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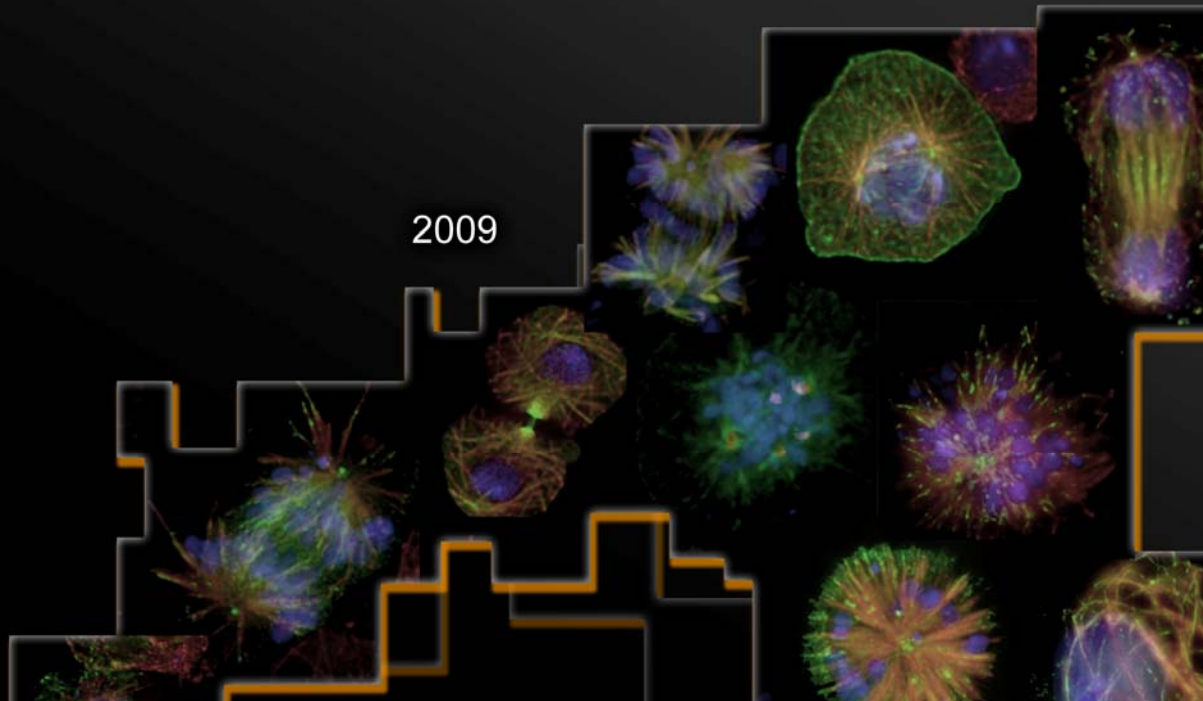
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
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The involvement of the protein Mast in microtubule dynamics and kinetochore-microtubule interactions

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in kinetochore-microtubule interactions**

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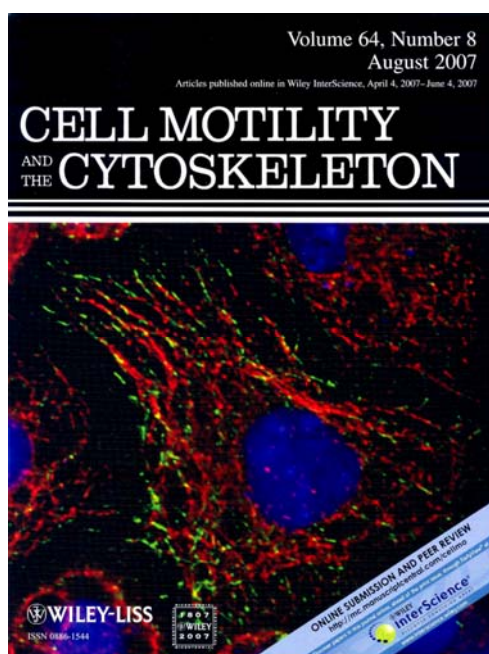
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SUMMARY

The dynamic behavior of microtubules is crucial for a normal cell division and so it must be highly regulated. One of the most important events in the cell cycle is the conversion of the interphase microtubule network into a mitotic bipolar spindle with microtubules capable of capturing, align and segregating sister chromatids to opposite poles of the dividing cell, generating two daughter cells with the same genetic material as the mother cell. The +TIPs (plus-end tracking proteins), have been described to modulate the dynamic properties of microtubules. Mast/Orbit/CLASP is a +TIP that has important roles in interphase and mitosis. The work presented in this thesis aims to further our understanding on the role of Mast in microtubule-kinetochore interaction using as a model *Drosophila melanogaster*. First, we dissect Mast into its functional domains through analysis of transient transfections of S2 cells. The results show that, the microtubule-binding domain of Mast is located at the central basic region of the protein and the kinetochore-binding domain at the C-terminal. The second part of the thesis, describes the behavior of Mast as a +TIP. *In vivo* studies of interphase cells expressing EGFP-Mast and EGFP-Mast-fragments show that Mast exhibits a typical +TIP behaviour, binding preferentially at the plus-ends of microtubules when present at normal levels, and along the entire length when overexpressed. Moreover, the data demonstrate that Mast moves at a velocity very similar to microtubule polymerizing rate. In addition, we found that although a small central region is sufficient to promote microtubule plus-ends accumulation, Mast requires the full-length sequence to display normal plus-end directed +TIP behavior. Finally, the last part of the work focuses on the role of Mast at kinetochores and explores the functional interplay with Dynein in this process using embryos and S2 cells. The results show that Mast has a dynamic localization at kinetochores, accumulating at high levels during prometaphase and decreasing at metaphase when starts to be removed along microtubules in a poleward streaming fashion, partially dependent on Dynein. In addition, the data suggests that Mast and Dynein have antagonistic roles in the regulation of microtubule plus-end dynamics at kinetochores contributing to spindle bipolarity. In the absence of Dynein, Mast-depleted cells form bipolar spindles without collapse, a rescue that is correlated with a rescue of microtubule polymerizing rate, which is lost upon depletion of Mast. These data suggest that Mast is not involved in the direct incorporation of tubulin at the kinetochore-microtubule plus-ends, but rather regulates this process. Together, the work presented in this thesis provides further insights into Mast interactions with microtubule plus-ends and kinetochores, and into involvement of Mast in kinetochore-microtubule interactions.

RESUMO

O comportamento dinâmico dos microtúbulos é crucial para uma correcta divisão celular, sendo por isso muito bem regulado. Um dos principais eventos do ciclo celular é a transformação da rede de microtúbulos interfásicos no fuso mitótico bipolar, com microtúbulos capazes de capturar, alinhar e segregar as cromátidas irmãs para pólos opostos da célula em divisão, gerando assim duas células filhas com o mesmo material genético que a célula mãe. As +TIPs (proteínas que se ligam às extremidades "+" dos microtúbulos) são descritas como moduladoras das propriedades dinâmicas dos microtúbulos. A Mast/Orbit/CLASP é uma +TIP com funções importantes tanto em interfase como em mitose. O trabalho apresentado nesta tese pretende esclarecer o papel da Mast nas interacções cinetocóro-microtúbulo usando como modelo a *Drosophila melanogaster*. Primeiro, através de transfecções transientes de células S2, dissecámos os domínios funcionais da Mast. Os resultados revelam que o domínio de ligação aos microtúbulos se localiza numa zona básica da região central da proteína e o domínio de ligação aos cinetocóros na região C-terminal. A segunda parte da tese descreve o comportamento da Mast como uma +TIP. Estudos *in vivo* de células interfásicas que expressam EGFP-Mast completa ou versões truncadas, mostram que a Mast tem um comportamento típico de uma +TIP, ligando-se preferencialmente às extremidades "+" dos microtúbulos quando presente em níveis normais, e ao longo de todo o comprimento quando sobreexpressa. Os resultados também revelam que a Mast se move a uma velocidade muito semelhante à taxa de polimerização dos microtúbulos e que apesar de uma pequena região central ser suficiente para promover a sua acumulação nas extremidades "+" dos microtúbulos, necessita da sequência completa para exibir a sua dinâmica normal. Por último, a terceira parte do trabalho foca-se no papel da Mast nos cinetocóros e explora a sua interacção funcional com a Dineína nesse processo usando embriões e células S2. Os resultados demonstram que a Mast tem uma localização dinâmica nos cinetocóros, acumulando durante a prometáfase e diminuindo em metafase quando começa a ser removida ao longo dos microtúbulos por um processo parcialmente dependente da Dineína. Os dados obtidos sugerem que as duas proteínas têm funções antagonistas na regulação da dinâmica das extremidades "+" dos microtúbulos nos cinetocóros, contribuindo assim para a bipolaridade do fuso. Na ausência de Dineína, células sem Mast são capazes de formar fusos bipolares sem colapsar, esta capacidade deve-se à recuperação da taxa de polimerização das extremidades "+" dos microtúbulos, que é praticamente nula em células depletadas de Mast. Os resultados sugerem que a Mast não está envolvida na incorporação directa de tubulina nas extremidades "+" dos microtúbulos nos cinetocóros, mas sim na regulação deste processo. No seu conjunto, o

trabalho apresentado nesta tese traz novas perspectivas sobre a interacção da Mast com as extremidades "+" dos microtúbulos e com os cinetocóros, e sobre o seu envolvimento nas interacções cinetocóro-microtúbulos.

RÉSUMÉ

Le comportement dynamique des microtubules est fondamental pour une division cellulaire correcte, donc, il est très bien régulé. Un des principaux événements du cycle cellulaire c'est la transformation du réseau de microtubules interphasiques dans le fuseau mitotique bipolaire, avec des microtubules capables de saisir, organiser et séparer les chromatides sœurs en direction des pôles opposés de la cellule en division, en créant ainsi deux cellules filles avec le même matériel génétique que la cellule mère. Les +TIPs (des protéines qui se lient aux extrémités "+" des microtubules) sont décrites comme modulatrices des propriétés dynamiques des microtubules. La Mast/Orbit/CLASP est une +TIP avec d'importants rôles soit en interphase soit en mitose. Le travail présenté dans cette thèse veut rendre clair le rôle de la Mast dans les interactions kinétochore-microtubules utilisant comme modèle la *Drosophila melanogaster*. La première partie de la thèse, décrit l'analyse aux domaines fonctionnels de la Mast à travers des transfections de cellules S2. Les résultats ont démontré que le domaine de liaison aux microtubules se situe dans une région centrale du noyau de la protéine et que le domaine de liaison aux kinétochores se situe dans la région C-terminale. Ensuite, la deuxième partie de cette thèse décrit le comportement de la Mast comme une +TIP. Des études *in vivo* des cellules interphasiques qui révèlent des EGFP-Mast entières ou des versions coupées, démontrent que la Mast a un comportement typique d'une +TIP, en se liant préférentiellement aux extrémités "+" des microtubules quand il est présent dans des niveaux normaux, et au long de toute la longueur quand elle y est surexprimée. Les résultats révèlent aussi que la Mast se déplace à une vitesse semblable au taux de polymérisation des microtubules et bien qu'une petite région centrale soit suffisante pour promouvoir son accumulation dans les extrémités "+" des microtubules, elle a besoin d'une séquence complète pour produire sa dynamique normale. Finalement, la troisième partie du travail met au point le rôle de la Mast dans les kinétochores et exploite son interaction fonctionnelle avec la Dineine dans ce processus, en utilisant des embryons et des cellules S2. Les résultats prouvent que la Mast a une localisation dynamique dans les kinétochores, en s'accumulant pendant la prométaphase et en diminuant en métaphase quand elle commence à être supprimée au long des microtubules par un processus partiellement dépendant de la Dineine. Les données obtenues suggèrent que les deux protéines ont des fonctions antagonistes dans la régulation de la dynamique des extrémités "+" des microtubules dans les kinétochores, en convergent vers la bipolarisation du fuseau mitotique. Lorsque la Dineine est absente, des cellules sans Mast sont capables de créer des fuseaux bipolaires sans s'effondrer, cette capacité se doit au rattrapage du taux de la polymérisation des extrémités "+" des microtubules qui sont

presque nulle dans les cellules où la déplétion de la Mast a eu lieu. Les résultats suggèrent que la Mast ne participe pas à l'incorporation directe de la tubuline dans les extrémités "+" des microtubules dans les kinétochores, mais elle contribue dans la régulation de ce processus. Dans son ensemble, le travail présenté dans cette thèse apporte de nouvelles perspectives sur l'interaction de la Mast avec les extrémités "+" des microtubules et avec les kinétochores, et sur sa participation dans les interactions kinétochore-microtubules.

PART I

GENERAL INTRODUCTION

1. CELL CYCLE

The first time the word “cell” was used in histological description was in 1663 when an English scientist, Robert Hooke, observing cork at the microscope saw tiny box-like structures and coined these structures as “CELLS” from the Latin “*cellula*” which means small compartment (Hooke, 1665). Nevertheless, it took almost 200 years until the formulation of the “Cell theory”, by Schleiden and Schwann in 1838 (Schwann, 1847), that conceptualizes the cell as the *quantum minimum* of life, capable of independent development, yet an integral part of the organism as a whole (Tavassoli, 1980). However, Schwann mistakenly thought that cells form *de novo* (free-cell formation) and only at 1858 Rudolf Virchow presented the correct interpretation of cell formation with “The law of continuous development” translated in the expression “*Ominis cellula e cellula*” (all cells only arise from pre-existing cells) (Turner, 1890). It was the beginning of a new era in biology, in which “Cell Division” would take a central stage.

In 1879, Walter Flemming was the first to describe in great detail cell division. For the first time he considers that the stainable substance in the interphase nucleus (Chromatin – “stainable material”) shortens and thickens originating the threads (Chromosomes – “stainable bodies”). But, Fleming's great achievement was that during cell division one longitudinal half of each chromosome goes in each direction, so that each daughter nucleus is formed from a complete set of longitudinal halves. In 1882 he termed this process Mitosis (from the Greek *mitos* “thread-metamorphosis”) (Baker, 1949; Paweletz, 2001).

1.1. OVERVIEW

Cell cycle can be defined as a sequence of events that leads to the reproduction of the cell. The generation of two identical daughter cells with the same genetic material as the parental cell is the basis for the development of multicellular organisms and for its maintenance as adults by continuous cell renewal and regeneration.

The cell cycle is a highly regulated process that can be simplified by dividing it into two major phases, Interphase, a long period during which the cell duplicates its contents and grow, and a shorter phase, M-phase, in which the cell contents are equally distributed into two new formed cells (Fig. 1). Interphase is the period between the end of one M-phase and the beginning of the next. During interphase, the chromatin is decondensed and cells appear morphologically uniform but at molecular level it is the time during which both cell growth and DNA replication occur preparing the cell for its final division. Although most of the cell's components like cytoplasmic organelles, membrane, proteins and RNAs,

are replicated continuously, resulting in the doubling of the cell size, the chromosomes are present in only single copies and must be duplicated only once per cycle, this occurs in the Synthesis phase (S-phase) so that at the end of this stage each chromosome has now two sister chromatids. This phase is preceded and followed by Gap phases, G1 and G2, respectively.

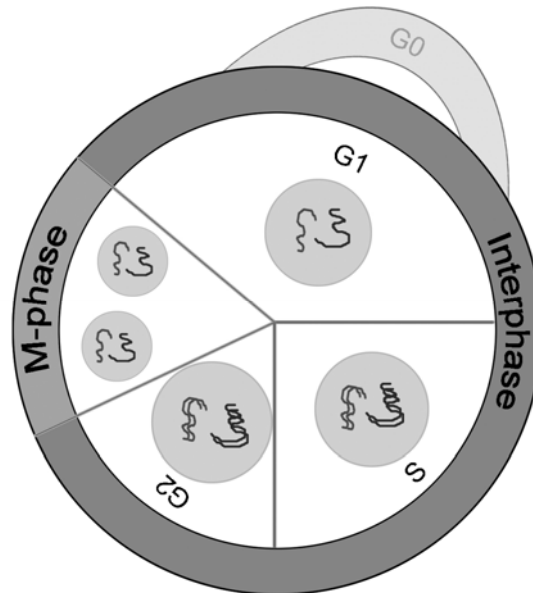


Figure 1. Illustration of the eukaryotic cell cycle. There are two major phases, Interphase (orange) and M-phase (green). Interphase is composed by two Gap-phases, G1 and G2, during which the cell grows by synthesizing components required for DNA synthesis or preparing for mitosis. At G1, cell can chose by exiting from the cell cycle (ex.G0) or continue to divide. If it chose to proliferate, cell proceeds to the next phase, S-phase, in which occurs the replication of the DNA. Finally, during M-phase, cell contents are equally distributed and two new daughter cells are formed.

G1 is the first phase of interphase, during which the biosynthetic machinery of the cell, after been slowed down during M-phase, is very active, in particular the synthesis of enzymes required for DNA replication. This Gap phase is a specially important regulatory period because here most cells choose its fate; they can proliferate (continuing dividing) or exit from the cell cycle and became quiescent (G0, reversible non-proliferating resting state), senescent (permanently retired out of the cell cycle, final resting state), differentiated (irreversibly acquire a specialization), or go into apoptosis (programmed cell death) (Blomen and Boonstra, 2007). The second Gap phase, G2, occurs between S and M-phase, during which cells continue to grow and proteins are synthesized in preparation for Mitosis. Finally, the second major phase of the cell cycle is M-phase, which is divided into two key events: nuclear division (mitosis) and cytoplasmic division (cytokinesis).

1.2. MITOSIS

Mitosis is a complex, highly regulated and beautiful process used by eukaryotes to generate two identical daughter cells from one original mother cell.

All somatic cells in G1 have two copies of the genome, one inherited from the father and one from the mother, being thus known as diploid cells. Mitosis, by ensuring that the cell faithfully segregates the sister chromatids of the duplicated chromosomes into the daughter cells, guarantees that when the somatic cell divides the resulting cells not only still diploid but also contain two complete copies of the mother cell genome. The central events of mitosis start with the formation of chromosomes, through condensation of the chromatin, followed by formation of mitotic spindle. This bipolar array of microtubules will attach to each sister chromatid and after their separation will be responsible for their segregation to opposite poles of the cell leading to the formation of two daughter nuclei. Although this series of events occur continuously, they are divided into five phases: Prophase, Prometaphase, Metaphase, Anaphase and Telophase (Fig. 2).

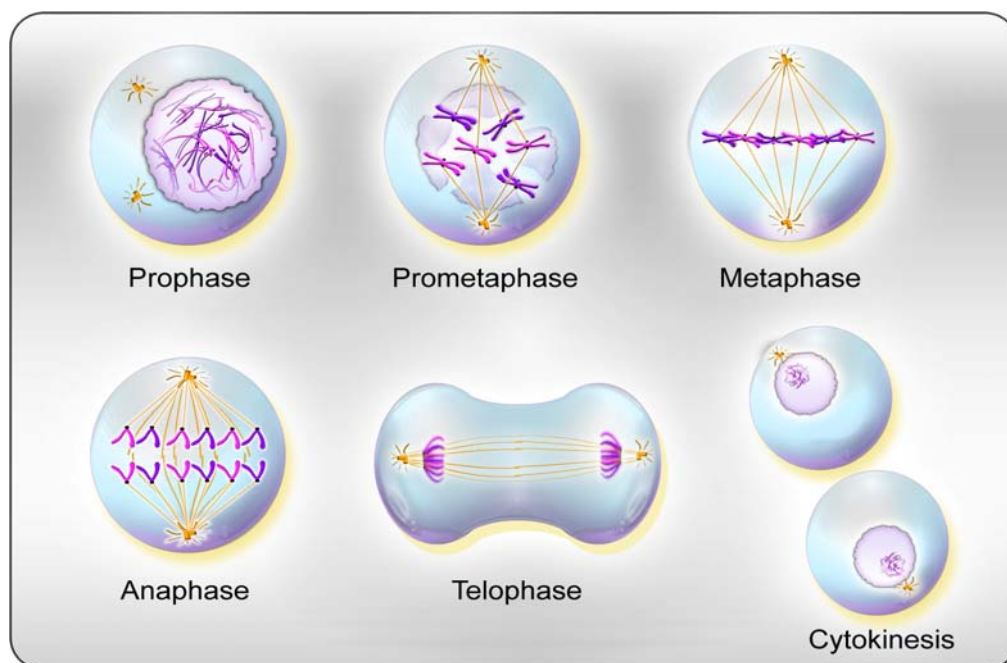


Figure 2. Schematic representation of the mitotic stages and cytokinesis of animal cells. The first stage of mitosis is *Prophase* during which chromatin condense into chromosomes and centrosomes start to separate. Nuclear envelope breakdown point out the beginning of *Prometaphase* when chromosomes are captured by microtubules that are growing from opposite poles of the spindle. At *Metaphase*, all chromosomes align at the equator of the cell and at *Anaphase*, sister chromatids move to opposite poles. Finally, at *Telophase*, sister chromatids reach the poles and decondense and nuclear membrane reforms around each identical daughter nuclei. Simultaneously, the division of the cytoplasm generates two independent daughter cells by a process called *Cytokinesis*.

Early mitosis includes *Prophase*, that is marked by the emergence of condensed chromosomes that appear as two identical filaments (sister chromatids, produced in S phase), held together by a constricted region, the centromere, where specific proteins bind and form the kinetochore (Kt) – the site where spindle microtubules attach. It is also during this phase that the centrosomes, the major microtubule-organizing center (MTOC), begin to separate to opposite sites, and initiate the spindle assembly serving as the two poles for the mitotic spindle. The next stage, *Prometaphase*, starts with the nuclear envelope breakdown (NEBD), allowing that microtubules from the opposite centrosomes occupy all the cytoplasmic space and fully organize the mitotic spindle. These microtubules bind the kinetochores of chromosomes in a manner that the kinetochores of sister chromatids are oriented and attached to opposite poles of the spindle. Finally, all chromosomes reach and align at the equatorial region, at this stage the cell has achieved the *Metaphase*. In this stage of mitosis, chromosomes are aligned at the middle of the spindle forming the metaphase plate, awaiting the signal to the next stage, *Anaphase*. The transition from metaphase to anaphase is the most dramatic event of the cell cycle. This event occurs when the cohesion between sister chromatids is abruptly dissolved and the separated sisters migrate to opposite poles of the spindle. In anaphase B the spindle poles also moves apart and the spindle elongates. The last stage of mitosis is *Telophase*; the chromosomes decondense, nuclear membrane reappears and like other nuclear components are repackaged into two identical daughter nuclei. Simultaneously with these later events occurs the second stage of M-Phase, Cytokinesis that involves the division of the cell cytoplasm and separation and formation of the two interphase daughter cells.

1.3. CELL CYCLE CONTROL SYSTEM

The most important events of the cell cycle are DNA replication (S-Phase) and its equal division into the daughter cells (Mitosis). If these events do not occur properly and in the right sequence, the newly formed cells either die or carry on genetic damages that can lead ultimately to cancer. Due to the dramatic consequences of a failure, the cell cycle must be tightly controlled and regulated.

1.3.1. CELL CYCLE REGULATION

Different cell cycle phases were linked together in a continuous sequence in which later events only occur when earlier events are successfully completed. Thus, the beginning of mitosis is dependent on completion of DNA synthesis and the completion of mitosis is dependent on proper mitotic spindle assembly and microtubule-kinetochore attachment. By ensuring this unidirectionality, the regulation of the cell cycle becomes a vital process for the normal development of living organisms (Murray and Kirschner, 1989). Nowadays it is clearly recognized that the master controller of the regulation of the cell cycle are a family of enzymes called *Cyclin-dependent kinases* (Cdks). Its importance is such that it was proposed that Cdks “*are the engines that drive the events of the eukaryotic cell cycle and the clock that times them*” (Morgan, 1997). Like other kinases, Cdks catalyze the attachment of phosphate groups to protein substrates, inducing changes in their enzymatic activity or interaction with other proteins.

Studies in many different organisms were required to conclude that the Cdks are the cell cycle controllers. Now it is well established that these kinases are composed by two components, a catalytic subunit that transfers the phosphate to the substrate and the regulatory subunit, cyclin. Complete activation of Cdk, requires both the binding of the cyclin and phosphorylation of a threonine residue adjacent to the kinase active site, catalyzed by CAKs (Cdk-activating kinases).

Cyclins were discovered using early sea urchin eggs in which differences between the patterns of protein synthesis before and after activation by sperm were analyzed. In this study, Evans et al. observed that some of the proteins whose synthesis was strongly activated after fertilization appear to be destroyed every time the embryos divided. As the concentration of these specific proteins cycle in phase with the cell cycle they call them *cyclins* (Evans et al., 1983).

A significant advance in the identification of factors that induces mitosis also came from physiological studies with frog oocytes. In *Xenopus*, fully grown oocytes (immature) in response to specific signals (ex. progesterone), were released from G2 arrest and start to mature. Oocyte maturation begins with the first meiotic division, meiosis I and is completed when the oocyte is arrested in metaphase of meiosis II and converted to an unfertilized egg. Masui et al. discovered the “entity” whose activity controls entry into M-phase, the Maturation-Promoting Factor, by observing that although the absence of progesterone, when cytoplasm from unfertilized eggs arrested in metaphase of meiosis II is injected into recipient G2-arrested oocytes (immature), these overcome the arrest and mature into eggs (Masui and Markert, 1971). Later, MPF was also called Mitosis-Promoting Factor since it was observed that its activity controls entry in mitosis.

In parallel, genetic studies in fission yeast designed to identify cell cycle control genes resulted in the identification of *cdc2* (cell division cycle). Mutants lacking this gene could not undergo S phase or mitosis and therefore never divide but showed continued to grow in size. This gene is also found in humans and it was seen that the human *cdc2* was able to complement yeast *cdc2* mutant, suggesting that the mechanisms that control the cell cycle are conserved from yeast to humans (Lee and Nurse, 1987; Nurse, 2000).

Now it is well-established that MPF is a heterodimer composed by one molecule of cyclin (B) and one of Cdc2. Later, Cdc2 became known as Cdk1 since it was established that “kinases that are associated with cyclins should be called ‘cyclin dependent kinases’ or Cdks” (Doree and Hunt, 2002). Each Cdk catalytic subunit can associate with different cyclins and the associated cyclin determines which proteins will be phosphorylated by the Cdk-Cyclin complex. In metazoan cells Cdks act in early G1 to activate E2F-dependent transcription of genes required for S phase, in late G1 to initiate S phase, and finally in G2 to initiate mitosis.

Different cyclins are produced at distinct cell cycle stages, resulting in the formation of a series of Cyclin-Cdk complexes (Fig. 3). Cyclins that directly regulate Cdks controlling cell-cycle progression can be divided into four classes: *G1* (*cyclin D* in vertebrates), contributes to the control of cell-cycle entry in response to extracellular factors. *G1/S* (*cyclin E* in vertebrates), rise in late G1 and falls in early S phase, formation of active G1/S-Cdk complexes triggers progression of the cell through the “restriction point” (the point of the cell that occurs the commitment to cell division, ‘start’ in yeast) and initiates the processes that lead to DNA replication and other cell cycle events such as duplication of the centrosome. *S cyclins* (*cyclin A* in vertebrates), remains high throughout S phase, G2 and early mitosis, cyclin A-Cdk complexes stimulate DNA replication and help to promote early mitotic events. Finally, at last appear *M cyclins* (*cyclin B* in vertebrates) whose concentrations rises as the cell approaches mitosis and reach a maximum at metaphase. Cyclin B-Cdk1 accumulation is essential for entry in mitosis and its activity leads to the phosphorylation of several substrates with important roles in early stages of mitosis, like nuclear lamins, kinesin-related motors and other microtubule binding proteins, condensins and Golgi matrix components. These proteins are involved in central mitotic events as nuclear envelope breakdown, centrosome separation, spindle assembly, chromosome condensation and Golgi fragmentation, respectively. The destruction of the Cyclin-Cdk1 complexes in anaphase is required for mitotic exit and cytokinesis (Nigg, 2001).

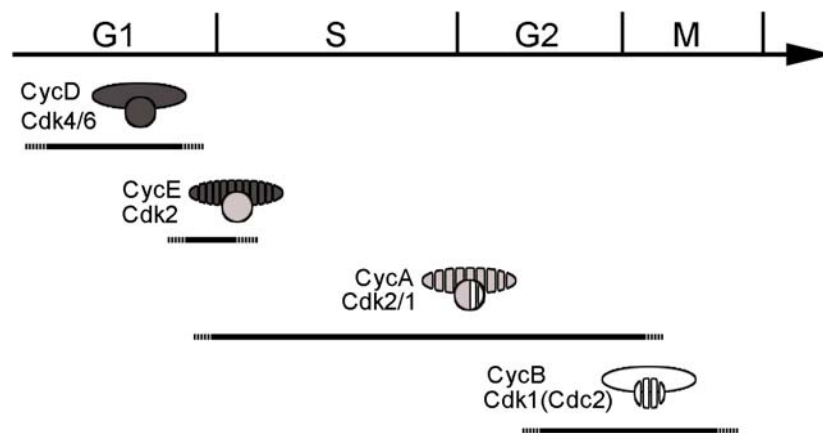


Figure 3. Cyclin-Cdk complexes along the different cell cycle stages in vertebrates. Cyclin D controls the cell cycle entry; Cyclin E initiates the process of DNA replication; Cyclin A stimulates DNA replication and promotes early mitotic events; finally, Cyclin B is essential for entry mitosis and its degradation necessary for mitotic exit (adapted from Morgan, 1997; Vermeulen et al., 2003).

Contrary to cyclins, whose function are primarily controlled by changes in protein levels, levels of Cdks tend to remain constant throughout the cell cycle and its activity is regulated by a combination of mechanisms. These includes changes in cyclin gene expression, rates of cyclin degradation, changes in phosphorylation of the catalytic site controlled by Wee1 through phosphorylation (inhibition) and Cdc25 through dephosphorylation (reactivation), and by changes in levels of Cdk inhibitor proteins (CKIs) (Morgan, 1995).

Other important regulatory processes for cell cycle progression are proteolysis and transcription. Besides the direct role in regulating Cdks by controlling cyclin levels, proteolysis drives cell cycle progression also by directly influence chromosome and spindle dynamics triggering some cell cycle key events such as sister chromatids separation at metaphase-anaphase transition, contributing therefore to the irreversibility of the cell cycle (King et al., 1996). Also, transcription is involved in regulating the cell cycle since progression in cell cycle is associated with periodical transcription of genes that can either control directly cell cycle progression, or function in metabolic processes linked to the cell cycle, such as nucleotide and DNA biosynthesis. Particularly E2F family is an important family of transcription factors that during G1 regulate genes required for the S phase onset, if these genes are activated earlier in G1 then S phase can be advanced (Muller, 1995).

1.3.2. CELL CYCLE CHECKPOINTS

Checkpoint was first defined by Hartwell and Weinert in 1989 as a “control mechanism enforcing dependency in the cell cycle” meaning that the cell ‘checks’ if an earlier event is successfully completed before proceeds to a later event (Hartwell and Weinert, 1989). These control points occur before key events like DNA replication and genome segregation, by delaying progression through the cell cycle they provide time to cell for repair. Thus checkpoints are fundamental for the maintenance of genomic stability since loss or attenuation of their function would allow cells to divide even with DNA damaged or incompletely replicated, or when chromosomes are incorrectly attached to the spindle, contributing to genomic instability and consequent generation of cancer cells (Kaufmann and Paules, 1996). There are three major cell cycle checkpoints: Restriction point (R or G1-phase checkpoint), DNA damage and Spindle Assembly Checkpoint (SAC) (Fig. 4).

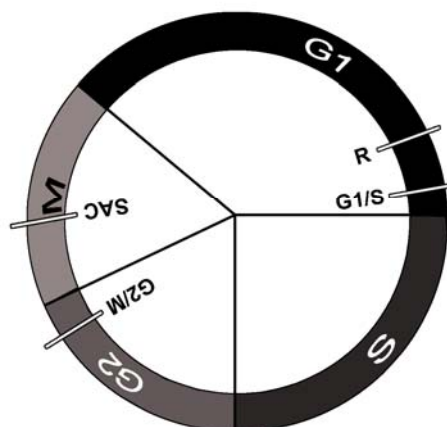


Figure 4. Cell cycle checkpoints. R represents the restriction point or G1-phase checkpoint, cells that overtake this point proceed to cell division; G1/S and G2/M compose the DNA damage checkpoints, which act before and after DNA replication, detecting DNA lesions; Spindle Assembly Checkpoint (SAC), ensures that chromosomes are aligned and correctly attached to the spindle microtubules before segregate.

Restriction point (Start in yeast) or *G1-phase checkpoint*, is defined as a point of no return because cells that transverse this point became committed to cell division. But when the cell is under growth limiting conditions G1-checkpoint is activated and the cell became arrested with a G1 amount of DNA (Cooper, 2006). There are experiments demonstrating that cells starved of serum before the restriction point enter in a G0 state while cells starved after this G1 checkpoint are unaffected and proceed through the cell cycle (Pardee, 1974).

DNA damage checkpoints, detect DNA lesions such as single strand DNA or DNA double-strand breaks, arrest the progression of the cell cycle and trigger DNA repair.

