 (Walczak et al., 1996) and the mammalian MCAK (Wordeman and Mitchison, 1995). Unlike other motors, MCAK/XKCM1 do not move along microtubules, but instead are thought to couple ATP hydrolysis with the bending of the protofilament at the plus ends, resulting in depolymerization (Desai et al., 1999; McNally, 1999;

Moores et al., 2002). Inactivation of XKCM1 in mitotic xenopus egg extracts results in large asters of long, nondynamic microtubules that are incapable of forming a mitotic spindle (Kline-Smith and Walczak, 2002). On the other hand, depletion of centromeric MCAK leads to chromosome congression and segregation defects due to improper kinetochore attachments (Kline-Smith et al., 2004). Localization and activity of MCAK are regulated by Aurora A kinase which inhibits MCAK's depolymerising activity (Andrews et al., 2004; Lan et al., 2004; Ohi et al., 2004).

1.5. Microtubule Plus End Tracking Proteins

The microtubule plus end tracking proteins (+TIPs) is a distinct class of MAPs that accumulate to the growing microtubule plus ends; they are implicated in local control of microtubule assembly and in the attachment of microtubules to the cell membrane or kinetochores (Schuyler and Pellman, 2001). However, the molecular mechanisms behind this tracking behaviour and the cellular significance of plus end tracking are still unknown. Plus end tracking may occur as a result of specific attachment of proteins to the polymerised microtubule tip coupled with their dissociation from the older part of the microtubule, the treadmilling model. In principle, treadmilling consists in a balance between binding and release of +TIPs from the MT leading to plus-end accumulation (Carvalho et al., 2003). Plus end tracking can also be caused by motor dependent transport to microtubule plus end or association of proteins with other +TIPs, named hitchhiking (Carvalho et al., 2003; Howard and Hyman, 2003; Akhmanova and Hoogenraad, 2005; Wittmann and Desai, 2005).

The first MAP to show +TIP behaviour was CLIP-170 (Perez et al., 1999). Analysis of the dynamic distribution of CLIP-170 expressed as a fusion protein with GFP (green fluorescence protein) and imaged by video microscopy in living cells showed GFP-CLIP-170 as a comet-like structure that accumulated only at the growing plus ends of microtubules. This unusual tracking behaviour of the protein was first explained as resulting from treadmilling (Diamantopoulos et al., 1999). On the other hand, Bik1 and tip1p, the CLIP-170 homologues from *S. cereviseae* and *S. pombe* form a complex with kip2 and tea2p kinesins, respectively, suggesting

that they reach the plus end of microtubules by motor dependent transport (Busch et al., 2004; Carvalho et al., 2004; Maekawa and Schiebel, 2004). Furthermore, tip1p was shown that although it binds directly to microtubules, its localization to the growing microtubules plus ends depends upon mal3p the *S. pombe* homologue of EB1, a MAP that itself displays +TIP behaviour suggesting that they form a complex (Busch and Brunner, 2004). It was also proposed that CLIP-170 targets to the microtubule plus ends by recognition of a specific structural feature of the plus end (the GTP cap or the open sheet of the polymer) or by co-polymerization with tubulin heterodimers during microtubule polymerization (Diamantopoulos et al., 1999). Recent studies suggest that CLIP-170 targets specifically at microtubule plus ends by co-polymerization with tubulin but these studies do not exclude the possibility of a direct recognition of structural features at MT ends (Arnal et al., 2004). The activity of CLIP-170 is regulated by phosphorylation, inhibiting the binding to microtubules (Rickard and Kreis, 1991). Therefore, phosphorylation has been proposed as the favourite mechanism to explain the release from the microtubule lattice. However, recent studies have shown that phosphorylation of CLIP-170 might also promote microtubule binding making it difficult to understand how this posttranslational modification affects the behaviour of CLIP-170 (Choi et al., 2002). In addition, a recent study found that CLIP-170 had intramolecular head-to-tail interactions and suggested that this conformational change that appears to be controlled by phosphorylation, inhibits the binding of CLIP-170 to microtubules (Lansbergen et al., 2004). Finally, it has also been shown that conformational changes of the microtubule can decrease the affinity of CLIP-170 to tubulin (Arnal et al., 2004). More work is clearly required in order to determine what are the molecular mechanisms involved in regulating the ability of CLIP-170 to interact with microtubules.

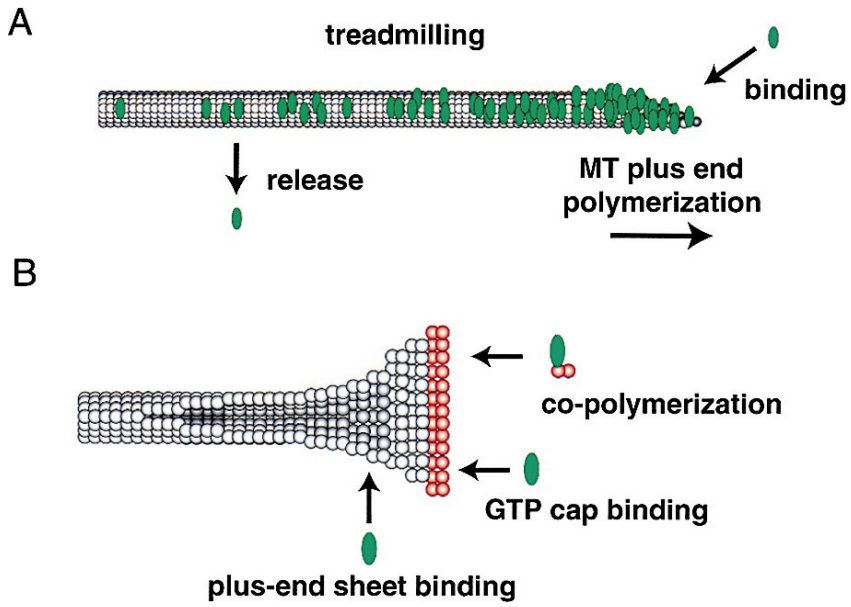

Figure 6. Microtubule plus end treadmilling. (A) Microtubule plus end tracking proteins are thought to bind the polymerizing end of the microtubule and then fall off behind the region of growth. (B) Three models exist to explain the treadmilling: the first two are based on the idea that these proteins recognize specific structural feature of the growing plus end, either the GTP-bound tubulin cap or the open sheet of the polymer. The third model proposes that these proteins bind to free tubulin heterodimers and are then co-assembled into the plus ends during microtubule polymerization (Schuyler and Pellman, 2001).

The APC (adenomatous polyposis coli) protein is the product of the tumor suppressor gene APC, which is mutated in familial adenomatous polyposis and during the progression of colorectal cancer (Nakamura, 1993). It is well established that APC regulates the transcription of target genes by promoting the destruction of β-catenin (Polakis, 1999), a key component of a multiprotein transcription factor complex. Mutations in β-catenin that stabilize this protein and prevent its destruction are known to cause colon cancer (Pellman, 2001). APC also localizes to the microtubule cytoskeleton, as well to the leading edges of migrating epithelial cells (Nathke et al., 1996). Analysis of GFP-tagged APC in living cells has revealed in detail the peculiar behaviour of APC (Mimori-Kiyosue et al., 2000a; Mimori-Kiyosue and Tsukita, 2001). It was shown that APC-GFP moved along microtubules towards their distal end in an ATP-dependent manner and accumulated at the growing plus ends, and when microtubules begin to shorten, the APC is released from the microtubules ends. APC provides the best example of motor-dependent transport to the plus ends, but also illustrate how a

combination of mechanisms might contribute to plus end targeting (Carvalho et al., 2003). APC interacts with microtubules in tree different ways: direct binding via a microtubule biding domain, hitchhiking by interaction with EB1 and motor-dependent transport (Askham et al., 2000; Mimori-Kiyosue et al., 2000a; Jimbo et al., 2002).

EB1 was initially identified in a yeast two hybrid screen as a protein that interacts with the C-terminal of APC (Su et al., 1995). The EB1–APC interaction is likely to be critical for tumorigenesis/metastasis of intestinal epithelial, probably through a loss of the normal polarity of the epithelia (Slep et al., 2005). EB1 is part of a conserved family of MAPs that include Bim1p from budding yeast (Schwartz et al., 1997), mal3p from fission yeast (Beinhauer et al., 1997) and dEB1 from *Drosophila* (Lu et al., 2001). It localizes to the centrosomes, the mitotic spindle and the distal tips of cytoplasmatic microtubules (Berrueta et al., 1998; Morrison et al., 1998). The analysis of the dynamic behaviour of EB1 shows that when the GFP-tagged protein is expressed in cells it concentrates at the growing ends of cytoplasmatic microtubules and disappears when microtubules start to shorten (Mimori-Kiyosue et al., 2000b). A recent study provided direct evidence for the treadmilling of EB1, suggested that plus end accumulation is independent of molecular motors and is due to a preferential binding to a special feature at microtubule plus end (Tirnauer et al., 2002). In addition, *in vitro* studies concluded that there is no association between EB1 and tubulin heterodimers (Gache et al., 2005).

1.6. MAST

The first mutant allele of the *mast* (*multiple asters*) gene (*mast*^{1}) was first identified from a collection of P-element insertion mitotic mutants in *Drosophila* melanogaster (Omel'ianchuk et al., 1997). Subsequently, two other P-element induced alleles, *mast*^{2} and *mast*^{3}, were identified from the Berkeley Drosophila Genome Project (BDGP) collection. A fourth allele, *mast*^{4}, an imprecise excision allele, was obtained after remobilization of the P-element in *mast*^{1} (Lemos et al., 2000). Cells carrying *mast* mutants alleles show severe mitotic abnormalities

including highly condensed chromosomes, highly polyploidy cells and few abnormal anaphases. Quantification of mitotic figures with respect to mitotic progression indicates that all mutant alleles cause a decrease in the number of cells in prophase, a significant increase of cells in prometaphase/metaphase and a decrease in the proportion of cells in anaphase or telophase. In conclusion, mutations in *mast* cause severe abnormalities in chromosome segregation leading cells to arrest at prometaphase/metaphase. However, the arrest can be overcome and cells undergo multiple rounds of proliferation since most of them are polyploidy (Lemos et al., 2000).

The *mast*[1] was mapped by in situ hybridisation to the 78C1-C2 cytological region of chromosome 3 (Lemos et al., 2000). This region of the genome produces basically a single transcript (cDNA LD11488) composed by 5938 bp, containing a single ORF of 1491 amino acids, encoding for a protein with 165.5 kD. This protein contains a centrally located highly basic region (pI of 11) of 472 amino acids, flanked on both sides by short stretches of acidic residues (Inoue et al., 2000). Analysis of the protein sequence showed that Mast contains, within the basic domain, a 170 amino acid region that shares limited homology with the proline-rich domain of MAP4 that is thought to be involved in the high efficiency binding to microtubules (Lemos et al., 2000). It also contains two regions with significant homology to the HEAT repeat at positions 169-207 and 1414-1452 amino acids. Within the basic domain there are two consensus sites for phosphorylation by cyclin-dependent kinase 1 (cdk1), and two putative GTP-binding motifs. The motif GGGTGTG (residues 544-550) closely resembles the glycine rich peptide, which interacts with the guanine or phosphate groups of the bound GTP in the β tubulin and in the E. coli FtsZ protein (Nogales et al., 1998). The sequence NKLD (residues 400-403) correspond to the NKXD (X for any amino acid residue) consensus motif which can interact with the purine base of the bound nucleotide in the GTPase superfamily (Inoue et al., 2000). Protein sequence analysis also indicates that Mast shares some similarity to proteins from the dis1-TOG family like ch-TOG (Charrasse et al., 1998), XMAP215 (Vasquez et al., 1994) and Msps (Cullen et al., 1999). In addition, the region between 609 and 742 amino acids of Mast shares identity with the basic domain of APC, which is responsible for the

interaction of APC with microtubules (Deka et al., 1998). Also, the region between 535 and 736 residues are 25% identical and 39% similar with the proline rich domain of MAP4 (Aizawa et al., 1990).