These checkpoints localize before the cell enters S phase (*G1/S checkpoint*) or after DNA replication (*G2/M checkpoint*). At *G1/S checkpoint*, cell cycle arrest is p53-dependent. Upon DNA damage, ATM and ATR kinases (transducers of DNA damage signals) phosphorylate p53 leading to increased levels of this transcription factor. As the key transcriptional target of p53 is the p21 Cdk inhibitor (CKI), which inhibits cyclin E-cdk2 activity, the G1/S transition is inhibited and thus the replication of damaged DNA prevented. When DNA damage occurs during G2, the *G2/M checkpoint* is activated and the cell cycle arrest is independent of p53. The progression to mitosis is prevented by maintaining Cdk1-Cyclin B complex inhibited by phosphorylation. DNA damage in an ATM-dependent manner activates the kinases Chk1 and Chk2 which phosphorylates Cdc25 inhibiting its activity and preventing it from activate the Cdk1-Cyclin B complex and mitotic entry (Elledge, 1996; Niida and Nakanishi, 2006; Sanchez et al., 1997; Vermeulen et al., 2003).

Spindle Assembly Checkpoint (SAC) stops mitosis before the sister chromatids separation at metaphase-anaphase transition. Proper chromatids segregation occurs only when several conditions are satisfied such as assembly of bipolar spindle, chromosomes attached by sister kinetochores to microtubules from opposite poles of the spindle and alignment on metaphase plate of properly attached chromosomes. The spindle assembly checkpoint prevents progression through mitosis until all these processes are concluded (Elledge, 1996). Separation of sister chromatids is then triggered by a protease called separase that is required to cleave the cohesion complex that holds sister chromatids together. Separase is kept inhibited by two mechanisms: by its association with an inhibitory protein called securin and due to phosphorylation by Cdk1-Cyclin B. Then, separase protease activity is turned on by the destruction of both securin and cyclin B. This process is mediated by the 26S proteasome, which is preceded by their ubiquitination by the ubiquitin protein ligase called anaphase promoting complex or cyclosome (APC/C), whose activity depends on the activator protein Cdc20 (Zachariae and Nasmyth, 1999). Checkpoint proteins involved in the spindle assembly checkpoint, like Mad2 and BubR1, tightly inhibit the APC/C by sequestering the co-factor Cdc20, maintaining it unable to activate APC. Thus SAC regulates negatively the ability of Cdc20 to activate the APC/C-mediated polyubiquitynation and consequent destruction of two key substrates, securin and cyclin B, hence preventing the action of separase and the inactivation of Cdk1-Cyclin B complex required to cell exit from mitosis (Musacchio and Salmon, 2007).

2. MITOTIC SPINDLE

Flemming saw that the nucleus did not divide directly into two, instead chromatin first condense and the resulting chromosomes moved apart to the two daughter cells by a transient fibrillar achromatic apparatus (Flemming, 1965). In 1953 Inoue using a polarizing microscope observed, in living cells, this fibrous apparatus composed of continuous fibers, chromosomal fibers and astral arrays (Inoue, 1953). Subsequent studies revealed several dynamic characteristics of spindle fibers indicating that they were not static structures, but rather exhibit a dynamic behavior: the birefringence of the spindle fiber could be reversibly reduced or totally abolished by low temperature or with colchicine, an anti-mitotic alkaloid; the fibers were organized by “centers” like centrioles and kinetochores that are able of nucleating the fibrils from a preformed pool of unassembled subunits; poleward chromosome movement are linked with the birefringent fibers (Inoue, 1981).

2.1. CENTROSOMES

Although Edouard van Beneden and A. Neyt were the first to observe the “corpuscule central” inside the asters, it was Boveri in 1888 that coined the name for this organelle, as the “centrosome”, and realized its importance for cell division and fertilization. Boveri described the centrosome as constituted by a pair of centrioles surrounded by a special material, which is capable to assemble a sphere of archiplasm (aster-forming material) containing all the components involved in the transient generation of an astrosphere. He also observed that the centrosomes are donated by the sperm and that the fibrils emerging from one centrosome can only contact one side of each chromosome, while the other side attaches to fibrils from the other centrosome. Also at that time Boveri recognized the major role of centrosomes in cancer (Maderspacher, 2008; Moritz and Sauer, 1996; Schatten, 2008).

2.1.1. COMPOSITION AND KEY FUNCTIONS

The centrosome of animal cells, in interphase, is located in the cytoplasm just outside the nuclear envelope. It is comprised of two centrioles positioned orthogonally to each other that are surrounded by an electron-dense matrix called pericentriolar material (PCM). Each centriole is a short cylindrical array of nine microtubule triplets and the PCM is a fibrous scaffolding lattice that consists of a large amount of proteins that docks regulatory components and molecules that mediate the anchoring and nucleation of

microtubules, some are permanently associated with the centrosome core structure such as γ -tubulin, γ -TuRC and centrin while others are only temporarily associated (Schatten, 2008).

The major function of the centrosome is to organize cellular microtubules and is thus known as the Microtubule (MT) Organizing Center (MTOC). This organelle is the primary MTOC of the mammalian cells. Through its microtubule-organizing function, the centrosome is also involved in some cell key events like organization of the interphase microtubule network and mitotic spindle, cytokinesis and formation of cilia and flagella. The involvement of the centrosome in these central cellular processes allow it to facilitate several activities including maintenance of cell shape, transport of vesicles, cell division, polarity, adhesion and cell motility. In addition to the MTOC function the centrosome seems to be also involved in regulating cell cycle progression from G2 into mitosis and G1/S phase transition (Kramer et al., 2004; Rieder et al., 2001; Schatten, 2008).

Although the MTOC functions of centrosomes are its most well-known activity, centrosomes are not essential for the survival and division of somatic cells since functional microtubule arrays can be organized during interphase and mitosis in the absence of this organelle. However, the centrosome is essential for the formation of primary cilia in somatic cells and flagella in sperm. In addition to the centrosomal pathway, cell possesses alternative and redundant mechanisms where nucleation of MTs occurs in the immediate vicinity of the chromatin. These pathways are not mutually exclusive but when centrosomes are present, they act in a dominant fashion. Contrary to what happens in individual cells, for an organism the centrosome is vital for proper development and healthy survival since formation of cilia and flagella derive from it and are essential to higher animals (Martinez-Campos et al., 2004; Rieder et al., 2001; Sluder and Nordberg, 2004).

2.1.2. CENTROSOME CYCLE

Centrosome cycle can be divided in four main phases: centrioles disengagement, nucleation of the daughter centrioles (pro-centrioles), elongation of the pro-centrioles and separation of the centrosomes (Fig. 5) (Bettencourt-Dias and Glover, 2007).

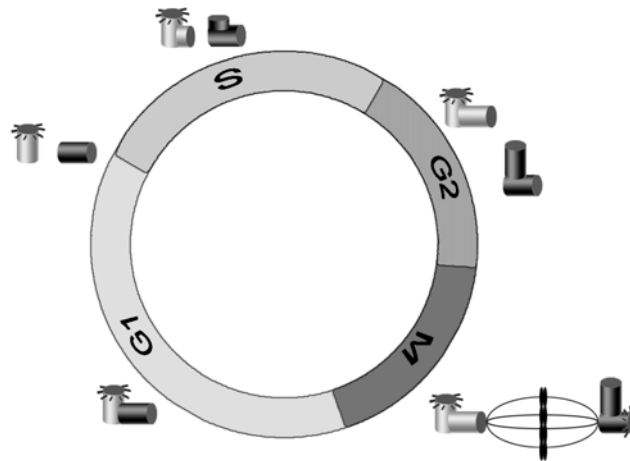


Figure 5. Centrosome duplication cycle. Centrioles are represented by dark and light grey cylinders, with distal appendages shown as rays. At G1, each cell contains one centrosome composed by two centrioles that in G1/S split apart. At S phase occurs the nucleation of pro-centrioles that elongate at the time of G2. At mitosis the new formed centrosomes migrate to form the poles of the mitotic spindle (adapted from Urbani and Stearns, 1999).

A G1 cell contains a single centrosome, with two centrioles. One is older, generated at least two cell cycles prior, whereas the other is newer, generated in the previous cycle. The older centriole has appendages and in many cell types serves as the basal body for a primary cilium. In late G1 when the cell becomes committed to a new cell cycle, the two centrioles lose their orthogonal orientation and split slightly apart (G1/S), but are still linked by the pericentriolar material (*centrioles disengagement*). In S phase, new centrioles begin to grow from the sides of the two original centrioles, like DNA replication centrosome duplication can be considered semi-conservative, with each centrosome receiving one old centriole and one new centriole (*nucleation of pro-centrioles*). In late S phase and during G2, elongation of the pro-centrioles results in the formation of new centrioles at right angles to the original centrioles (*elongation of the pro-centrioles*). At the onset of mitosis, the amount of pericentriolar material increases, along with the number of nucleated microtubules. The first step in spindle assembly is the severing of the connections between the two centrosomes that begin to separate, and a microtubule array forms between them (*separation of the centrosomes*) and ultimately form the two poles of the mitotic spindle. Mitosis and cell division result in the distribution of one centrosome to each daughter cell (Stearns, 2001 ; Morgan, 2007).

2.1.3. CENTROSOME-MICROTUBULE ORGANIZING CENTER

Microtubule nucleation is defined as the *de novo* formation of microtubules from tubulin. *In vitro*, the tubulin subunits self-assemble into a stable aggregate from which a

microtubule grows however, this spontaneous nucleation besides being very slow is almost absent in cells. *In vivo*, microtubule nucleation takes place primarily at morphologically distinct structures, coined MTOC (Microtubule-Organizing Center) (Pickett-Heaps, 1969), that catalyze nucleation at tubulin concentrations that are too low to promote spontaneous microtubule nucleation. The pericentriolar material (PCM) present at MTOCs is responsible for microtubule nucleation (Gould and Borisy, 1977), in fact a particular tubulin, γ -tubulin (Oakley and Oakley, 1989), present at PCM (Stearns et al., 1991) was found to be the major player of microtubule nucleation from fungi to mammals (Job et al., 2003). γ -tubulin form complexes containing several copies of γ -tubulin and at least five further proteins (Moritz et al., 2000). By electron microscopy (EM), a purified γ -tubulin complex is seen to have an open ring structure that resembles a microtubule cross-section and as been called the γ -tubulin ring complex (γ -TuRC) (Moritz et al., 2000; Zheng et al., 1995).

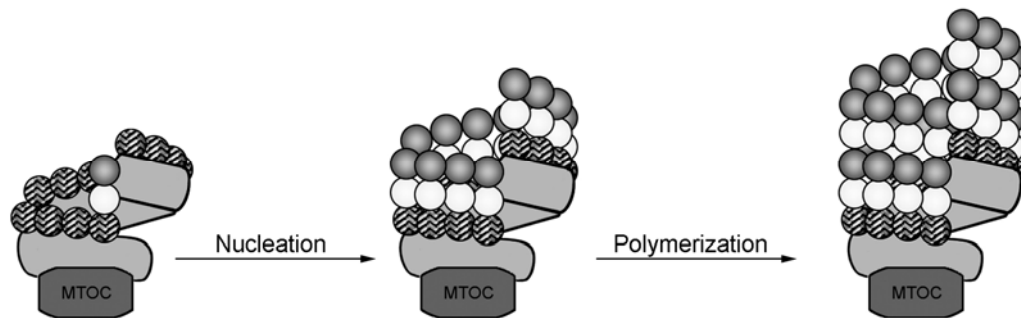


Figure 6. Template model for microtubule nucleation. γ -Tubulin complex proteins constitute a scaffold (grey stripe) on which γ -tubulin subunits align (textured circles) serving as template for the assemble of tubulin subunits (α -tubulin, white circles and β -tubulin, grey circles) (adapted from Pereira and Schiebel, 1997).

Based on the structure of γ -tubulin Zheng et al. proposed a model for microtubule nucleation called "seeded nucleation model" that was posteriorly confirmed by other studies and called also "template model" (Moritz et al., 2000; Zheng et al., 1995) (Fig. 6). This model is an extension of what Oakley et al. had suggested, it postulates that γ -TuRC serves as a template for the assemble of tubulin subunits, with adjacent γ -tubulin subunits interacting laterally with each other forming a circular array that caps the minus-end of the nucleated microtubule (Oakley et al., 1990; Zheng et al., 1995).

2.2. KINETOCHORES

Metzner, in 1894, was the first investigator to describe a specific chromatin area, located at the primary constriction on each side of the paired chromatids, which contains a small, discrete structure that leads the way during poleward chromosome motion (Metzner, 1894; Schrader, 1944). Metzner already had a very explicit idea of function of these bodies, as the name "Leitkörperchen" (the leading body) suggests. However, later investigators have proposed other terms such as "spindle fiber attachment" (many geneticists), "insertionsstelle" (Belar and others), "attachment body" (Schrader), "spindle fiber locus" (Metz), "commissure" (Lorbeer) and others. But it was the name "kinetochore" (from the Greek 'kineto-' meaning 'move' and '-chore' meaning 'means for distribution') used by Sharp in 1934, which attributes to these bodies a dynamic role, that became adopted (Schrader, 1936; for review Rieder, 2005).

2.2.1. STRUCTURE AND FUNCTION

The mammalian kinetochore structure was first described by Bill Brinkley in the 1960s. He saw, by electron microscopy, a trilaminar proteinaceous disc structure that flanked the centromere (Brinkley and Stubblefield, 1966). An electron-dense inner plate is located on the surface of the centromeric heterochromatin and it is separated from an electron dense outer plate by a lighter middle layer. Moreover Jokelainen in 1967 call attention to the existence of a corona of electron opaque substance that covers the outer kinetochore layer (Jokelainen, 1967) (Fig. 7). However using high-pressure freezing/freeze substitution, the light-staining electron-translucent middle layer is not visible suggesting that it is an artifact produced during the conventional fixation and/or dehydration procedures (McEwen et al., 1998). Kinetochores acquire this mature laminar structure only in prometaphase after nuclear envelop breakdown. In early G1 these plate conformation disappears giving rise to a condensed structure that in late G1 unfolds revealing linear, bead-like organization of the CREST-stain region (mammal kinetochore marker), and this conformation persists until S-phase where it transforms into a loose fibrous bundle that duplicates at late S-phase. In late G2 pre-kinetochores refold into two separated condensed structures and during prophase these duplicated pre-kinetochores differentiate at the primary constriction of the sister chromatids and originate the kinetochore plates at the time of nuclear envelop breakdown, completing the cycle (He and Brinkley, 1996).

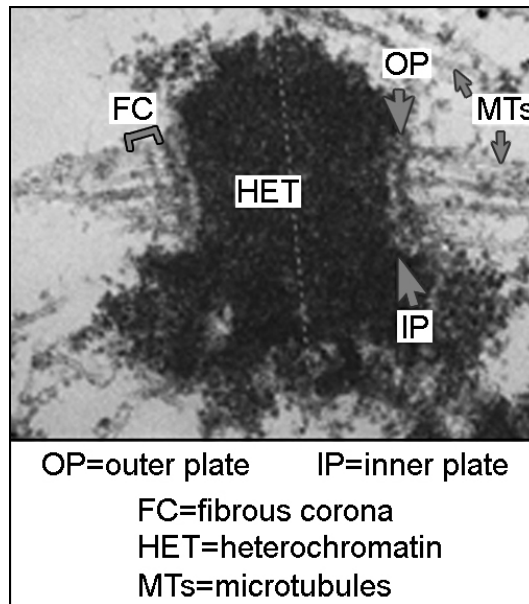


Figure 7. Kinetochore structure. Kinetochore pair of a metaphase chromosome from a HeLa cell, showing the different kinetochore constituent layers, microtubules and heterochromatin (from Earnshaw's Laboratory site).

The inner kinetochore layer is assembled on specialized nucleosomes containing α -satellite DNA and a histone H3 variant called CENP-A in humans, which is associated with numerous heterochromatin and other proteins (Vafa and Sullivan, 1997). The outer kinetochore layer is a proteinaceous DNA free structure that in absence or with few microtubules attachments expands a fibrous corona (Cooke et al., 1993; Rieder, 1982). The fibrous corona and the outer plate contain a dynamic network of permanent but mainly transient proteins essential for kinetochore functions. Upon microtubule binding, many proteins of the corona dissociate from the outer plate and this fibrous structure becomes hardly detectable by EM. Kinetochores with its numerous proteins are responsible for several key events during mitosis namely, attachment of chromosomes to spindle microtubules, monitorization of the integrity of those kinetochore-microtubule attachments, maintenance of an active spindle checkpoint avoiding mitotic progression until all chromosomes align properly at metaphase plate, regulation of microtubule dynamics and help on the movement of chromosomes at the spindle (Maiato et al., 2004a).

2.2.2. MOLECULAR ORGANIZATION

It is thought that the number of microtubule attachments depends on the surface area of the kinetochore. In fact the number of microtubule attachments as well as the size and complexity of kinetochores vary considerably among different species; the budding

yeast kinetochore binds just one microtubule, *S.pombe* bind up to four, *Drosophila* on average bind eleven and vertebrate kinetochores typically bind fifteen to thirty microtubules (Maiato et al., 2006; Maiato and Sunkel, 2004). In yeast, a unit length of centromeric DNA organize a unit block of proteins that binds a single microtubule, by analogy in vertebrates a kinetochore is composed by several repeat microtubule-binding units, where each repeat might reflect the unit module of the yeast kinetochore. This "repeat unit model" acquired another dimension when Zinkowsky in 1991 with two different techniques, caffeine-induced detachment of unreplicated kinetochores and stretching of kinetochores by hypotonic treatment, obtained strong data supporting this model (Zinkowski et al., 1991). Analysis of detached unreplicated kinetochores reveals a large number of spots staining for CREST (seven kinetochores gives rise to 80-100 spots) that represent fragments with approximately equal length and volume, derived from whole kinetochores. Also, while immunostaining with CREST antibodies against normal unstretched kinetochores appear as a single pair of lines on each side of chromosome, after hypotonic treatment stretched kinetochores exhibit a linear array of fluorescent subunits arranged in a repetitive pattern that are able to bind tubulin and contain dynein, a protein that localizes to the kinetochore outer plate (Zinkowski et al., 1991). Taken together these and other posterior studies support the repeat unit model in which the plate like structure of eukaryotic kinetochores is formed by the folding of a linear DNA containing tandem repeat subunits of kinetochore organizer sequences, which bind kinetochore proteins (microtubule-binding units) that during chromosome condensation folds bringing these microtubule-binding units together (Brinkley et al., 1992; Cleveland et al., 2003; He and Brinkley, 1996; Hejnowicz and Feldman, 2000; McEwen et al., 1993).

Currently, the human kinetochore is thought to contain some eighty different proteins and besides some minor organism-specific differences, the kinetochore composition and organization appear to be conserved among eukaryotes (Fig. 8). Purification of CENP-A nucleosomes from human cells identified several constitutive proteins such as CENP-C and 13 interacting proteins present throughout the cell cycle (CENP-H, CENP-I, CENP-K-U; CENP for centromere-associated proteins). Cheeseman call this group of proteins CCAN for constitutive centromere-associated network. Some proteins from CCAN namely CENP-C and CENP-H/I/K, in combination with the multiple-protein complexes Mis12 and KNL1 (also known as SPC105) are thought to be necessary to kinetochore assembly. Besides Mis12 and KLP1, the Ndc80 complex is also part of the structural core of the kinetochore. Ndc80 is a four subunit complex with two proteins that localize to the outer region and bind directly to microtubules (Ndc80 - human Hec1 and Nuf2), and two that are located at the inner kinetochore region (Spc24 and Spc25). A recent study suggests that this core also attach to microtubules through KNL1, both

kinetochore microtubule interactions via Ndc80 and KNL1 are of low affinity although the microtubule-binding affinity of Ndc80 increases when it associates with Mis12 and KNL1.

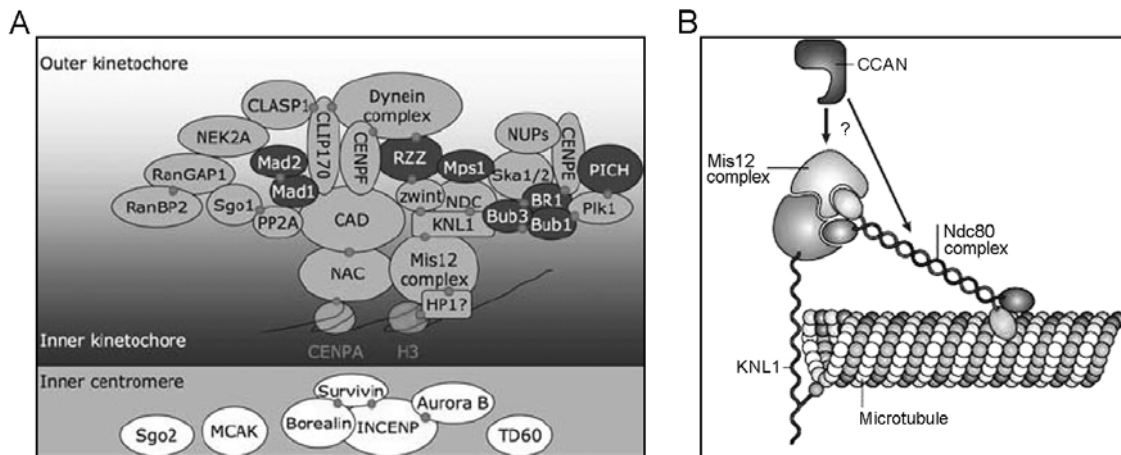


Figure 8. Kinetochore composition and core interaction with microtubules. (A) Proteins that compose an unattached human kinetochore. Checkpoint proteins are shown in dark grey and direct interactions represented by dots. (B) Core-attachment of microtubule binding. The association between Mis12 and KNL1 creates a binding site for the Ndc80 complex and both, KNL1 and Ndc80 complex bind directly to MTs (adapted from Cheeseman and Desai, 2008; Kops, 2008).

Once assembled, in mitosis this core kinetochore allows the loading of a myriad of proteins involved in essential functions like microtubule regulation and interaction (such as CENP-E, dynein/dynactin complex, CLASP, CLIP170, EB1) and spindle checkpoint signaling (such as Mad2, Bub1, Bub3, BubR1, RZZ complex) (Fig. 8) (Cheeseman and Desai, 2008; Przewłoka et al., 2007).

2.3. MICROTUBULES

Microtubules represent one of the types of filamentous structures involved in cellular and subcellular movements and in the determination of cell shape. During mitosis the spindle, essential for chromosome segregation, is composed by a large dynamic array of MTs and during interphase and in non-dividing cells microtubules are involved in the organization of the cytoplasm, position the nucleus and organelles, transport of vesicles, and serve as the principal structural elements of flagella and cilia. Almost all the microtubule functions result predominantly from its capacity of polymerize and depolymerize making it a highly dynamic structure.

2.3.1. STRUCTURE AND BEHAVIOR

Microtubules are made of tubulin subunits that exist as heterodimers of α and β -tubulin. These basic building blocks bind head-to-tail to form a linear protofilament, lateral aggregation of 13 protofilaments form a cylindrical wall of a 25 nm wide hollow polymer made of $\alpha\beta$ -tubulin: the microtubule (Tilney et al., 1973) (Fig. 9). Because in each protofilament all tubulin dimmers are oriented in the same way, the microtubule has a polar nature with β -tubulin exposed at one end (plus-end) and α -tubulin at the other end (minus-end) (Desai and Mitchison, 1997 ; Lodish, 1998) (Fig. 9).

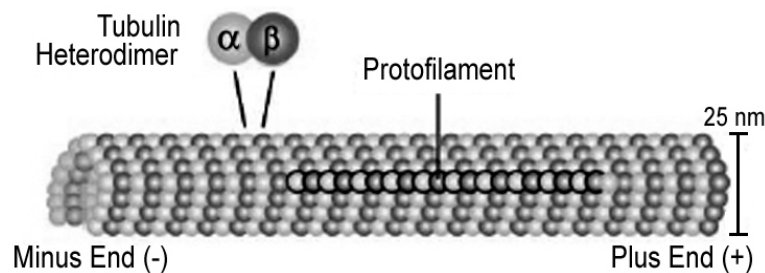


Figure 9. Microtubule structure. Illustration of a 25 nm wide polymer composed by α - (light circles) and β -tubulin (dark circles) heterodimers oriented in the same way creating a polar character, with β -tubulin at the plus-end and α -tubulin at the minus end (adapted from Kline-Smith and Walczak, 2004).

The monomers α and β tubulin have the same overall structure and both bind a guanine nucleotide thus, each heterodimer binds two molecules of GTP although, α -tubulin binds GTP irreversibly and does not hydrolyze it (N-site for Non-exchangeable) whereas, β -tubulin bind GTP reversibly and can hydrolyze it to GDP, as GTP and GDP can exchange, this site is called E-site (for Exchangeable). This occurs because the nucleotide localizes at the interface between monomers so that N-site of α -tubulin is buried into the dimer while the E-site of β -tubulin is exposed although, upon polymerization this E-site also becomes buried and thus non-exchangeable (Downing and Nogales, 1998). Like other GTPases, tubulin by hydrolyzing GTP, is able to change its conformation and consequently its affinity for microtubules, with the GTP conformation having higher affinity than the GDP-conformation. During polymerization the GTP from β -tubulin of the heterodimer is hydrolyzed and converted to GDP, originating a microtubule lattice of GDP-tubulin and therefore less stable. However, as the hydrolysis does not occur within the dimer being added to the microtubule plus-end but in the penultimate dimer, there will always be a cap of GTP-tubulin at the plus-end ensuring the stability of whole microtubule (Downing, 2000; Tran et al., 1997a).

Assembled microtubules assume different conformations depending on the state of polymerization or depolymerization. Heterodimers of GTP-tubulin are thought to have a

“straight” conformation while heterodimers of GDP-tubulin display a curved conformation, thus during polymerization the GTP-hydrolyzes should cause a strait-to-curved change of the dimer however in the microtubule lattice, bound to its neighbor dimmers, they are forced to remain in a straight conformation. So, during elongation microtubules assume a structure of a strait open sheet that later close into a tube that is stabilized by the terminal cap of GTP-tubulin. Depolymerising microtubules lose their GTP-cap and this allows GDP-tubulin dimmers to achieve their natural curved conformation, peeling away from the microtubule lattice and acquire a ram-horn-shaped structure at the microtubule end (Desai and Mitchison, 1997; Mandelkow et al., 1991; Tran et al., 1997a) (Fig. 10).

The alternation between phases of polymerization and depolymerization is an intrinsic property of microtubules and is called "dynamic instability" (Mitchison and Kirschner, 1984) (Fig. 11A, B). These transitions are abrupt and stochastic and are defined as "catastrophe" for the transition from growth to shortening and "rescue" for the switch from shortening to growth. Microtubule ends can also "pause", a behavior often observed in living cells, in which microtubules are neither polymerizing nor depolymerising (Schulze and Kirschner, 1988; Walker et al., 1988).

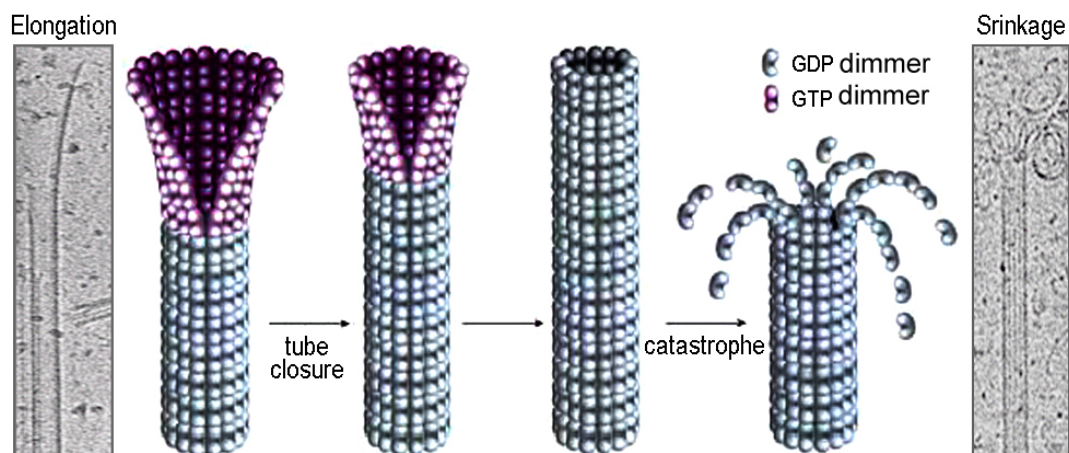


Figure 10. Microtubule conformations. During elongation, microtubules assume a strait open sheet conformation, stabilized by a GTP-cap (pink), that later close into a tube. During depolymerization, the GTP-cap is lost and the GDP dimmers (blue) acquire their natural curved conformation producing a ram-horn-shaped structure at the depolymerising tip (adapted from Lodish, 1998; Hyman and Karsenti, 1996).

Studies *in vitro* demonstrate that although polymerization and depolymerization phases can occurs at both ends, the kinetic parameters are different for plus and minus-ends, with the plus-end polymerizing at approximately a two fold higher rate than the minus-end (Walker et al., 1988). However, in all different cell types studied, polymerization of the minus-end never occurs, suggesting the existence of a specific cellular mechanism

that prevents the growth of minus-end, hence *in vivo* minus-ends are either stable or depolymerize persistently (Dammermann et al., 2003). This intrinsic property of microtubules, higher rate of polymerization at the plus-end and depolymerization of the minus-end, results in a microtubule behavior called "treadmilling" in which new subunits are added to the plus-ends and old subunits released at the minus-end so that the whole MT changes in a poleward direction (Margolis and Wilson, 1981) (Fig. 11B).

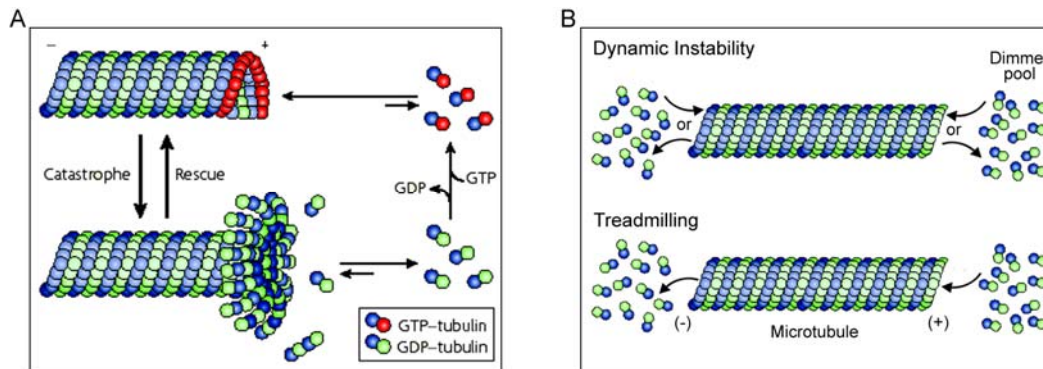


Figure 11. Microtubule dynamics. (A) Scheme of microtubule dynamic instability. Transition from polymerization to depolymerization is called catastrophe and the reverse transition, depolymerization to polymerization, is a rescue. (B) Comparison of dynamic instability and treadmilling mechanisms. While dynamic instability is characterized by abrupt changes between states of grow and shrink at both ends of microtubule, treadmilling is characterized by a polarized movement of tubulin dimers that polymerize at one end and depolymerize at the other end (adapted from Cheeseman and Desai, 2008; Grego et al., 2001).

2.3.2. SPINDLE MICROTUBULES

Microtubules of the spindle are organized in a symmetric and fusiform structure with a uniform polarity, in which the minus-ends are oriented toward the poles and the plus-ends point to the cell cortex or chromosomes (Euteneuer and McIntosh, 1981; Heidemann and McIntosh, 1980; Telzer and Haimo, 1981). Depending on the position of the microtubule plus-ends, spindle microtubules are divided into three classes (Fig. 12): kinetochore-microtubules (kMTs), interpolar-microtubules (ipMTs) and astral-microtubules.

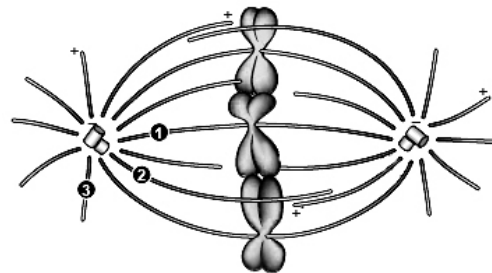


Figure 12. Spindle structure and composition. Spindle illustration showing its fusiform shape, in which the minus-ends of microtubules concentrate at the poles and the plus-ends are oriented to chromosomes or the cell cortex. Mitotic spindle is composed by three different classes of microtubules: kinetochore-microtubules (1), interpolar-microtubules (2) and astral-microtubules (3) (adapted from Wittmann et al., 2001).

Kinetochore microtubules connect the spindle poles to chromosomes, binding laterally or end-on, specifically to the kinetochores (Hayden et al., 1990; Merdes and De Mey, 1990; Rieder and Alexander, 1990). They form a morphologically distinct bundle denominated K-fiber (kinetochore-fiber) which allows chromosome to align at the metaphase plate and posteriorly segregate the sister chromatids to originate the daughter cells (Rieder and Salmon, 1998). Comparing the different classes of microtubules, the K-fibers are more stable (resist to cold treatment) and the microtubules less dynamic than interpolar or astral microtubules (Mitchison et al., 1986; Rieder, 1981; Salmon and Begg, 1980). Interpolar microtubules extend from the spindle poles to the spindle midzone where they form an interdigitating system that connects the two spindle poles. However, contrary to kMTs whose minus-ends are near the poles, most minus-ends of ipMTs are spread over half of the spindle length. At the midzone, ipMTs interact with antiparallel neighbors from opposite poles, although it is more evident on anaphase progression, these interactions occur throughout mitosis and help to stabilize the bipolar spindle and to separate the spindle poles by antiparallel microtubule sliding (Mastronarde et al., 1993; Sharp et al., 2000c). Finally, the third class of microtubules appears in systems with centrosomes or spindle pole bodies, astral microtubules emanate from the spindle poles and radiate out into the cytoplasm with the plus-ends interacting with the cell cortex. These interactions are required for spindle orientation and proper cytokinesis (Hyman, 1989; Khodjakov and Rieder, 2001).