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   1 MAYRKPSDLD GFIQQMPKAD MRVKVQLAED LVTFLSDDTN SIVCTDMGFL IDGLMPWLTG
  61 SHFKIAQKSL EAFSELIKRL GSDFNAYTAT VLPHVIDRLG DSRDTVREKA QLLLRDLMEH
 121 RVLPPQALID KLATSCFKHK NAKVREEFLQ TIVNALHEYG TQQLSVRVYI PPVCALLGDP
 181 TVNVREAAIQ TLVEIYKHVG DRLRPDLRRM DDVPASKLAM LEQKFDQVKQ EGLLLPSALK
 241 NTNGNGVGLD EADNIGLRER PTRMIKRPLH SAVSSSLRPK PNVNDVTGDA GAVTMESFES
 301 SFEVVPQLNI FHAKDMDDIY KQVLVIISDK NADWEKRVDA LKKIRALLIL SYHTQPQFVA
 361 VQLKELSLSF VDILKEELRS QVIREACITI AYMSKTLRNK LDAFCWSILE HLINLIQNSA
 421 KVIASASTIA LKYIIKYTHA PKLLKIYTDT LNQSKSKDIR STLCELMVLL FEEWQTKALE
 481 RNATVLRDTL KKSIGDADCD ARRHSRYAYW AFRRHFPELA DQIYGTLDIA AQRALERERE
 541 GGGGGGTGTG TGTAPETRRT VSRIGRTPGT LQKPTPSMRS ISAVDTAAAQ RAKVRAQYTL
 601 YSRQRKPLGP NNSNQASMTG AAASGSLPRP RLNSNSGGTP ATTPGSVTPR PRGRAGVSQS
 661 QPGSRSTSPS TKLRDQYGGI GNYYRGATGA IPKKASGIPR STASSRETSP TRSGGGLMKR
 721 SMYSTGAGSR RTPERNNPVR PSAAARLLAQ SREAEHTLGV GDDGQPDYVS GDYMRSGGMR
 781 MGRKLMGRDE SDDIDSEASS VCSERSFDSS YTRGNKSNYS LSGSHTRLDW STQRAPFDDI
 841 ETIIQFCAST HWSERKDGLI SLTQYLADGK ELTQQQLKCV LDMFRKMFMD THTKVYSLFL
 901 DTVTELILVH ANELHEWLFI LLTRLFNKLG TDLLNSMHSK IWKTLQVVHE YFPTQLQLKE
 961 LFRIISDSTQ TPTTKTRIAI LRFLTDLANT YCKSSDFPSD QSQACERTVL KLAQLAADQK
1021 SMELRSQARS CLVALYNLNT PQMTLLLADL PKVYQDSARS CIHSHMRRQS QSCNSGANSP
1081 SSSPLSSSSP KPLQSPSVGP FASLQSHHHQ LSISSTSPRS RQSSVEQELL FSSELDIQHN
1141 IQKTSEEIRH CFGGQYQTAL APNGFNGHLQ YHDQGQQDSC ASLSSNSKTQ SSANTTQSNT
1201 PESATMRLDN LERERTTQNA KSPTDDAKVI TVSINMAENG ELILASNLME SEVVRVALTL
1261 TKDQPVELLQ TSLTNLGICI KGGNCELPNK HFRSIMRMLL NILEAEHTDV VIAGLHVLSK
1321 IMRSNKMRHN WMHFLELILL KIIQCYQHSK EALRDIDSMI PRIAPSLPLD LSINIVNPVI
1381 ATGEFPTNLC AIKILLEVTE HHGSEITDAH LDIVFPNLAR SADDTQSMVR KAAVFCIVKL
1441 YFVLGEEKVK PKLSVLNPSK VRLLNVYIEK QRNCISGGGS STKNSSAASS S
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Figure 8. Amino acid sequence of Mast. Red regions represent HEAT repeats; green regions represent the putative GTP binding motifs and the blue regions the predicted sites of phosphorylation by cdk1. The yellow region defines the conserved MAP4 microtubule binding domain.

Mast protein is evolutionary conserved since it shares significant identity with proteins encoded by two human cDNAs, KIAA0622 and KIAA0627 (Ishikawa et al., 1998), three putative proteins in *C. elegans*, CO7H6.3, R107.6 and ZC84.3 (Wilson et al., 1994) and also limited identity with Stu1p from *S. cerevisiae* (Pasqualone and Huffaker, 1994) and its putative homologue in *S. pombe*, SpStu1p. Therefore, Mast and its homologues define a new evolutionary conserved protein family, that was named Mast/Orbit since the same gene was independently described as Orbit (Inoue et al., 2000).

At interphase, Mast localises to centrosomes and shows a punctuate pattern of co-localizing with α tubulin (Lemos et al., 2000). During mitosis, Mast is localized to the mitotic spindle, centrosomes and kinetochores, ending up accumulating in the central region of the spindle and at the midbody (Inoue et al., 2000; Lemos et al., 2000). Mast was classified as a microtubule associated protein (MAP) by its ability to bind microtubules *in vitro* and *in vivo* (Lemos et al., 2000).

The human homologues of Mast, CLASPs (CLIP-associated proteins), were initially identified from a yeast two-hybrid screen using as bait a conserved region from CLIP-115 (Akhmanova et al., 2001; McNally, 2001). Both CLASP1 and CLASP2 localize to the plus ends of growing microtubule in living interphase and mitotic cells suggesting that CLASPs belong to the +TIP protein family (Maiato et al., 2003a). Akhmanova and colleagues suggested that microtubule binding by CLASPs is negatively influenced by phosphorylation (Akhmanova et al., 2001). A model proposed for CLASPs suggested that localization at the microtubule plus ends is by recognition of the open sheets or by copolymerization with the tubulin heterodimer due to direct interaction with β-tubulin bound to GTP (Maiato et al., 2003a) and even by direct binding (Mimori-Kiyosue et al., 2005).

To further elucidate the function of Mast during mitosis, Maiato and colleagues performed *in vivo* analysis of mitotic progression in *mast* mutant embryos and in *Drosophila* S2 cells after Mast RNA-mediated interference (RNAi) (Maiato et al., 2002; Maiato et al., 2003b). Mutant embryos in mitosis were characterized by the presence of monopolar spindles organized from one or two centrosomal foci with chromosomes disperse within the aster. *In vivo* analysis of these mutant embryos showed that in the absence of Mast the bipolar spindle forms, chromosomes never congress properly and then the spindle collapses giving rise to the monopolar configuration. After Mast RNAi, most mitotic cells were in a prometaphase-like stage, displaying either monopolar spindles, with chromosomes buried close to the centre of the aster or bipolar spindles but with chromosomes that were not aligned at the metaphase plate. Immunofluorescence of Mast RNAi cells with kinetochores markers like BubR1 and Cid revealed that BubR1 was present in all chromosomes, clearly indicating that sister chromatids did not separate before migrating to the poles (Maiato et al., 2002). Furthermore,

when microtubule dynamics was suppressed by treatment with low taxol doses, Mast depleted cells formed a monopolar aster but this time chromosomes localized at the periphery of the aster associated with the plus ends of microtubule bundles. Thus, Mast appeared to be required for proper chromosome congression, for proper microtubule-kinetochore attachments and for the stability of the bipolar spindle (Maiato et al., 2002; Sharp, 2002).

Recent studies showed that Mast has an important role in maintaining the microtubule poleward flux, by allowing the incorporation of microtubule subunits at the kinetochore during metaphase (Maiato et al., 2005). Maiato and colleagues depleted Mast by RNAi from *Drosophila* S2 cell expressing GFP-α-tubulin and showed by photobleaching that mature K-fibres do not show poleward flux and that in these cells the spindle collapses as the k-fibres loose subunits preferentially at the minus ends. Analysis of k-fiber growth after severing with a high power laser also revealed that in the absence of Mast kinetochore bound microtubules are unable to grow. Taken together, these results suggest that Mast is essential to allow incorporation of tubulin subunits at the microtubules bound to the kinetochores. As kinetochore-attached microtubules continue to depolymerize at the minus ends the spindle collapses and the centrosomes move progressively towards the cell centre. Depleting Mast did not affect the targeting of other kinetochore proteins, and the same experiment realized in cells depleted of either dynein or EB1 showed that photobleached marks exhibited poleward motion similar to controls. Thus, the absence of microtubule subunit flux is due to direct involvement of Mast (Maiato et al., 2005).

Mast/Orbit is also implicated in *Drosophila* oocyte differentiation (Mathe et al., 2003). In dividing germline cysts Mast/Orbit is initially found on the mitotic spindle concentrated at the poles, then progresses onto the spindle remnant, from where it moves to the arrested cleavage furrow and fusome, suggesting it participates in interactions between these structures (Mathe et al., 2003). The localization of CLIP-190 to microtubules and to the fusome is Mast/Orbit dependent with which it forms a complex. In Mast/Orbit mutants, the stem cells loose their associated fusomes and the mitotic spindles of those that do divide are either diminutive or monopolar and do not make contact with the fusome. The

results suggested that Mast/Orbit appears to facilitate multiple interactions of the fusome with the mitotic spindle ensuring the polarized growth of the fusome and the correct division of the germline cells. Finally, Mast/Orbit is required during mid-oogenesis for the organization of the polarized microtubule network inside the cells that ensures oocyte differentiation (Mathe et al., 2003).

More recently, the role of Mast/Orbit in cytokinesis of *Drosophila* primary spermatocytes was addressed (Inoue et al., 2004). They showed that the central spindle is comprised of two microtubule populations: one located at the interior of the central spindle found within the spindle envelope and a separate one that they called peripheral, which included astral microtubules that appear to be involved in initiating the cleavage furrow at the site they contact the cortex. Mast/Orbit concentrates at the interior rather then the peripheral microtubules. In hypomorphic *mast*/*orbit* mutants the interior central spindle fails to form or is unstable; in contrast, peripheral microtubules still probe the cortex. Analysis of male meiotic divisions in *mast*/*orbit* mutants revealed cytokinesis defects and aberrant central spindles. The interior central spindle microtubules are more severely affected than those of the periphery and the furrow initiates but then may regress and fail to complete division. In addition, mutants had disorganized or incomplete anillin and actin rings and fail to recruit Pav-KLP and aurora B to interior central spindle microtubules (Inoue et al., 2004).

Since Mast appears to affect a number of microtubule-based phenomena, its role in neuronal growth has also been studied. Axon guidance requires coordinated remodelling of actin and microtubule polymers, however, very little progress has been made in identifying MAPs that participate in these specific pathways. From a genetic screen designed to identify neuronal defects, Mast/Orbit was identified as a partner of the Abelson (Abl) tyrosine kinase, a protein required for the accurate guidance of axon growth and that also modulates the function of several axonal receptors (Lee et al., 2004). Identical axon guidance abnormalities were found in *mast*/*orbit* and *Abl* mutants at the midline, where the repellent Slit restricts axon crossing. Genetic interactions and epistasis assays indicate that Mast/Orbit mediates the action of Slit and its receptors, acting downstream of Abl.

In addition, Mast/Orbit localizes to *Drosophila* axons and growth cones (Lee et al., 2004).

It is well established that Mast bind microtubules both *in vitro* and *in vivo* and this interaction seems to be independent of other factors. However, studies in Mast/Orbit suggested that it could bind microtubules in a GTP dependent manner (Inoue et al., 2000). In addition, Mast/Orbit has two putative GTP-binding motifs, which led these authors to speculate that Mast/Orbit could be itself a GTPase (Inoue et al., 2000; Kline-Smith and Walczak, 2000).

1.7. GTPases

GTPases are a large family of enzymes that can bind and hydrolyse GTP. GTPases play an important role in signal transduction, cell motility and intracellular trafficking (Liang et al., 2000). The GTPase superfamily is divided in three major groups: the heterotrimeric G proteins, the small GTPases and other less abundant special GTPases, the atypical GTPases, like tubulin.

Heterotrimeric G proteins consist of three different subunits, designated alpha (α), beta (β) and gamma (γ). The alpha subunit typically 39-52 kD performs the GTPase activity (binding and hydrolysis of GTP) of the heterotrimer. The beta (~35-38 kD) and gamma (~5-8 kD) subunits associate tightly with each other to form a protein complex. These proteins can be classified by sequence homology of the alpha subunit into four families: G_s, G_i, G_q and G_{12} and are activated by the interaction of the heterotrimer with a receptor. The immediate result of this interaction is displacement of GDP (the hydrolyzed form of GTP) from the alpha subunit by a molecule of GTP. Subsequently, the heterotrimer dissociates into an activated alpha subunit (with GTP) and the beta-gamma complex. Depending on the type of G-protein, the alpha subunit, the beta-gamma complex, or both may in turn bind to cellular target sites, thus passing the signal on to a diversity of intracellular effectors. Such effectors include protein kinases, ion channels, enzymes and cytoskeleton proteins.

Small GTPases are monomeric guanine nucleotide-binding proteins with molecular masses of 20-25 kD and serve as molecular switches to regulate growth, morphogenesis, cell mobility, axonal guidance, cytokinesis and trafficking. The first small GTPase to be discovered was Ras (Shih et al., 1980), and there are now approximately 60 different small GTPases that have been identified in mammalian cells. Based on structure, sequence and function, the Ras superfamily is divided into eight main families, each of which is further divided into subfamilies: Ras, Rho, Rab, Arf, Ran, Rheb, Rad and Rit. The small GTPases cycle between inactive (GDP-bound) and active (GTP-bound) states (Sprang, 1997; Wittinghofer, 1998). GTPases normally are GDP-bound and therefore inactive. Upon stimulation, GTPases release GDP and bind to GTP, a reaction accomplished by guanine nucleotide exchange factors (GEFs). In their active GTP-bound state, small GTPases interact with a variety of effector proteins to promote cellular responses. The active state of GTPases is transient because of their intrinsic GTPase activity, which is stimulated further by GTPase activating proteins (GAPs)(Vetter and Wittinghofer, 2001).

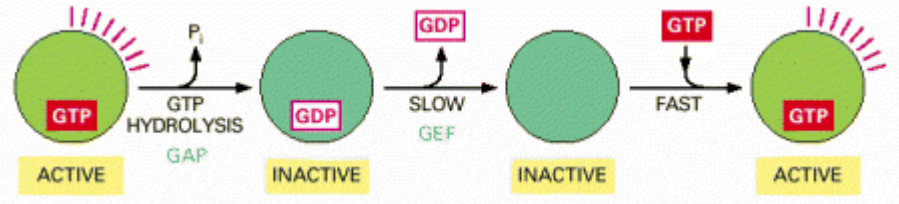

Figure 7. GTP-binding proteins as molecular switches. The activity of a GTP-binding protein (also called a GTPase) generally requires the presence of a tightly bound GTP molecule (switch "on"). Hydrolysis of this GTP molecule produces GDP and inorganic phosphate (P_i), which is stimulated by GAPs and it causes the protein to convert to a different, usually inactive, conformation (switch "off"). As shown here, resetting the switch requires the tightly bound GDP to dissociate, a slow step that is greatly accelerated by GEFs; once the GDP has dissociated, a molecule of GTP is quickly rebound (adapted from Molecular Biology of the Cell, 2002).

Analysis and sequence comparison of GTPases has yielded further information on the general characterization of the GTP-binding domain, including three different consensus sequences. The GXXXXGKS/T motif, whereas X is any amino acid, called the P-loop; the NKXD motif that interacts with the nucleotide base and the DXXG motif that is specific for the guanine (Dever et al., 1987; Vetter and Wittinghofer, 2001).

Tubulin is a GTPase in which the GTP-bound form has a higher affinity for microtubule ends and promote microtubule stabilization and growth, whereas the GDP-bound form favours microtubule disassembly (Burns and Farrell, 1996). However, tubulin is an atypical GTPase for two reasons. First, Tubulin primary sequence lacks the consensus sequences of the conventional GTPase superfamily members. Nevertheless, a consensus sequence, GGGTGSG, called the tubulin signature is thought to be responsible for the GTPase activity of tubulin (Nogales, 2000). Second, tubulin is it own GAP, since interaction between tubulin heterodimers stimulated GTP hydrolysis.

2. Results

2. Results

2.1. Cell cycle distribution and +TIP behaviour of Mast

Human CLASPs show +TIP behaviour both during interphase and mitosis (Akhmanova et al., 2001; Maiato et al., 2003a; Mimori-Kiyosue et al., 2005), however the dynamic behaviour of Mast has not yet been reported. To address this issue, we expressed the full Mast protein in *Drosophila* S2 cells, as an N-terminal tagged EGFP fusion protein and analyzed the distribution of EGFP-Mast during mitosis and interphase (Figure 1). We found that EGFP-Mast follows a pattern of localization that is in all respects similar to that previously described (Inoue et al., 2000; Lemos et al., 2000) and also similar to CLASP1 (Maiato et al., 2003a). Indeed, during interphase, Mast co-localizes with microtubules and as the cell enters mitosis at prophase, Mast localizes preferentially to the centrosomes and with astral microtubules. During Prometaphase/Metaphase, Mast co-localizes with spindle microtubules, kinetochores and centrosomes. Throughout anaphase, Mast is found on microtubules, centrosomes and the central spindle. In telophase, Mast accumulates in the midbody but can be also seen associated with microtubules. Interestingly, we also observed that EGFP-Mast localizes to the plus ends of astral microtubules suggesting that it might display +TIP behaviour (see insert in Figure 1).

In order to analyze the dynamic localization of Mast to the microtubule plus-ends, we observed S2 cells expressing EGFP-Mast by time-lapse fluorescence imaging (Figure 2). The results show that Mast tracks along growing microtubules and is never associated with depolymerising ends. However, +TIP behaviour was only observed when EGPF-Mast was expressed at relatively low levels, since at high levels the protein was found to bind throughout the microtubule lattice and cause the formation of microtubule bundles which failed to display dynamic behaviour.

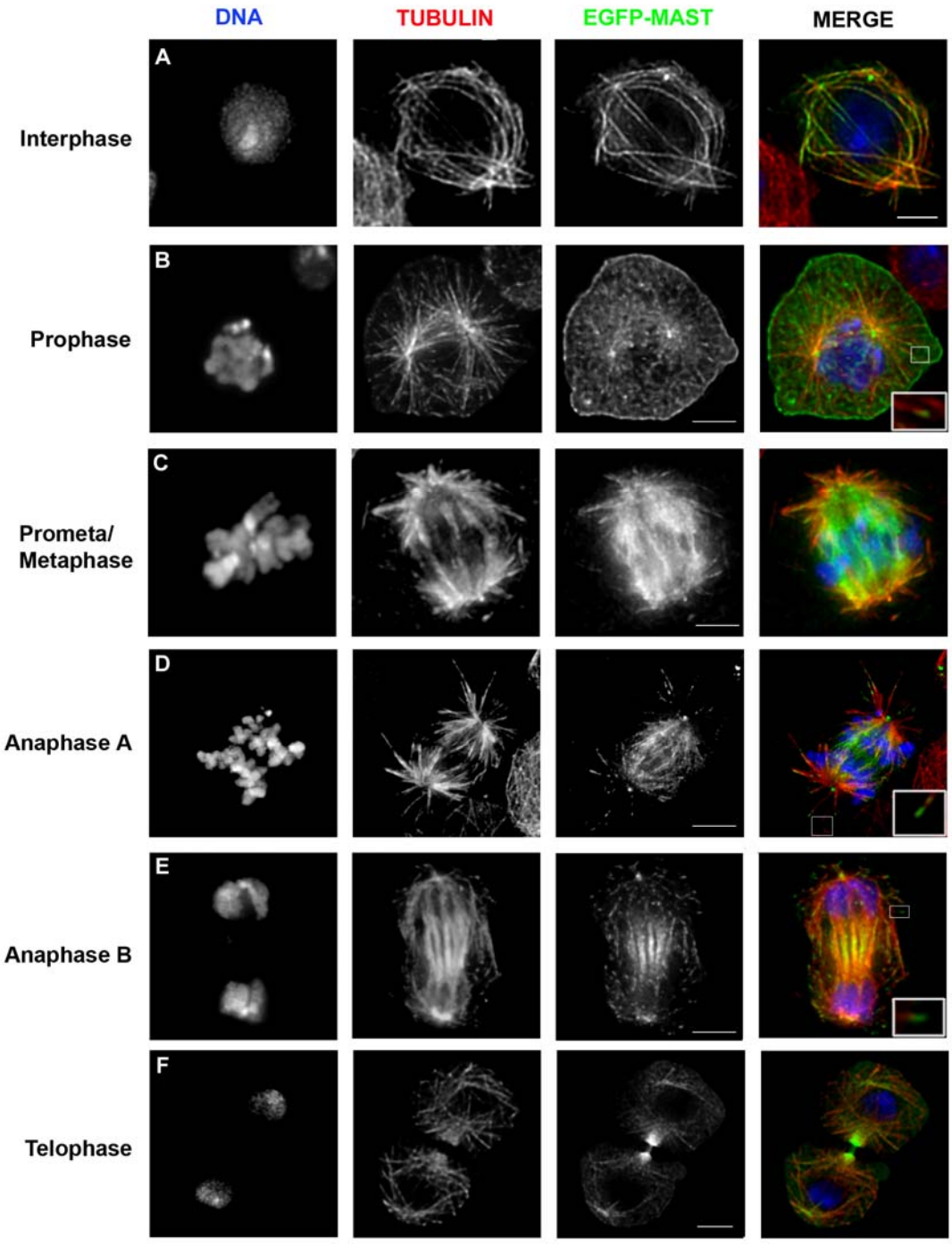

Figure 1. Cell cycle localization of EGFP-Mast. *Drosophila* S2 cells expressing EGFP-Mast were fixed and immunostained with anti-tubulin antibody and DAPI to show the DNA. (A) During interphase, Mast co-localizes with microtubules. (B) At prophase, Mast localizes preferentially to the centrosomes but can also be seen at the tips of astral microtubules (see insert). (C) During Prometaphase/Metaphase, Mast co-localizes with spindle microtubules and centrosomes. (D and E) Throughout anaphase, Mast is found on microtubules, centrosomes and the central spindle. Mast can also be easily detected at the plus ends of astral microtubules (see insert). (F) In telophase, Mast accumulates in the midbody but can be also seen associated with microtubules. Scale bar is 5 μm. Courtesy of Rita Reis.

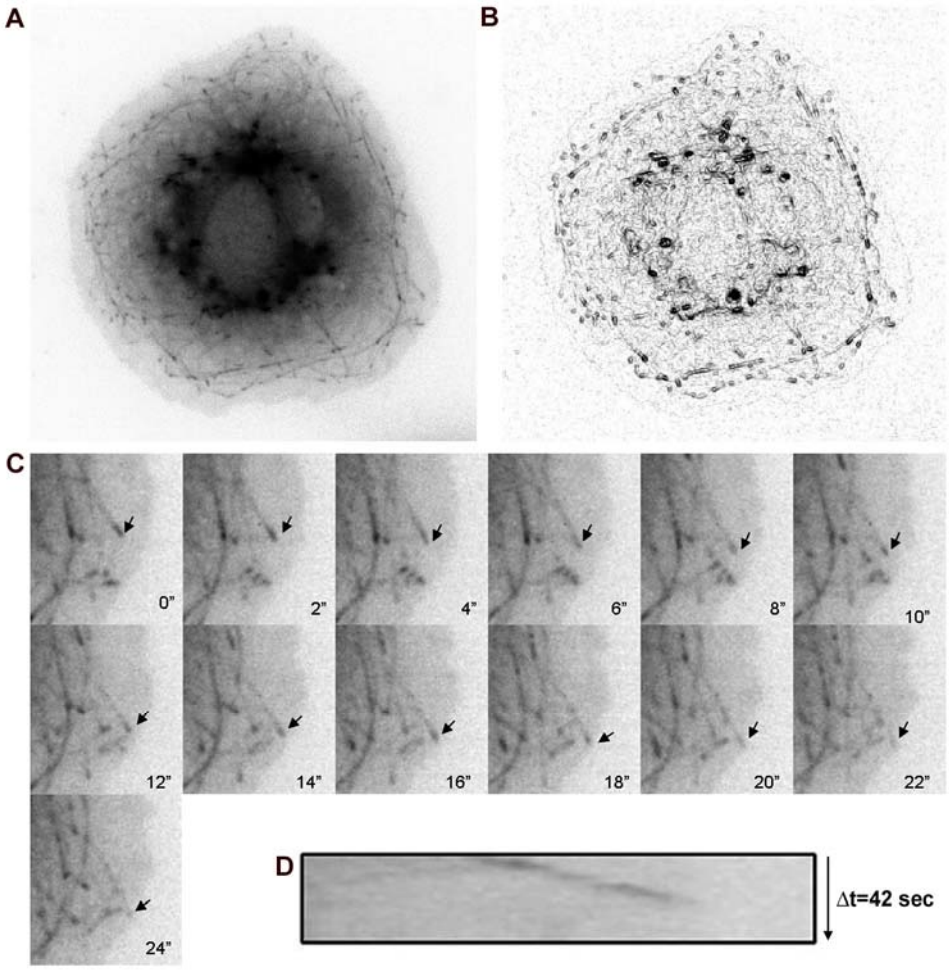

Figure 2. Mast is a plus-end tracking protein. Full length EGFP-Mast was expressed in *Drosophila* S2 cells and its distribution analysed *in vivo* by time-lapse fluorescence microscopy. (A-B) Image from movie 1 in which EGFP-Mast is clearly visible in an interphase S2 cell. (A) Inverse colouring highlights the comet-like structures present near the edges of the cell. (B) Edge enhancement of the same image shown in A shows the cap-like EGFP-Mast signal at the tip of microtubules. (C) Sequential images of a comet-like signal (arrow) over time. (D) Preferential accumulation of EGFP-Mast to the growing plus end is clearly illustrated by the kymograph. Courtesy of Paula Sampaio.