2.4. MECHANISMS OF SPINDLE ASSEMBLY

A key event in cell division is the dismantling of the interphase microtubule network and its transformation into a bipolar spindle with microtubules capable of capturing, aligning and segregating the chromosomes. Thus, in addition to changes in microtubule organization, at the onset of mitosis there are remarkable changes in microtubule dynamics with long and stable interphase microtubules replaced by shorter, less stable and highly dynamic microtubules. Microtubule turnover increases dramatically and there is also an increase in the frequency of microtubule catastrophes and a decrease in rescue events and time spent in pause state, these changes in dynamic behavior allow spindle microtubules to assemble and disassemble more quickly during mitosis, the ideal behavior to capture a chromosome (Rusan et al., 2001; Saxton et al., 1984). The favored model for spindle assembly in systems with centrosomes is based on this microtubule dynamic instability and is known as "search and capture" (Kirschner and Mitchison, 1986) (Fig. 13A).

In centrosome containing cells, a dense array of microtubules radiate from each centrosome and when nuclear envelop breaks down these very dynamic microtubules have access to the chromosomes. The continuous cycling of growing and shrinking allow aster microtubules to randomly explore the cytoplasm until capture a kinetochore, laterally or with the plus-end, forming an attachment that stabilizes the microtubule (Mitchison et al., 1986; Nicklas and Kubai, 1985). Sometimes the plus-ends of microtubules contact directly the kinetochore, but usually binding occurs laterally. After binding one of the two unattached kinetochores, the chromosome is rapidly transported along the side of microtubule towards the pole in a mechanism independent of microtubule depolymerization (Rieder and Alexander, 1990). Later studies have reveled that the minus-end directed motor protein dynein is responsible for this rapid poleward motion (Yang et al., 2007). Since microtubule density is highest near the spindle pole additional microtubules from the same pole are then attached to the kinetochore, the plus-ends of microtubules that are laterally associated remain dynamically unstable and will shorten until reach the end-on binding, resulting in the formation of a kinetochore-fiber containing several stabilized microtubules (Rieder, 2005).

In cells lacking centrosomes, like higher plants and many animal oocytes, the formation of kinetochore-microtubules and consequently the mitotic spindle must rely in other mechanism than "search and capture". Studies with *Xenopus* extracts revealed that although anastral, a normal bipolar spindle is formed in absence of centrosomes and kinetochores in which microtubules grow close to chromatin coated beads (Heald et al., 1996). Further studies demonstrated that chromatin does not nucleate microtubules *per se* but modify locally the cytoplasm to favor microtubule growth. Indeed, it is already known that mitotic chromosomes are surrounded by a Ran-GTP gradient that in turn induces a gradient of proteins that regulate microtubule dynamics and organization. This Ran-GTP gradient occurs due to mitotic chromosome localization of RCC1 (guanine-nucleotide exchange factor that converts Ran-GDP into Ran-GTP - active form) creating a high concentration of Ran-GTP in the vicinity of chromosomes. This active Ran-GTP causes local activation of microtubule-stabilizing proteins by dissociating them from importin- β , promoting spindle assembly around the chromosomes (Kalab et al., 2002). Depletion studies using RNAi technique show that γ -tubulin, like for centrosome-nucleated microtubules, is essential for the formation of chromatin-nucleated microtubules (Luders et al., 2006; Mahoney et al., 2006). After nucleation, these chromatin-nucleated microtubules are stabilized and organized into a bipolar array by a process called "spindle self organization" (Fig. 13B). In this process microtubules nucleated near chromatin with random polarity elongate and then, helped by motor proteins, are sorted into a bipolar array with the proper polarity and focused at the poles (Walczak et al., 1998).

By selectively destroying centrosomes in living mammalian cells, Khodjakov and colleagues show for the first time, that cells that usually have centrosomes are also able to nucleate microtubules in a centrosome-independent way (Khodjakov et al., 2000). Later, Maiato et al. demonstrated that, besides the search and capture mechanism, the chromosome-driven K-fiber formation, also contributes for spindle assembly during normal mitosis in centrosomal *Drosophila* S2 cells. However, in these "combined" system, the K-fibers nucleated from chromosomes are integrated at the spindle helped by astral microtubules that search, capture and transport them toward the pole in a dynein dependent mechanism (Khodjakov et al., 2003; Maiato et al., 2004b) (Fig. 13C).

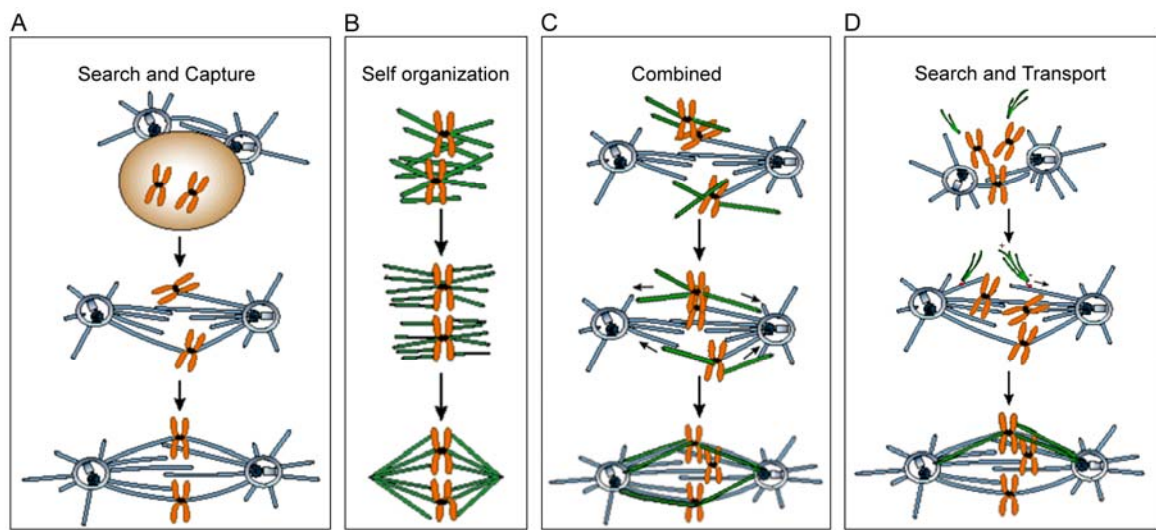


Figure 13. Mechanisms of spindle assembly. (A) In the "search and capture" mechanism, microtubules nucleate from centrosomes and randomly explore the cytoplasm until capture a kinetochore; (B) the "self organization" model postulate that microtubules assemble in the vicinity of chromatin and helped by motor proteins, organize a bipolar array; (C) in the "combined" model, microtubules nucleated from chromosomes (green) are captured by centrosomal-nucleated microtubules (blue) and incorporated into the forming spindle; (D) in the "search and transport" mechanism, peripheral microtubules (green) are captured by aster microtubules, transported to poles and incorporated into the forming spindle (adapted from Gadde and Heald, 2004; Scholey et al., 2003; Tulu et al., 2003).

In fact, studies using GFP- α -tubulin reveal another mechanism for spindle formation the "search and transport" of peripheral-microtubules (Fig. 13D). In this mechanism peripheral microtubules are transported through aster microtubules to the poles, probably in a dynein dependent manner, where are incorporated into the spindle (Tulu et al., 2003). Other studies in S2 cells also show that even without asters, these pre-formed microtubules still being incorporated contributing to spindle assembly (Mahoney et al., 2006). Thus spindle formation is a process that results from several redundant

mechanisms including centrosome and chromatin-nucleated and incorporation of pre-formed microtubules.

3. MICROTUBULE ASSOCIATED PROTEINS

The key features of microtubules are their ability to rapidly assemble and disassemble creating structures with different shapes and stabilities, and their capacity to bind specific structures at their ends. These features coupled with the properties of dynamic instability suggest that MTs can perform work through a regulated process of assembly and disassembly. However, Mts require other proteins to help them regulate their behavior properly so that functions in cell shape, cell motility, intracellular transport, chromosome search, capture and segregation, can be accomplished. These proteins, called Microtubule Associated Proteins (MAPs), execute a wide range of tasks including regulating microtubule dynamics, guiding microtubules toward specific positions, cross-linking microtubules and mediating the interactions of microtubules with other proteins. MAPs were first defined on the basis of their ability to bind microtubules (Olmsted, 1986) and were originally identified as proteins that co-purified with tubulin during the process of tubulin isolation even after several cycles of assembly and disassembly, suggesting that MAPs were not contaminants but rather attach specifically to tubulin. Nowadays MAPs are defined as proteins that specifically bind to microtubules, at least transiently, either *in vitro* or *in vivo*. The targets are soluble tubulin subunits, microtubule lattice and/or microtubule ends and can be divided into two major groups: motor and non-motor MAPs (Akhmanova and Steinmetz, 2008; Maiato et al., 2004c).

3.1. NON-MOTOR MAPs

Non-motor microtubule associated proteins are responsible for numerous structural and regulatory functions throughout the cell cycle. They are involved in several processes as the control of cell cycle progression, directly or indirectly influence motor function and regulation of microtubule nucleation, organization and dynamics. These proteins are present at different structures in particular to microtubule lattice, microtubule ends, kinetochores, centrosomes, spindle poles, central spindle, and midbody (Fig. 14).

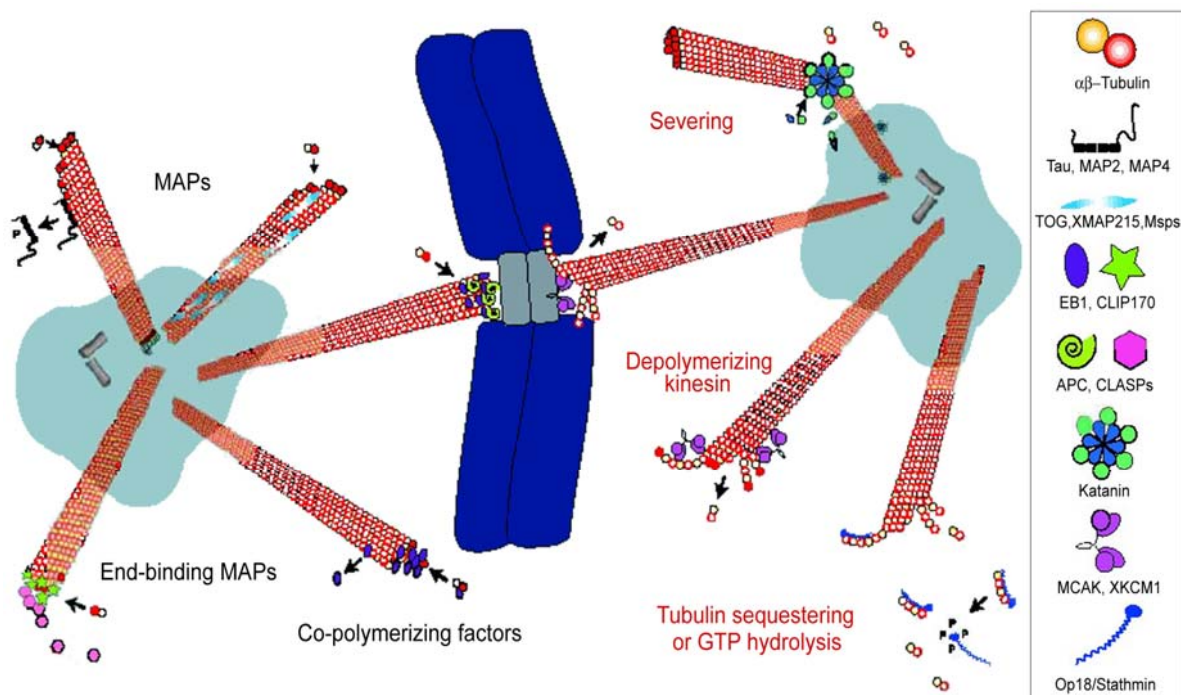


Figure 14. Non-motor MAPs. Spindle localization of some non-motor microtubule associated proteins (adapted from Heald and Nogales, 2002).

3.1.1. MICROTUBULE STABILIZING FACTORS

In early 1970's it was discovered that microtubules could assemble *in vitro* and the conditions to isolate them were developed, providing the basis for the identification of the first MAPs (Shelanski et al., 1973; Weisenberg, 1972). Since the majority of these studies were done using brain cell extracts, because brain tissue was the most abundant source of microtubules, the first MAPs were found in axons and dendrites of nerve cells. These microtubule associated proteins were termed more specifically "Structural MAPs" due to their capacity to bind, stabilize and promote polymerization of microtubules. They included MAP1, MAP2, Tau and MAP4 (Mandelkow and Mandelkow, 1995).

The firsts microtubule associated proteins reported were MAP1 and MAP2 of high molecular mass and Tau of lower molecular mass (Murphy and Borisy, 1975; Sloboda et al., 1976; Weingarten et al., 1975). All of these proteins are neuronal MAPs, but latter it was found that MAPs were also distributed in other cells and tissues. The first to be described with a ubiquitous distribution was MAP4 (Bulinski and Borisy, 1979; Bulinski and Borisy, 1980; Parysek et al., 1984). These MAPs are composed of two main domains, an acidic projection domain and a basic, cationic microtubule-binding domain. The projection domain is involved in MAP interaction with other components of the cytostructure (including other microtubules) and with cell membranes. Based on sequence

analysis, these MAPs were divided into two groups that differ in their microtubule-binding domains: Type I, includes the family of MAP-1 proteins, contain several repeats of the amino acid sequence KKEE or KKE(I/V) that binds to the negatively charged tubulin, neutralizing the charge repulsion between tubulin subunits and stabilizing the polymer. And Type II, that includes MAP2, MAP4 and Tau, and are characterized by three or four repeats of an 18-residue sequence in the microtubule binding domain located near the COOH-terminus of the molecule (Maccioni and Cambiazo, 1995) (Lodish, 1998).

MAP1A and MAP1B are multimeric protein complexes composed by one heavy chain (HC) and several light chains (LC) derived from proteolytic cleavages of a polyprotein precursor. They bind to microtubules inducing rapid polymerization of tubulin and can form cross-bridges between the tubulin polymers. Moreover, it was shown that the light chains of these MAPs can bind to actin filaments suggesting that these LC are potential linkers involved in regulation of neuronal microtubules and microfilaments interactions, which is believed to be essential for neuronal morphogenesis and function (Noiges et al., 2002).

MAP2, Tau and MAP4 belong to the same family, although MAP2 and Tau are found in neurons whereas MAP4 in many other tissues but is generally absent from neurons. Members of this family are detected along the side of microtubules and are known to increase their rigidity, induce bundles and stabilize the microtubules by altering their dynamic behavior. Tau and MAP2 increase the rate of polymerization, inhibits the rate of depolymerization and decrease the catastrophe frequency (Drechsel et al., 1992; Kowalski and Williams, 1993). On the other hand, MAP4 increases the rescue frequency without decreasing the catastrophe frequency (Ookata et al., 1995). Besides its microtubule stabilizing activity there are evidences suggesting the involvement of these MAPs in other functions such as binding to actin filaments (MAP2), recruitment of signaling proteins and regulation of microtubule-mediated transport (Dehmelt and Halpain, 2005). Also miss-expression/regulation of these proteins are related with some disease for instance the abnormal phosphorylation of Tau is associated with Alzheimer's disease and numerous neurodegenerative disorders known as Tauopathies (Buee et al., 2000).

The constant optimization of methods and discovery of new tools allowed the identification of novel proteins that also associate with microtubules and stabilize them but do not satisfy the classical criteria for MAP definition. Initially they were classified as a different group denominated Microtubule Interacting Proteins (MIPs) (Maccioni and Cambiazo, 1995) but currently all of these proteins belong to the same broad group of MAPs. Stabilizing non-motor microtubules associated proteins are now found in several species, tissues, and cell structures and the number is still increasing with the majority of them having a preponderant role in mitosis namely in formation, maintenance and

progression of the mitotic spindle. These proteins can stabilize the spindle by different ways namely by regulating microtubule dynamics, crosslinking microtubules and by interacting with other proteins, in fact there are MAPs involved in more than one stabilizing pathway: TPX2 (*Drosophila* Asp1) and Astrin, besides crosslinking microtubules also promote microtubule nucleation and stabilize microtubules at the kinetochores, respectively. Others like PRC1 (*Drosophila* Feo) and NuMA, provide structural support to the spindle by crosslinking microtubules at spindle midzone and spindle poles, respectively; and some as TACC, target another stabilizing protein, TOG (*Drosophila* Msps) to centrosomes, promoting microtubule nucleation and stabilization (Manning and Compton, 2008b).

3.1.2. MICROTUBULE DESTABILIZING FACTORS

Microtubule destabilizing factors act by altering microtubule dynamic parameters in a way that leads to the reduction of the net tubulin assembly. They can promote microtubule destabilization by directly or indirectly increasing the catastrophe frequency, increase the rate of depolymerization, decrease rescue frequency and/or inhibit the polymerization rate.

3.1.2.1. MICROTUBULE-SEVERING PROTEINS

Initial observations of microtubule severing activity immediately suggested an important participation on critical cellular events. In fact, it was shown that microtubule severing activity was involved in creation of non-centrosomal microtubules (critical for the activity of differentiated cells, ex. structural support and transport of material), on regulation of microtubule dynamics and also in certain physiological events as deflagellation in *Chlamydomonas* (McNally et al., 1996; Quarmby and Lohret, 1999).

The first microtubule-destabilizing factor discovered was the microtubule-severing protein Katanin. This enzyme is an ATPase that severs and disrupts bonds between tubulin dimers within the microtubule lattice using the energy generated by the ATP hydrolysis (McNally and Vale, 1993). *In vitro* studies suggest a model in which Katanin oligomerizes in an ATP and microtubule dependent manner using the microtubule as a scaffold to form a ring. Once completed, this ring stimulates the ATPase activity of Katanin that undergoes a conformational change and destabilizes tubulin-tubulin contacts leading to microtubule disassembly (Quarmby, 2000). Katanin has been found to target to centrosomes and/or spindle poles in different cell types suggesting a role in minus-end microtubule depolymerization (Hartman et al., 1998; McClinton et al., 2001; McNally et al., 1996; McNally and Thomas, 1998; McNally et al., 2000; Srayko et al., 2000; Zhang et al., 2007). Later studies reveal that other two proteins closely related with Katanin, Spastin

and Fidgetin, also localizes to spindle and have *in vivo* microtubule severing activity (Evans et al., 2005; Roll-Mecak and Vale, 2005; Zhang et al., 2007) (Fig. 15).

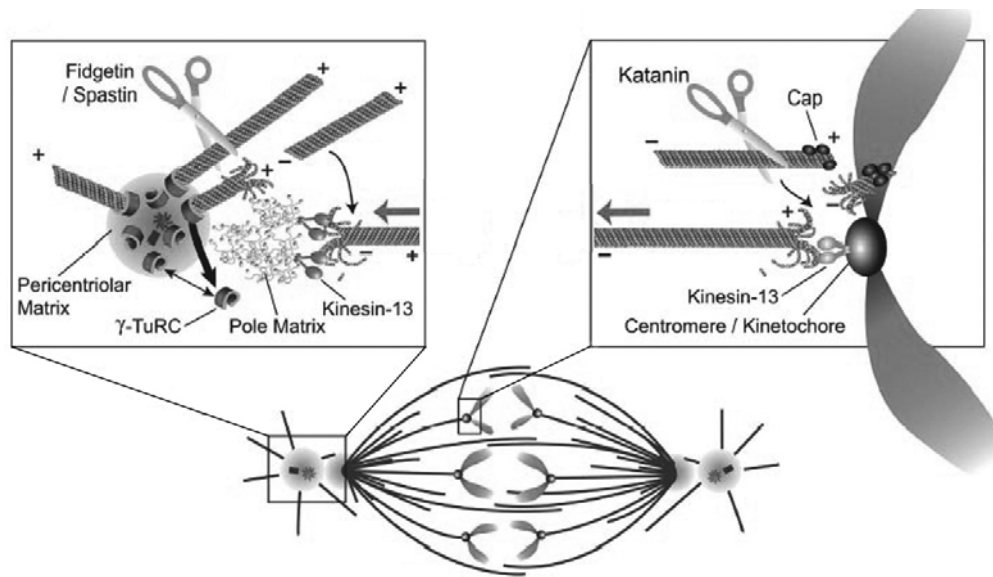


Figure 15. Contribution of microtubule-severing proteins in anaphase chromosome movement. Fidgetin and Spastin, at centrosomes, induce microtubule minus-end depolymerization whereas katanin, at kinetochores, stimulates microtubule plus-end depolymerization; both contribute to the anaphase chromosome movement (adapted from Zhang et al., 2007).

Indeed a study in *Drosophila* S2 cells demonstrate that all three proteins localize not only to centrosomes but also to chromosomes. Both Spastin and Fidgetin target to chromosomes near kinetochores during prometaphase/metaphase but decrease substantially at anaphase onset, whereas Katanin is widely dispersed on chromosomes throughout mitosis and during anaphase some clearly target to kinetochores. Beside its common localization on centrosomes and chromosomes, these proteins have different functions in S2 cells; both Spastin and Fidgetin stimulate microtubule minus-end depolymerization whereas Katanin stimulates plus-end depolymerization playing an important role in anaphase chromosome movement. The general mechanisms of action of these microtubule-severing proteins could be through the rapid generation of free microtubule ends that became exposed to the action of other regulatory proteins (Zhang et al., 2007).

3.1.2.2. OP18/STATHMIN

Another microtubule destabilizing factor is a conserved small protein highly expressed in acute leukemia known as oncoprotein 18/Stathmin (Op18) (Belmont and Mitchison, 1996). In contrast to microtubule-severing proteins that cause internal breaks into microtubules, the Op18/Stathmin promote endwise disassembly of microtubules, it increases the catastrophe rate and decreases the polymerization rate (Belmont and

Mitchison, 1996). Stathmin was found to directly interact with tubulin dimmers and to act on GTP-tubulin at microtubule ends stimulating the GTP hydrolysis (Howell et al., 1999; Jourdain et al., 1997; Larsson et al., 1999; Segerman et al., 2000) (Fig. 16). Based on these observations, were proposed two models for Stathmin-dependent microtubule destabilization: 1) Stathmin sequesters the tubulin dimmers preventing microtubule polymerization and/or 2) induces catastrophe events at microtubule plus-ends by hydrolyzing the GTP from the stabilizing GTP-Cap (Steinmetz, 2007).

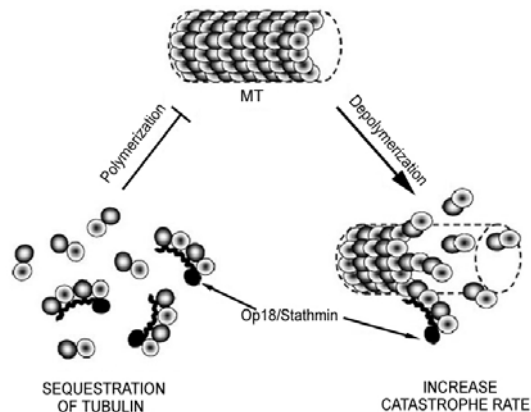


Figure 16. Models for Stathmin-dependent microtubule destabilization. Stathmin can destabilize microtubules by blocking the polymerization through sequestration of free tubulin, or/and can promote catastrophes by binding to the plus-ends of microtubules inducing a conformal change that promotes microtubule depolymerization (adapted from Rubin and Atweh, 2004).

The subcellular localization of Stathmin in HeLa cells reveal that during interphase these destabilizing protein does not bind to the microtubule network, but in mitosis although detected throughout the cell, it accumulates at the mitotic spindle (Gavet et al., 1998). The activity of this destabilizing factor is subjected to spatial and temporal regulatory phosphorylation and is essential for orderly progression through cell cycle. Stathmin needs to be down-regulated by phosphorylation for G2/M transition and to allow microtubule polymerization for mitotic spindle assembly (Larsson et al., 1997; Marklund et al., 1996), on the other end it needs to be dephosphorylated in late mitosis for its microtubule destabilizing activity disassemble the mitotic spindle and cell exit properly (Iancu et al., 2001; Rubin and Atweh, 2004).

3.1.2.3. KIN I DEPOLYMERISING KINESINS

Kin I, "internal kinesins" or Kin M, "middle kinesins" have this name since the motor domain, which contains the microtubule and ATP-binding sites localize in the middle of the protein sequence (Vale and Fletterick, 1997). This subfamily of kinesins, later called kinesin-13 (Lawrence et al., 2004), has a unique feature among other kinesins because rather than walk on microtubules, they use the ATP hydrolysis energy to depolymerize

them (Desai et al., 1999). Kin I kinesins operate at both ends of microtubules inducing bent-conformations that favor depolymerization (Desai et al., 1999). The targeting of these kinesins to microtubule ends is extremely rapid and it can occur by both ways direct binding and interaction with the microtubule lattice. In the latter case it reaches the ends through one dimensional diffusion, once there, the ATP hydrolysis of Kin I kinesin is coupled to a processive depolymerization of the microtubule ends (Helenius et al., 2006; Hunter et al., 2003). Members of this family include mammalian Kif2a, Kif2b and Kif2c/MCAK (Kif – kinesin family member, MCAK – mitotic centromere associated kinesin); *Xenopus* XKCM1 and XKif2 (XKCM - *Xenopus* kinesin central motor); *Drosophila* KLP10A, KLP59C and KLP59D (KLP - kinesin like protein) (Moores and Milligan, 2006). The kinesin-13 family can be used just to depolymerize microtubules or to allow the coupling of depolymerization with the movement of cargos to which is attached. Particularly in mitosis it has been implicated in several critical events like spindle assembly, proper chromosome congression and segregation and correction of improper microtubule-kinetochore attachments (Maiato et al., 2004c).

The ability of Kin I kinesins to destabilize microtubules directly at their ends confers them a crucial role during mitosis, in particular when they localize to the centromeric region and centrosomes/spindle poles (Maney et al., 1998; Rogers et al., 2004a; Walczak et al., 1996). These kinesins work at both ends of kinetochore microtubules, MCAK (mammal) and KLP59C (*Drosophila*) work at the centromere; Kif2a (mammal) and KLP10A (*Drosophila*) at the centrosomes/spindle poles (Fig. 17). Depletion of Kin I kinesins that work preferentially at the spindle poles result in monopolar spindles or abnormally long and dense bipolar spindles, whereas the ablation of the centromeric ones have no perceptible impact on spindle structure (Ganem and Compton, 2004; Goshima and Vale, 2003; Kline-Smith et al., 2004; Rogers et al., 2004a).

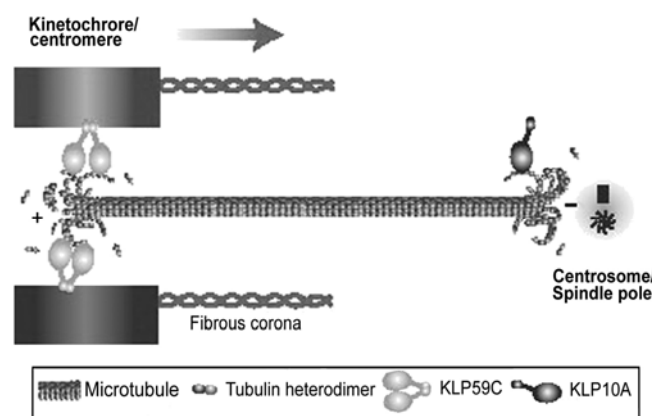


Figure 17. Kinesin-13 localization. These kinesins destabilize both ends of KMTs. In *Drosophila*, KLP59C localizes at the kinetochore, destabilizing microtubule plus-ends, and KLP10A act at the spindle pole, depolymerising the minus-ends of microtubules (adapted from Rogers et al., 2004a).

KLP10A concentrates to mitotic centrosomes, spindle poles and centromeres throughout mitosis but its centromeric localization decreases at anaphase on-set. Besides the assembly and maintenance of proper spindle structure, this protein is also required for normal poleward anaphase chromosome motion. In S2 cells and *Drosophila* embryos that lack KLP10A the poleward translocation rate of chromatids during anaphase is significantly reduced (Buster et al., 2007; Goshima and Vale, 2005; Rogers et al., 2004a). On the other hand, KLP59C localizes to centromeres, with identical levels at metaphase and anaphase, and depleting this protein results in a deficient alignment of chromosomes at metaphase plate and in problems of chromatid separation such as defects on segregation and decrease in the poleward rate. Taken together, Kin I-induced disassembly at the spindle poles (KLP10A) allows chromosomes to be driven into the poles, and at the centromere (KLP59C) promotes disassembly at the plus-ends, resulting in the shortening of kinetochore-microtubules (Rogers et al., 2004a; Sharp and Rogers, 2004). Evidence suggests that these kinesins work by affecting different dynamic parameters, KLP10A stimulates catastrophes and KLP59C inhibits rescue events (Buster et al., 2007; Civelekoglu-Scholey et al., 2006; Mennella et al., 2005).

3.2. MICROTUBULE ASSOCIATED MOTORS

Microtubule associated motors undergo chemico-mechanical cycles converting chemical energy of ATP hydrolysis into mechanical work. Microtubule motors are usually homodimers composed by two globular heads (ATPases) or motor domain that bind directly to microtubules, and other associated subunits that link the motor unit to a protein cargo or function as an anchor. The hydrolysis of ATP induces a conformational change in the globular motor domain that with the help of the accessory structural motifs is amplified and converted into movement (Schliwa and Woehlke, 2003 ; Morgan, 2007). These proteins are responsible for most forms of movement in a cell and particularly at mitosis are known to be involved in centrosome separation, assembly and maintenance of bipolar spindle, spindle pole formation, chromosomes movement at prometaphase, metaphase and anaphase, spindle elongation and assembly and function of central spindle and midbody (Loughlin et al., 2008). To achieve these functions mitotic motors use three distinct mechanisms: transport cargoes along the microtubule lattice, cross-bridge and slide microtubules relative to adjacent microtubules or other structures, and regulate and couple movements to microtubule dynamics. Microtubule associated motors are divided into two families: kinesins and dyneins (Maiato et al., 2004c; Sharp et al., 2000c).

3.2.1. KINESINS

'Kinesin' comes from the Greek 'kinein' (to move) and it was the name of the first described superfamily member (Vale et al., 1985). The ability of motor proteins to recognize microtubule polarity results in a unidirectional movement. Most kinesins move along microtubules towards the plus-ends usually bringing cargos to cell periphery and at mitosis move away from the spindle poles. Some kinesins are particularly important in spindle assembly and function and in chromosome movement (Fig. 18).

Kinesin-5 proteins (bipolar kinesins), like vertebrate Eg5 or KLP61F from *Drosophila*, are homotetrameric plus-end directed motors containing two complete motor domains capable of bind microtubules. By cross-linking microtubules, kinesin-5 plus-end directed motility is activated and induces antiparallel microtubule sliding using energy from ATP hydrolysis (Kapitein et al., 2008; Kapitein et al., 2005; Kashina et al., 1996). KLP61F localizes to centrosomes before NEBD and to spindle microtubules from prometaphase to anaphase concentrating in the midzone at anaphase B and midbody at telophase (Barton et al., 1995; Cheerambathur et al., 2008; Goshima and Vale, 2005; Sharp et al., 1999). Members of this family are essential for bipolar spindle formation in all eukaryotes, in its absence or lack of function, centrosomes do not separate and result in monopolar spindles (Blangy et al., 1995; Goshima and Vale, 2003; Heck et al., 1993; Hoyt et al., 1992; Sawin et al., 1992). Also one of the main roles attributed to kinesin-5 is its involvement in microtubules poleward translocation contributing to chromatid movement toward the spindle poles (Cameron et al., 2006; Goshima et al., 2005b; Mitchison et al., 2004; Miyamoto et al., 2004). Generally, it is assumed that due to its ability of "sliding filaments" this family as an important role throughout mitosis. At the beginning is involved in centrosome separation and formation of the bipolar spindle, then by crosslinking antiparallel microtubules at the midzone contributes to the integrity of the spindle structure and by moving to the plus-end of antiparallel microtubules induces a poleward force that result in spindle elongation at the time of anaphase B. However a continuous study in this field reveals that the possible roles of this tetrameric motor family depend on the organism or system (Civelekoglu-Scholey and Scholey, 2007).

Kinesin-14 proteins (C-terminal kinesins), as *Drosophila* Ncd and mammalian HSET, unlike conventional kinesins have the motor domain at C-terminal and are minus-end directed proteins (Ovechkina and Wordeman, 2003). Ncd a well studied *Drosophila* member of kinesin-14 family has two microtubule-binding domains, one at C-terminal, dependent of ATP and other at N-terminal, independent of ATP (Chandra et al., 1993). This feature combined with its motile activity allow Ncd to exert its function by bundling and sliding microtubules against each other (McDonald et al., 1990; Oladipo et al., 2007). The absence of kinesin-14 induces the formation of bipolar spindles with unfocused poles

and/or multipolar spindles indicating that these kinesins are essential for normal bipolar spindle formation (Endow et al., 1994; Goshima et al., 2005a; Goshima and Vale, 2003; Morales-Mulia and Scholey, 2005; Zhu et al., 2005). Kinesin-14 were shown to localize to centrosomes and along the spindle with a slight enrichment at microtubule plus-ends (Endow and Komma, 1996; Goshima et al., 2005a; Goshima and Vale, 2005; Hatsumi and Endow, 1992). Since these kinesins are minus-end directed motors and are able to crosslink and slide microtubules it was proposed that kinesin-14 are required to tether microtubules together at the spindle poles and help in generating inward forces upon antiparallel microtubules, counteracting the outward forces exerted by other motors like kinesins-5 (Sharp et al., 2000a; Sharp et al., 2000c; Tao et al., 2006). Also its presence at the plus-ends of microtubules suggests a role in facilitating the capture and transport of non-centrosomal K-fibers along centrosomal-microtubules integrating them into the spindle and driving into the pole (Goshima et al., 2005a).