2.2. Identification of the microtubule binding domain of Mast

In order to analyse further the interaction between Mast and microtubules, we mapped the microtubule binding domain of Mast by expressing in *Drosophila* S2 cells different regions of the protein fused to EGFP (Figure 3).

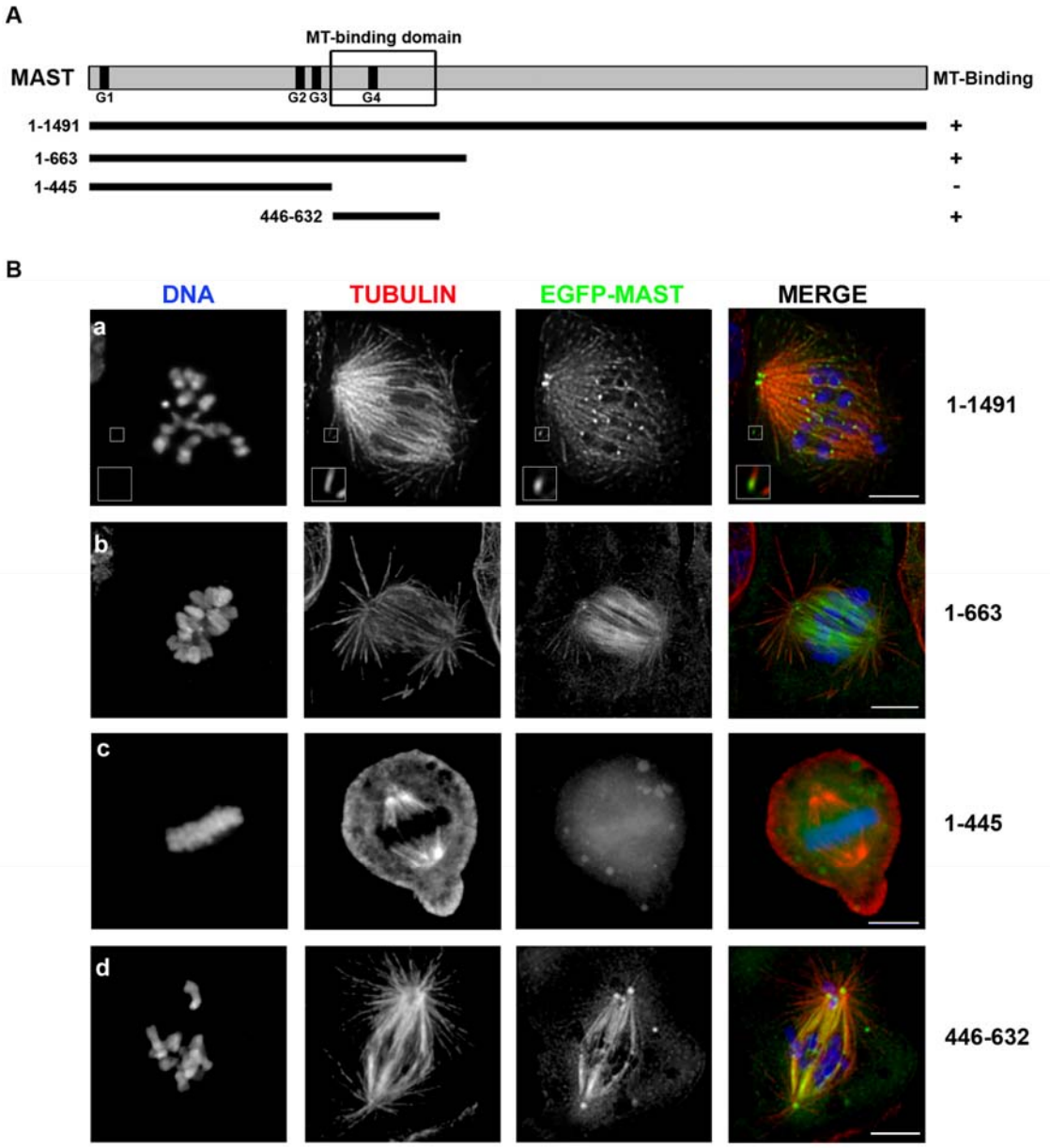

Figure 3. Mapping the microtubule binding domain of Mast. (A) Schematic drawing of the Mast protein with the N-terminus located at the left hand side and the different constructs that were transfected into *Drosophila* S2 cells shown below by the solid lines. The putative GTP binding domains of Mast (G1-4) are indicated (see also Supplemental Figure S4). Each construct contained EGFP at the N-terminus and was cloned in frame with the fragment indicated by a solid line. The numbers correspond to the amino acids in Mast. Binding to microtubules after transfection is indicated on the right hand side. (B) Immunofluorescence analysis of S2 cells expressing the complete Mast protein or different deletion constructs fused to EGFP, stained for DNA and tubulin. Scale bar is 5 μm. Courtesy of Rita Reis.

The results indicate that the microtubule binding domain of Mast is contained within a relatively small region of 186 residues (amino acids 446-632) (Figure 3A and B). This region is sufficient for microtubule binding during mitosis (Figure 3A and B) but we could only show that is required during interphase because this fragment showed strong nuclear accumulation (Figure 4).

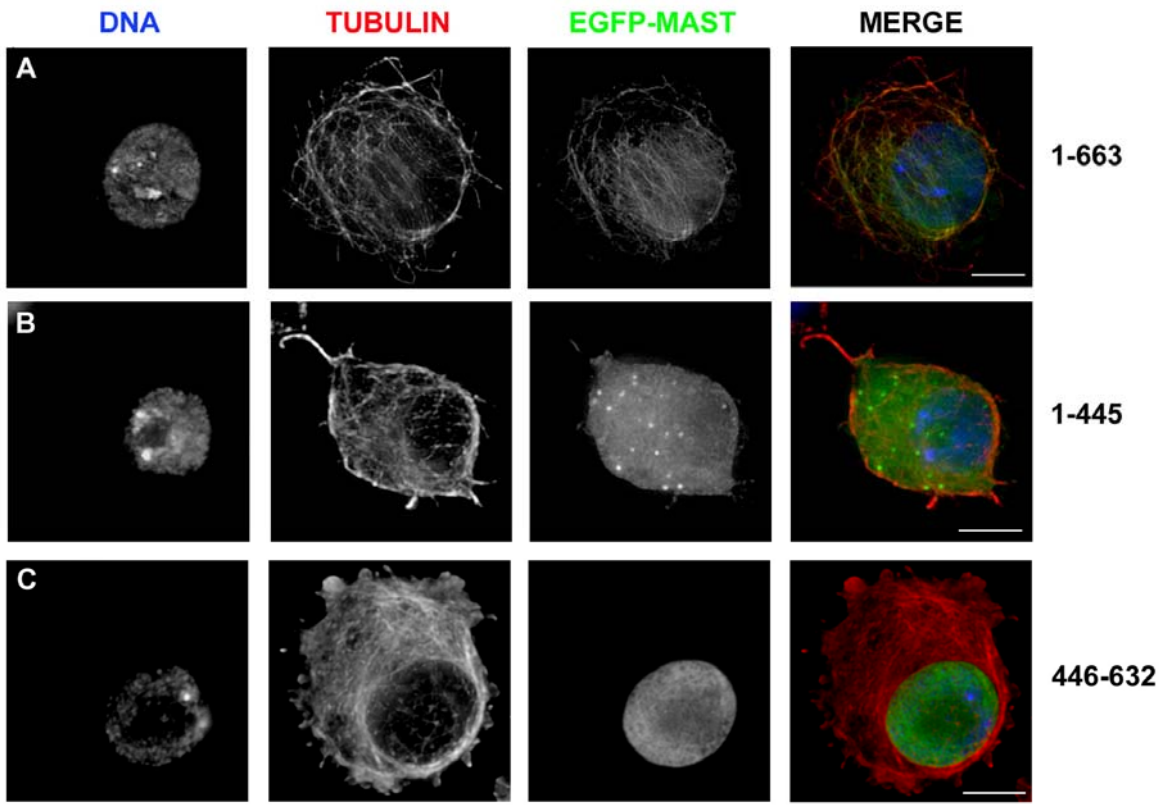

Figure 4. Mapping the microtubule binding domain of Mast during interphase. Deletion mutants from the full length of Mast were transiently expressed in *Drosophila* S2 cells tagged at the N-terminus by EGFP and their localization analysed during interphase after immunostaining with anti-tubulin antibodies and for DNA. (A) Mast 1-663 co-localize with microtubules. (B) However, Mast 1-445, forms small aggregates on the cytoplasm and does not appear to bind microtubules. This suggests that deletion of the 186 C-terminal amino acids from Mast 1-663 abolishes its ability to bind microtubules. (C) The smallest fragment of Mast (446-632) could not be tested for its ability to bind interphase microtubules because it shows strong nuclear accumulation. Scale bar is 5 μm. Courtesy of Rita Reis.

Protein sequence analysis of this region revealed a high degree of conservation (Figure 5), including the microtubule binding domain of STU1p, the *S.cerevisiae* homologue (Yin et al., 2002), suggesting that this domain might define the microtubule binding motif for this protein family.

Figure 5. Sequence alignment of the microtubule binding domain. Sequence alignment of the microtubule binding domain identified in Mast with homologous regions of other members of this protein family. Sequences are from *Drosophila melanogaster* (DM Mast), *Anopheles gambiae* (Ag Mast), *Brachydanio rerio* (Br CLASP), *Homo sapiens* (Hs CLASP1), *Mus musculus* (MM CLASP1), *Homo sapiens* (Hs CLASP2), *Rattus norvegicus* (Rn CLASP2), *Oryza sativa* (Os CLASP1), *Caenorhabditis elegans* (Ce Cls-2), *Neurospora crassa* (NC Stu1), *Aspergillus fumigatus* (Af Stu1) and *Saccharomyces cerevisiae* (SC STU1p).

2.3. Interaction of Mast with microtubules and tubulin heterodimers

In order to study the interactions of Mast with microtubules we produced Mast recombinant proteins in *E. coli*. Attempts to produce the complete protein failed due to the largely insoluble protein products. Then we subcloned the microtubule binding domain of Mast, Mast446-632, into pGEX-5X1 vector as a C-terminal GST-tag protein. Attempts to purify this fusion protein failed because the protein could not be eluted from the columns. Therefore, we subcloned an N-terminal fragment of Mast consisting of amino acids 1-663 (Mast1-663) into pET 23b vector and expressed as a C-terminal His tagged protein (Figure 6A). Mast1-

663 was purified by Hitrap affinity columns in non-desnaturated conditions and eluted with imidazole (Figure 6B).

We also expressed a C-terminal fragment of Mast (Mast1218-1491) as a negative control for microtubule binding.

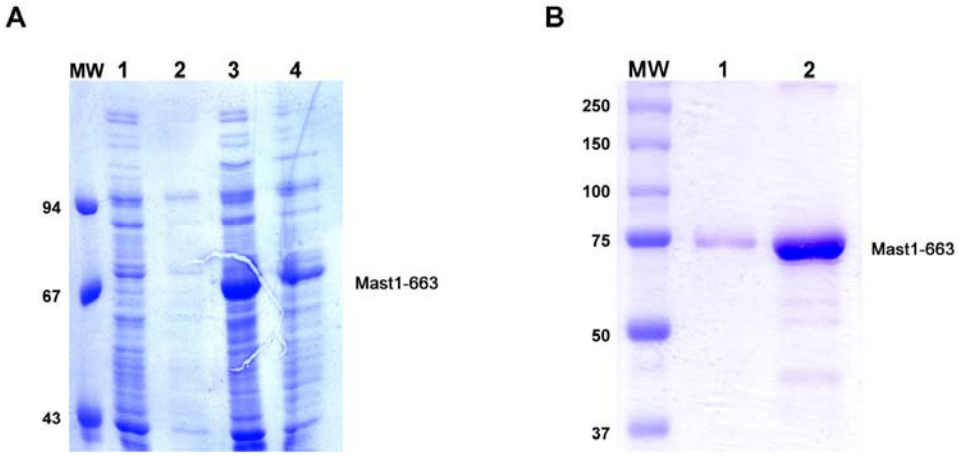

Figure 6. Mast 1-663 expression and purification. (A) Coomassie-stained gel showing the N-terminal fragment of Mast, comprising amino acids 1-663 (Mast1-663) expressed in bacteria, show the non induced fraction, soluble (lane 1) and insoluble (lane 2); and the IPTG induced fraction. Mast1-663 was expressed in the soluble fraction (lane 3) and a small amount in insoluble fraction (lane 4). (B) Coomassie-stained gel showing the fusion protein Mast1-663 purified by affinity chromatography: 1 µl (lane 1) and 10 µl (lane 2). Purified Mast1-663 was used in all tubulin overlay assays.