Kinesin-8 family members, like *Drosophila* KLP67A and mammalian Kif18A, are plus-end directed proteins and plus-end depolymerases (Gandhi et al., 2004; Mayr et al., 2007; Pereira et al., 1997; Walczak, 2006). In all systems studied, the loss of these kinesins lead to an excess of microtubule polymerization resulting in abnormally long spindles (Walczak, 2006). Kif18A was found to be also required to proper chromosome alignment and metaphase to anaphase transition (Mayr et al., 2007; Zhu et al., 2005). In agreement, absence of KLP67A, besides atypical long spindles, results in failure of chromosome congregation and separation (block anaphase entry and the ones that progress exhibit abnormal segregation and cytokinesis). In addition, KLP67A is also involved in centrosome separation, *Drosophila* embryos lacking these kinesin exhibit an incomplete centrosome separation and S2 cells form monopolar spindles that generally organizes into monastral bipolar structures (Gandhi et al., 2004; Gatt et al., 2005; Goshima and Vale, 2003; Goshima et al., 2005b; Savoian et al., 2004). Kinesin-8, in *Drosophila*, localizes to kinetochores and faintly at spindle microtubules from prometaphase until anaphase, afterwards the kinetochore signal decreases and accumulates at the spindle midzone and later at the midbody (Goshima and Vale, 2005; Savoian et al., 2004). In mammalian cells, Kif18A is present along spindle microtubules and at metaphase concentrates near the plus-ends of kinetochore-microtubules decreasing gradually at anaphase on-set (Mayr et al., 2007). The location and depolymerising ability of kinesin-8 members gives them the capacity to balance microtubule dynamics maintaining a proper spindle length and chromosome movement, in fact it was shown that Kif18A and Kip3p (yeast) depolymerize longer microtubules faster than shorter ones (Mayr et al., 2007; Varga et al., 2006).

Kinesin-10 (Chromokinesins), which contains vertebrate Kid and *Drosophila* Nod have a characteristic motif at C-terminal tail, a DNA binding domain. Members of this family localizes to chromosome arms and spindle microtubules (Afshar et al., 1995; Tokai et al., 1996) and have been reported to be involved in chromosome congression, segregation and in generating the polar ejection force that antagonizes poleward forces preventing premature chromosome movement towards the poles (Mazumdar and Misteli, 2005; Miki et al., 2005).

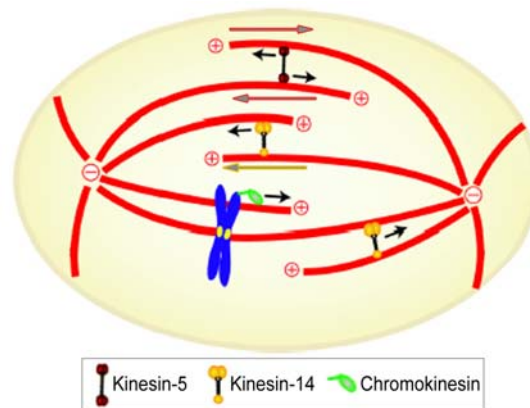


Figure 18. Different motor activities of diverse kinesins in mitotic spindle. Kinesin-5 (red), a plus-end directed motor, crosslinks MTs and slides antiparallel MTs outward (red arrows). Kinesin-14 (yellow), a minus-end directed motor, crosslinks MTs and produces inward forces upon antiparallel MTs (yellow arrow). Chromokinesins (green), can mediate chromosome attachment and plus-end directed movement (adapted from Gadde and Heald, 2004).

3.2.2. DYNEINS

Although there are fourteen classes of kinesins, dyneins are divided only into axonemal and cytoplasmic. Axonemal dyneins were discovered in axonemes of cilia and flagella where by minus-end directed movement generate microtubule sliding and consequently ciliary and flagellar beating. In contrast, cytoplasmic dyneins are found in all microtubule-containing cells and are involved in a vast number of functions. Both are minus-end directed motors composed by multiple subunits as, heavy chains, that contain the motor domain, and accessory subunits including intermediate, light intermediate and light chains, that are usually associated with the cargo-binding (Hook and Vallee, 2006).

Cytoplasmic dynein localizes to a variety of subcellular sites, including kinetochores, microtubules, centrosomes, cell cortex and has been also found at microtubule plus-ends (Dujardin et al., 2003; Dujardin and Vallee, 2002; Hays et al., 1994; Pfarr et al., 1990; Sharp et al., 2000a; Siller et al., 2005; Steuer et al., 1990; Vaughan et al., 1999; Xiang et al., 2000). This wide spread location allows cytoplasmic dynein to be involved in so distinct processes as intracellular transport, mitosis, cell polarization and directed cell movement (Hook and Vallee, 2006). Particularly at mitosis, this minus-end directed motor

is required for the organization of the minus-ends of the spindle and their tethering to the centrosomes or focusing acentrosomal spindle poles (Compton, 1998; Maiato et al., 2005; Maiato et al., 2004b), target proteins to kinetochores (Dujardin et al., 1998; Dzhindzhev et al., 2005) and remove others from kinetochores to the spindle poles (Howell et al., 2001; Wojcik et al., 2001), facilitate proper microtubule capture and stabilize kinetochore-microtubule attachments (Li et al., 2007; Yang et al., 2007), and in some cells is required for normal chromosome congression and alignment (Li et al., 2007; Yang et al., 2007). Also, its absence causes a metaphase delay (Goshima and Vale, 2003; Harborth et al., 2001) and a slow down in poleward chromosome movement at anaphase (Savoian et al., 2000; Sharp et al., 2000b). In addition, it was proposed that cytoplasmic dynein at the cortex, can pull on astral microtubules exerting a poleward force and at kinetochores, can push microtubules toward the spindle equator and operate as a "feeder", driving the K-fiber towards a "chipper" (ex. KLP59C) (Gadde and Heald, 2004; Sharp et al., 2000c) (Fig. 19). Its presence at kinetochores is of extreme importance and so is highly regulated, it target to kinetochores in a RZZ (Rod-ZW10-Zwilch complex) dependent manner and its dissociation from these structures is mediated by microtubule attachment and interaction with other proteins (Karess, 2005; Liang et al., 2007; Starr et al., 1998).

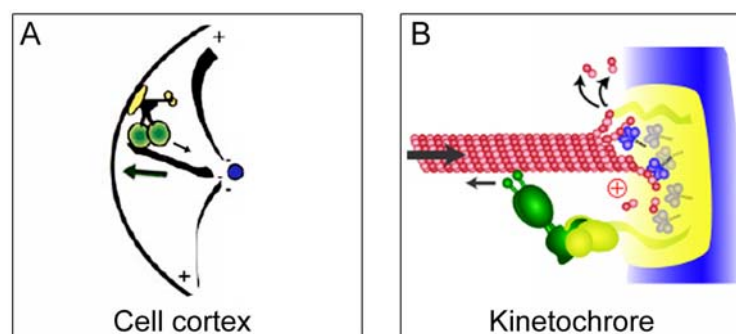


Figure 19. Dynein localization and mechanisms of action. (A) At cell cortex, this minus-end directed motor can pull on aster microtubules contributing to spindle expansion and positioning. (B) At kinetochores, dynein (green) may drive KMTs plus-ends towards depolymerising kinesins (violet) (adapted from Gadde and Heald, 2004; Heald, 2000).

3.3. MICROTUBULE PLUS-END TRACKING PROTEINS: +TIPs

Microtubule plus-end tracking proteins (+TIPs) are a particular class of MAPs conserved in all eukaryotes that accumulates specifically at the plus-ends of microtubules (Schuyler and Pellman, 2001). Although some +TIPs associate with plus-ends of depolymerising microtubules, the big majority associate with only growing microtubule plus-ends (Akhmanova and Steinmetz, 2008). Due to its plus-end accumulation, +TIPs have the ideal localization to carry out several functions like regulating microtubule plus-

ends dynamics, attaching microtubules to the cell cortex, kinetochores or different cellular organelles and by coupling these two roles, the dynamic behavior with the attachment of microtubules to cellular structures, they can also be involved in force generation. Thus, +TIPs are important for a variety of cellular processes like chromosome segregation, cell migration, vesicle and organelle intracellular transport and protein delivery, serving as cargo-loading sites for minus-end directed transport or delivering cargos to particular cell regions. In addition, these particular MAPs have important roles in cell differentiation and organ development and maintenance, malfunction of +TIPs are implicated in several human malignancies as some types of cancer and neuronal diseases (Carvalho et al., 2003; Honnappa et al., 2006; Howard and Hyman, 2003; Jaworski et al., 2008; Lansbergen and Akhmanova, 2006; Schuyler and Pellman, 2001). +TIPs belong to several structurally unrelated families including microtubule plus and minus-end directed motor proteins and non-motor proteins (Akhmanova and Steinmetz, 2008) (Table 1).

The first identified plus-end tracking protein was CLIP-170 that fused to the green-fluorescent protein (GFP) appears to “surf” at the plus-ends of microtubules (Carvalho et al., 2003; Perez et al., 1999). The mechanism for +TIPs plus-end targeting is not yet completely clarified however, there are some proposals, these include treadmilling, motor-dependent transport and hitchhiking, and the same protein can use more than one mechanism to reach the microtubule plus-end (Fig. 20 and Fig. 21) (Carvalho et al., 2003).

The treadmilling is favored for several mammalian +TIPs such as CLIP-170 and EB1 (Jaworski et al., 2008). In this mechanism, proteins are added to the plus-ends of growing microtubules and almost immediately are released behind the region of new growth, this coupling of association and rapid dissociation creates an optical illusion of plus-end tracking movement but in fact, these proteins remain stationary with respect to the microtubule lattice (Carvalho et al., 2003). The most likely mechanism for +TIP binding to the plus-ends is co-polymerization with tubulin dimmers, which is the case of CLIP-170 (Diamantopoulos et al., 1999; Folker et al., 2005). Alternatively, recognition of unique structural features at plus-ends could also be involved. These features could include the GTP cap, the shape of tubulin sheets or even specific tubulin sites that are hidden inside the microtubule lattice and become exposed at the tip. However, in order to accumulate only at the plus-end these proteins must dissociate rapidly from microtubule lattice (Fig. 20). Several mechanisms have been proposed to account for this event including, phosphorylation or auto-inhibition of +TIPs and tubulin changes as α -tubulin tyrosination-detyrosination or conformational changes during tube formation (Akhmanova and Steinmetz, 2008; Carvalho et al., 2003).

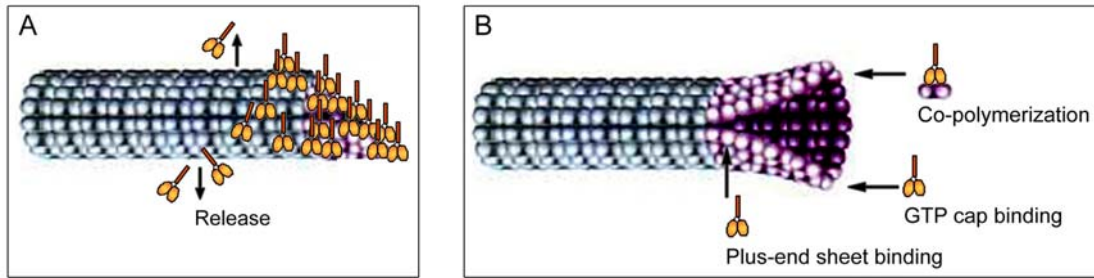


Figure 20. Treadmilling of +TIPs. (A) The +TIP (orange) binds the plus-ends of polymerizing microtubules (pink) and remains attached until it is released behind the region of growth, creating the illusion of plus-end directed movement. (B) Models for +TIPs binding to microtubule plus-ends. +TIP (orange) can co-polymerize with tubulin dimers or recognize specific structural features as, microtubule GTP-cap (pink) or the shape of tubulin sheet (adapted from Schuyler and Pellman, 2001).

Although treadmilling is the most common mechanism, +TIPs can also reach the plus-ends being actively transported by plus-end directed motors, a mechanism that is frequently used in fungi but it seems to be rare in animals (Fig. 21). The only known cases are APC that is transported to microtubule tips by kinesin-2 and *Drosophila* kinesin Khc-73 that is likely to transport +TIP partners to microtubule plus-ends (Akhmanova and Steinmetz, 2008; Jimbo et al., 2002; Siegrist and Doe, 2005). Another target mechanism commonly used by +TIPs is "hitchhiking" in which the protein reach the microtubule plus-ends by binding to another +TIP (Fig. 21). One key protein for this plus-end targeting mechanism is EB1; it was shown that several proteins as APC, spectraplakins, CLIP-190, KLP10A/MCAK and Ncd accumulate on microtubule plus-ends through hitchhiking on this +TIP (Dzhindzhev et al., 2005; Goshima et al., 2005a; Lansbergen and Akhmanova, 2006; Lee et al., 2008; Mennella et al., 2005; Slep et al., 2005). However there are other proteins also involved in this mechanism, for instance dynein/dynactin target the plus-ends of microtubules by interacting with CLIP-170 (Lansbergen et al., 2004; Valetti et al., 1999). Finally some plus-end tracking proteins like MCAK, XMAP215 and dynactin, can also reach the microtubule tips by one-dimensional diffusion along the microtubule lattice (Fig. 21) (Brouhard et al., 2008; Culver-Hanlon et al., 2006; Helenius et al., 2006).

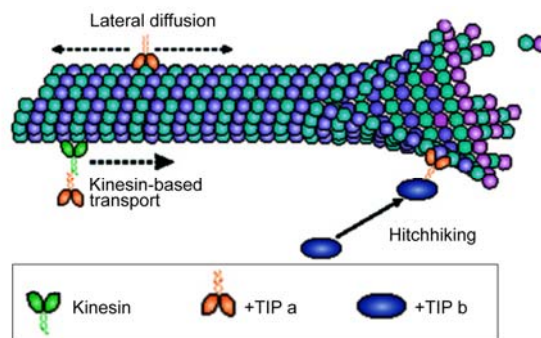


Figure 21. Mechanisms for +TIPs plus-end targeting. Besides the treadmilling, plus-end tracking proteins can arrive at the plus-ends of microtubules through transport by plus-end directed motors (kinesins); by diffusion along the MT lattice; or more common, they can reach the plus-ends by hitchhiking, binding to another +TIP (adapted from Akhmanova and Steinmetz, 2008).

Independently of how +TIPs associate and dissociate from microtubule plus-ends, their functions are extremely important since its disruption is connected with abnormal cell cycle progression and several diseases.

Table 1. Plus-end tracking proteins (Akhmanova and Steinmetz, 2008)

+Tip family (mammalian)	Homologues	+TIP Interaction partners	Main functions
EB1 family EB1, EB2, EB3	Mal3 (Sp) Bim1 (Sc)	Most known +TIPs (CAPGly domains, basic/serine rich stretches)	Promotion of MT growth and dynamicity Anti-catastrophe activity Targeting of other +TIPs to MT ends
CAP-Gly family CLIP170 CLIP115 p150glued (dynactin subunit)	Tip1 (Sp) Bik1 (Sc) CLIP-190 (Dm) Ssm4 (Sp) Nip100(Sc) NudM (An)	EB proteins CLASPs CLIP170 p150Glued Stu2 Kar9 Tea1 Tea2 (Kip2) LIS1 (Pac1) XKCM1 Cytoplasmic dynein CLIP170	MT rescue and stabilization Targeting of dynein to MT ends MT interaction with the cell cortex, kinetochores and vesicles Binding dynein to cargo Regulation of dynein processivity
CLASPs CLASP1/2	Orbit/MAST(Dm) Peg1(Sp) Stu1(Sc) Cis-2 (Ce)	EB proteins CLIP170 CLIP115	MT rescue and stabilization MT interaction with kinetochores, cell cortex and the Golgi
APC	dAPC1/ 2 (Dm)	EB proteins XKCM1	MT stabilization, anti-catastrophe activity Interactions with the cell cortex and kinetochores MT-unrelated functions in Wnt signalling, etc
XMAP215/Dis1 family ChTOG	XMAP215(XI), Dis1, Alp14 (Sp) Stu2(Sc) Msps (Dm) DdCP224 (Dd) ZYG-9 (Ce)	EB proteins Bik1 Kar9 XKCM1	MT stabilization, promotion of MT growth MT-cortex interactions
Spectraplakins MACF2 (ACF7)	Shot/Kakapo (Dm)	EB proteins	MT stabilization, linking MTs to actin and the cell cortex
LIS1	Pac1(Sc) NudA (An)	CLIP170 (Bik1) Dynein Dynactin	Dynein activation, interaction with the cell cortex
Melanophilin		EB1	Melanosome transport
Neuron Navigator Navigator-1,2,3	Unc-53 (Ce)		Regulation of neuronal migration
Rho GEF2 (Dm)	Many related Rho-GEFs, but no direct +TIP homologue	EB1	Regulation of Rho activity
STIM1		EB1	Calcium sensing and activation of calcium channels in store-operated calcium entry; Extension of ER tubules driven by microtubule growth
Cytoplasmic dynein Dynein heavy chain	Dyn1 (Sc) Dhc1(Sp)	Dynactin (p150 ^{Glued}) LIS1	MT minus-end-directed transport of macromolecular complexes and organelles Pulling at MTs at the cortex, transport of MTs
Kinesin-13 MCAK (KIF2C)	KLP10A (Dm) XKCM1 (XI)	EB proteins XMAP215 CLIP170 APC	 MT depolymerization, induction of catastrophes
Kinesin-14 Ncd (Dm)	KLP2 (Sp) ATK5 (At) Kar3 (in complex with Cik1) (Sc)	EB proteins	MT minus-end-directed transport Antiparallel MT sliding MT depolymerization

4. SPINDLE MICROTUBULE DYNAMICS AND CHROMOSOME MOTION

The interaction between chromosomes and microtubules is essential for normal cell division, however, the detailed mechanism by which kinetochores and dynamic K-fibers generate chromosome motion while maintaining their attachment remains a mystery. It is recognized that kinetochores are key players in this process, besides providing attachment sites between chromosomes and microtubules (through Ndc80 complex, CENP-E, dynein, etc) they also generate or transduce forces required for chromosomal movement. There are two main force-generating mechanisms acting at kinetochore-microtubule interface involved in this movement: "microtubule flux", which consists in a flow of tubulin subunits from the spindle equator to the spindle poles as consequence of polymerization at the plus-ends and depolymerization at minus-ends. At anaphase, the decrease of tubulin entrance and the continuous minus-end microtubule depolymerization coupled to motor-dependent poleward microtubule translocation result in the movement of the entire microtubule and its attached chromatid toward the spindle pole. The other major force is generated by the "Pacman" mechanism, in which the kinetochores catalyze the depolymerization of kinetochore-microtubule plus-ends resulting in chromosomal movement to the poles by "chewing up" the microtubule track (Fig. 22).

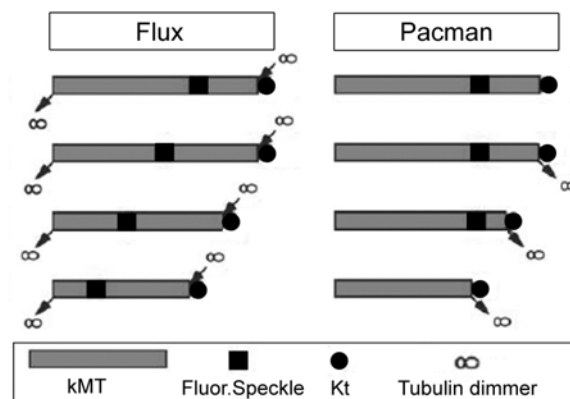


Figure 22. Force-generating mechanisms acting at kinetochore-microtubule interface. There are two main forces involved in poleward chromosomal movement: Flux, in which MTs polymerize at the plus-ends (near kinetochore), depolymerize at the minus-ends (near the centrosome) and the Mt as a whole moves toward the spindle pole; and Pacman, in which kMT plus-ends are "chewing up" by kinetochore-depolymerising proteins (adapted from Maddox et al., 2003).

The contribution of each force to chromosome segregation is system-dependent. Pacman generated force is particularly important in yeast which does not have microtubule flux (Maddox et al., 2000; Sagolla et al., 2003), in contrast in crane-fly spermatocytes flux seems to contribute with 100% for K-fiber shortening (LaFountain et al., 2004). But there are several systems in which the two mechanisms contribute to chromosome poleward movement: in *Drosophila* S2 cells the two forces have roughly the

same participation (~50%) (Zhang et al., 2007) whereas in *Drosophila* embryos flux as a minor involvement (~40%) (Brust-Mascher and Scholey, 2002; Rogers et al., 2004a). In mammalian cells flux also as a minor contribution (20%-45%) compared with Pacman mechanism (80%-55%) (Ganem et al., 2005; Mitchison and Salmon, 1992; Zhai et al., 1995). In contrast, in *Xenopus* egg extracts, flux has a major contribution (~60%) for poleward chromatid movement (Maddox et al., 2003).

4.1. MICROTUBULE FLUX

Poleward microtubule flux is the movement of tubulin subunits from microtubule plus-ends at the spindle equator to the microtubule minus-ends at the spindle poles (Mitchison, 1989). It occurs both at kMTs and ipMTs however the poleward flux rate is slightly slower on kMTs (Maddox et al., 2003). Flux requires that microtubules polymerize at the plus-ends, depolymerize at the minus-ends and that microtubules in their entirety moves toward the spindle pole (Cassimeris, 2004). Conventionally flux models relay in three main activities: a) microtubule poleward translocation, thought to be carried out in *Xenopus*, *Drosophila* and mammal cells, by kinesin-5 (KLP61F, Eg5) through its antiparallel microtubule-sliding activity (Cameron et al., 2006; Goshima et al., 2005b; Mitchison et al., 2004; Miyamoto et al., 2004); b) minus-end depolymerization at the spindle poles, a task shown to be performed by members of kinesin-13 family in human cells and *Drosophila* (Kif2a, KLP10A) (Ganem et al., 2005; Goshima and Vale, 2005; Rogers et al., 2004a) and c) plus-end polymerization of MTs at spindle equator, a process achieved by Mast protein (Clasp family) in *Drosophila* S2 cells (Maiato et al., 2005). Spindle microtubule flux occurs throughout metaphase/anaphase A isometric state however, the flux rate decreases after metaphase-anaphase transition and at anaphase B onset ipMTs flux is suppressed resulting in coupling of sliding forces with spindle elongation (Brust-Mascher et al., 2004; Brust-Mascher and Scholey, 2002; Buster et al., 2007; Mitchison and Salmon, 1992; Zhai et al., 1995).

The variation of flux velocities within a spindle is larger than between different spindles suggesting that microtubule flux is not a homogeneous process and that it involves different contributions from different proteins adjusted to a particular moment or situation (Cameron et al., 2006). Beside the contribution of kinesin-5 in microtubule poleward translocation, kinesin-13 in minus-end depolymerization and Mast in plus-end polymerization, other proteins were found to alter the microtubule flux rate. *Drosophila* proteins Spastin and Fidgetin were found to induce flux by allowing minus-end depolymerization. As both are severing proteins, localize at the centrosomes and catalyze

γ -tubulin turnover, Zhang et al. proposed that they operate by releasing the stabilizing γ -tubulin ring complexes from microtubule minus-ends. The free minus-end can now be depolymerized by kinesin-13 proteins inducing microtubule flux and the small microtubule stub attached to γ -tubulin ring complex undergoes plus-end depolymerization allowing γ -TuRC turnover (Zhang et al., 2007). Flux rates in *Drosophila* are also affected by other proteins including one that localizes at the minus-end and promotes microtubule flux: Msps (mini-spindles - the only *Drosophila* member of XMap215/Tog family) and one positioned at the plus-end that inhibits flux: the depolymerising kinesin-8 KLP67A (Buster et al., 2007). The presence of proteins with opposite functions at the plus-end, one promoting and other inhibiting the flux, Mast and KLP67A respectively, suggests that the regulation of flux at the plus-end is achieved by a balance between antagonistic contributions. Indeed, cells depleted of Mast have a flux rate near zero (Maiato et al., 2005) contrasting with cells depleted of KLP67A that exhibit a flux rate near twice faster than the control ones, but when these two proteins are simultaneously depleted the flux rate is restored to control values (Buster et al., 2007). Buster and colleagues also saw that the centromeric kinesin-13, KLP59C, although does not affect directly the flux seems to antagonize Mast since cells lacking these two proteins exhibit normal flux rates. At minus-ends, flux requires both KLP10A (by depolymerization) and Msps (maybe by enabling dynamic behavior), since depletion of these proteins inhibits the flux rate and it seems that could not be restored, it was proposed that inhibition of minus-end factors leads to a flux arrest whereas inhibition of the relative activities of plus-end factors regulate changes in the flux rate (Buster et al., 2007).

Although the conventional models do not take it into account that a non-microtubule spindle matrix might also be involved in flux generation, other flux models assume this as a possibility. In a simplistic definition, spindle matrix constitute a stationary or elastic molecular network providing a substrate for motor and non-motor proteins to interact with during microtubule sliding and which can stabilize the spindle during force production (Forer et al., 2008; Johansen and Johansen, 2007). Proteins thought to belong to a spindle matrix are able to retain the spindle shape even after spindle microtubules are depolymerized (Walker et al., 2000). Indeed, *Drosophila* S2 cells lacking one of those proteins, Chromator, originate abnormal spindles and problems in chromosome segregation (Rath et al., 2004). Also, inhibition of actin and myosin, considered for some as matrix network proteins, induces a microtubule flux arrest in crane-fly spermatocytes (Forer et al., 2008; Forer et al., 2007; Silverman-Gavrila and Forer, 2000).

Since the mechanisms involved in generating flux as well as its contribution to chromatid segregation differs widely in different cell types and systems it is difficult to clarify the flux origin and function. Indeed it was speculated that in some systems were it

at has a minor contribution for poleward chromatid movement, besides acting as a redundant mechanism for this motion, flux may be also involved in other functional roles however these remain unclear (Ganem and Compton, 2006).

4.2. PACMAN MECHANISM

The Pacman mechanism postulates that kinetochores induce poleward chromatid movement by chewing up the plus-ends of kMTs (Gorbsky et al., 1987; Mitchison et al., 1986). In this model, at the same time that a kinetochore induces plus-end microtubule disassembly it must maintain the linkage with the K-fiber. The mechanism behind this process is not yet been solved however, it is known that catastrophe proteins from kinesin-13 family have a preponderant role in the plus-end depolymerization and that some conventional motors like Cenp-E and dynein seem to be involved in maintaining the kinetochore-microtubule attachment (Maiato et al., 2004a).

The first protein identified to be required for pacman mechanism was the *Drosophila* kinesin-13 KLP59C, inhibition of this protein has no effect on microtubule flux but decreases significantly (~60%) the rate of anaphase chromatid movement (Rogers et al., 2004a). In order to keep on the K-fiber shortening, at the same time that the plus-end is depolymerized the entire microtubule must be pulled to kinetochore-depolymerising factors, a task thought to be carried on by the minus-end directed motor dynein, its inhibition also slows down anaphase A without affecting the rate of poleward microtubule flux (Maiato et al., 2005; Savoian et al., 2000; Sharp et al., 2000b; Yang et al., 2007). Also, the plus-end directed motor protein Cenp-E seems to contribute to this mechanism as coupler to the depolymerising microtubules (Lombillo et al., 1995; McEwen et al., 2001). Although some proteins are known to be involved in this process, their precise roles in the pacman mechanism remain unclear. However, a "feeder and chipper" model was proposed, in which dynein and other proteins act as a "feeder", attaching and driving the microtubule plus-ends to the kinetochore, allowing the depolymerization and concomitant poleward movement, by the kinesin-13 proteins (ex. KLP59C), the "chipper" (Gadde and Heald, 2004).

5. CLASP FAMILY

CLASPs are evolutionary conserved proteins found in a wide range of organism, from human to yeast and in plants. This family is composed by *Drosophila* Orbit/Mast (*Multiple asters*) (also known as *Chromosome Bows* [Chb]) (Fedorova et al., 1997; Inoue et al.,

2000; Lemos et al., 2000), mammalian Clasp1 and Clasp2 (*Clip-associated proteins*) (Akhmanova et al., 2001), *Xenopus* Xorbit (Hannak and Heald, 2006), *S. cerevisiae* Stu1 (Pasqualone and Huffaker, 1994; Yin et al., 2002), *S. pombe* Peg1 (Grallert et al., 2006), *C.elegans* Clasp^{clis2} (Cheeseman et al., 2005; Gonczy et al., 2000) and the plant *Arabidopsis thaliana* AtClasp (Ambrose et al., 2007; Gardiner and Marc, 2003). CLASP proteins belong to the +TIP subfamily of microtubule associated proteins and generally are shown to be involved, both in interphase and mitosis, in the regulation of microtubule plus-end dynamics and stabilization.

Studies in motile fibroblasts demonstrated that Clasp associates with the distal ends of microtubules at the leading edge of the cell and suggest that this asymmetric distribution is mediated by glycogen synthase kinase-3 β (GSK3 β). When this kinase is overexpressed, Clasp binding to distal ends is reduced and when GSK3 β is inactivated, Clasp binding is stimulated, resulting in a symmetric association throughout the cell. In agreement, *in vitro* assays also indicate that Clasp microtubule binding is negatively affected by phosphorylation (Akhmanova et al., 2001). Indeed another study, using migrating PtK1 epithelial cells, suggests that the affinity of Clasp for microtubule lattice is spatially regulated by GSK3 β , resulting in tracking microtubule plus-ends in the cell body and in a dynamic decoration of the microtubule lattice in the leading edge lamella, which is consistent with the low GSK3 β activity in the lamella and high activity in the cell body (Wittmann and Waterman-Storer, 2005).

Overexpression of Clasp in interphase cells was found to result in global microtubule stabilization and in contrast, Clasp inhibition leads to a loss of stabilized microtubules (Akhmanova et al., 2001; Maiato et al., 2003a; Sousa et al., 2007). Indeed, in diverse organisms Clasp was shown to stabilize microtubules by promoting the pause state being classified as the first pause promoter factor (Grallert et al., 2006; Mimori-Kiyosue et al., 2005; Sousa et al., 2007). A study in HeLa cells revealed that Clasp require both its EB1/MT binding domain (middle region) and the cortex binding domain (C-terminal) to promote microtubule stabilization (Mimori-Kiyosue et al., 2005). Indeed, this physical interaction of Clasp/EB1 is also seen in other organisms (Grallert et al., 2006; Rogers et al., 2004b). Since these two proteins have opposite roles in microtubule dynamics, with Clasp as a pausing factor and EB1 as an anti-pausing factor, it was proposed that Clasp stabilize microtubules by mediating interactions between microtubule plus-ends and the cell cortex and by forming a complex with EB1 at the microtubule plus-ends (Mimori-Kiyosue et al., 2005; Rogers et al., 2002; Sousa et al., 2007; Tirnauer et al., 2002; Tirnauer et al., 1999). The interaction of Clasp with the cell cortex was also seen in plant cells in which the absence of this +TIP results in partial detachment of microtubules from

the cortex (Ambrose and Wasteney, 2008). Some studies have appeared highlighting this interaction, indeed it is already known that Clasp can bind directly to the cortical protein LL5 β and that the actin-microtubule crosslinking factor ACF7 regulates cortical Clasp localization in HeLa cells (Drabek et al., 2006; Lansbergen et al., 2006).

Besides being a plus-end tracking protein, Clasp also localizes to kinetochores, in a microtubule-independent manner, where it starts to accumulate soon after NEBD reaching the highest level during prometaphase while at metaphase and anaphase Clasp levels are significantly reduced. Clasps also localize to centrosomes, central spindle at anaphase, the midbody (Lemos et al., 2000; Maiato et al., 2003a; Pereira et al., 2006), the cell cortex (Mimori-Kiyosue et al., 2005) and Golgi (Akhmanova et al., 2001; Efimov et al., 2007). In mitosis, it was shown to be essential for normal kinetochore-microtubule attachment, chromosome congression and maintenance of spindle bipolarity (Hannak and Heald, 2006; Lemos et al., 2000; Maiato et al., 2003a; Maiato et al., 2002). Mast mutants or RNAi treated cells form monopolar spindles due to failure in sustain spindle bipolarity. Experiments in *Drosophila* S2 cells demonstrate that in the absence of Mast, tubulin poleward flux is strongly reduced (Buster et al., 2007; Maiato et al., 2005). Accordingly, it was proposed that the collapse of the spindle in the absence of this MAP, results from the inability to incorporate tubulin subunits at the plus-ends of mature K-fibers while depolymerization of microtubule minus-ends still occur at the poles, resulting in the shortening of the K-fibers and consequent collapse of the spindle (Maiato et al., 2005). In agreement, co-depletion of Mast with the minus-end depolymerising kinesin KLP10A originates non-fluxing bipolar spindles (Buster et al., 2007; Laycock et al., 2006). As the studies of this MAP proceed, the roles attributed to Clasps increase. They are also involved in microtubule nucleation at the Golgi apparatus in human cells (Efimov et al., 2007), in mitochondrial distribution in *S. pombe*, acting as a molecular link between microtubules and mitochondria (Chiron et al., 2008), in the formation or stabilization of the interior central spindle in *Drosophila* primary spermatocytes, ensuring a normal cleavage (Inoue et al., 2004) and is shown to be required for asymmetric stem cell and cystocyte divisions and development of the polarized microtubule network during *Drosophila* oogenesis, ensuring normal oocyte differentiation (Mathe et al., 2003).

+TIPs are multi-domain proteins which enable them to interact with several subcellular structures and proteins. Analysis of the Clasp domains reveal the presence of Heat repeats, thought to participate in protein-protein interactions, a Dis1/Tog domain at the N-terminal and a Ser-Arg rich region at the central part of the protein, implicated in microtubule binding. Functional dissection studied revealed that Clasps bind to microtubules and EB1 through its middle region, can bind to actin also by this central part and by the N-terminal Dis1/TOG domain and interact with Clips, Cenp-E, kinetochores,

cell cortex and Golgi by the C-terminal region (Akhmanova et al., 2001; Hannak and Heald, 2006; Maiato et al., 2003a; Mathe et al., 2003; Mimori-Kiyosue et al., 2005; Tsvetkov et al., 2007). Although Clasp can bind microtubules directly, studies in *C.elegans* revealed that Clasp^{cls2} targeting to kinetochores is dependent on the Cenp-F (mitosin)-like proteins HCP-1/2, a protein shown to be required for proper kinetochore-microtubule interactions in HeLa cells probably by coupling kinetochores to the minus-end-directed motor activities (Akhmanova et al., 2001; Cheeseman et al., 2005; Galjart, 2005; Vergnolle and Taylor, 2007).

6. OBJECTIVES

The work reported in this thesis aims to understand the involvement of the protein Mast/Orbit/CLASP in microtubule dynamics and in kinetochore-microtubule interactions. We have explored how it reaches these structures, how it is released, the interaction with other proteins and possible mechanisms of actions. In Chapter 1, we dissect the functional domains of Mast, delimitating the microtubule-binding domain and the kinetochore binding domain. In Chapter 2, we analyze the +TIP behaviour of the full-length and several truncated forms of Mast in interphase, and in Chapter 3 we investigate the dynamic behaviour of Mast along the cell cycle, interaction with the motor protein Dynein and involvement in spindle stability and microtubule dynamics.

PART II

EXPERIMENTAL WORK

CHAPTER 1

Molecular dissection of the functional domains of Mast

1. INTRODUCTION

The gene *mast*, multiple asters, or *chb*, for chromosome bows as it was first denominated, was identified through *Drosophila* mutational analysis (Fedorova et al., 1997). The first mutant allele, *mast*^{P1} (chbv40), was produced by P-element insertion mutagenesis and it was found during the identification of novel insertions that resulted in abnormal mitosis in third larval neuroblasts (Omeliianchuk et al., 1997). This mutant exhibited highly polyploid cells, monopolar spindles and circular mitotic figures in which circles of chromosomes had centromeres pointing inward and chromatid arms pointing outward (Fedorova et al., 1997). Later, two other P-element-induced alleles, *mast*^{P2} and *mast*^{P3}, were identified from the Berkeley Drosophila Genome Project (BDGP) database and a fourth allele, *mast*^{P4}, was generated after remobilization of the P-element in *mast*^{P1} producing an imprecise excision allele (Lemos et al., 2000). Neuroblasts from larvae carrying *mast* mutant alleles displayed highly polyploid cells, highly condensed chromosomes frequently organized in circular arrangements and very few and abnormal anaphases. Taken into account the effects on viability, mitotic phenotype and mitotic progression, *mast*^{P4} was classified as the more severe allele. Homozygosity for this allele resulted in late larval/pupal lethality and cell progression analysis revealed a very high mitotic index with cells arrested at prometaphase/metaphase, however this arrest is overcome since most of them were polyploid (Lemos et al., 2000). *Mast* localizes at the cytological region 78C1-C2 on chromosome 3, the cDNA (LD11488) is composed of 5938 bp and contains a single open reading frame (ORF) of 1491 amino acids (a.a.) coding for a protein with 165.5 kDa (Lemos et al., 2000). Sequence analysis (Fig. 1.1) show that the protein contains a highly basic central region which includes a sequence that shares limited homology with the MAP4 microtubule-binding domain (Aizawa et al., 1991; Aizawa et al., 1990; Inoue et al., 2000; Lemos et al., 2000); two consensus sites for phosphorylation by p34^{cdc2}; two regions with significant homology to HEAT repeats, a motif that is also present in proteins from the Dis1-TOG family and form rod-like helical structures that participate in protein-protein interactions (Andrade and Bork, 1995; Tournebize et al., 2000); and four GTP-binding motifs, ⁵⁴⁴GGGTGTG⁵⁵⁰, ³⁹⁹NKLD⁴⁰², ⁴¹⁹SAK⁴²¹ and ⁹⁷DRLG¹⁰⁰ (Inoue et al., 2000).

Mast was shown to be a microtubule-associated protein that associates also with kinetochores. Although the putative sites, given by sequence analysis and by comparison with other species, the specific microtubule or kinetochore-binding domains of *Mast* remained unidentified. Therefore, in order to define the functional domains of *Mast*, we

constructed several truncated fragments fused to the reporter molecule EGFP (enhanced green fluorescent protein) (Tsien, 1998), transfected the constructs into *Drosophila* S2 cells, induced their expression and analyzed the localization of the mutant proteins during the cell cycle.

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1  MAYRKPSDLD GFIIQMPKAD MRVKVQLAED LVTFLSDDTN SIVCTDMGFL IDGLMPWLTG SHFKIAQKSL EAFSELIKRL
81  GSDFNAYTAT VLPHVIDRLG DSRDTVREKA QLLLRDLMEH RVLPPQALID KLATSCFKHK NAKVREEFLQ TIVNALHEYG
161  TQQLSVRVYI PPVCALLGDP TVNVREAAIQ TLVEIYKHVG DRLRPDLRRM DDVPASKLAM LEQKFDQVKQ EGLLLPSALK
241  NTNGNGVGLD EADNIGLRER PTRMIKRPLH SAVSSSLRPK HNVDVTGDA GAVTMESFES SFEVVPQLNI FHAKDMDDIY
321  KQVLVIISDK NADWEKRVDA LKKIRALLIL SYHTQPQFVA VQLKELSLSF VDILKEELRS QVIREACITI AYMSKTLRINK
401  LDAFCSWILE HLINLIQNSA KVIASASTIA LKYIIKYTHA PKLLKIYTDI LNQSKSKDIR STLCELMVLL FEEWQTKALE
481  RNATVLRDTL KKSIGDADCD ARHRSRYAYW AFRRHFPOLA DQIYGTLDIA AQRALERERE GGGGGGTGTG TGTAPETRRT
561  VSRIGRTPGT LQKPTPSMRS ISAVDTAAAG RAKVRAQYTL YSRQRKPLGP NNSNQASMTG AAASGSLPRP RLNSNSGGTP
641  ATTPGSVTPR PRGRAGVSQS QPGSRSTSPS TKLRDQYGGI GNYYRGATGA IPKKASGIPR STASSRETSP TRSGGGLMKR
721  SMYSTGAGSR RTPERNHPVR PSAAARLLAQ SREAEHTLGV GDDGQPDYVS GDYMRSGGMR MGRKLMGRDE SDDIDSEASS
801  VCSERSFDSS YTRGNKSNYS LSGSHTRLDW STQRAFPDDI ETIIQFCAST HWSERKDGLI SLTQYLADGK ELTQQQLKCV
881  LDMFRKMFMD THTKVYSLFL DTVTELILVH ANELHEWLFI LLTRLFNKLG TDLNLSMHSK IWKTLQVVEH YFPTQLQLKE
961  LFRIISDSTQ TPTTKTRIAI LRFLTDLANT YCKSSDFPSD QSQCERTVL KLAQLAADQK SMELRSQARS CLVALYNLNT
1041  PQMTLLADL PKVYQDSARS CIHSHMRQSQ QSCNSGANSP SSSPLSSSSP KPLQSPSVGP FASLQSHHQ LSISSTSPRS
1121  RQSSVEQELL FSELDIQHN IQKTSEEIRH CFGGQYQAL APNGFNHGLQ YHDQGGQDSC ASLSSNSKTQ SSANTTQSN
1201  PESATMRLDN LERERTQNA KSPTDDAKVI TVSINMAENG ELILASNLME SEVVRVALTL TKDQPVELLQ TSLTNLGICI
1281  KGGNCELPNK HFRSIRMLL NILEAEHTDV VIAGLHVLSK IMRSNKMRRN WMHFLELILL KIIQCYQHSK EALRDIDSMI
1361  PRIAPSLPLD LSINIVNPVI ATGEFPTNLC AIKILLEVTE HHGSEITDAH LDIVFPNLAR SADDTQSMVR KAAVFCIVKL
1441  YFVLGEEKVK PKLSVLNPSK VRLNLYIEK QRNCISGGGS STKNSSAASS S

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Figure 1.1. Amino acid sequence of Mast. HEAT repeats are represented in blue and putative GTP-binding motifs in red. The box delimits the basic region within which Mast displays homology to the conserved MAP4 microtubule-binding domain (violet) and the two predicted sites for phosphorylation by p34^{cdc2} are underlined.

In summary, this in vivo analysis revealed that, the microtubule-binding domain of Mast localizes at the basic central region of the protein defined by a fragment of 187 amino acids, Mast⁴⁴⁶⁻⁶³², and the kinetochore-binding domain is composed by the last 271 amino acids of the C-terminal region, Mast¹²²⁰⁻¹⁴⁹¹. Both sequences are highly conserved amongst the homologs of other species.

2. RESULTS

Mast is a protein with a broad pattern of localization, during mitosis it localizes to centrosomes, kinetochores, microtubules, central spindle and midbody (Lemos et al., 2000). In order to establish the regions responsible for each localization and define specifically the microtubule-binding domain (MBD) and the kinetochore-binding domain (KBD) we created several Mast truncated fragments linked to the reporter gene EGFP (Fig.1.2) and analyzed the pattern of localization by transfecting them transiently in S2 cells.



Figure 1.2. Full length Mast and truncated proteins transfected into S2 cells. The grey bar represents the full length protein in which the HEAT repeats (blue), the putative GTP-binding motifs G₁, G₂, G₃ and G₄, starting from the N-terminal (red), the consensus sites for phosphorylation by p34^{cdc2} (yellow), the putative microtubule-binding domain (violet) and the basic region (open box in green) are indicated. Black bars represent the full length (top) and fragments of Mast (below) tagged with EGFP at the N-terminus expressed in S2 cells and used to dissect the protein.

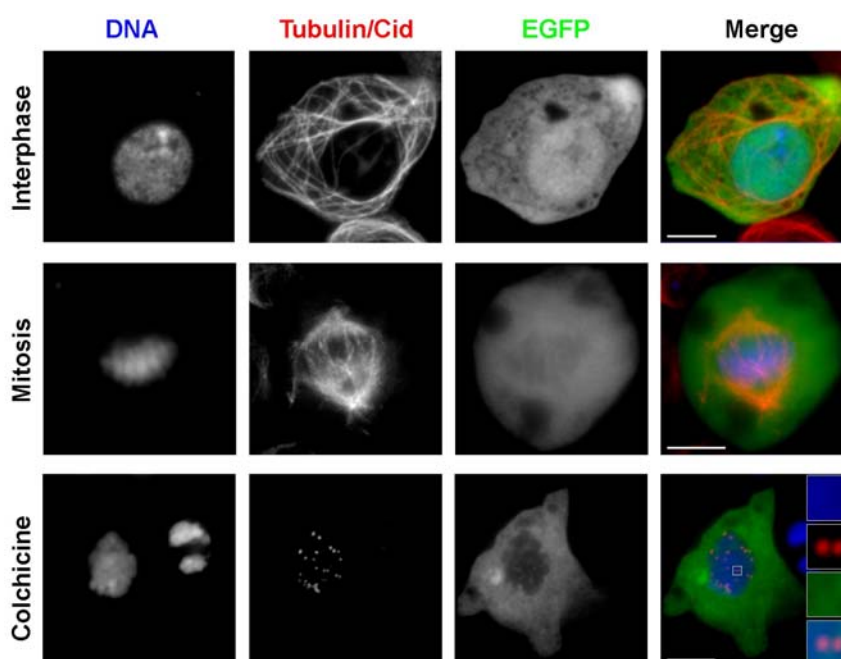


Figure 1.3. Cellular distribution pattern of EGFP. S2 cells transfected with EGFP (green) and immunostained with α -tubulin or cid (last row) (red) and labeled for DNA (blue). Scale bar: 5 μ m.

Since all fragments transfected are fused to EGFP, it was possible to evaluate their cellular localization. As control we expressed EGFP alone and found that it does not concentrate to any specific structure (Fig. 1.3). At mitosis, EGFP exhibits a diffuse pattern over the cytoplasm and at interphase, it is also present at the nucleus. To analyze EGFP localization specifically at kinetochores, cells were incubated with colchicine, a microtubule depolymerising drug that leads to a cellular arrest in prometaphase increasing the mitotic population, the results show that like in other cellular structures EGFP is completely absent from kinetochores (Fig. 1.3, high magnifications).

2.1 Cellular localization of Mast throughout mitosis

To test if the complete fusion protein EGFP-Mast has the same distribution as the endogenous protein, S2 cells were transfected with EGFP-Mast or immunostained with the antibody anti-Mast (Fig. 1.4 and Fig. 1.5).

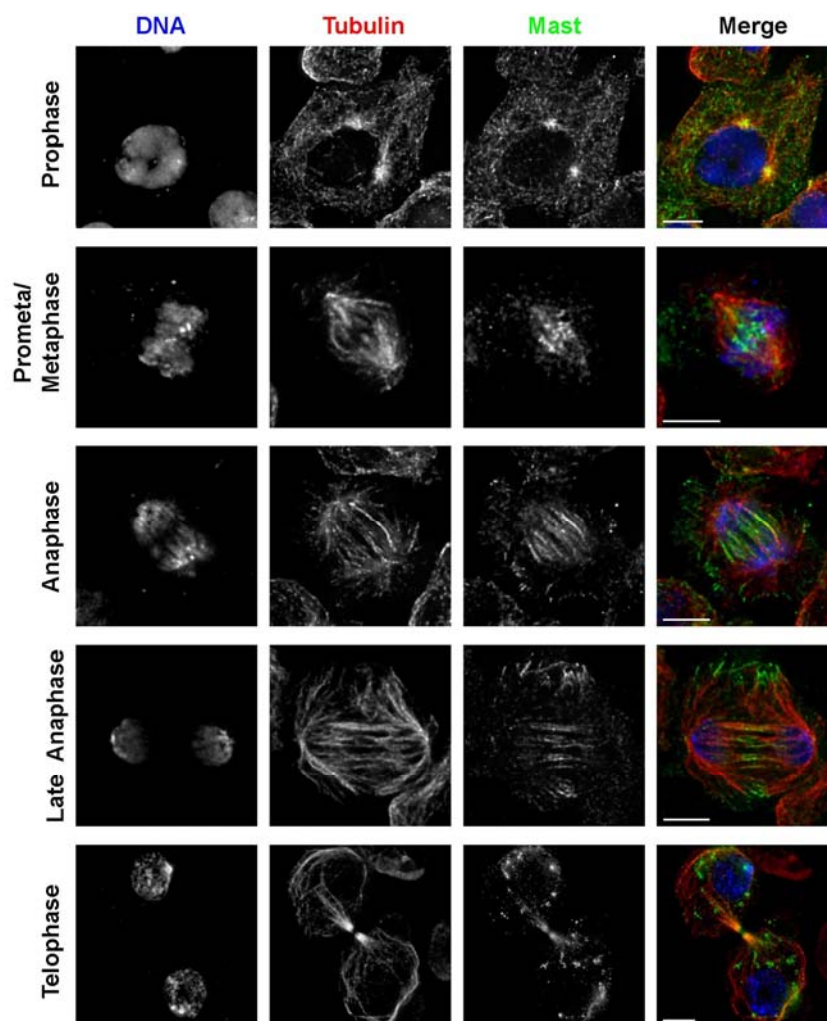


Figure 1.4. Cellular localization of endogenous Mast during mitosis in S2 cells. Immunofluorescence images labeled with anti-Mast (green), anti- α -tubulin (red) and stained with DAPI (blue) to visualize the DNA. Scale bar: 5 μ m.

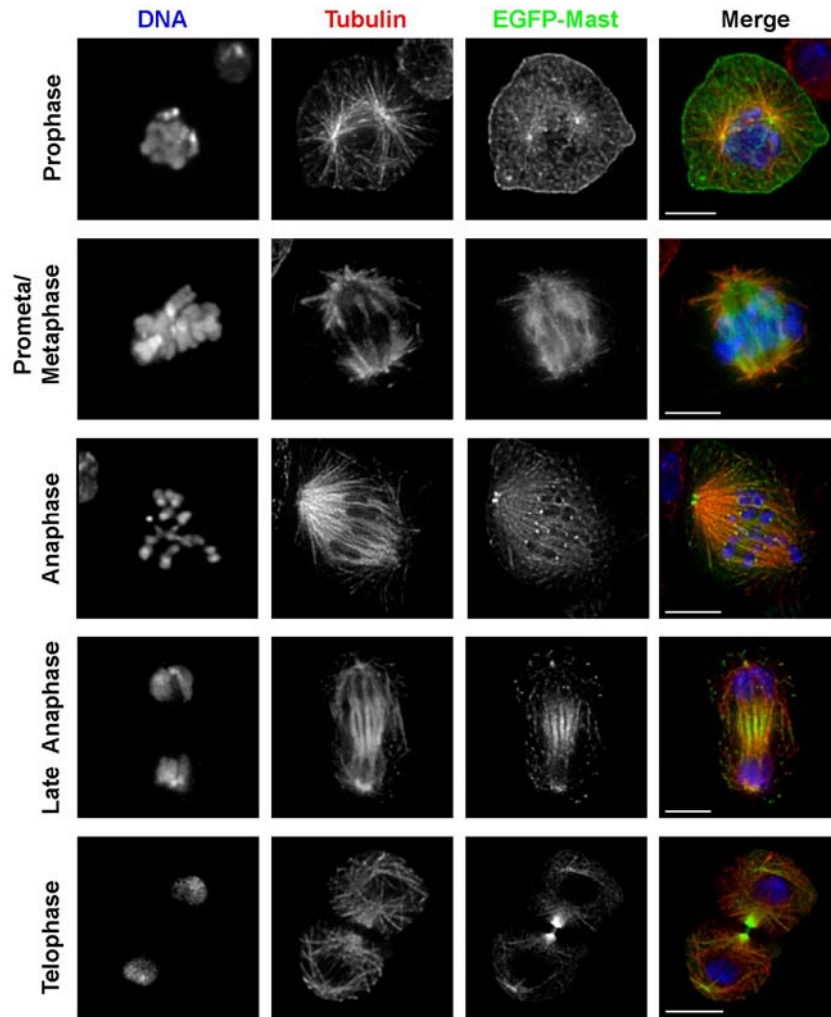


Figure 1.5. Cellular localization of the fusion protein EGFP-Mast (EGFP-Mast¹⁴⁹¹) throughout mitosis. S2 cells transfected with EGFP-Mast (green) and stained with α -tubulin (red) and DAPI (blue). Scale bar: 5 μ m.

Our results show that Mast, both endogenous and the fusion protein concentrate to the same cellular structures. In agreement to what as been described, we observed that Mast co-localizes with microtubules, accumulating at the plus ends; centrosomes; kinetochores and at later stages it concentrates to the central spindle in anaphase and to the midbody in telophase. In addition, at least in prometaphase, Mast seems to localize to the cell cortex.

2.2 Microtubule-Binding Domain

It is well established that proteins from the CLASP family (human homologue of Mast) bind microtubules both *in vivo* and *in vitro*. In human cells, the domain responsible

for microtubule-binding of CLASP was mapped to a basic/serine rich central area, a region often involved in mediating interactions with microtubules (Maiato et al., 2003a; Mimori-Kiyosue et al., 2005). Therefore, to define the microtubule-binding domain of Mast we transfected S2 cells with several EGFP-Mast-fragments containing the basic region and analyzed their ability to bind microtubules (Fig. 1.6).

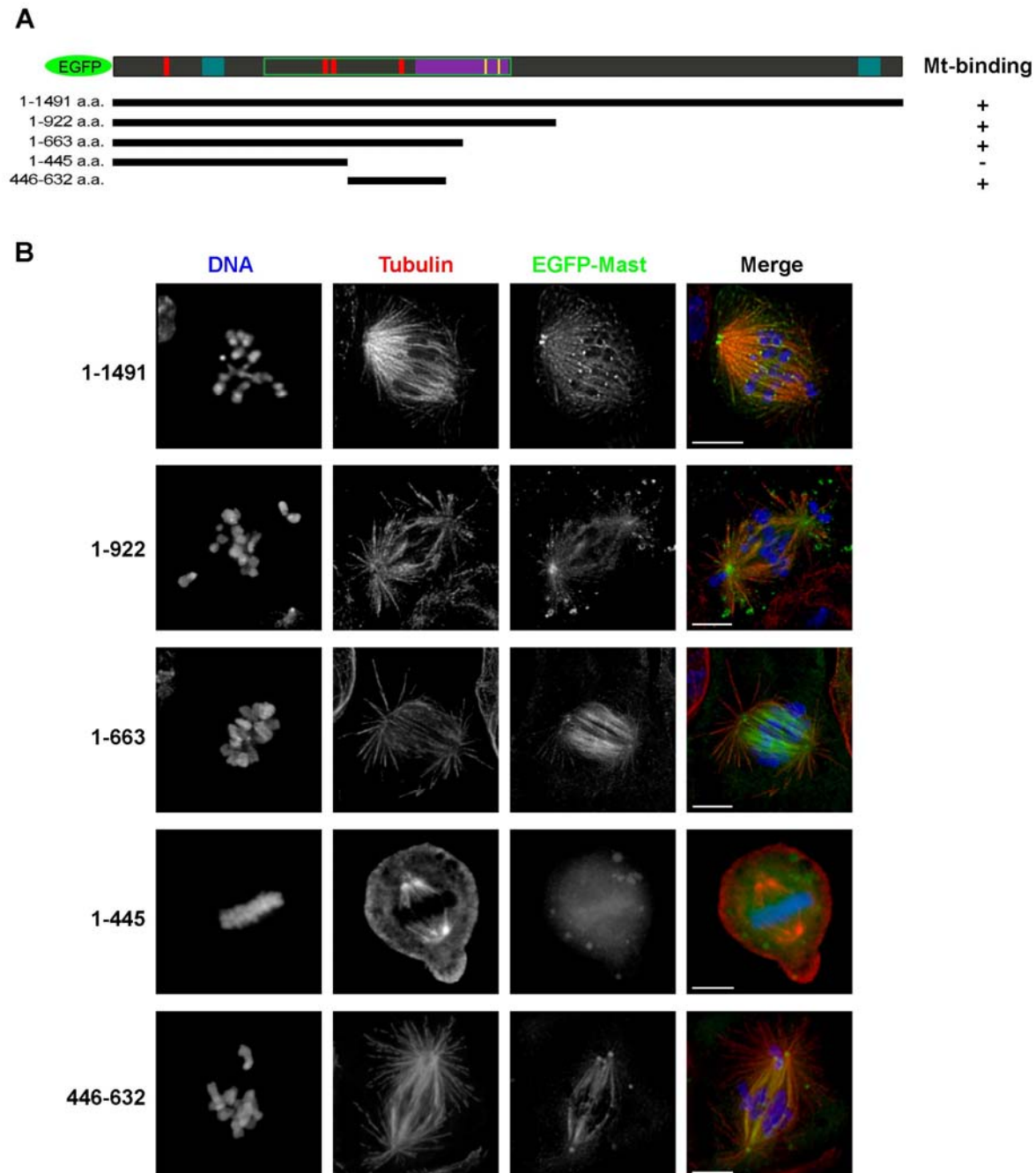


Figure 1.6. Identification of the microtubule-binding domain of Mast. (A) Schematic drawing of Mast protein and EGFP-fragments used to delimitate the microtubule-binding domain. (+) indicate the fragments that bind to microtubules. (B) S2 cells transfected with the different truncated proteins (green), immunostained with α -tubulin to visualize the microtubules (red) and incubated with DAPI to stain the DNA (blue). Scale bar: 5 μ m.

The results indicate that the microtubule-binding domain of Mast is contained within the fragment 446-632 a.a. However, during interphase and prophase this fragment does not bind to microtubules instead, it is sequestered into the nucleus and it co-localizes with microtubules only after NEBD until late telophase/cytokinesis, when it concentrates again into the nucleus (Fig. 1.7). Thus, to confirm that this region is required for Mast to bind microtubules during the entire cell cycle we analyzed the fragments Mast¹⁻⁶⁶³ and Mast¹⁻⁴⁴⁵ and found that while the larger fragment is able to bind microtubules, the smaller, which lacks the amino acids 446-632, is not (Fig. 1.8). Taken together our results define the microtubule-binding domain to a fragment of 187 amino acids delimited by the amino acids 446-632, located upstream of the putative MBD but within the central basic region.

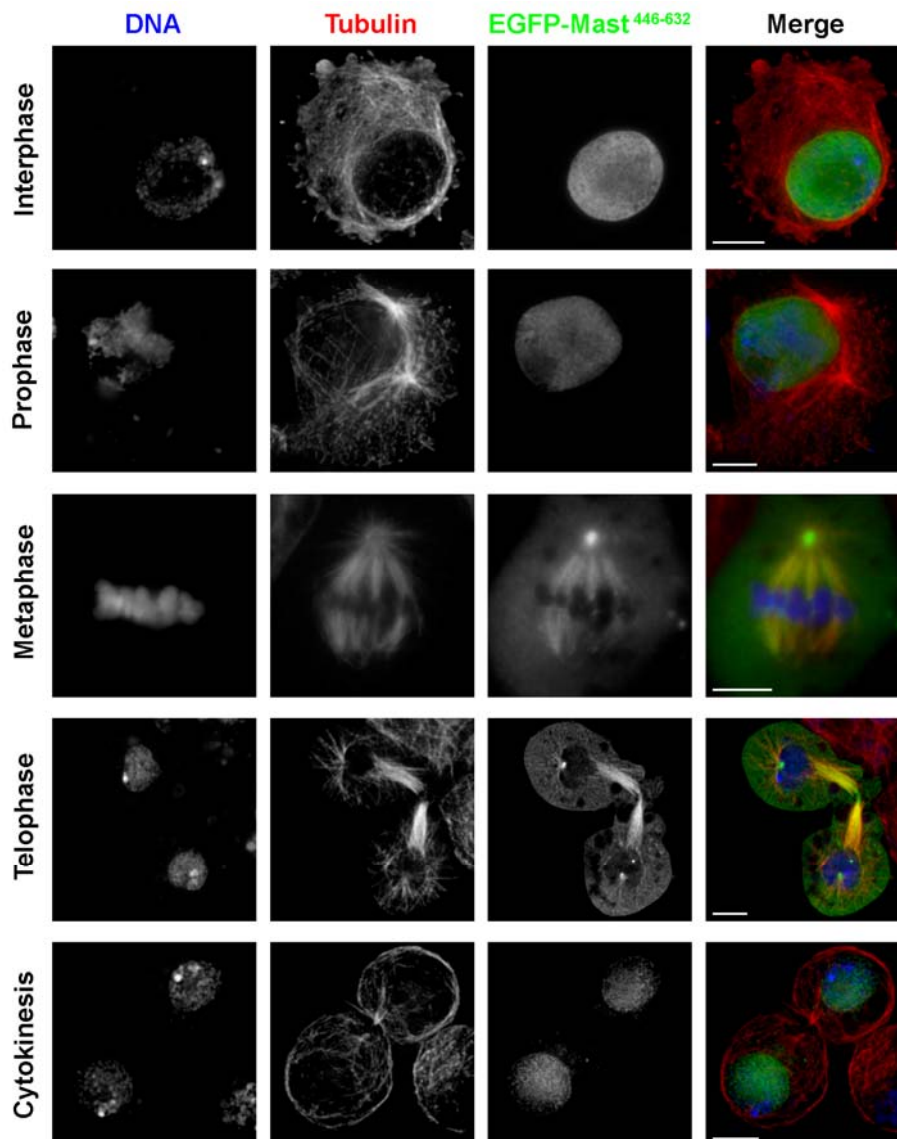


Figure 1.7. Cellular distribution of the microtubule-binding domain of Mast during cell cycle. S2 cells were transfected with the fragment Mast⁴⁴⁶⁻⁶³² (green), immunostained with α -tubulin to visualize the microtubules (red) and incubated with DAPI to stain the DNA (blue). Scale bar: 5 μ m.

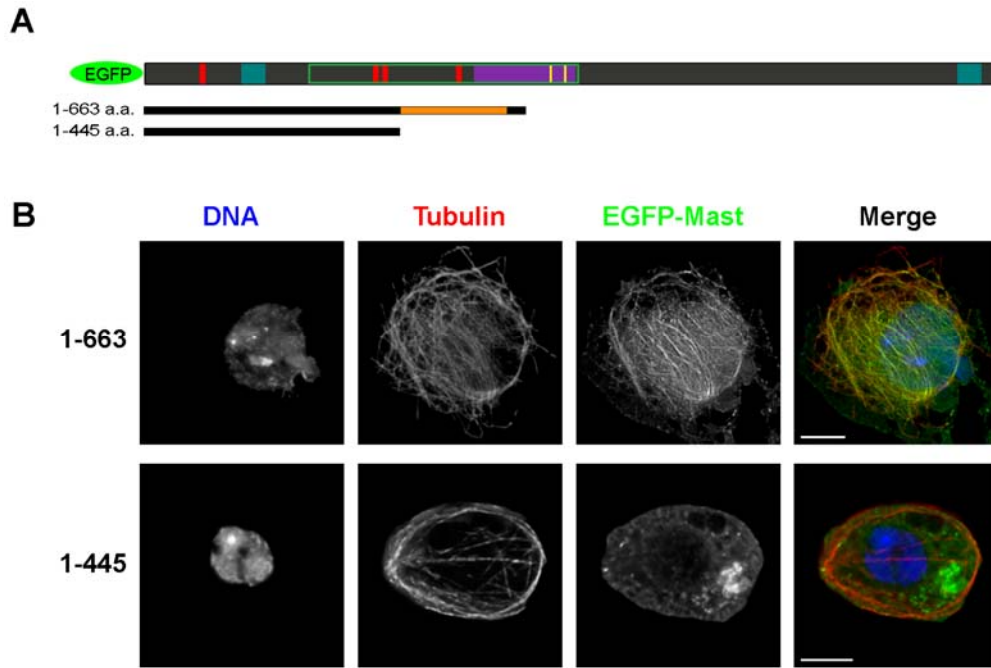


Figure 1.8. Mapping the microtubule-binding domain in interphase. (A) Scheme of Mast fragments used to determine the MBD at interphase. The orange bar represents the Mast⁴⁴⁶⁻⁶³². (B) S2 cells transfected with Mast¹⁻⁶⁶³ and Mast¹⁻⁴⁴⁵ (green), with microtubules in red and DNA in blue. Scale bar: 5 μ m.

As the microtubule-binding domain of Mast is within the central region of the protein, like in CLASP, and usually these basic-rich sequences are involved in microtubule binding, we wanted to know if this domain is conserved among species. Indeed, the analysis of sequence alignment of Mast⁴⁴⁶⁻⁶³² with the homologous regions of other species revealed that this domain is highly conserved across species, from humans to yeast (Fig. 1.9).