To study the binding of Mast to microtubules we used these three fusion proteins: purified Mast1-663, Mast446-632 and Mast1218-1491 in microtubule overlay assay, as previously described (Inoue et al., 2000). In short, the fusion proteins were separated by SDS-PAGE, transferred to nitrocellulose, renatured, incubated with microtubules, and the binding detected with anti-tubulin antibodies. Mast1-663 and Mast446-632 showed strong binding to microtubules, and the Mast fragment that did not contain the microtubule binding domain was unable to bind microtubules (Figure 7).

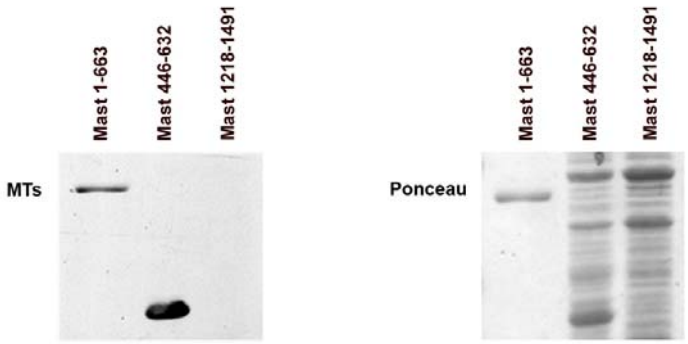

Figure 7. Binding of Mast to microtubules. Purified Mast1-663, Mast446-632 and Mast1218-1491 were separated by SDS-PAGE, transferred to nitrocellulose, renatured in appropriate buffers, incubated with microtubules, washed and probed with anti-tubulin antibodies. Mast1-663 and Mast446-632 bind to microtubules while Mast1218-1491 does not. The membrane was stained with ponceau to determine loading levels.

Previous sequence analysis (Inoue et al., 2000), identified two putative GTP-binding motifs, a β-tubulin-like signature (GGGTGTG) and a motif (NKLD) found in the GTPase super family (Sprang, 1997). These motifs were thought to be important because it was observed that Mast/Orbit associates with microtubules in a GTP dependent manner (Inoue et al., 2000). Protein sequence analysis revealed that Mast contains at least two more putative GTP-binding motifs that are specific for guanine binding, a DXXG-like motif and a SAK motif (Vetter and Wittinghofer, 2001) (Figure 8). However, the β-tubulin signature (GGGTGTG) present in Mast is not conserved.

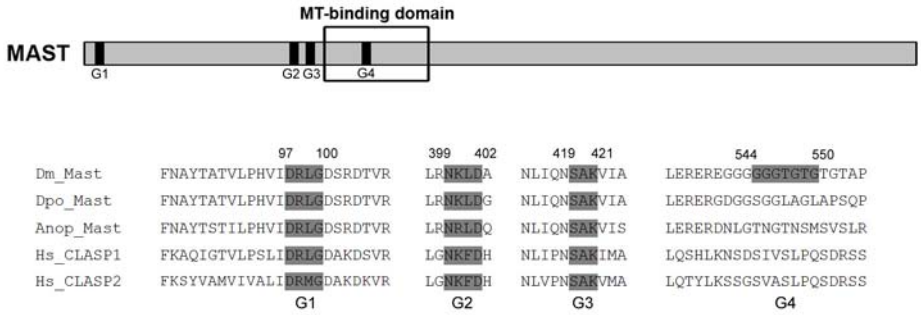

Figure 8. Mast contains three highly conserved putative GTP binding sites. Protein sequence alignment of Mast/Orbit homologues showing the three highly conserved putative GTP binding motifs (G1-3) and the poorly conserved β-tubulin motif (G4) present only in *Drosophila* melanogaster. The sequences include, Dm_Mast (*Drosophila melanogaster*), Dpo_Mast (*Drosophila peusoobscura*), Anop_Mast (*Anopheles gambiae*), Hs_CLASP1 and Hs_CLASP2 (*Homo sapiens*).

To study in more detail whether Mast binds microtubules in the presence or absence of guanine nucleotides, we did microtubule overlay assays with Mast1-663 preincubated with or without nucleotides before incubation with microtubules. Again, Mast1-663 showed strong binding to microtubules and, in contrast with previous results (Inoue et al., 2000), this fragment binds microtubules independently of GTP (Figure 9A). To determine whether Mast can also bind tubulin heterodimers, the overlays assays were incubated at 4ºC with GTP-tubulin heterodimers (Figure 9B). The results show that Mast1-663 can bind tubulin heterodimers with higher affinity after preincubation with GTP.

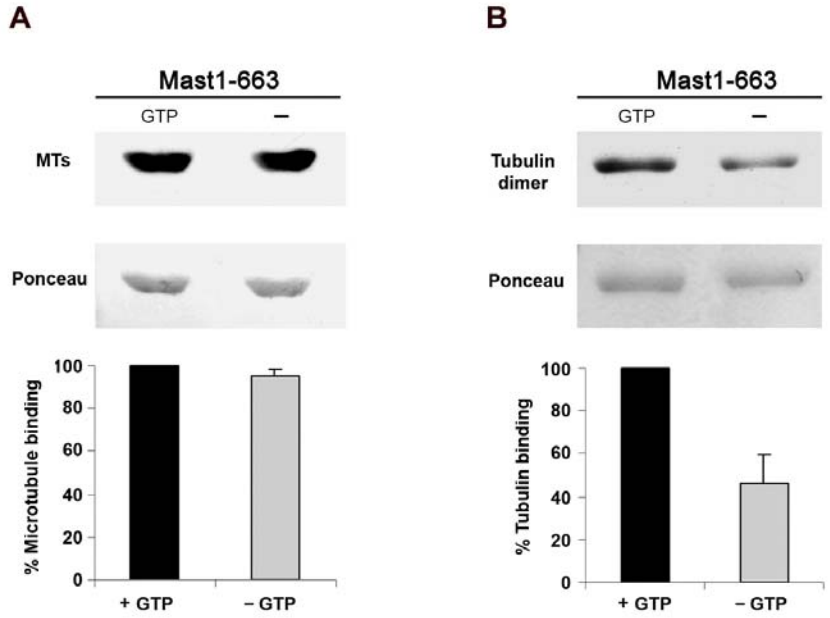

Figure 9. Binding of Mast to microtubules and tubulin heterodimers is independent of GTP. Mast1-663 was separated by SDS-PAGE, transferred to nitrocellulose, renatured in appropriate buffers, pre-incubated in the presence or in the absence of GTP, incubated with either microtubules or tubulin heterodimers, washed and probed with anti-tubulin antibodies. (A) Mast1-663 was preincubated with GTP (+) or without (-) before addition of microtubules. Taxol-stabilized microtubules were washed after polymerization to remove free GTP. (B) Mast1-663 was preincubated with GTP (+) or without (-) before addition of tubulin heterodimers. In all overlay assays, after Mast1-663 was trasnferred to nitrocellulose and the membrane was stained with ponceau as loading control. Each overlay assay is representative of three independent experiments and the quantification corresponds to the mean of tubulin signals normalised by the ponceau signal.

In the absence of GTP or in the presence of GDP, binding of Mast1-663 to tubulin heterodimers, decreases significantly (Figure 9B and 10A), suggesting that Mast-GTP strongly favours binding to tubulin heterodimers. Furthermore, while GTP is not required for binding of Mast to microtubules, GDP strongly inhibits microtubule binding (Figure 10B).

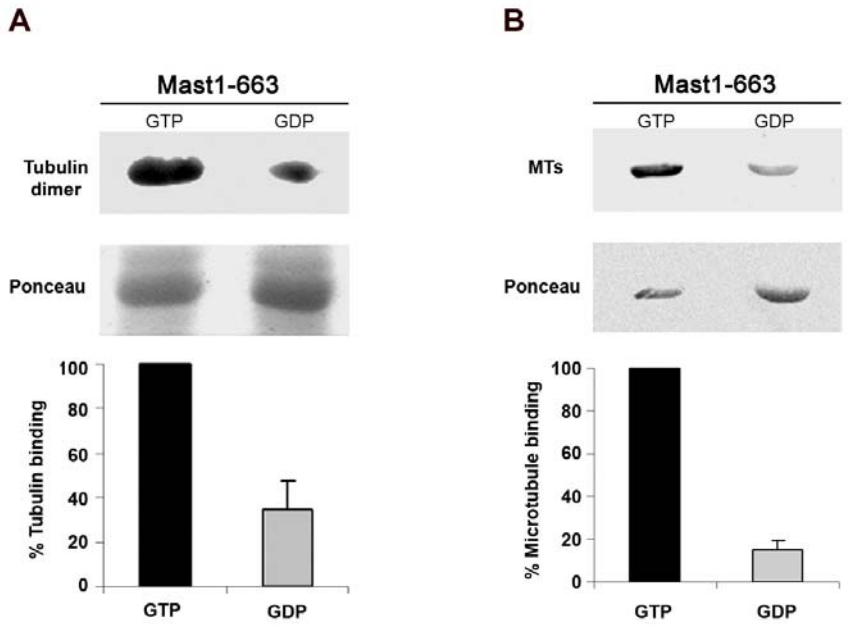

Figure 10. Binding of Mast to microtubules and tubulin heterodimers is dependent of GDP. Mast1-663 was separated by SDS-PAGE, transferred to nitrocellulose, renatured in appropriate buffers, pre-incubated in the presence of GTP or GDP, incubated with either microtubules or tubulin heterodimers, washed and probed with anti-tubulin antibodies. (A) Mast1-663 was preincubated with GTP or GDP before addition of tubulin heterodimers. (B) Mast1-663 was preincubated with either GTP or GDP before addition of taxol-stabilized microtubules. In all overlay assays, after Mast1-663 was trasnferred to nitrocellulose the membrane was stained with ponceau as loading control. Each overlay assay is representative of three independent experiments and the quantification corresponds to the mean of tubulin signals normalised by the ponceau signal.

Mast binds microtubules so tightly that it remains bound even after incubation in 2% SDS. However, addition of GDP to the overlay assay can easily displace microtubules bound to Mast (Figure 11). Overall, the data shows that the affinity of Mast for tubulin heterodimers is enhanced in the presence of GTP, while GDP can strongly inhibit the association of Mast with microtubules. These results are fully consistent with a model in which binding of Mast to either GTP or GDP

alters its conformation and consequently its affinity for microtubules or tubulin heterodimers.

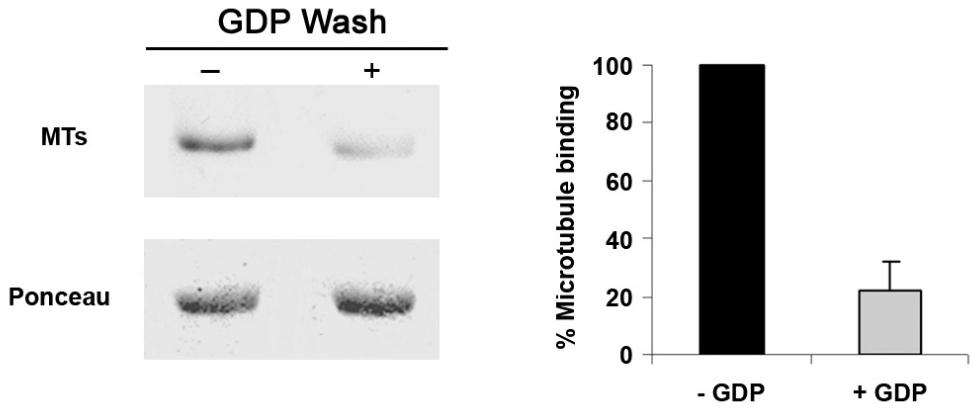

Figure 11. Binding of Mast to microtubules and the effect of GDP. Mast1-663 was separated by SDS-PAGE, transferred to nitrocellulose, renatured in appropriate buffers, incubated with taxol-stabilized microtubules and then washed in the absence (-) or in the presence (+) of 2 mM GDP. In all overlay assays, after Mast1-663 was trasnferred to nitrocellulose the membrane was stained with ponceau as loading control. Each overlay assay is representative of three independent experiments and the quantification corresponds to the mean of tubulin signals normalised by the ponceau signal.