DM Mast	446-IYDTLNQSKSDIRSTLCELMVLLFEWQTKALERNAVLRDYLKKSIGDADCDARRHRYAYWAFRRHFFELADQIYGTLDIAAQALERER
Ag Mast	446-ILTQNLMLKSKDIRSTLEMLGLLFEWPTKLEKHNLLEALRKGMDADNDARRHRCAPWSPFRHFFPDLDNLVGLDITQALERER
Br CLASP	216-PLIANCTKSVAVRRRYEFLDLLLQEWQTHLERHAAVLVEIKKGIHDADAARVEARKATYGLRAHFFGEAEHLNLESSYQRLQCL
Hs CLASP1	447-PVITSNCTKSVAVRRRYEFLDLLLQEWQTHLERHIIVLAEIKKGIHDADAARVEARKCTYGFHFFGEAEHLNLESSYQKALQHL
MM CLASP1	221-PLITSNCTKSVVRRRYEFLDLLLQEWQTHLERHAAVLVEIKKGIHDADAARVEARKTYMGLRNHFFGEAEHLNLEPSYQKLQTYL
Hs CLASP2	221-PLITSNCTKSVVRRRYEFLDLLLQEWQTHLERHAAVLVEIKKGIHDADAARVEARKTYMGLRNHFFGEAEHLNLEPSYQKLQTYL
Rn CLASP2	221-PLITSNCTKSVVRRRYEFLDLLLQEWQTHLERHAAVLVEIKKGIHDADAARVEARKTYMGLRNHFFGEAEHLNLEPSYQKLQTYL
Os CLASP1	410-IADAKNDRAAILRARCSEYAILILEYADAFIQRASDIYEDLIKLVADAMSEVRATARSCTRMFTKTWPPERRLFMSPFAVORIINDED
Ce Cls-2	211-CIASTYTKDKNQRRQLCALLEIVLEHNEKIKRVLPQIGELIKAAICDADPETRVAGRKAFKLDALHSTEADKLFVDSKKQKMLRAD
NC Stu1	433-AQGDKNVQPRLYAEWLTMLKMAHHKQVEHGGGLDMEKVKKCLADANFGVREKMRATYWTFFGIWPNRANHIMNLDLTAQKLLQ-KD
Af Stu1	553-GACQDKIVQLRFAAGLKLKQKQHHKQSEHGGGLDMEKVKKCLADANFGVREKMRATYWTFFGIWPNRANHIMNLDLTAQKLLQ-KD
SC STU1p	455-RPCDAILLRFLIKFNDNLNNNTTSTPSKLENNIYIEELKKGISDQTTVREAMRLTFWTFYKCYPTNAKRLSSSPQPKKATELA

DM Mast	541-EGGGGGGCTGCTGAPETRRIVRIGRTFGLOKPTPMRISAVDTAAQRAKVRAQTYLYRQRKFLGPNNSNQAMTGAAAGSLPRFRL
Ag Mast	541-DNLGTNGTNSMVELRG-SNSSLNVS GGVI SMAGG-FRVSVDVTAQAQARARAYSTLARLKVTSGTALPRAKKTPSSAATSSSAN
Br CLASP	331-KSSGVASLPQDRSSSSSQEELNRF-LKNSAAAGRVFAGKSSG-----FASLQR-RSDVDVNAAGAKARH-SGAGGAGRVTTGLTF
Hs CLASP1	542-KNSDSIVSLPQDRSSSSSQEELNRF-LAKRPTGTTS-RASTVSTKVSSTGSLQR-RSDIDVNAAGAKARHAKKVSSSGSTFPSSAALPP
MM CLASP1	316-KSSGVASLPQDRSSSSSQEELNRFSSSKNSTANPSTVAGRVVGGSKANPLFGSLQR-RSDIDVNAAGAKARHAAQAVR-GRLGAGALN
Hs CLASP2	316-KSSGVASLPQDRSSSSSQEELNRFSSSKNSTANPSTVAGRVVGGSKASLFGSLQR-RSDIDVNAAGAKARHAAQAVR-GRLGAGALN
Rn CLASP2	316-KSSGVASLPQDRSSSSSQEELNRFSSSKNSTANPSTVAGRVVGGSKASLFGSLQR-RSDIDVNAAGAKARHAAQAVR-GRLGAGALN
Os CLASP1	505-GGLKRYFSPSLREKGVQLHASSHAGTHLAGYSTSAIVAMDKAAI SSESSLSS-RLLSQSKKIGRTAERIEISVLSKSKQKVATIESLL
Ce Cls-2	306-AASSSTINSEGTAFGRKLKLAGIGGIRNAPHISKFLAQRASAIIDTKQVFMATSVRPNIRPMTTTLKIDITSGGKFAFPVGA
NC Stu1	528-PHNPAHRTETGGARFGMGLSKVMGAKPSPVRDAIIAQKRMASSKMQPFRFGAMAHFSPVGTFRVVSSTSQASVAASAVPAFK
Af Stu1	648-PANPAHQAFKDGPRKGLANGTSSLAGRSALKEAIAAQKARLAPAKAVPPRPESQAQALDKHSSAPSSSKSVRVVPTGTSLSLSS
SC STU1p	550-IPAHLLINYQVRSSTASATSLYSHSSNNSSRKTSLEQKRNYPYAQPTQSSSTSLNAPAVTAGG-VIAKLSNKLKTLNRSTSEY

Figure 1.9. Sequence alignment of microtubule-binding domain. Sequences are from *Drosophila melanogaster* (DM), *Anopheles gambiae* (Ag), *Brachydanio rerio* (Br), *Homo sapiens* (Hs), *Mus musculus* (MM), *Rattus norvegicus* (Rn), *Oryza sativa* (Os), *C. elegans* (Ce), *Neurospora crassa* (NC), *Aspergillus fumigatus* (Af) and *S. cerevisiae* (SC). Aminoacids are shaded according to their polarity characteristics and chemical structure (adapted from Gouveia et al., in preparation).

Interestingly, the region of Mast shown to be sufficient to bind microtubules, Mast⁴⁴⁶⁻⁶³², does not include the sequence found to be responsible for microtubule plus-end binding in CLASP (Fig. 1.10), a small region placed downstream of Mast-microtubule binding domain, that is also able to interact with EB1 and EB3 (Mimori-Kiyosue et al., 2005). Particularly a small sequence of four aminoacids within this region, the SxIP motif, has been shown to control microtubule plus-end localization of diverse +TIPs, including CLASPs, in an EB1 dependent manner (Honnappa et al., 2009).

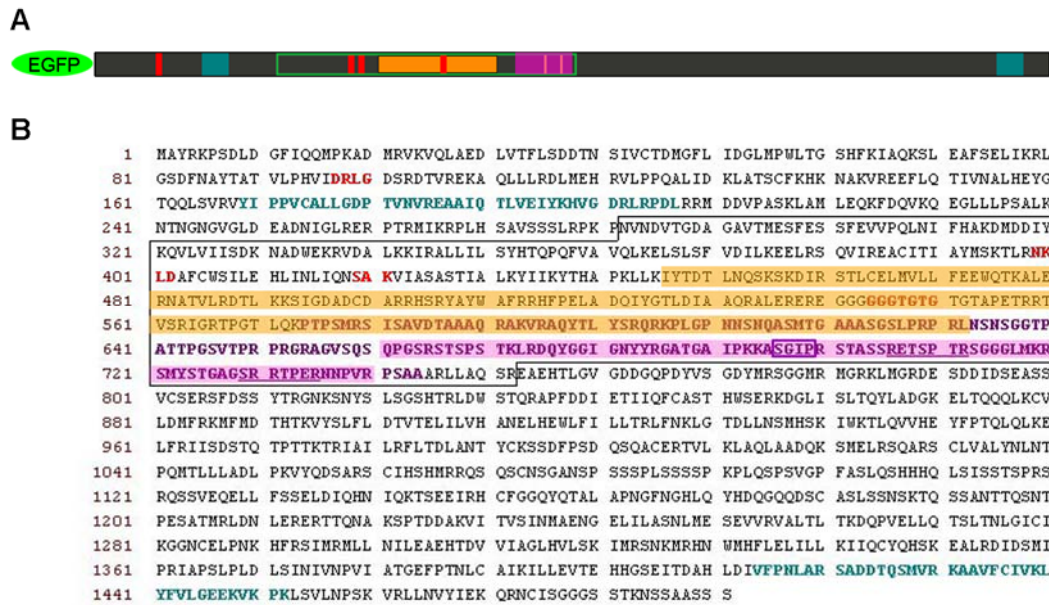


Figure 1.10. Localization of the newly defined MBD and the putative +TIP sequence. (A) Schematic representation of Mast and its sequence (B) showing the MBD (orange), the corresponding region of the microtubule plus-end binding found in CLASP (pink) that includes the SxIP motif (open violet box)

2.3 The role of GTP-binding sites of Mast in microtubule binding

One of the first studies with Mast/Orbit suggests that this MAP binds directly to microtubules in a GTP-dependent manner and identified two GTP-binding motifs, one related to the FtsZ and β -tubulin signatures, ⁵⁴⁴GGGTGTG⁵⁵⁰, and other found in the GTPase superfamily, ³⁹⁹NKLD⁴⁰² (Inoue et al., 2000). However, a more extensive protein sequence analysis revealed that Mast possesses two additional GTP-binding motifs, the ⁹⁷DRLG¹⁰⁰ and ⁴¹⁹SAK⁴²¹ specific for guanine binding (Vetter and Wittinghofer, 2001). Moreover, analysis of the protein sequence homology demonstrated that three of the four GTP-binding motifs are conserved among Mast homologues while ⁵⁴⁴GGGTGTG⁵⁵⁰, located at the MBD, is present only in *Drosophila melanogaster* (Fig. 1.11) (Gouveia et al., in preparation).

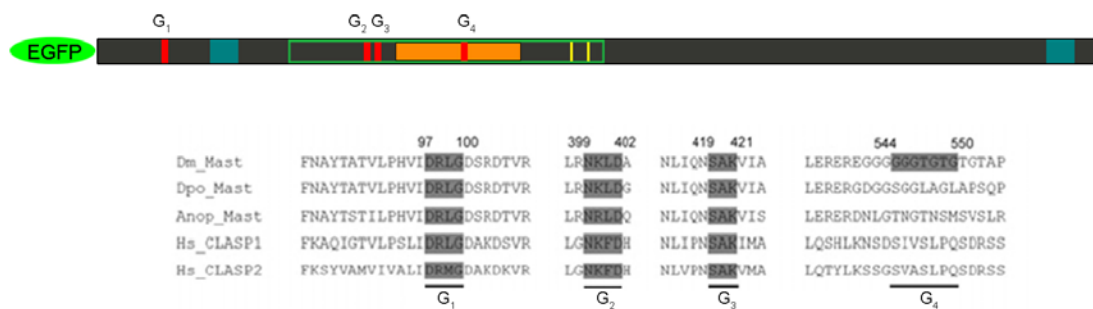


Figure 1.11. Sequence homology of GTP-binding motifs in CLASP family. Alignment of sequences from *Drosophila melanogaster* (Dm), *Drosophila pseudoobscura* (Dpo), *Anopheles gambiae* (Anop) and *Homo sapiens* (Hs). Sequences in grey boxes are the GTP-binding motifs also represented by red bars in the Mast protein scheme where the orange bar represents the newly defined MBD (adapted from Gouveia et al., in preparation).

In vitro studies indicated that besides binding to GTP, Mast is also able to hydrolyze this nucleotide (Gouveia et al., in preparation). In order to determine the involvement of the different GTP binding sites in Mast GTPase activity a number of point mutations in each motif (G₁, G₂, G₃ and G₄) were made and their ability to hydrolyze GTP was analyzed (Gouveia unpublished). The data revealed that while mutations in G₁ and G₄ have no significant effect, mutations in G₂ and G₃ result in a significant decrease of Mast GTPase activity (Gouveia unpublished). Given these results, we wanted to analyze the relationship between GTPase activity and the ability of Mast to bind microtubules. Therefore, S2 cells were transfected with EGFP-Mast, EGFP-Mast_{G2} and EGFP-Mast_{G3}, which have the GTP hydrolysis capacity compromised, and their subcellular distribution analyzed for co-localization with microtubules by immunofluorescence and their dynamic behavior during interphase studied using time-lapse microscopy (Fig.1.12).

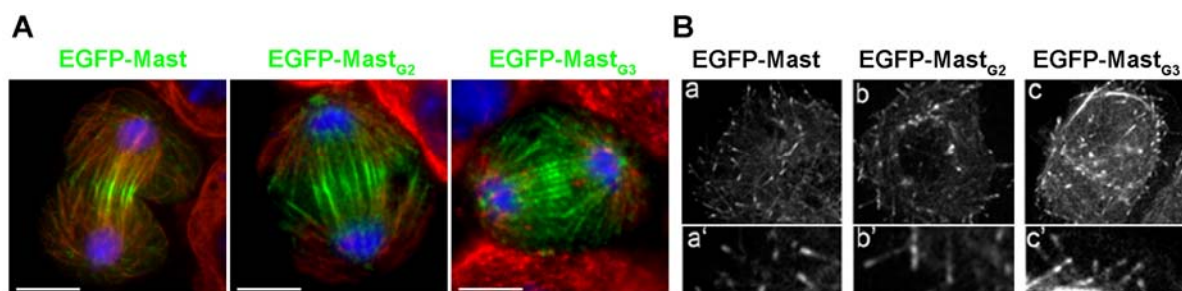


Figure 1.12. Mast-GTPase activity and microtubule association. (A) S2 cells transfected with EGFP-Mast, EGFP-Mast_{G2} and EGFP-Mast_{G3} (green), fixed and labeled for microtubules (red) and DNA (blue). Scale bar: 5 μm. (B) Still images from time-lapse movies of interphasic cells expressing either EGFP-Mast, EGFP-Mast_{G2} or EGFP-Mast_{G3} (adapted from Gouveia et al., in preparation).

Analysis of fixed cells shows that both the control and the mutated forms of Mast bind to microtubules in a very similar manner. Also, the decrease in GTP hydrolysis (Mast_{G2} and Mast_{G3}) does not seem to interfere with the ability of Mast-microtubule dissociation since the pattern of microtubule co-localization is identical to the control and there are no abnormal figures expected from an over-accumulation of Mast (Fig. 1.12A) (Maiato et al., 2003a; Sousa et al., 2007). In addition, live-cell analyses show that independently of the decreased in GTPase activity, EGFP-Mast_{G2} and EGFP-Mast_{G3} are able to display normal +TIP behavior, accumulating at the plus ends of growing microtubules in a comet-like fashion (Fig. 1.12B). Taken together these results indicate that there are no differences between Mast and Mast_{G2} or Mast_{G3} concerning their dynamic behavior and microtubule interaction.

2.4 Expression of Mast basic region causes a dominant-negative effect

Using *Drosophila* mutants Lemos et al. (2000) show that Mast is essential for the maintenance of spindle bipolarity, a result confirmed later by Maiato et al. (2002) using S2 cells, which demonstrated that in absence of Mast the spindle at first forms normally but then collapses during prometaphase, originating monopolar figures with chromosomes buried inside this structure (Lemos et al., 2000; Maiato et al., 2002). During our studies towards the identification of the microtubule-binding domain of Mast, several EGFP-Mast-fragments were expressed in S2 cells and we identified an internal region to contain the necessary motif for microtubule binding. Interestingly, when this basic domain of the protein, Mast²³⁴⁻⁸⁶⁶, was expressed it was shown to behave as a dominant negative (Fig.1.13). The expression of Mast²³⁴⁻⁸⁶⁶ induces the formation of monopolar spindles, with string accumulation of the Mast fragment at the plus-ends of microtubules, as well as all the chromosomes located close to the center, a phenotype very similar to one seen in *mast* mutants (Fig. 1.13). Similarly, HeLa cells expressing the microtubule binding region of CLASP also form monopolar spindles with the protein associated to the plus-ends of microtubules and the chromosomes located in the interior of the aster (Maiato et al., 2003a). In addition, similar to what was seen in Mast depleted cells and in antibody CLASP-injected cells, S2 cells expressing Mast²³⁴⁻⁸⁶⁶ when incubated with taxol at concentrations that induce microtubules to grow (Jordan and Kamath, 2007), exhibit chromosomes at the plus ends of microtubules, distal to the center of monopolar spindle, suggesting that kinetochore-microtubule attachment is unaffected (Fig. 1.14) (Maiato et al., 2003a; Maiato et al., 2002). Taken together, these results show that the basic region of Mast, fragment Mast²³⁴⁻⁸⁶⁶, has a dominant negative effect mimicking the absence of the protein.

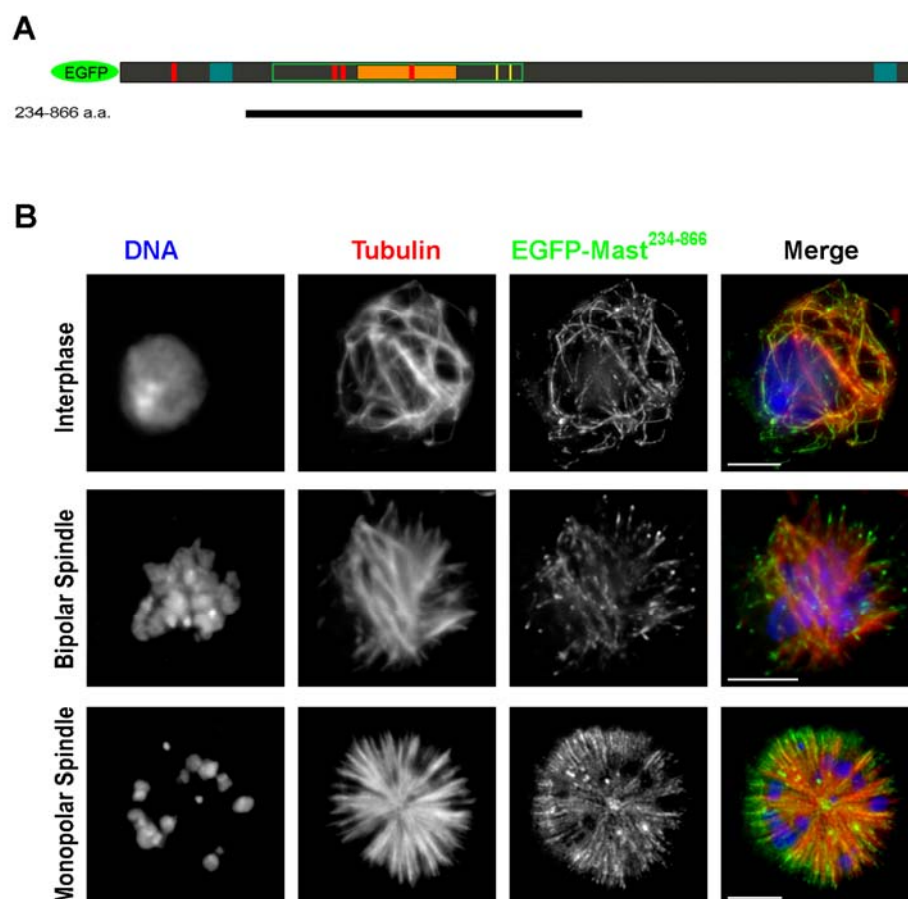


Figure 1.13. Expression of the basic region of Mast results in a Dominant negative phenotype
 (A) Scheme of Mast showing that Mast²³⁴⁻⁸⁶⁶ fragment comprises the basic region of the protein (green open box). (B) S2 cells transfected with Mast²³⁴⁻⁸⁶⁶ fragment (green), with microtubules in red and DNA in blue. Scale bar: 5 μ m.

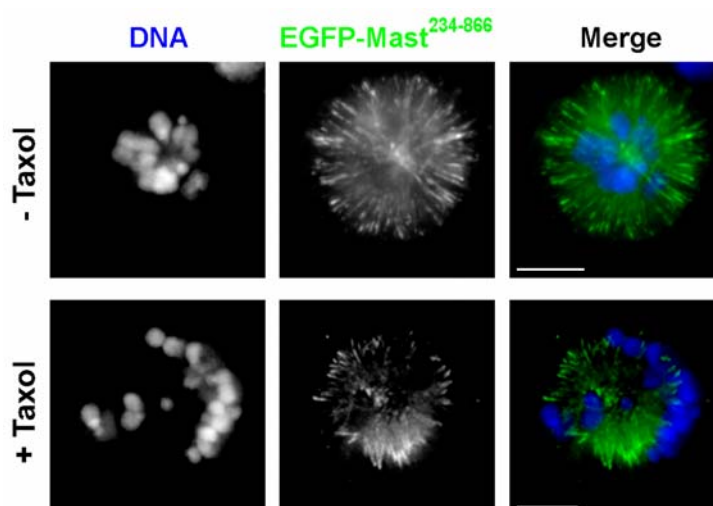


Figure 1.14. Taxol drives chromosome localization to the periphery of the monopolar spindle.
 S2 cells overexpressing the dominant negative fragment EGFP-Mast²³⁴⁻⁸⁶⁶ (green) after 30 minutes incubation with 10 μ M of Taxol. Scale bar: 5 μ m.

2.5 High levels of EGFP-Mast/fragments result in monopolar spindles

Mast/CLASP is a microtubule stabilizer that acts as a pause promoter protein (Mimori-Kiyosue et al., 2005; Sousa et al., 2007). Studies in cells during interphase demonstrate that when expressed at low levels EGFP-Mast accumulates at the plus-ends of growing microtubules but if expressed at high levels, the protein binds throughout the microtubule lattice abolishing the dynamic behaviour of the polymer (Sousa et al., 2007). Similar results were found by overexpressing EGFP-CLASP1 in HeLa cells (Maiato et al., 2003a). In order to evaluate the effect of high levels of the complete Mast protein or proteins fragments in mitosis, S2 cells were transfected with EGFP-Mast constructs and cells expressing high levels of protein were analyzed (Fig. 1.15).

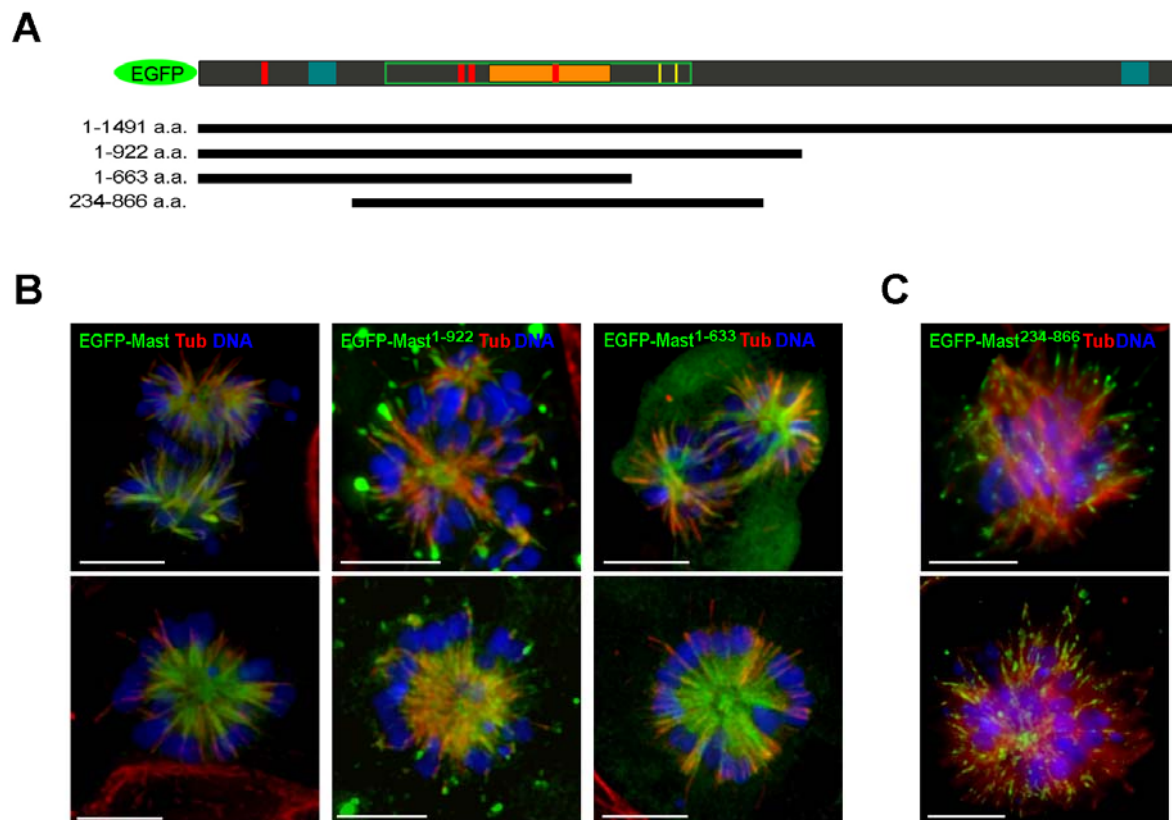


Figure 1.15. High levels of full length Mast or Mast fragments cause monopolar spindles. (A) Schematic representation of Mast protein and EGFP-fragments that can form monopolar spindles. (B) S2 cells transfected with EGFP-Mast or EGFP-fragments (green), and labeled for α -tubulin (red) and DNA (blue) showing an intermediate phenotype and the monopolar spindles caused by overexpression (C) S2 cell transfected with Mast²³⁴⁻⁸⁶⁶ exhibiting a monopolar spindle characteristic from dominant negative phenotype. Scale bar: 5 μ m.

Our results show that at high levels both, EGFP-Mast or fragments of the protein that contain the MBD, result in the formation of monopolar spindles, similar to what was

found in absence of Mast (Fig. 1.15B). However, when compared with the mono-asters characteristic of Mast deficiency (or dominant negative effect) the phenotype is not identical. While low levels of EGFP-Mast²³⁴⁻⁸⁶⁶ are sufficient to induce the dominant negative effect and it accumulates at the plus ends of microtubules, EGFP-Mast/fragments need to be expressed at very high levels to induce the phenotype and accumulate along the entire microtubule lattice. In addition, chromosomes in monopolar spindles induced by overexpression of EGFP-Mast/fragments localize to the external surface of the aster rather than being buried near the centrosomes, a behavior very similar to what was seen by addition of taxol to Mast depleted cells (Fig. 1.15B and C). These results show that at mitosis the effect of overexpression or depletion of Mast, although not identical does result in the formation of monopolar spindles.

2.6 Kinetochore-Binding Domain

Previous work with CLASP1 has determined that its kinetochore-targeting domain is localized at the C-terminal region of the protein (Maiato et al., 2003a). Therefore, we used several deletion constructs of the C-terminus of Mast fused with EGFP to determine its kinetochore-targeting domain (Fig. 1.16). S2 cells were transfected with the different fragments and incubated with colchicine to increase the population of mitotic cells. The results indicate that the kinetochore-targeting domain of Mast is located within the last 271 amino acids of the C-terminal region, Mast¹²²⁰⁻¹⁴⁹¹, a region that is highly conserved amongst several species (Fig. 1.17). In addition, C-terminal Mast-fragments are able to localize to kinetochores in absence of microtubules as was previously shown for the complete Mast protein (Lemos et al. 2000). Taken together these results show that, similar to the human CLASP1, Mast targeting to kinetochores is microtubule-independent and requires the conserved C-terminal region of the protein.

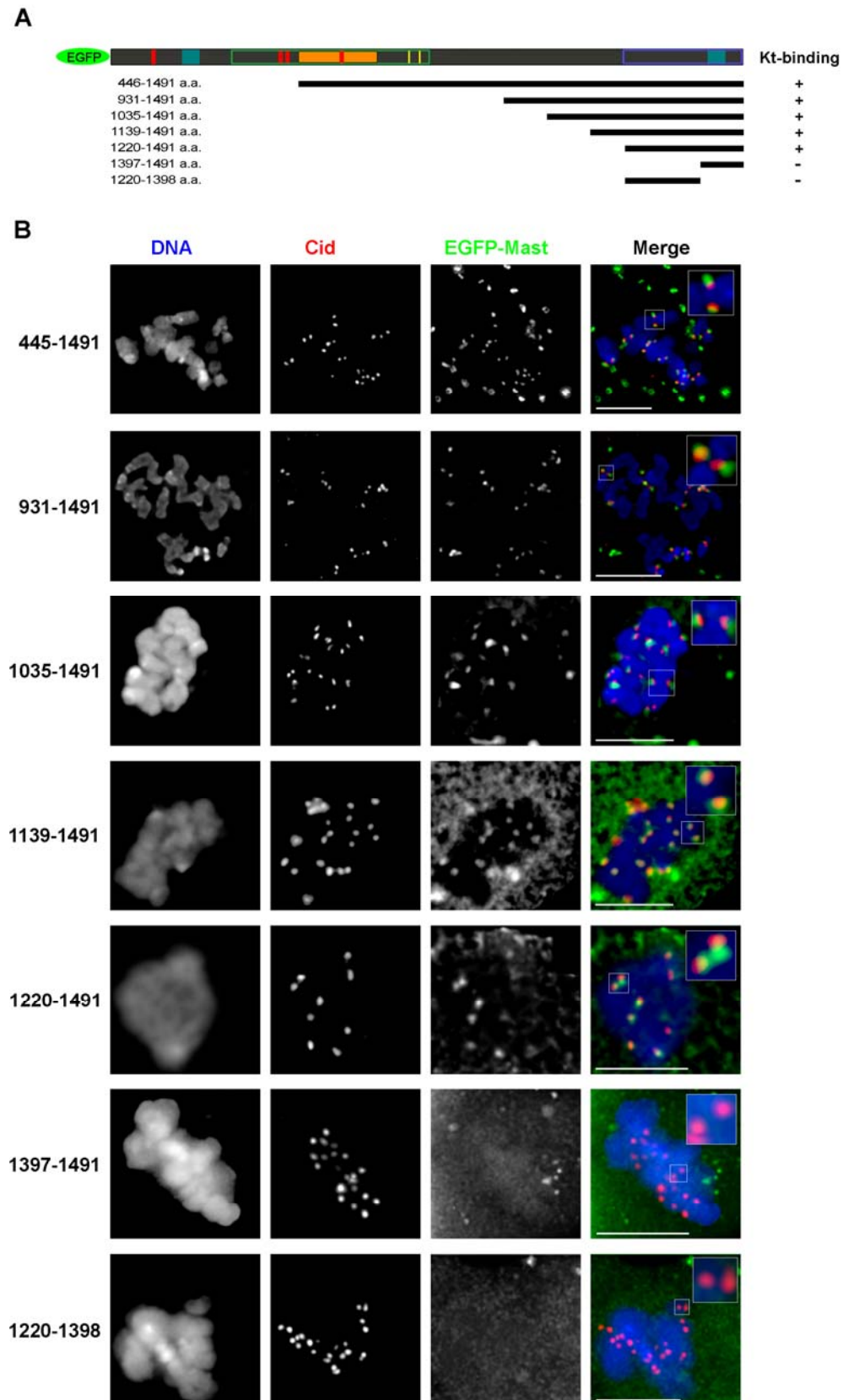


Figure 1.16. Identification of kinetochore-binding domain of Mast. (A) Illustration of Mast protein and EGFP-fragments used to delimitate the kinetochore-binding domain (open blue box), (+) indicate the fragments that bind to kinetochores. (B) S2 cells transfected with the different truncated proteins (green) and treated with 30 μ M of colchicine. Kinetochore where immunolabeled with anti-cid (red) and DNA visualized with DAPI (blue). Scale bar: 5 μ m.

Dm Mast	-----	ly-mlntp	-----	smllldpdkvyd	-----	dear	-----	schshmr	33
Hs CLASP1	laqmndptlf-vnseetrivsvrtwtwtepkssdvrrkaagvllsif-qlntp	-----	efmligalpktfa	-----	agat	-----	klhnhlr	78	
Hs CLASP2	laqmndptlf-insseetrivsvrtwtwtepkssdvrrkaagvllsif-qlntp	-----	efmligalpktfa	-----	agat	-----	klhnhlr	78	
S.Cerevisiae STU1	stepgepvdi-pneagpp	-----	step--delaksagsevhvte-ahied	-----	pfg--	-----	dalpkkgs	67	
S.Pombe PEG1	lnegkfengfllglsaaakvlnkdtldhvsqgnssvsssfkfttrcpfkisngeretgf	-----	lfsyfnalskddainvqktenvdesvqln	-----	amfndvsn	-----	79		
Arabidopsis CLASP	fnryagnpdi-sgnsilklwlaqltplrdknklkeasitciisvynhydsa	-----	glnylfslsveea	-----	hslr--	-----	ralkytvp	79	
Dm Mast	rqsgscnsgans-----	pssslpssesp	-----	kplqspsgp-faslgshhhlsissprsrqssvea	-----	e	-----	195	
Hs CLASP1	nsntsvgspen-----	tgrtpsrhtssrsltsptncshgglspsmldvdehlseaiysilrgvteadek	-----	f	-----	149			
Hs CLASP2	ntngvgssmgs-----	ptrtpsrpanvsspltsptnsqntlsasfaydtemussediysilrgvteadek	-----	f	-----	149			
S.Cerevisiae STU1	snntedgvpvshdesepmehcdsdndavdngnndtkcqqdqtsttp--mefshhldkldntsspseltalispdqykeldhvpaeifllcviif	-----	165						
S.Pombe PEG1	qdslnsvdqsag--kdkllltststpnkpttfmg-----	aneeflgsapkaydlhgsgysvhelhsenmrenngas	-----	168					
Arabidopsis CLASP	rievdllynmqss--kkeqgrksydpadaigtsssegayagaskkniflg--rvaggsisdsgrkwsssqeptmitgg	-----	v	-----	154				
Dm Mast	lssselqihhngqdtseei--rhcfaggyqtala-----	pngfnshlqyhdg	-----	g	-----	qgdscaelssns	-----	153	
Hs CLASP1	sarsgeclnepkkrdkke-cdivsrdggaasatategrggsevegrtalnd	-----	-----	-----	-----	-----	-----	216	
Hs CLASP2	sarsgeclnepkkrdkkddgdsncgpgmsdrra-----	ggdatdssatadn	-----	-----	-----	-----	-----	212	
S.Cerevisiae STU1	sehvmdogevsrdnla-----	lstssmvmvcardvypedsrwsqatveelkhvtvtclewltklvnesseastlatnkryrtslvhlstsv	-----	el	-----	259			
S.Pombe PEG1	inrardymtmmndfslf--seikgilesrve	-----	smptstisplda	-----	desvlhdipaye--sl	226			
Arabidopsis CLASP	gqnvssgtelkilyqvtrtg-----	issdillnkd--sdyfssagqnsr	-----	-----	-----	-----	-----	221	
Dm Mast	-----k-tssanc	-----	qentpsatmldnlerer--ttqnakspddakvltvsnnmngslilssnmlse--vrvr-altltkdqpv	-----	ellg	236			
Hs CLASP1	rard--vypypyd	-----	ontyaktllkeavfdddm-eql-rdvpdhsdlvadllkeshnherveerkqell	-----	ellk	287			
Hs CLASP2	rsrd--vypypyd	-----	sapfnksllkeamfddda-dqfpddisldhsdlvaellkeshnherveerkqaly	-----	ellk	284			
S.Cerevisiae STU1	hkqk--sgdvthnslivhsamklagppdkrtcr--afleapspdsllvedtdkshvtvkefktlrlppglasgqlfpgsevtwsklele	-----	355						
S.Pombe PEG1	nkseknkpegayst--plhhtlnvlpknkwlslrm-hkmengsnhvdknlddvaeeavkelndgsyntk	-----	tlkf	-----	cl	304			
Arabidopsis CLASP	ekng--liltsvd	-----	legrhneersldlghymtsikvntpspspllhmgngsgspssskksal-q	-----	cl	295			
Dm Mast	teitnlgticaggnccelpkhk	-----	rsarmulmhaehtdyvnael	-----	hvlekmsnkmrhnwmhflleil	-----	305		
Hs CLASP1	tedslgyweehi	-----	ktillliltgdkhsiralal	-----	rvlrelan--gparfknyaeit	-----	346		
Hs CLASP2	td-----	geesfsvdehi	-----	ktillliltgdkqptiralal	-----	kvirelsh--gparfknyaeit	-----	343	
S.Cerevisiae STU1	la-----	spscdsdeaildavsrdkvevtqlstlasripelledvltatlayhis	-----	hpalttaalvaikqvlikpelklsmghvteifst	-----	445			
S.Pombe PEG1	vc-----	ketpsmlysh	-----	glipailhynesnhsamhisdcl--ilheflvggygdvdmthhnic--il	-----	366			
Arabidopsis CLASP	se-----	vaneesvrtkyf	-----	nqiltvilevddedfsikelel	-----	stseelss--akdamedsveiv	-----	354	
Dm Mast	llqicqyqh-skealrdlqsmprhpsplpllsninyvpvlatgefpnlca	-----	skillevtelhgsaitdehldifnrlarsaddtqsmvrkaev	-----	400				
Hs CLASP1	mttleahkdkshkvrraeaeastlssshpqcikylcpigtadypinlaa	-----	kmqdkvverlakesllqllvdiipglggydntessvrkaev	-----	442				
Hs CLASP2	mttleahkdkshkvrraeaeastlssshpqcikylcpigtadypinlaa	-----	kmqdkvverlakesllqllvdiipglggydntessvrkaev	-----	439				
S.Cerevisiae STU1	lnhnhshlrspplaaaheladlhtdssemlefl--lrsrdsktqnk	-----	lilntvwhvtpallpsyetqlalalalissapplvrrty	-----	539				
S.Pombe PEG1	iekakkkk-epvlagiednllieciadlgllyeftgarlqaintetgksapll	-----	mliseimrikleletkdilrhvlykyishanpelrkattf	-----	465				
Arabidopsis CLASP	edlhvskdtkvpkqsteaenclttvlgqydpfrslsivplvdektlacc	-----	inciklvgrlsqeeimdglsflbavfaefggsadrvktv	-----	450				
Dm Mast	fcvkllyfvlgeekvkkklavnpakvlllnvyskgrncisggssstknssaaase	-----	457						
Hs CLASP1	fcvkllyfvlgeekvkkklavnpakvlllnvyskgrncisggssstknssaaase	-----	495						
Hs CLASP2	fcvkllyfvlgeekvkkklavnpakvlllnvyskgrncisggssstknssaaase	-----	492						
S.Cerevisiae STU1	glvrvvrlr-spe-megthelkikskmdlvryymga	-----	573						
S.Pombe PEG1	nvclavnth-vnn-vdetfslgglneggrllfmhyllkmsdekn	-----	508						
Arabidopsis CLASP	fcvkllyfvlgeekvkkklavnpakvlllnvyskgrncisggssstknssaaase	-----	499						

Figure 1.17. Comparison of Mast C-terminal amino acid sequence to those of CLASP in different species. Residues that are identical among the sequences have a black background, and those that are similar among the sequences have a grey background. The sequence correspond to Mast¹⁰³⁵⁻¹⁴⁹¹ fragment and the open boxes, rose and blue, represent Mast¹¹³⁹⁻¹⁴⁹¹ and Mast¹²²⁰⁻¹⁴⁹¹ (Kt-binding domain), respectively.

3. DISCUSSION

3.1 Mast binds to microtubules by a central basic domain

Mast binds to centrosomes, kinetochores and microtubules during various stages of mitosis. At anaphase, Mast accumulates to the central spindle and at telophase, to the midbody (Lemos et al., 2000). By analysis of S2 cells expressing low levels of EGFP-Mast, we observed that Mast also accumulates at the plus ends of microtubules and at the cell cortex, a behaviour also seen with the human homolog, CLASP (Akhmanova et al., 2001; Mimori-Kiyosue et al., 2005).

Previous work with CLASPs has determined that its microtubule-binding domain localizes at the central region of the protein. (Maiato et al., 2003a; Mimori-Kiyosue et al., 2005). Therefore, we used several deletion constructs of Mast fused to EGFP to

determine its microtubule-binding domain. The results delimitate the MBD to a 187 amino acid sequence placed at the amino acids 446-632 at the central basic region. Sequence alignment and analysis of this fragment revealed a high degree of conservation across species, suggesting that it might define the microtubule-binding motif of the CLASP family. Interestingly, this region does not contain the small repetitive sequence responsible for microtubule plus-end targeting found in human CLASP. Without this sequence, that is simultaneously the EB1-binding domain, the association of CLASP to the plus-ends depends on CLIPs (Mimori-Kiyosue et al., 2005). Recent studies found within this region, a conserved small sequence also present in Mast, which acts as a general microtubule plus tip localization signal, the SxIP motif. This motif targets different +TIPs, including CLASP2, to growing microtubule ends in an EB1-dependent manner. In addition, it was found that this interaction is controlled by phosphorylation of serine residues surrounding the SxIP motif (Honnappa et al., 2009). In agreement, another study indicate that the interaction of CLASP2 with EB1 and microtubule tips is directly inhibited by GSK3 β phosphorylation of several residues in the vicinity of the SxIP sequence (Kumar et al., 2009). Although, it was shown that without the EB1-binding domain, CLASP still bind to the plus-ends of microtubules but in a CLIP-dependent manner (Mimori-Kiyosue et al., 2005). Taken together these results suggest that the domains of microtubule-binding and plus-end targeting are independent and that the plus-end accumulation of Mast/CLASP relies on other +TIPs, namely EB1 or/and CLIP. In agreement, experiments with CLASPs demonstrate that the plus-end targeting depends on the presence of EB1-binding (central region) or CLIP-binding domain (C-terminal region), even when the corresponding Mast-MBD region is present (Mimori-Kiyosue et al., 2005), suggesting that, like many other +TIPs, one of the mechanisms for Mast/CLASP for plus-end accumulation is by 'Hitchhiking'.

3.2 Mast GTPase activity is not involved in microtubule binding or +TIP behavior

One of the first studies with Mast/Orbit suggests that this protein binds directly to microtubules in a GTP-dependent manner and identify two GTP-binding motifs, in which one is at the MBD (Inoue et al., 2000). Later results showed that Mast not only binds to GTP but also hydrolyze it (Gouveia, unpublished). An extensive protein sequence analysis revealed that indeed, Mast contains four GTP-binding motifs and surprisingly, the motif at MBD (G₄) is the only one that is not conserved among other species. Accordingly, this motif is unlikely to be involved in Mast GTPase activity, but mutations in the ³⁹⁹NKLD⁴⁰² (Mast_{G2}) and ⁴¹⁹SAK⁴²¹ (Mast_{G3}) GTP-binding sites were shown to result in a

decrease in GTP hydrolysis. Nevertheless, our transfection studies with these point mutants demonstrated that this decrease in GTP hydrolysis apparently does not affect the ability of Mast to bind microtubule or its dynamic +TIP microtubule binding suggesting that there is no relationship between GTPase activity and the ability of Mast to bind microtubules or display its +TIP behavior. The functional significance of the GTP-binding and hydrolysis ability of *Drosophila* Mast remain unresolved.

3.3 Mast is not required for kinetochore-microtubule attachment

Several studied in different organisms have shown that Mast/Xorbit/CLASP is essential for the maintenance of spindle bipolarity suggesting that it might be involved in kinetochore-microtubule attachment. In the absence of Mast, cells at prometaphase display problems in chromosome alignment and the spindle begins to reduce in length until eventually collapses, resulting in a monopolar figure with chromosomes buried near the center (Hannak and Heald, 2006; Lemos et al., 2000; Maiato et al., 2003a; Maiato et al., 2002). Accordingly to what was observed in HeLa cells, we found that the expression of the basic domain of Mast causes the same phenotype as seen in the absence of the protein. This result can be explained if the EGFP-Mast²³⁴⁻⁸⁶⁶ fragment was able to sequester the endogenous Mast protein preventing its normal localization, or by displacement/substitution of the endogenous protein from its functional location. Our results favor the second hypothesis since EGFP-Mast²³⁴⁻⁸⁶⁶ localizes to the plus-ends of microtubules just like the endogenous protein. Similarly, it was shown that the central domain of CLASP1, with dominant negative properties, was able to displace the endogenous Claps1 from kinetochores in HeLa cells (Maiato et al., 2003a). Taken together, these data indicate that the expression of the basic domain of Mast mimics the absence of the protein possibly by binding with higher affinity to the normal sites than the endogenous Mast protein.

Studies in S2 cells lacking Mast and HeLa cells injected with CLASP antibody reveal that when incubated with taxol, chromosomes of the monopolar spindles are associated to the plus ends of long microtubule bundles rather than buried at the center (Maiato et al., 2003a; Maiato et al., 2002). In agreement, our results show that after incubation with taxol, S2 cells overexpressing EGFP-Mast²³⁴⁻⁸⁶⁶ exhibit chromosomes at the periphery of the aster. These results indicate that in the absence of Mast, kinetochores are efficiently bound to microtubule bundles at the monopolar spindle, and when microtubule polymerization is induced by taxol, chromosomes are able to follow the growing microtubules until reach the periphery of the monopolar spindle, suggesting that Mast is

not required for an efficient Kt-Mt attachment but rather to polymerization, as it was demonstrated later by Maiato and colleagues (Maiato et al., 2005).

3.4 Both Mast depletion and overexpression result in monopolar spindles

The +TIP behaviour is a very fragile process that rely in a delicate balance between binding and release, essential for the selective plus-end accumulation of proteins and when overexpressed this balance is destroyed and the +TIP proteins bind along the entire tubulin polymer (Carvalho et al., 2003). Similar to what was seen for other +TIPs, when expressed at low levels, EGFP-Mast accumulates at the plus ends of interphasic microtubules but high levels result in a massive binding throughout the polymer, abolishing its dynamic behaviour (Sousa et al., 2007). However, the effect of overexpression of Mast in mitosis was unknown. Surprisingly, our results from overexpression assays with EGFP-Mast and EGFP-Mast fragments containing the MBD reveal that, the phenotype is very similar to the phenotype found in absence of the protein or overexpression of dominant negative that is, formation of monopolar spindles. The main difference is that chromosomes in monopolar spindles generated by overexpression are at the periphery of the aster rather than near the center as was observed by depletion, mutation or expression of the dominant negative.

It is already known that in absence of Mast the spindle collapses due to a lack of plus-end polymerization associated to a constant minus-end depolymerization, originating monopolar spindles with chromosomes near the center of the aster attached to the plus-ends of very short K-fibers (Maiato et al., 2005). However, the mechanism of monopolar spindles generation in cells overexpressing Mast/fragments remains unknown. In different organisms, Mast was shown to stabilize microtubules by promoting the pause state, being classified as the first pause promoter protein (Grallert et al., 2006; Mimori-Kiyosue et al., 2005; Sousa et al., 2007). Therefore, we reasoned that high levels of Mast might over-stabilize microtubules resulting in a total failure of bipolar spindle formation, with no centrosome separation but with an efficient kinetochore-microtubule attachment as demonstrated by chromosome localization to the plus-ends of microtubules at the periphery of the aster. Alternatively, the spindle might first form but subsequently collapses due to a highly abnormal spindle dynamics, while the overstabilized microtubules keep chromosomes attached to the tips decorating the periphery of the monopolar spindle. In agreement, S2 cells treated with the microtubule stabilizing drug Taxol, after incubation with MG132 (to arrest cells at metaphase) exhibit similar results, bipolar spindle collapses with chromosomes at the border of the mono-aster. (Maia et al.,

2007; Maresca and Salmon, 2009). Taken together these results are more consistent with the second hypothesis in which Mast, like Taxol, when overexpressed binds along the entire microtubule resulting in its stabilization and at the same time keeps the chromosomes at the plus ends at the periphery of the aster. However, the molecular mechanism responsible for the collapse of the spindle in these circumstances remains unknown.

3.5 Mast target kinetochores through its C-terminal domain, independently of microtubules

In addition to Microtubule-binding domain, Mast also possesses a Kinetochore-binding domain (KBD). Localization studies using several deletion constructs allow us to delimitate the KBD of Mast to the last C-terminal 271 amino acids, a region highly conserved amongst several species. We also demonstrate that this targeting to kinetochores is microtubule independent, like in CLASP1 (Maiato et al., 2003a). In human cells, it was shown that this COOH-terminal domain is also involved in association of CLASP with the cell cortex, Golgi apparatus and with CLIP-170 (Akhmanova et al., 2001; Mimori-Kiyosue et al., 2005).

CHAPTER 2

Mast dynamics in interphasic microtubules

1. INTRODUCTION

Almost all microtubule functions rely on the facility that these polymers have of rapidly assembly and disassembly, making them highly dynamic structures capable of assuming variable lengths with different stabilities. Microtubules are composed of $\alpha\beta$ -tubulin heterodimer subunits oriented in the same way creating a polar polymer with different polymerization rates at the two ends, in which the plus-end is the faster growing end and the minus-end the slower growing end (Desai and Mitchison, 1997). In fact, some studies demonstrate that *in vivo*, the polymerization of the minus-ends never occurs, being always in a stable or depolymerising state (Dammermann et al., 2003). Microtubules can experience three dynamic states, depolymerization, polymerization, and can be in a pause state when they are neither shrinking nor growing. The transitions between the growth and shrink phase are classified as "rescue" when microtubule switches from shortening to growth and "catastrophe" when the transition is from growth to shortening (Schulze and Kirschner, 1988; Tran et al., 1997b; Walker et al., 1988). Some proteins, known as MAPs, are known to be intimately involved in regulating the dynamic behavior of microtubules leading either to stabilization or destabilization.

A particular class of MAPs, the microtubule plus-end tracking proteins (+TIPs), have a prominent role in affecting the dynamic behavior of microtubules during both interphase and mitosis (Schuyler and Pellman, 2001). These proteins accumulate specifically at the plus-ends of microtubules and although the majority associate with only growing microtubule ends, some +TIPs also associate with plus-ends of depolymerising microtubules. Distinct +TIPs are known to physically interact with one another suggesting that they can form different molecular complexes at the microtubule plus-ends where besides affecting the dynamics they can also regulate microtubule interactions with different cellular structures (Akhmanova and Steinmetz, 2008). Some of the best known +TIPs are the End Binding protein family (EB1, EB2 and EB3), Adenomatous Polyposis Coli (APC), Cytoplasmic Linker Proteins (CLIPs), CLIP-Associating Proteins (CLASPs), Lis1, Cytoplasmic Dynein, among others.

In general, +TIPs have been shown to be required to promote the stability of microtubules, for instance, EB1 acts as an anti-pause promoting factor inducing microtubule dynamicity and growth (Rogers et al., 2002; Tirnauer et al., 2002), CLIP-170 enhances polymerization by stimulating rescues (Komarova et al., 2002), APC stabilizes microtubules by promoting growth and slowing shortening (Kita et al., 2006). However, there are also some +TIPs that stimulate microtubule destabilization, members of the depolymerising kinesin-13 family, MCAK in mammals and KLP10A and KLP59C in

Drosophila were shown to localize to microtubule plus-tips (Mennella et al., 2005; Moore et al., 2005). These depolymerising proteins affect the dynamic instability parameters in different ways, MCAK and KLP10A stimulate catastrophes, while KLP59C destabilizes microtubule plus-ends by suppressing rescue events (Mennella et al., 2005; Newton et al., 2004).

CLASP family members were also shown to exhibit +TIP behavior enriching the plus-ends of growing microtubules in a comet like fashion (Akhmanova et al., 2001; Maiato et al., 2003a; Mimori-Kiyosue et al., 2005; Sousa et al., 2007). Mast/CLASP was the first +TIP classified as a pause promoter factor since studies in HeLa and *Drosophila* S2 cells demonstrate that during interphase this MAP is required for microtubule stabilization, reducing the dynamic behavior of microtubules by promoting the time microtubules spend in pauses (Mimori-Kiyosue et al., 2005; Sousa et al., 2007).

Studies with *Drosophila* S2 cells show that during interphase Mast is present at plus-ends of growing microtubules and absent of depolymerising ends (Sousa et al., 2007). However, nothing is known about the rate that Mast moves or if it requires specific regions of the protein for this effect. To address all these issues, we transfected EGFP-Mast/fragments into S2 cells and follow *in vivo* their dynamic behavior by time-lapse microscopy. Our results show that Mast moves at a velocity very similar to microtubule polymerizing rate, moving usually in plus-end direction but also experiencing pause moments. In addition, we found that although a small central region is sufficient to promote microtubule plus-ends accumulation, Mast requires the full-length sequence to display normal +TIP behavior.

2. RESULTS

2.1 Interphasic dynamic behavior of Mast

In interphase mammalian cells, CLASPs have been shown to display +TIP behavior (Akhmanova et al., 2001; Maiato et al., 2003a). A plus-end tracking protein typically accumulates at the plus-ends of microtubules and when overexpressed decorates the entire polymer. To evaluate if Mast also displays +TIP behavior, first we analyze the localization of the endogenous protein to see if there was an enrichment at the plus-ends of microtubules. Then, as all +TIPs bind along the entire length of microtubule when overexpressed, we transfected S2 cells with EGFP-Mast and analyze the localization of the fusion protein in cells with high levels of expression (Fig. 2.1). The results show that while the endogenous Mast accumulates preferentially to the plus-ends of microtubules (Fig. 2.1A), when expressed at higher levels, Mast binds along the whole length of microtubules without any particular preference, as demonstrated by EGFP-Mast overexpression (Fig. 2.1B). In addition, we also observe that cells expressing high levels of Mast exhibit abnormal microtubule arrays, in which microtubules are in a reduced number and arranged in bundles, compared with the vast and intricate microtubule network seen in control cells.

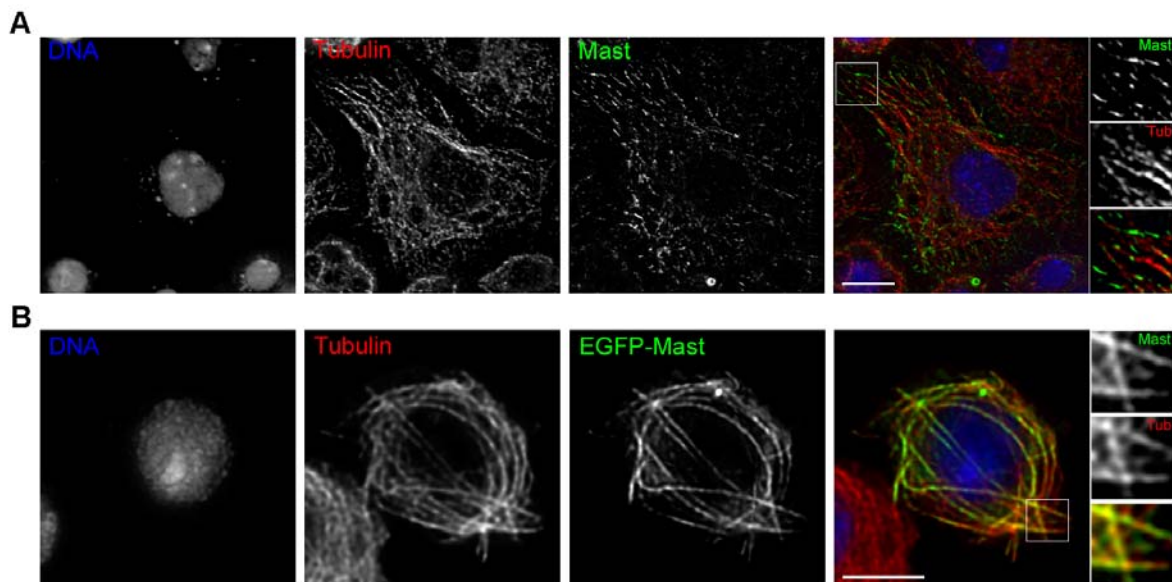


Figure 2.1. Cellular localization of endogenous Mast and overexpressed EGFP-Mast in interphasic S2 cells. (A) Immunofluorescence images labeled with anti-Mast (green), anti- α -tubulin (red) and stained with DAPI (blue) to visualize the DNA. (B) S2 cell overexpressing EGFP-Mast (green) and labeled for tubulin and DNA. Scale bar: 5 μ m.

The name "plus-end tracking proteins" or "+TIPs" emerged because these particular class of proteins localize to the microtubule plus-ends and in living cells, mark the growing tips giving the appearance of "tracking". By live-cell microscopy, +TIPs appear to be able to surf on polymerizing microtubule ends forming comets at all growing ends creating an effect similar to that of fireworks (Carvalho et al., 2003; Schuyler and Pellman, 2001). In order to determine if Mast also have these comet like behavior we analyze, by time-lapse microscopy, S2 cells transiently expressing EGFP-Mast (Fig. 2.2 and Movie 2.1). The results show that like other +TIPs, Mast have a comet-like behavior, accumulating at the plus ends of growing microtubules. In addition, we observed that this dynamic behavior fails to occur when the protein is present at high levels, when the excess of Mast binds throughout the microtubule lattice causing formation of bundles that seem overstabilized (Fig. 2.1B and Movie 2.2).

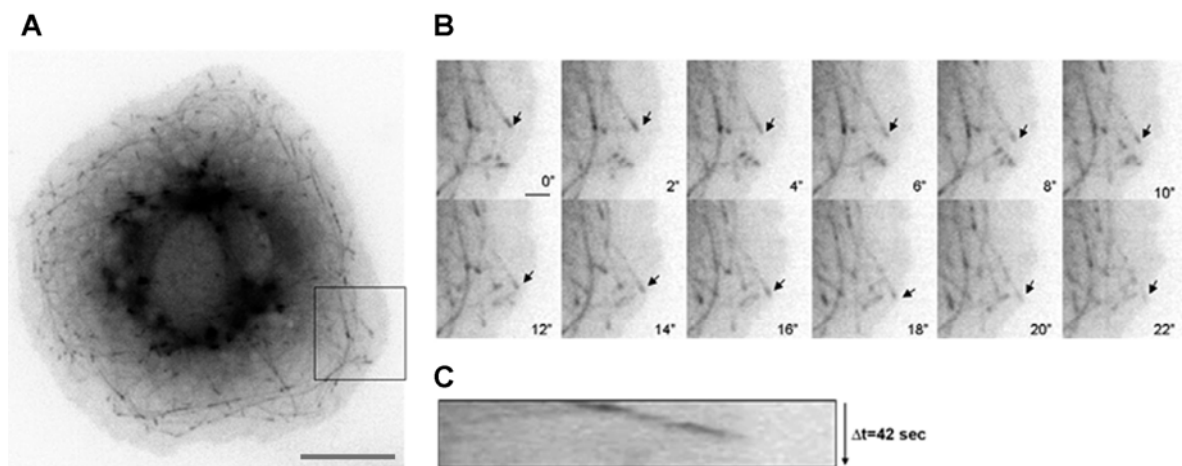


Figure 2.2. Dynamic behavior of Mast during interphase. (A) Still image from a time-lapse movie of a S2 cell transiently expressing EGFP-Mast. Scale bar: 5 µm (B) Sequential images of the insert in A highlighting the comet like signal (arrows) over time. Scale bar: 1 µm. (C) Kymograph from the time-lapse movie showing the EGFP-Mast enrichment at the growing plus-end (adapted from Sousa et al., 2007).

Taken together these results demonstrate that Mast is a classical +TIP, which accumulates preferentially at the plus-ends of growing microtubules and when overexpressed, binds along their entire length. At normal levels, Mast exhibits a dynamic comet like behavior that is lost when it is present at high levels, a state that induces the formation of microtubule bundles that seem overstabilized.

2.2 Mast moves with a velocity identical to the polymerizing rate of microtubules

After show that Mast is a +TIP we wanted to characterize its dynamic behavior in interphase. Therefore, we used S2 cells transiently expressing EGFP-Mast and quantified the velocity of EGFP-Mast movement by tracking the bright comets (Fig. 2.3). The results show that EGFP-Mast moves towards the cell cortex with irregular velocities in which the same comet could travel at velocities that vary from 0.07 to 0.42 $\mu\text{m}/\text{sec}$ (Fig. 2.3C and data not shown). In general, EGFP-Mast seems to move faster near the interior of the cell, slows down as goes to cell periphery, and then disappears (Fig. 2.3B).

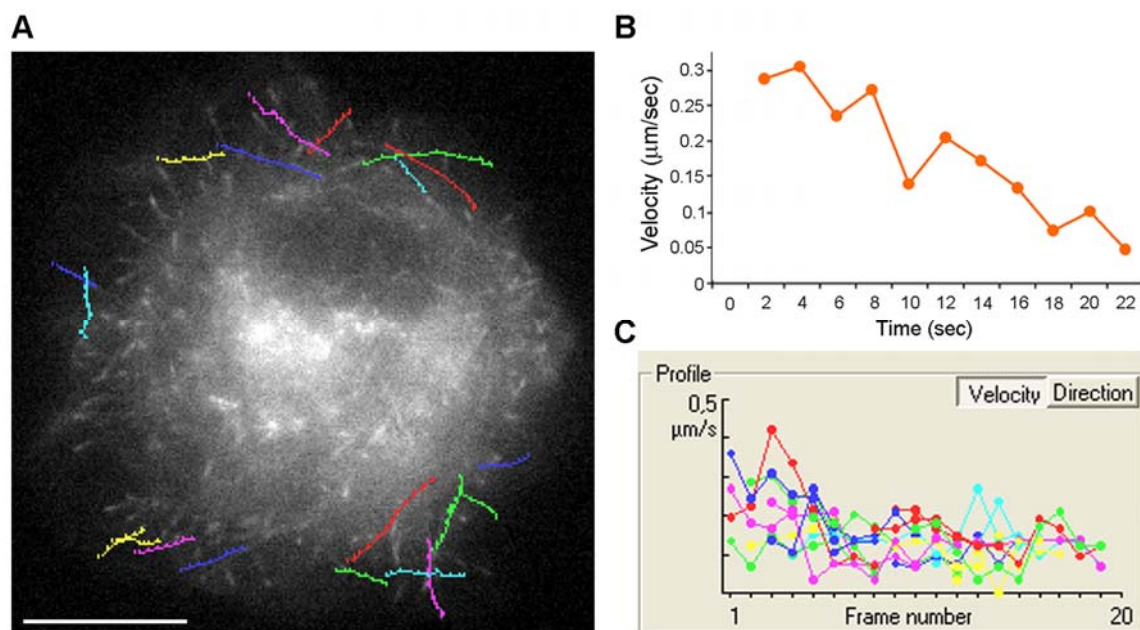


Figure 2.3. Tracking of EGFP-Mast comets. (A) Picture of an S2 cell expressing EGFP-Mast, showing the tracking comets analyzed with the software Retrac 2.10. (B) Quantification of velocities over time from an EGFP-Mast comet. (C) Chart from Retrac obtained by the tracking data from comets present in A.

By tracking comets from forty microtubules of two different cells with an appropriate software, *Retrac 2.10* (Fig. 2.3A and C), we determined that EGFP-Mast moves towards cell periphery at a velocity of $8.19 \pm 2.89 \mu\text{m}/\text{min}$ ($0.14 \pm 0.05 \mu\text{m}/\text{sec}$). The same rate was found for EGFP-CLASP1 ($\sim 0.14 \mu\text{m}/\text{sec}$) in mammalian cells (Maiato et al., 2003a). These values are quite similar to the microtubule polymerizing rate reported for S2 cells, $8.75 \pm 3.31 \mu\text{m}/\text{min}$ (Sousa et al., 2007). Therefore, these results suggest that the dynamic behavior of Mast is identical to the dynamic behavior of polymerizing microtubules.

2.3 Plus-end dynamics of Mast and Mast-fragments at interphase

In order to investigate the effect of different regions of Mast in plus-tip accumulation and dynamics, we transfected some EGFP-Mast-fragments in S2 cells and, as for the full length Mast, we analyze their behavior using time-lapse microscopy (Fig. 2.4 and Movies 2.3, 2.4 and 2.5).

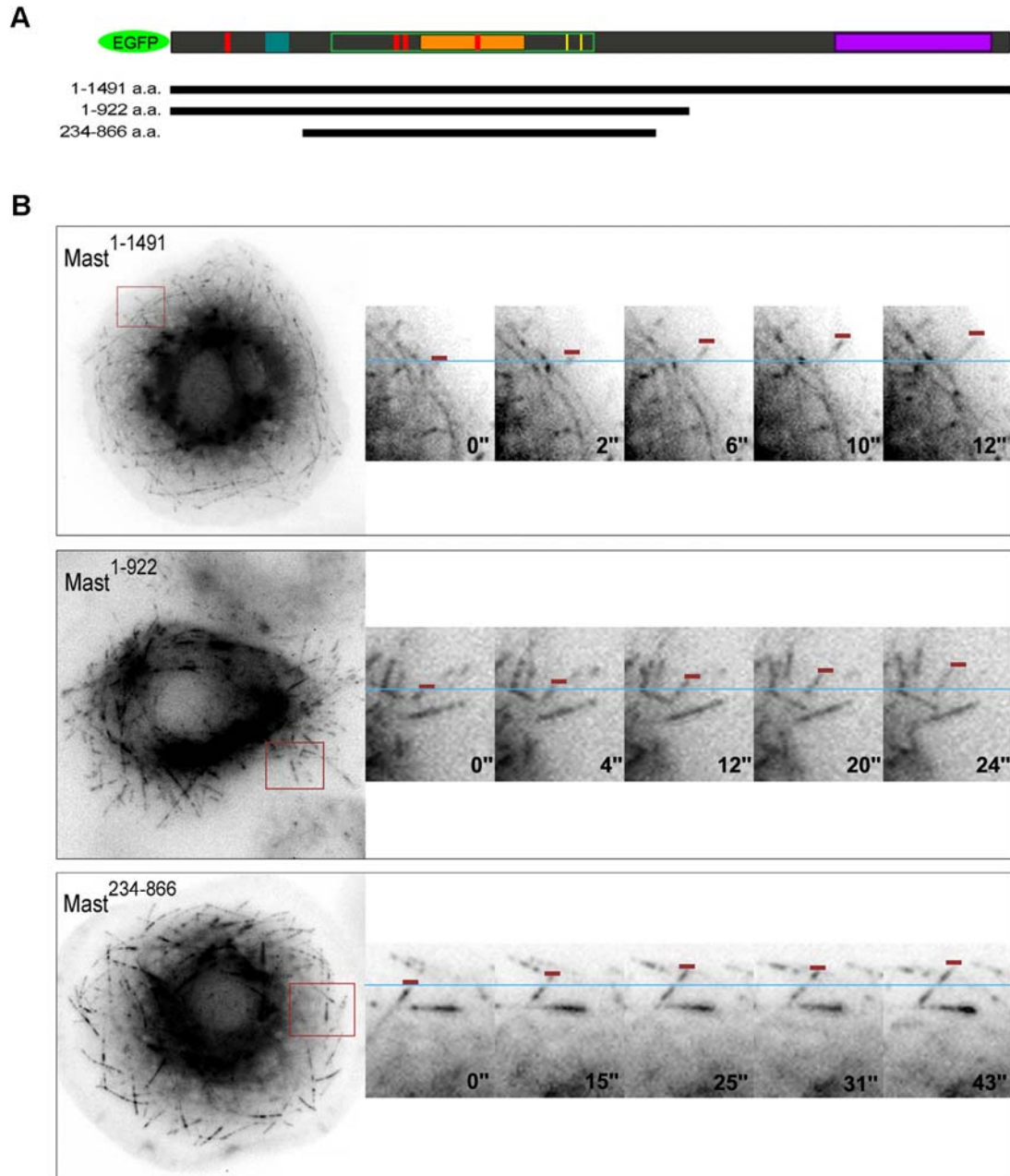


Figure 2.4. +TIP behavior of Mast and Mast-fragments (A) Scheme of Mast showing the determined Microtubule Binding Domain at orange, the Kinetochore Binding Domain at violet and the GTP-binding domains at red (G₁-G₄ from left to right). Black bars represent the EGFP-Mast/fragments transiently transfected in S2 cells. (B) Still images from time-lapse movies of interphasic cells expressing, EGFP-Mast, EGFP-Mast¹⁻⁹²² or EGFP-Mast²³⁴⁻⁸⁶⁶. Red squares represent the high-magnified areas, red bars mark the front of the growing comets and the blue lines correspond to position zero.

S2 cells expressing different EGFP-Mast fragments, reveal that like the full-length protein these truncated forms also exhibit a +TIP behavior, accumulating at the plus ends of growing microtubules. However, the velocity at which EGFP-Mast¹⁻⁹²² and EGFP-Mast²³⁴⁻⁸⁶⁶ move towards the cell periphery is much slower when compared to the full-length protein ($3.29 \pm 1.67 \mu\text{m/min}$, $1.99 \pm 1.35 \mu\text{m/min}$ and $8.19 \pm 2.89 \mu\text{m/min}$, respectively) (Fig. 2.4 and Fig. 2.5A).

In addition, by tracking comets, we also detect that both Mast and Mast-fragments, besides moving from the interior to the periphery of the cell, sometimes stay in the same position, in a pause state. While full length Mast was rarely observed to remain in pause ($1.62 \% \pm 1.36$), comets of Mast²³⁴⁻⁸⁶⁶ spend much more time in this pause state ($22.8 \% \pm 0.52$). Also, although not as significant as Mast²³⁴⁻⁸⁶⁶, Mast¹⁻⁹²² pauses more than the full-length protein ($14.25 \% \pm 5.56$) (Fig. 2.5B). Further work needs to be carried out in order to fully explore these preliminary observations.