2.4. Mast binds GTP and displays GTPase activity

The presence of putative GTP-binding motifs, the GTP-dependent increase affinity to tubulin heterodimers and the strong inhibition of binding to microtubules and tubulin heterodimers caused by GDP raised the possibility that Mast could itself bind GTP. To test this hypothesis directly, we performed pull down assays using GTP-agarose beads. The results show that Mast binds specifically to GTP-agarose and that this interaction is competed by free GTP (Figure 12).

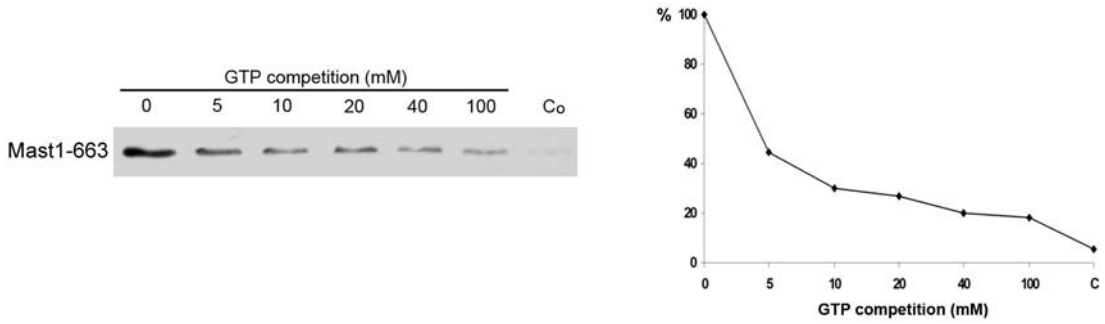

Figure 12. Mast is a GTP-binding protein. Mast1-663 was incubated with different concentrations of free GTP for 30 min at 4ºC prior to the addition of GTP-agarose. Mast1-663 bound to GTP was then pulled down, separated by SDS-PAGE, blotted to nitrocellulose and detected with anti-Mast antibodies. Quantification of the autoradiograph shows that more than 50% of GTP-bound Mast1-663 is competed out by addition of 5 mM GTP. As control, a pull down with Protein A-agarose was also carried out (Co).

Pull down assays with GDP-agarose also show that Mast is able to bind GDP specifically (Figure 13).

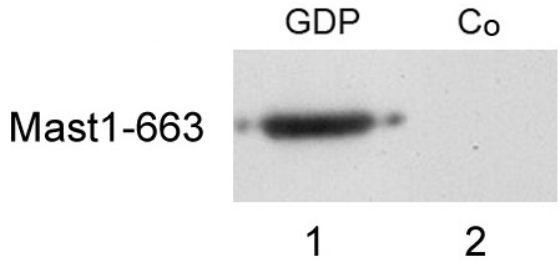

Figure 13. Mast binds GDP. Mast1-663 was incubated for 30 min with GDP-agarose (lane 1). As control, a pull down with Protein A-agarose was also carried out (lane 2).

Since GDP has a strong effect on the ability of Mast to bind microtubules and heterodimers, we sought to determine whether this nucleotide also affects the ability of Mast to bind GTP. Accordingly, we incubated Mast1-663 at 4ºC in the presence of 2 mM GTP or GDP and then added GTP-agarose. Preincubation with GDP reduced significantly the recovery of Mast1-663 from the GTP-agarose pull

down assay, indicating that in the presence of GDP the ability of Mast to bind GTP is significantly reduced (Figure 14).

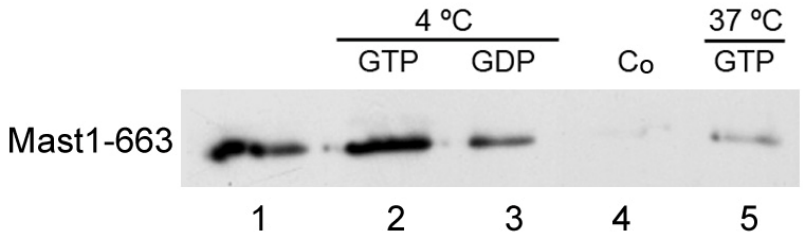

Figure 14. Mast binding to GTP is modulated by temperature. Mast1-663 was pre-incubated for 30 min, prior to the addition of GTP-agarose, with buffer alone (lane 1), 2 mM GTP (lane 2) or GDP (lane 3) at 4ºC, and 2 mM GTP (lane 5) at 37ºC. As control, a pull down with Protein A-agarose was also carried out (lane 4).

Previously, it has been suggested that Mast/Orbit proteins could have GTPase activity (Inoue et al., 2000; Kline-Smith and Walczak, 2000). To begin addressing this question we carried out a simple competition assay in which we preincubated Mast 1-663 with GTP at 37ºC and tested whether it could bind GTP-agarose (Figure 14). Under these conditions, the ability to recover Mast1-663 in the GTP-agarose bound fraction was substantially reduced. Since Mast shows strong binding to GDP (Figure 13), one possible interpretation of this result is that during the incubation at a higher temperature Mast1-663 was able to hydrolyse free GTP into GDP, which then competed out the interaction of Mast 1-663 with the GTP-agarose.

Therefore, we performed a direct test for the ability of Mast1-663 fusion protein to hydrolyse radioactively labelled GTP in solution (Figure 15). The results show that purified Mast1-663 is able to hydrolyse GTP (Figure 15), while heat inactivated Mast1-663 does not (Figure 16A).

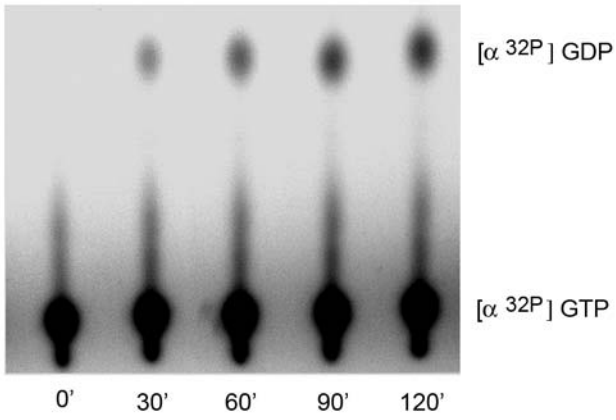

Figure 15. Mast has GTPase activity. Freshly purified Mast1-663 was incubated with [α^{32P}] GTP for different periods. Samples of each time point were analysed by thin layer chromatography followed by autoradiography.

Quantitative analysis (Figure 16B) shows that Mast has a very high rate of GTPase activity (~ 4,8 s^{-1}) when compared with tubulin (8 X 10 $^{-5}$ s^{-1}) (Mejillano et al., 1996) or Ras (3 X 10 $^{-5}$ s^{-1}) (Gibbs et al., 1988).

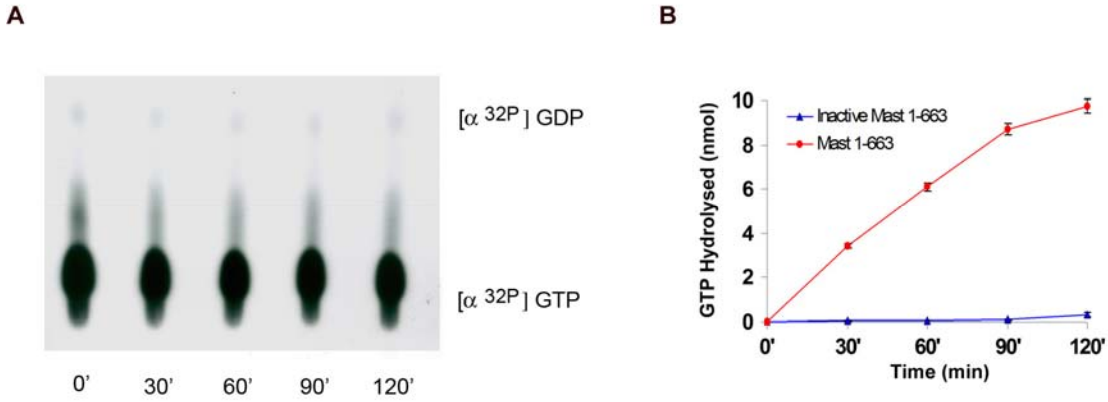

Figure 16. Heat inactivated Mast is unable to hydrolyse GTP. (A) Heat inactivated purified Mast1-663 was incubated with [α^{32P}] GTP for different periods. Samples of each time point were analysed by thin layer chromatography followed by autoradiography. (B) Quantification of autoradiography signals from Mast GTPase activity assays of three independent experiments.

In order to test if the GTP-binding motif (GGGTGTG) present in Mast is in any way involved in the GTPase activity, a mutated form of Mast1-663 in which the first three Glycine were changed to Alanine (AAATGTG), was expressed and purified as before and tested for its ability to hydrolyse radiolabelled GTP (Figure 17). The GTPases assays showed that this mutation did not affect the GTPase activity of Mast1-663, suggesting that this motif is not involved in binding of GTP or the enzymatic activity of Mast, accordingly this motif is not conserved amongst the Mast/Orbit family.

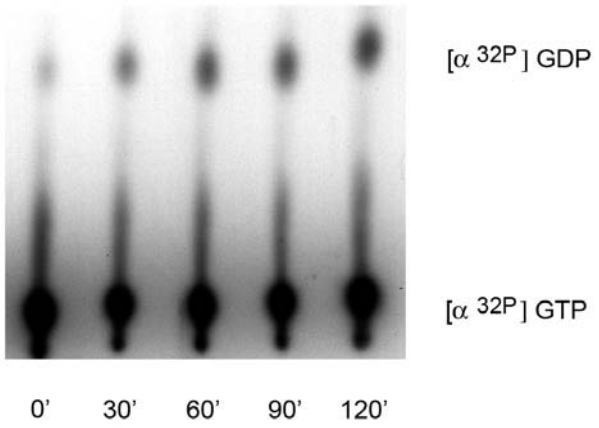

Figure 17. The Mast1-663 mutated form is able to hydrolyse GTP. Freshly purified Mast1-663 mutated form was incubated with [α^{32P}] GTP for different periods. Samples of each time point were analysed by thin layer chromatography followed by autoradiography.

Even though the mutation in Mast1-663 did not affect the ability to hydrolyse GTP, we wanted to know if the interaction with microtubules is affected. Therefore, we performed microtubule overlay assays with the mutated form and used Mast1-663 wild type as positive control (Figure 18). The results show that the mutated form is still able to bind microtubules, thus, we decided to study its behaviour in the presence of nucleotides. Pre-incubation of Mast1-663 or the mutated form with GTP does not affect microtubule binding significantly and both show strong microtubule binding. Nevertheless, the mutated Mast1-663 form binds microtubules ~20% less than control. However, when we preincubated either wild type or mutated Mast1-663 with GDP, the mutated form is still able to bind

microtubules and therefore GDP is not able to compete out the binding (Figure 18A). Moreover, the mutated Mast1-663 protein binds GDP ~50% stronger than GTP. To test if this had any functional consequence mutated Mast1-663 was bound to microtubules on an overlay assay and the effect of washing in the presence of GDP upon release of microtubules analyzed. As shown above, GDP displays the microtubules already bound to the wild type Mast1-663, but interestingly GDP was not able to release microtubules bound to the mutated form of Mast1-663 (Figure 18B). Therefore, the mutation appears to cause alterations in the way Mast interacts with microtubules.

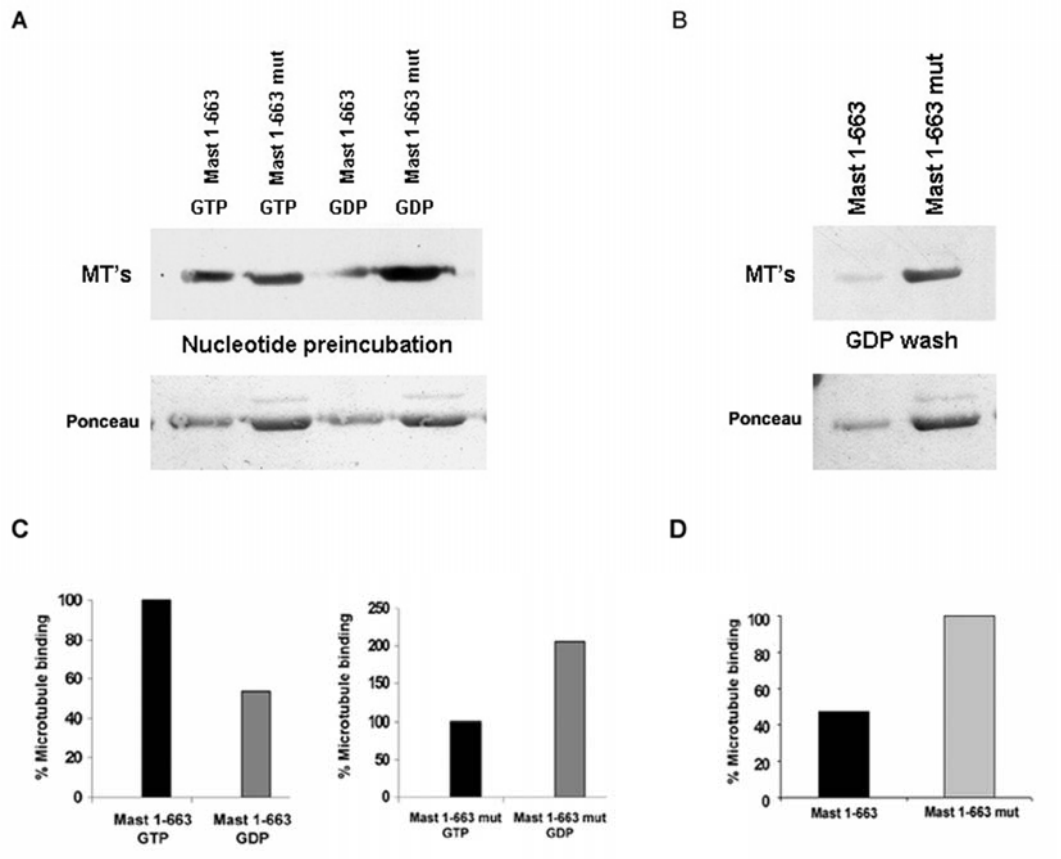

Figure 18. Microtubule binding of mutated Mast1-663 in the presence of nucleotides. (A) Mast1-663 and the mutated for Mast1-663 (mut) were separated by SDS-PAGE, transferred to nitrocellulose, renatured in appropriate buffers, pre-incubated in the presence of GTP or GDP, incubated with microtubules, washed and probed with anti-tubulin antibodies. (B) Mast1-663 and the mutated form of Mast1-663 were separated by SDS-PAGE, transferred to nitrocellulose, renatured in appropriate buffers, incubated with taxol-stabilized microtubules and then washed in the absence (-) or in the presence (+) of 2 mM GDP. (C-D) quantification of the mean of tubulin signals normalised by the ponceau signal.

3. Discussion

3. Discussion

3.1. +TIP behaviour of Mast

Mast is a microtubule associated protein that has an important role in the organization and function of the mitotic spindle and microtubule-kinetochore attachments (Inoue et al., 2000; Lemos et al., 2000; Maiato et al., 2002; Sharp, 2002). The human homologues of Mast, CLASPs also have also been shown to have a role in the regulation of microtubule dynamics (Akhmanova et al., 2001; Maiato et al., 2003a; Mimori-Kiyosue et al., 2005). Importantly, they show + TIP behaviour both during interphase and mitosis (Carvalho et al., 2003) and it has been suggested that the *Drosophila* homologue Mast should also display +TIP behaviour (Mimori-Kiyosue and Tsukita, 2003). Indeed, our *in vivo* results show that Mast tracks along growing microtubules and is never associated with depolymerising ends. However, +TIP behaviour was only observed when EGPF-Mast was expressed at relatively low levels, since at high levels the protein was found to bind throughout the microtubule lattice and cause the formation of microtubule bundles which failed to display dynamic behaviour. These results are similar to those obtained for CLASP1 (Maiato et al., 2003a) and all +TIPs (Carvalho et al., 2003) which when overexpressed bind along the whole microtubule lattice and increase microtubule stability. This suggest that the level and/or microtubule binding properties of these proteins must be carefully controlled otherwise the dynamic properties of microtubules are severely affected. In addition, other factors like cell cycle stage and growth conditions appear to be important for this fragile balance. Our results confirm that all members of Mast/Orbit/CLASPs protein family that have been tested show +TIP behaviour.

3.2. Identification of the microtubule binding domain of Mast

Early studies showed that Mast/Orbit binds microtubules possibly through a highly basic central region that shares limited homology with the microtubule

binding domain of MAP4 (Inoue et al., 2000; Lemos et al., 2000). More recently, the microtubule binding domain of CLASP1 was mapped to a large internal region (Maiato et al., 2003a) that includes a small domain responsible for plus end accumulation (Mimori-Kiyosue et al., 2005). We have shown that Mast446-632, a small region of 187 amino acids, is sufficient for microtubule binding both *in vivo* and *in vitro*. Protein sequence analysis of this region revealed a high degree of conservation, including with the microtubule binding domain of STU1p, the *S.cerevisiae* homologue (Yin et al., 2002), suggesting that this region might define the microtubule binding domain for this protein family. Interestingly, this motif does not include the domain responsible for plus end accumulation recently identified in Human CLASPs (Mimori-Kiyosue et al., 2005), indicating that members of Mast/Orbit family do not require +TIP association for microtubule binding. Microtubule binding and plus end accumulation could exist as two independent regions, suggesting that the microtubule binding domain it is not sufficient for + TIP behaviour. However, we cannot exclude the possibility that the fragment Mast446-632 has +TIP behaviour, because we cannot perform *in vivo* studies with this fragment due to it nuclear localization during interphase.

3.3. Interaction of Mast with tubulin is GDP sensitive

Inoue and colleagues identified two putative GTP binding motifs, GGGTGTG and NKLD in Mast/Orbit sequence and suggested that Mast/Orbit binds microtubules in a GTP dependent manner (Inoue et al., 2000). However, our results show that the binding of Mast to microtubules is independent of GTP. Mast binds directly to microtubules and does not require previous binding to GTP. We also found that Mast binds tubulin heterodimers but in contrast with binding to microtubules, binding to heterodimers is enhanced in the presence of GTP. Furthermore, while the interaction between Mast and microtubules is independent of GTP, our results showed that GDP strongly inhibits Mast binding to microtubules and tubulin heterodimers. In agreement, it was previously reported that Orbit binds microtubules with reduced affinity in the presence of GDP (Inoue

et al., 2000). Interestingly, even though Mast interacts very strongly with microtubules, these interactions are easily disrupted in the presence of GDP.

The association of many MAPs with microtubules is known to be regulated by ATP, typical of kinesins (Bringmann et al., 2004; Sablin and Fletterick, 2004) but regulation by guanine nucleotides is not well recognized. CLIP-170 was initially reported as a nucleotide sensitive protein that can be eluted from microtubules by ATP and 50% less by GTP (Rickard and Kreis, 1990). However, this interaction between CLIP-170 and GTP was not studied further. Nevertheless, more recent studies have shown that the elution profile of native proteins obtained after imunoprecipitation with GTP-tubulin or GDP-tubulin did not show significant differences, suggesting that proteins that associate with tubulin are not able to distinguish the different tubulin conformations (Gache et al., 2005). In addition, the interaction between the mitotic associated GTPase Gie1 and microtubules is independent of whether GTP or GDP are present (Okai et al., 2004). Therefore, the observation that GDP strongly inhibits the ability of Mast to bind microtubules appears to be a unique feature among MAPs, +TIPs and also GTPases.

3.4. Mast has GTPase activity

Pull down assays revealed that Mast binds GTP very specifically while in the presence of GDP the ability of Mast to bind GTP is significantly reduced. This suggests that Mast binds both GTP and GDP through the same site but that the dissociation rate of GDP is very slow, similarly to what has been already described for other GTP-binding proteins (Vetter and Wittinghofer, 2001). Taken as a whole, our results are fully consistent with a model in which binding of Mast to either GTP or GDP alters its conformation and consequently its affinity for microtubules or tubulin heterodimers.

Previously, it has been suggested that Mast/Orbit proteins could have GTPase activity (Inoue et al., 2000; Kline-Smith and Walczak, 2000). Indeed, our results show that Mast has GTPase activity with a very high rate when compared to tubulin or Ras. However, Mast does not share the common features of typical

GTPases such as the three subunits found in heterotrimeric G proteins or the low molecular weight of small GTPases (Sprang, 1997). Thus, Mast is appears to be a highly atypical GTPase similarly to tubulin or dynamin. Interestingly, Mast has the tubulin signature motif (GGGTGS/TG), which is known to be responsible for tubulin and FtsZ GTPase activities (Burns and Farrell, 1996). Mutations within the tubulin signature motif abolish the GTPase activity of tubulin and FtsZ (de Boer et al., 1992; RayChaudhuri and Park, 1992; Dai et al., 1994; Burns and Farrell, 1996; Dougherty et al., 2001; Addinall et al., 2005). Accordingly, we mutated the GGGTGTG motif in Mast and analyzed both its ability to bind microtubules and its GTPase activity. The results showed that the mutation did not affect the GTPase activity of Mast. In fact, the β-tubulin signature (GGGTGTG) present in Mast is not conserved among other proteins of the Mast/Orbit family, suggesting that it is unlikely that this motif is essential for a conserved GTPase activity within the Mast/orbit protein family. Nevertheless, the mutation affects the binding of Mast to microtubules since the protein becomes insensitive to the presence of GDP. We hypothesise that the mutation in the GGGTGTG motif could compromise an eventual GDP dependent conformational change of Mast that reduces it affinity to microtubules.

Extensive studies have shown that the enzymatic activity of most GTPases is regulated by the interaction with activating proteins (GAPs), that increase the GTP hydrolysis rate and guanine nucleotide-exchange factors (GEFs), that accelerate the intrinsically slow dissociation of protein-bound GDP (Vetter and Wittinghofer, 2001). Generally, GTPases contain at least two switch regions whose conformation is regulated by the bound guanine nucleotide. Alterations in these two regions results in changes in the affinity of GTPases for their effectors or regulatory proteins, such as GEFs and GAPs. Since association of Mast to microtubules is dependent on the nucleotide bound forms, altering their affinity to them is likely that specific GAPs and GEFs or other proteins could also regulate the GTPase activity of Mast and in this way control microtubule binding. Indeed, the ability of effector proteins to regulate microtubule binding of other +TIPs has recently been demonstrated (Rogers et al., 2004; Watanabe et al., 2004). It was shown that IQGAP1, an effector of Rac1 and Cdc42, interacts directly with APC

and CLIP-170 and is required for their localization during cell polarization. Indeed, Rho GTPases and GAP-like proteins are though to be involved in the regulation of microtubule dynamics and capture in close association with +TIPs like CLIP-170, Dynactin and EB1 (Gundersen, 2002). More recently, it was shown that *Drosophila* RhoGEF2 travels to the cell cortex on the tips of growing microtubules by interaction with EB1. Interesting, in pull down assays that detect the interaction of EB1 and RhoGEF2, Mast was also found, suggesting that RhoGEF2 could be a good candidate to be a GEF regulator of Mast (Rogers et al., 2004). In addition, Rho GTPases like Cdc42 and its downstream effector mDia3 are involved in bi-orientation and stabilization of spindle microtubules attachment to kinetochores (Narumiya et al., 2004). Given that Mast/CLASPs are required for the dynamic behaviour of kinetochore microtubules (Maiato et al., 2002; Maiato et al., 2003a), it is possible that GAP and GEF like proteins could regulate their function at kinetochores during mitosis.

3.5. Model for plus end tracking behaviour of Mast

Plus end tracking proteins were first described as a group of MAPs that localize to the growing tip of microtubules and that are then released giving a comet-like appearance (Carvalho et al., 2003). Plus end tracking behaviour was discovered by live-cell microscopy of tissue culture cells expressing GFP-CLIP-170 (Diamantopoulos et al., 1999; Perez et al., 1999). Subsequently, several other proteins including EB1 (Mimori-Kiyosue et al., 2000b), APC (Mimori-Kiyosue et al., 2000a), LIS-1 (Faulkner et al., 2000), $p150^{Glued}$ (Vaughan et al., 2002), cytoplasmatic dynein (Xiang et al., 2000) and CLASPs (Akhmanova et al., 2001; Maiato et al., 2003a) were shown to display +TIP behaviour.

Three potential mechanisms have been proposed to explain plus-end tracking (Carvalho et al., 2003). Treadmilling was initially proposed for the behaviour of CLIP-170 (Perez et al., 1999). The protein would bind the growing plus end, remain bound and shortly after dissociate behind the region of polymerization as the microtubule closes into tube (Schuyler and Pellman, 2001).

Targeting of these proteins to the plus ends can occur by co-polymerization with tubulin as CLIP-170 (Arnal et al., 2004). Alternatively, hitchhiking (Carvalho et al., 2003) is thought to occur when a +TIP carries a protein to the plus end as in the case of Kar9p by Bim 1 (Korinek et al., 2000), or by selective bind to a plus end protein as EB1 (Tirnauer et al., 2002). Finally, a +TIP could be actively transported to the plus end by a motor protein (Carvalho et al., 2004). APC was shown to be transported along microtubules by KAP3, a member of the kinesin superfamily (Jimbo et al., 2002). Several mechanisms were already proposed for release, post-translational modifications like phosphorylation, in the case of CLIP-170 (Rickard and Kreis, 1991) and $p150^{Glued}$ (Vaughan et al., 2002) or a conformational change in the MT and /or in the +TIP (Galjart and Perez, 2003).

In this context, our results suggest a new model for the interaction between Mast and microtubules (Figure 19). As microtubules grow, Mast-GTP specifically binds tubulin heterodimers, most likely through β-tubulin like STU1p (Yin et al., 2002), and is incorporated at the plus end by copolymerization. The ability of Mast-GTP to bind tubulin heterodimers and the recent results showing that Mast is essential for the incorporation of heterodimers into kinetochore fibres allowing both flux and growth of bound microtubules (Maiato et al., 2005) strongly supports this aspect of the model. However, we cannot exclude a direct binding of Mast to microtubule growing ends. Also Mast could interact at the plus ends with EB1, since the region of CLASPs suggested for plus end binding also binds EB1 (Mimori-Kiyosue et al., 2005). Once bound, Mast is released behind the microtubule region of new growth. Given that Mast displays GTPase activity and that GDP affects its ability to bind microtubules, release of Mast from the microtubule lattice could be the result of enhancement of its enzymatic activity by GAP-like proteins present on the microtubule lattice behind the plus end. Microtubules could also act as GAPs for Mast as they are to the tubulin heterodimer. Exchange of GTP by GDP would cause Mast to alter its conformation and loose its affinity for microtubules, promoting the release. In conclusion, our data suggests for the first time that a microtubule associated protein could use its GTPase activity to regulate the ability to bind microtubules and in this way directly control the dynamic properties of microtubules. This model indicates a novel

mechanism of action for +TIP behaviour and may allow new findings addressing the unknown mechanisms of plus end tracking and the mechanism by which +TIPs control microtubule dynamics.

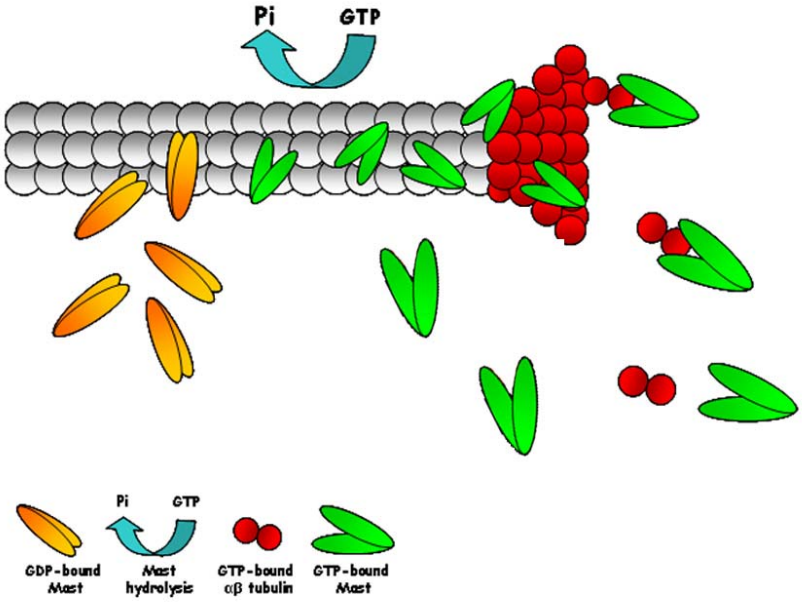

Figure 19. Model for the +TIP behaviour of Mast. Mast-GTP could be incorporated to the plus-ends of growing microtubules by binding to tubulin heterodimers in a copolymerization mechanism. Activation of the GTPase activity of Mast would lead to hydrolysis of GTP into GDP causing a conformational change of the protein that promotes its release from the microtubule lattice. The balance between binding and release of Mast would generate its tracking behaviour.

4.Experimental Procedures

4. Experimental Procedures

4.1. Constructs and Transfections

pMTEGFP-Mast was obtained previously (Lemos et al., 2000). pMTEGFP-Mast1-445 was obtained by removing a BglII and SacII fragment from pMTEGFP-Mast and religation. pMTEGFP-Mast1-663 was obtained by removing a BstXI and BglII fragment from pMTEGFP-Mast and religated. pMTEGFP-Mast446-632 was obtained by inserting a BglII EcoRI fragment from the cDNA LD11488 into pMTEGFP-C1 (Clonetch).

Transfections of various constructs expressing the wild type or mutant form of Mast as a fusion protein with EGFP were performed as described by Lemos *et al.* (2000). Transfections were done by calcium-phosphate method. $3x10^6$ *Drosophila* S2 cells were grown in 3 ml of Schneider's medium (Sigma) with 10% fetal bovine serum (FBS), at 25ºC over 24 h. Cells were incubated 16 h at 25ºC with a transfection mix obtained from a solution A (19 μg of plasmid, with 36 μl of $CaCl_2$ 2 M and 245 μl of tissue culture sterile water) and a solution B (300μl of 2x Hepes-Buffered Saline – 50 mM HEPES, 1.5 mM Na_2HPO_4, 280 mM NaCl, pH 7.1). Calcium phosphate solution was remove by centrifugation at 1000 g over 2 min. Cells were then washed and re-suspended with complete medium and after 12 h, expression of EGFP-Constructs was induced from the metallothionein promoter by 1.0 mM $CuSO_4$ for 16 h at 25 ºC. Cells were then analyzed by immunofluorescence.

4.2. Immunofluorescence

Drosophila S2 cells were centrifuged onto slides at 1000 r.p.m. for 5 min, fixed in 3.7% formaldehyde in PHEM (60 mM PIPES, 25 mM HEPES pH 7.0, 10 mM EGTA, 4 mM $MgSO_4$) for 12 min and detergent-extracted with 0.5 % Triton X-100 in PBS (PBST 0.5%) three times for 5 min. Blocking was performed in PBST 0.1% with 10 % of FBS for 30 min at room temperature. Cells were then incubated

with a mouse anti-α-tubulin antibody clone B-5-1-1 (Sigma) at a 1:4000 dilution in PBST 0,1 % with 10% FBS for 1h at room temperature. After washing with PBST 0.1 %, cells were incubated with secondary antibody Alexa 568 anti-mouse (Molecular Probes) at a dilution of 1:2000 in PBST 0,1 % with 10% FBS for 45 min at room temperature. After washing with PBST 0.1% preparations were mounted in Vectashield medium containing DAPI (Vector) to label DNA and observed with a Zeiss Axiovert microscope and images collected using an AxioCam camera (Zeiss). All figures were deconvolved using Axio Vision 4.2 software, projected with ImageJ software (http://rsb.info.nih.gov/ij/) and processed with Photoshop CS (Adobe systems).

4.3. Recombinant protein expression and purification

Mast446-632 fusion protein was obtained by expressing a BglII-EcoRI fragment from the cDNA of Mast (LD11488) in pGEX-5X1 (Amersham). The fusion protein Mast446-1491 was obtained by expressing a StuI-XhoI fragment of LD11488 in pGEX-4T3 (Amersham). Mast1-663 was obtained by inserting a fragment from the cDNA clone LD11488 by PCR using as a template the full-length MAST, a 5' primer containing an *NdeI* site (5'-CGAATAGACATTGCACATATGGCCTATCGG-3') and a 3' primer containing a *XhoI* site (3'-GAGGTGGATCTCGAGTCCTGGCTGTGACTG-5'). The amplified product was subcloned into *NdeI* and *XhoI* sites of pET 23b vector (Novagen). The fusion proteins were transformed in *E. coli* strain BL21, inoculated with overnight culture and incubated at 37ºC until reaching OD_{600} = 0.7. Then expression was induced with 0.4 mM of IPTG and incubated for a further 3 hours. Cell were harvested by centrifugation and ressuspended in the appropriated buffer. Cells were then sonicated, centrifuge again and the supernatant save for protein purification.

Mast1-663 was purified using *Hitrap affinity columns* (Amersham Pharmacia Biotech) according to manufacturer's instructions. Briefly, the column was equilibrated with10 ml of binding buffer (20 mM phosphate buffer, 0.5 M NaCl and

20 mM imidazole). Then the pre-filtered sample was applied to the column followed by a wash with 30 ml of binding buffer and finally the purified protein was eluted with elution buffer (binding buffer with 500 mM of imidazole)

4.4. Overlay assays

Microtubule overlay assays were performed as previously described (Inoue *et al*, 2000). Mast recombinant fusion proteins (500 ng/lane) were fractionated in a 10 % SDS-PAGE and then transferred into PROTAN® nitrocellulose membranes (Schleicher & Schuell BioScience). To determine loading levels, the membranes were stained with Ponceau S. Then the membranes were blocked overnight in TBST buffer (150 mM NaCl, 50 mM Tris, pH 8, 0.05% Tween 20) plus 5% powdered milk. Membranes were then washed 3 times 15 min with lysis buffer (100 mM Pipes, 5mM EGTA, 1mM $MgSO_4$, 0.9M glycerol, 1mM DTT and protease inhibitor cocktail tablets (Complete, Roche) and, depending on the assay, preincubated for 30 min with lysis buffer containing 2mM nucleotides (GTP or GDP). At this stage tubulin (Molecular Probes) was polymerized for 30 min at 37ºC in lysis buffer containing 1 mM GTP. Then excess solution was removed from the membranes and the blots were incubated with microtubules in lysis buffer during 1 hour at 37ºC, followed by a 30 min incubation in which taxol was added to a final concentration of 10 μM. Then blots were washed 2 times 15 min with TBST buffer and the bound tubulin detected using standard Western Blots procedures with mouse monoclonal anti α-tubulin antibody, clone DM1A (Sigma) 1:4000, anti-mouse HRP (Amersham Biosciences) 1:2500 and ECL™ detection reagents (Amersham Biosciences).

4.5. GTP agarose assays

To determine whether Mast binds GTP, 50 μl GTP-agarose beads (Sigma) were equilibrated with binding buffer (20 mM Tris pH 7.5, 200 mM NaCl, 50 mM

KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.01% Triton X-100, protease inhibitor cocktail tablets (Complete, Roche)). After equilibration, beads were incubated with 250 ng of purified recombinant Mast1-663 protein during 1 hour at 4ºC. Beads were then centrifuged and washed three times with binding buffer and bound protein were eluted by boiling for 5 min in 2x SDS. Samples were analysed by SDS-PAGE and western blot with anti-Mast.

4.6. GTPase assays

All GTPase assays were done at 37ºC. Purified Mast1-663 (10 μg) were incubated in buffer GTPase (50 mM Tris-HCL pH 8, 5 mM $MgCl_2$, 100 mM KCL, 10% glycerol, 1mM DTT) and then reactions (in 30 μl) were initiated by the addition of GTP (1 mM) and 10 μCi ml^{-1} [α^{32P}] GTP. The reactions were stopped at various time points (0, 30, 60, 90 and 120 min) by adding an equal volume of a solution containing 2mM EDTA and 0.5% SDS and then boiled for 2 min at 65ºC. Samples were spotted onto a PEI cellulose thin-layer chromatography plate (Merck) and resolved with a solution containing 1M acetic acid and 1M LiCl. The signals were analysed by autoradiography and phosphorimager and quantified using ImageJ software (http://rsb.info.nih.gov/ij/).

4.7. Site direct mutagenesis

Mast1-663 was mutated with QuikChange ® XL site-directed mutagenesis kit (Stratagene). The primers used to insert the mutation were (5'-CGAGAGGGCGGCGGAGCAGCAGCAACTGGTACTGGGACTGGG-3') and (5'-CCCAGTCCCAGTACCAGTTGCTGCTGCTCCGCCGCCCTCTCG-3'). After the PCR reaction with pET-Mast 1-663 as template, 1μl of the DpnI enzyme was added directly to the amplified product and incubated at 37ºC during one hour. 2 μl of the DpnI treated DNA was used to transform ultracompetent cells and single colonies were tested by digestion and sequencing.

5. References

5. References

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