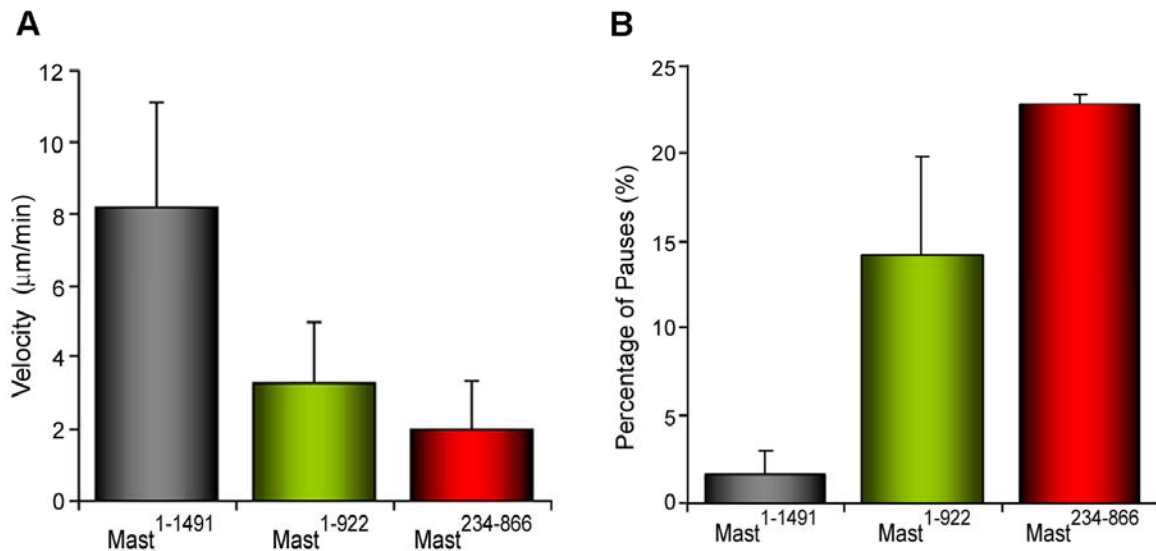


Figure 2.5. Dynamics of EGFP-Mast and EGFP-Mast-fragments at interphase. (A) Comet velocities of complete Mast, Mast¹⁻⁹²² and Mast²³⁴⁻⁸⁶⁶. These data was acquired using the *Retrac 2.10* software, it were analyzed 40 comets from 2 different cells for Mast, 40 comets from 3 different cells for Mast¹⁻⁹²² and 20 comets from 2 different cells for Mast²³⁴⁻⁸⁶⁶, in all cases $P < 0.001$. (B) Percentage of pauses from the total movements analyzed from two different cells for each condition, cells expressing EGFP-Mast, EGFP-Mast¹⁻⁹²² or EGFP-Mast²³⁴⁻⁸⁶⁶.

In mammalian cells, it is known that CLASPs need only a small central region to accumulate at the plus-ends of microtubule, the same region responsible for interaction with EB1 and EB3 (Mimori-Kiyosue et al., 2005). In order to investigate if the equivalent region of Mast as the same function, we transfected S2 cells with a fragment lacking that sequence, EGFP-Mast¹⁻⁶⁶³, and analyze its localization in interphase cells by time-lapse

microscopy (Fig. 2.6). The results show that, the fragment lacking the small central region is not able to accumulate at the plus tips of microtubules instead it binds along the entire length of the polymer (Fig. 2.6B). Therefore, like in CLASPs, this small central region is required for normal +TIP behavior of Mast.

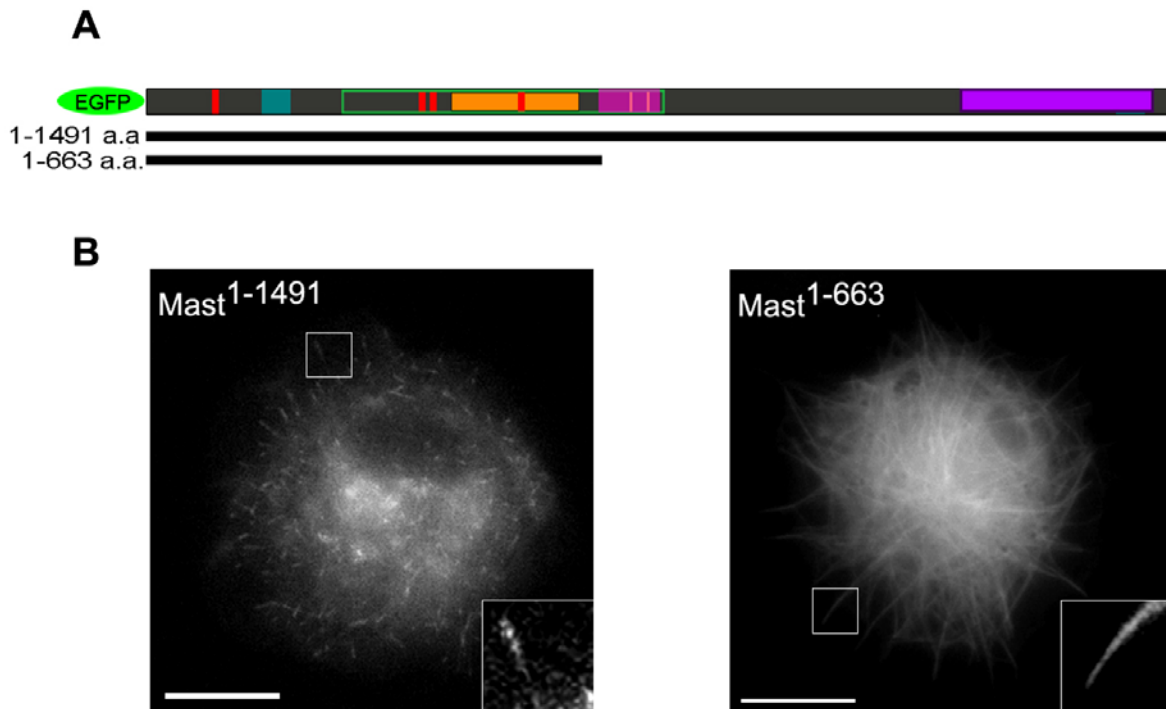


Figure 2.6. EGFP-Mast¹⁻⁶⁶³ binds along the entire microtubule length (A) Schematic representation of EGFP-Mast, showing the MBD (orange), KBD (violet) and the central fragment shown to be responsible for CLASPs plus-end accumulation (dark pink). (B) Still images from time-lapse movies of interphase S2 cells expressing either EGFP-Mast or the truncated protein EGFP-Mast¹⁻⁶⁶³. The inside box is a high magnification of the white square area. Scale bar: 5 μ m.

Taken together, our data show that the truncated proteins are less dynamics than the full length Mast. Particularly, the fragment EGFP-Mast²³⁴⁻⁸⁶⁶, which in mitosis induces the dominant negative effect, is four-fold slower than the complete protein. Furthermore, we demonstrate that although infrequently, Mast besides accumulating to the plus-ends of growing microtubules can also experience some pauses. This behavior increases significantly when the protein is truncated at C-terminal, Mast¹⁻⁹²², and reaches the maximum with the dominant negative fragment, Mast²³⁴⁻⁸⁶⁶, in which almost a quarter of the movements analyzed were pauses. Finally, our results also suggest that the small region located at 661-739 a.a. is responsible for the microtubule plus-end accumulation of Mast, a result also observed for CLASPs.

2.4 Dynamic behavior of Mast⁴⁴⁶⁻¹⁴⁹¹, a fragment that lacks N-terminus

After exploring the effect of the microtubule-binding domain, the dominant negative fragment and the putative sequence for +TIP behavior on Mast plus-end dynamics we sought to analyze the contribution of the kinetochore-binding domain (KBD). Therefore, we transfected S2 cells with a fragment containing the MBD, the +TIP sequence and the KBD, and follow its dynamic behavior in interphase cells by time-lapse microscopy (Fig. 2.7 and Movie 2.6). Surprisingly, the results show that this fragment, contrary to the full-length protein and other fragments that we analyzed, in addition to the microtubule plus-end directed movement towards the periphery of the cells, it also exhibits retrograde motion (Fig. 2.7B).

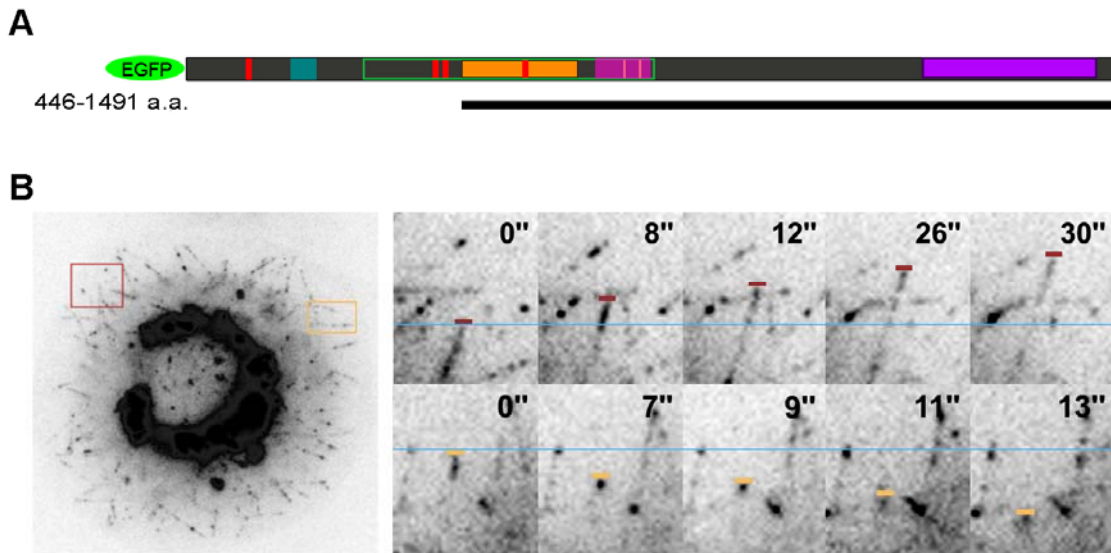


Figure 2.7. EGFP-Mast⁴⁴⁶⁻¹⁴⁹¹ exhibits retrograde movement (A) Schematic representation of EGFP-Mast, showing the MBD (orange), +tip sequence (dark pink), KBD (violet) and the GTP-binding domains at red (G₁-G₄ from left to right). Black bar is the transfected fragment. (B) Still images from a time-lapse movie of a cell expressing EGFP-Mast⁴⁴⁶⁻¹⁴⁹¹. The red square represents the high-magnified area of a periphery directed movement, with red bars highlighting the front of the comets; the orange square represent a high-magnified area of a retrograde movement with orange bars highlighting the front of the retrograde comets; the blue lines correspond to position at time zero.

As for the full-length protein and other Mast-fragments, we calculate the velocities and the percentage of pauses of both, plus-end directed (+) and retrograde (-) Mast⁴⁴⁶⁻¹⁴⁹¹ comets, by tracking them with *Retrac 2.10* software. The results show that, EGFP-Mast⁴⁴⁶⁻¹⁴⁹¹ moves forward and backward at the same velocity ($4.65 \pm 1.85 \mu\text{m}/\text{min}$ and $5.08 \pm 1.66 \mu\text{m}/\text{min}$; $P > 0.1$; respectively) but at a slower rate than the full length Mast ($8.19 \pm 2.89 \mu\text{m}/\text{min}$, $P < 0.001$) (Fig. 2.8A). However, Mast⁴⁴⁶⁻¹⁴⁹¹⁽⁺⁾ and Mast⁴⁴⁶⁻¹⁴⁹¹⁽⁻⁾ are

significantly faster than the fragments lacking the KBD, Mast¹⁻⁹²² and Mast²³⁴⁻⁸⁶⁶ ($3.29 \pm 1.67 \mu\text{m}/\text{min}$ and $1.99 \pm 1.35 \mu\text{m}/\text{min}$; $P < 0.001$; respectively).

In relation to pauses, the data obtained for the forward comets, Mast⁴⁴⁶⁻¹⁴⁹¹⁽⁺⁾, was similar to the full length Mast but interestingly, the retrograde comets, Mast⁴⁴⁶⁻¹⁴⁹¹⁽⁻⁾ were never seen in pause state (Fig. 2.8B). However, as for all the other Mast fragments analyzed the functional significance of these results remains to be determined.

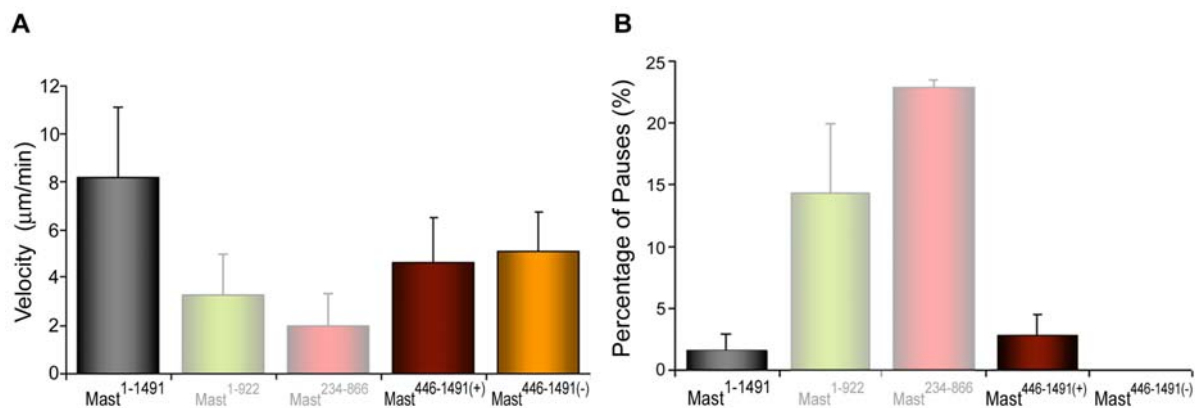


Figure 2.8. Dynamics of Mast⁴⁴⁶⁻¹⁴⁹¹⁽⁺⁾ and Mast⁴⁴⁶⁻¹⁴⁹¹⁽⁻⁾ at interphase. (A) Comet velocities of complete Mast, Mast¹⁻⁹²², Mast²³⁴⁻⁸⁶⁶ and Mast^{446-1491(+)/(-)}. These data is from 50 comets from 2 different cells for Mast⁴⁴⁶⁻¹⁴⁹¹⁽⁺⁾ and from 30 comets from 2 different cells for Mast⁴⁴⁶⁻¹⁴⁹¹⁽⁻⁾. (B) Percentage of pauses from the total movements analyzed from two different cells for each condition.

Overall, these data indicate that the N-terminal truncated Mast containing the MBD, +TIP motif and the KBD, is able to move in both directions, forward, in direction of growing microtubules, with some pauses and backwards, in direction of shrinking microtubules, without pausing. In addition, although the kinetochore-binding domain contributes to an increase on Mast velocity, it is not sufficient to reach the same rate as the full-length Mast, suggesting an involvement of N-terminal region both, in increasing Mast velocity and in forcing Mast to move only in plus-end microtubule direction.

3. DISCUSSION

3.1 Mast is a classic plus-end tracking protein (+TIP)

Typically, +TIPs are seen at growing microtubule plus-ends, a localization that by time-lapse microscopy gives the illusion that the protein is surfing at the plus-ends of microtubules creating an overall cell image of fireworks. This pattern relies on a delicate balance between rapid binding and release of the proteins and is lost when the protein is present at high levels, so that the +TIP binds along the entire length of microtubules (Carvalho et al., 2003). Live-cell microscopy studies with S2 cells expressing EGFP-Mast allow us to demonstrate that, like CLASPs in mammalian cells, Mast display a classic +TIP behavior accumulating preferentially at the plus-ends of microtubules in a comet-like fashion, and when overexpressed binds unselectively along the length of microtubules, inducing the formation of overstabilized bundles (Akhmanova et al., 2001; Maiato et al., 2003a; Sousa et al., 2007).

3.2 Mast dynamics follows growing microtubule ends

By tracking EGFP-Mast in interphase S2 cells, we found that the velocity of Mast is not constant in fact, the range of velocity is very large and individual velocities can vary between 4.2 and 25.2 $\mu\text{m}/\text{min}$. Indeed, it is not the first time that this behavior is described for a +TIP, the same irregularity was also found for CLIP170 (Perez et al., 1999). However, although the velocities of the comets are not constant they seem to follow a pattern. At the cell interior EGFP-Mast moves at a certain rate, slows down as it reaches the periphery and then near the cortex disappears. This behavior is very similar to that found for EB1, which was also visualized using a GFP tag, in live *Xenopus* A6 epithelial cells (Mimori-Kiyosue et al., 2000). In addition, by direct microtubule observation it was found that the rate of microtubule growth was higher at the cell interior than at the cell edge (Mimori-Kiyosue et al., 2005).

The average velocity calculated for EGFP-Mast in interphase was 8.19 ± 2.89 $\mu\text{m}/\text{min}$, the same value found for EGFP-CLASP1 in mammalian cells and very similar to the microtubule polymerizing rate of interphase S2 cells (Maiato et al., 2003a; Sousa et al., 2007). Taken together these results suggest that Mast, similarly to other +TIPs, has dynamic properties virtually identical to the dynamic properties of polymerizing microtubules.

3.3 Mast needs the full-length sequence for normal +TIP behavior

After studying the +TIP behavior of the complete Mast protein, we wanted to analyze the behavior of some truncated proteins in interphase cells, namely Mast²³⁴⁻⁸⁶⁶, that in mitosis causes a dominant negative phenotype. Therefore, we transfected S2 cells with full-length EGFP-Mast as control, EGFP-Mast²³⁴⁻⁸⁶⁶ and EGFP-Mast¹⁻⁹²², and analyzed them by time-lapse microscopy. All fragments were able to accumulate at microtubule plus-ends, however, careful quantification of their +TIP behavior revealed that the plus-end dynamics was very different from that of control Mast protein. The full-length is much more dynamic than the truncated proteins, Mast comets move twice faster than Mast¹⁻⁹²² and four times faster than the dominant negative fragment Mast²³⁴⁻⁸⁶⁶.

Interestingly, our tracking data also revealed that although comets of the complete Mast protein were usually moving in direction of growing microtubules, there are moments that they were in a pause state. In fact, this behavior was already seen for other +TIPs like mammalian EB1, APC or Bik1 (the CLIP-170 homolog in budding yeast) (Carvalho et al., 2004; Green et al., 2005; Kita et al., 2006). We observe that although Mast has few pausing episodes, almost a quarter of the movements analyzed for Mast²³⁴⁻⁸⁶⁶, which induces dominant negative phenotype in mitosis, were pauses.

Taken together, our results show that although Mast does not need the full-length sequence to accumulate to microtubule plus-ends that is not sufficient to display its normal dynamic behavior. The truncated forms generate less dynamic comets, with slow velocities and more pauses. Given that, we propose that as Mast is in close association with microtubule plus-ends, the increased number of paused EGFP-Mast²³⁴⁻⁸⁶⁶ comets and the slow velocity can reflect changes in the underlying microtubule dynamics.

3.4 Mast requires a specific sequence for microtubule plus-end accumulation

Previous studies, in mammalian cells, have reported a small sequence as being responsible for CLASP plus-end binding and simultaneously for the interaction with the end binding proteins EB1 and EB3. The same studies demonstrated that without this EB-binding domain, the association of CLASP to the microtubule plus-ends depends on CLIPs through the CLIP-binding domain located at C-terminal (Mimori-Kiyosue et al., 2005). Accordingly, our results show that EGFP-Mast¹⁻⁹²², which lacks the C-terminal region, can still target the microtubule plus-ends but EGFP-Mast¹⁻⁶⁶³, that lacks the C-terminal and the putative EB-binding sequence, fails to accumulate at the plus-ends, binding along the entire microtubule. These results show that the putative EB-binding domain is essential for Mast plus-end targeting at least when C-terminal is not present.

However, in order to evaluate if, as for CLASP, Mast lacking the EB-binding domain still associate with microtubule ends through the C-terminal region (CLIP-binding domain in humans), we still need to analyze S2 cells expressing EGFP-Mast^{ΔEB}, lacking the small central region. In addition, it would be interesting to analyze Mast plus-end targeting in cells depleted for EB1 since in *Drosophila* S2 cells, it was reported that the target of CLIP-190 to microtubule plus-ends is via EB1 (Dzhindzhev et al., 2005).

Overall, these results show that similarly to CLASP, Mast requires a small central region (EB-binding domain) for microtubule plus-end accumulation in absence of C-terminal region, suggesting that Mast targets to microtubule plus-ends by 'Hitchhiking' through EB1 or if C-terminal is sufficient to plus-end accumulation, through CLIP-190.

3.5 Kinetochore-binding domain contributes to Mast +TIP dynamics

In HeLa cells, it was described that CLASPs bind to CLIPs through its C-terminal region, the same region known to be required for kinetochore targeting (Akhmanova et al., 2001; Maiato et al., 2003a). In addition, it was reported that in the absence of the EB-binding domain (+TIP sequence), CLASPs depend on CLIPs to localize to microtubule plus-ends (Mimori-Kiyosue et al., 2005). Therefore, we decide to evaluate the role of the C-terminal region of Mast, defined as the kinetochore-binding domain, upon the microtubule plus-end dynamics behavior of the protein. By analyzes of S2 cells expressing EGFP-Mast⁴⁴⁶⁻¹⁴⁹¹, which contains the KBD, the MBD and the +TIP sequence, we show that the C-terminal region in combination with the other functional domains increases Mast velocity, however, it is not sufficient to reach the normal rate, suggesting that the regulation of Mast +TIP velocity depends on several factors and it could only be achieved with the full protein sequence.

Surprisingly, the tracking analyzes of EGFP-Mast⁴⁴⁶⁻¹⁴⁹¹ reveal that, contrary to the full-length and the other Mast-fragments analyzed, the comets observed after transfection with these truncated proteins are able to move in both directions, forward (EGFP-Mast⁴⁴⁶⁻¹⁴⁹¹⁽⁺⁾) and backward (EGFP-Mast⁴⁴⁶⁻¹⁴⁹¹⁽⁻⁾). Interestingly, although the velocity of the opposite movements is similar, the frequency of pauses is very different, as EGFP-Mast⁴⁴⁶⁻¹⁴⁹¹⁽⁺⁾ is comparable to the full-length, while EGFP-Mast⁴⁴⁶⁻¹⁴⁹¹⁽⁻⁾ seems to never pause. These results suggest different mechanisms of motion depending on the direction.

Traditionally, +TIPs are thought to associate only with growing microtubule plus-ends, however the number of +TIPs that can bind also to pausing or shrinking microtubules is increasing, indicating that contrary to what was thought, +TIPs exhibit heterogeneous behavior concerning the microtubule plus end dynamics. In budding yeast this behavior is relatively common, some +TIPs such as Stu2 (XMAP215 homolog) can

bind to the tips of stable microtubules or Bik1 (CLIP-170 homolog) and dynein that track growing but also shrinking microtubules (Akhmanova and Steinmetz, 2008). Although it is more frequent in yeast, there are some +TIPs in other organisms that can bind to stable or depolymerising microtubules, for example in mammalian cells, APC was found to remain associated with microtubule plus-ends during growth, shortening and pauses (Kita et al., 2006), EB1 comets were seen in a pause state in human cells (Green et al., 2005) and KLP59C associates with depolymerising microtubule plus-ends in *Drosophila* S2 cells (Mennella et al., 2005). Our results suggest that Mast is present only at growing and pausing microtubule plus-ends. In addition, N-terminal region seems to have an important role in this plus-end direction. Since there are two GTP binding sites in this region present in all fragments except in Mast⁴⁴⁶⁻¹⁴⁹¹, G₂ and G₃, we speculate that they could be responsible for ensuring the plus-end direction of Mast. However, it is known that they exhibit normal plus-end behavior (Gouveia et al., in preparation) suggesting that if they have any role in microtubule plus-end regulation they do not act individually.

CHAPTER 3

**Dynein and Mast/Orbit/CLASP have
antagonistic roles in regulating
kinetochore-microtubule
plus-end dynamics**

1. INTRODUCTION

Mitotic spindles are fusiform structures whose principal function is to carry out proper segregation of chromosomes during mitosis. Spindles are composed of highly dynamic microtubules that constantly exchange tubulin heterodimers with a soluble pool. This exchange results in some particular features of microtubules, such as dynamic instability, a behaviour in which individual microtubule ends alternate stochastically between prolonged phases of polymerization and depolymerization (Mitchison and Kirschner, 1984) and poleward flux (Mitchison, 1989). Flux requires that microtubules polymerize at the kinetochores and depolymerize at the pole, while translocating poleward (Cassimeris, 2004). Several factors have been implicated in this complex dynamic behaviour, which are required for the coordination of polymerization (Maiato et al., 2005), depolymerization (Rogers et al., 2004a) and sliding of microtubules (Miyamoto et al., 2004).

Mast/Orbit/CLASP (Akhmanova et al., 2001; Inoue et al., 2000; Lemos et al., 2000) are conserved non-motor microtubule-associated proteins (MAPs) with essential roles in mitosis. They belong to the plus-end tracking proteins (Carvalho et al., 2003) which transiently accumulate at microtubule growing plus ends (Akhmanova et al., 2001; Sousa et al., 2007). They also localize to kinetochores of mitotic chromosomes, centrosomes, the central spindle region and the midbody (Lemos et al., 2000; Maiato et al., 2003a; Pereira et al., 2006). In interphase Mast was shown to have a stabilizing role on microtubules by promoting the pause state (Sousa et al., 2007). In mitosis, it is required for functional kinetochore-microtubule attachments, chromosome congression and maintenance of spindle bipolarity (Lemos et al., 2000; Maiato et al., 2002). Mast mutants or siRNA-treated cells form monopolar spindles because of their inability to sustain spindle bipolarity. In S2 cells, the absence of Mast results in a strong reduction in microtubule poleward flux (Buster et al., 2007; Maiato et al., 2005). Accordingly, Mast was proposed to ensure spindle bipolarity by regulating the incorporation of microtubule subunits at attached plus-ends of mature kinetochore fibers (K-fibers), counterbalancing the activity of microtubule depolymerases at the minus-ends (Maiato et al., 2005).

Interestingly, a recent study revealed that in *S. pombe* many of the phenotypes associated with interphase microtubule dynamics arising from the loss of *peg1*, the Mast homologue, are seen only if Dynein is present (Grallert et al., 2006), suggesting a functional interplay between these two proteins. Dynein is a minus-end-directed microtubule associated motor protein (Paschal and Vallee, 1987) that localizes to a variety of subcellular sites, including kinetochores, microtubules, cell cortex, centrosomes and

microtubule plus ends (Dujardin and Vallee, 2002; Hays et al., 1994; Pfarr et al., 1990; Sharp et al., 2000a; Siller et al., 2005; Steuer et al., 1990; Vaughan et al., 1999; Xiang et al., 2000). This widespread localization allows Dynein to be involved in several distinct processes, including the organization of the minus ends of the spindle and their tethering to the centrosomes (Compton, 1998; Maiato et al., 2005; Maiato et al., 2004b). Loss of Dynein causes a metaphase delay (Goshima and Vale, 2003) and slows down poleward chromosome motion in anaphase (Savoian et al., 2000; Sharp et al., 2000b) without affecting the rate of microtubule poleward flux (Maiato et al., 2005; Yang et al., 2007). The localization of Dynein at the kinetochore relies on ZW10 (Scaerou et al., 1999; Starr et al., 1998) and Spindly (Griffis et al., 2007) where it has a dual role in facilitating force generation and also in mediating the poleward transport of spindle checkpoint proteins from kinetochore, thereby promoting the inactivation of the checkpoint and mitotic exit (Howell et al., 2001; Wojcik et al., 2001). The removal of Dynein from kinetochores requires at least partial microtubule-kinetochore attachment and also requires other proteins, including Nudel (Hoffman et al., 2001; King et al., 2000; Liang et al., 2007).

In this study, we investigate the role of Mast at kinetochores and explore the functional interplay with Dynein in this process using *Drosophila* embryos and S2 cells. We found that Mast accumulates at kinetochores soon after NEBD where it remains at high levels until metaphase. During mid metaphase, Mast starts to be removed along microtubules in a poleward streaming fashion, which is partially dependent on Dynein. We then analyzed whether the presence of Dynein affects spindle organization in cells where Mast is depleted. Surprisingly, we observed that after co-depletion of Mast and Dynein spindle bipolarity is maintained, suggesting that the collapse of the spindle resulting from depletion of Mast requires Dynein activity. Moreover, in contrast to Mast-depleted cells where microtubule poleward flux is severely affected, we found that in mitotic cells depleted of both Mast and Dynein, spindle microtubules displayed normal plus-end polymerization rates at kinetochores. Taken together, our results suggest that Dynein has an important role in regulating the kinetochore levels of Mast at the metaphase-anaphase transition and that Mast and Dynein have antagonistic roles in the regulation of microtubule plus-end dynamics at kinetochores.

2. RESULTS

2.1 Mast shows a highly dynamic pattern of kinetochore localisation during mitosis

Mast binds to centrosomes, microtubules and kinetochores during various stages of mitosis (Lemos et al., 2000). We found strong accumulation of Mast at the outer kinetochores by co-localization with the centromere marker Cid, from early prometaphase soon after NEBD, until early anaphase when it starts to accumulate also at the central spindle region (Fig. 3.1A). Similarly to CLASPs in HeLa cells (Maiato et al., 2003a; Pereira et al., 2006), the level of Mast at kinetochores was found to be higher at prometaphase when compared with metaphase and anaphase (Fig. 3.1B).

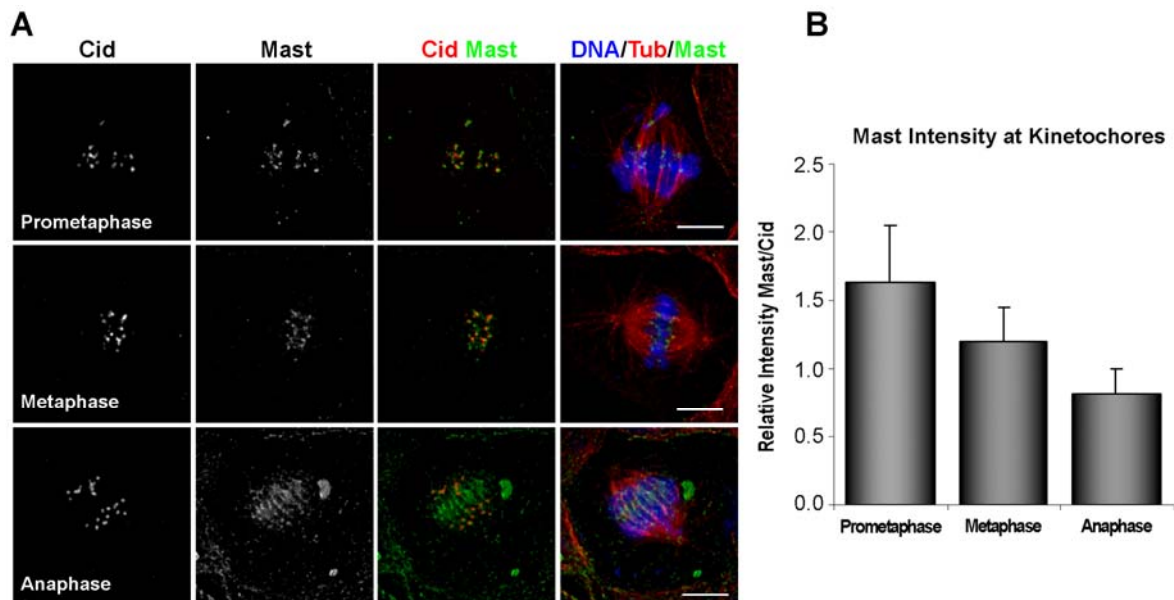


Figure 3.1. Localization of Mast at kinetochores through different stages of mitosis. (A) Immunostaining of Mast (green), Cid (red) and α -tubulin (red, right panel) in S2 cells during different stages of mitosis. (B) Quantification of fluorescence intensity of kinetochore-associated Mast relative to Cid at different stages of mitosis (prometaphase, $n=80$; metaphase, $n=79$; anaphase, $n=87$; error bars represent s.d.; $P<0.001$; n =number of kinetochores). Scale bar correspond to 5 μ m.

We then analyzed the dynamic behavior of Mast during mitosis using transgenic *Drosophila* lines in which either the full-length EGFP-Mast protein or the C-terminal fragment EGFP-Mast-Kt2 (containing the kinetochore-targeting domain, but not the microtubule binding domain), were expressed using the UAS-GAL4 system (Brand and Perrimon, 1993). To determine whether the full-length EGFP-Mast protein was functional, it was expressed in a *mast*⁴ mutant background (Lemos et al., 2000) under the control of either a specific neuroblast promoter (MZ1061) (Fig. 3.2 A-G) or a ubiquitous driver Act-

Gal4 (Fig. 3.2H). We found that full-length EGFP-Mast expressed specifically in neuroblasts could fully rescue the mitotic phenotype caused by mutations in the endogenous mast gene (Fig. 3.2A-G). Moreover, if expressed using the actin-Gal4 driver, most mutant cells formed normal bipolar spindles and other mitotic phenotypes associated with mutation of mast were substantially reduced (Fig. 3.2H). However, this ubiquitous driver did not rescue the lethality of mast, probably because of inappropriate control of EGFP-Mast expression in certain vital tissues. However, at the cellular level, EGFP-Mast could replace the function of the endogenous protein, suggesting that the presence of the EGFP-tag does not significantly affect the protein function and therefore its dynamic behavior probably reflects that of the endogenous protein.

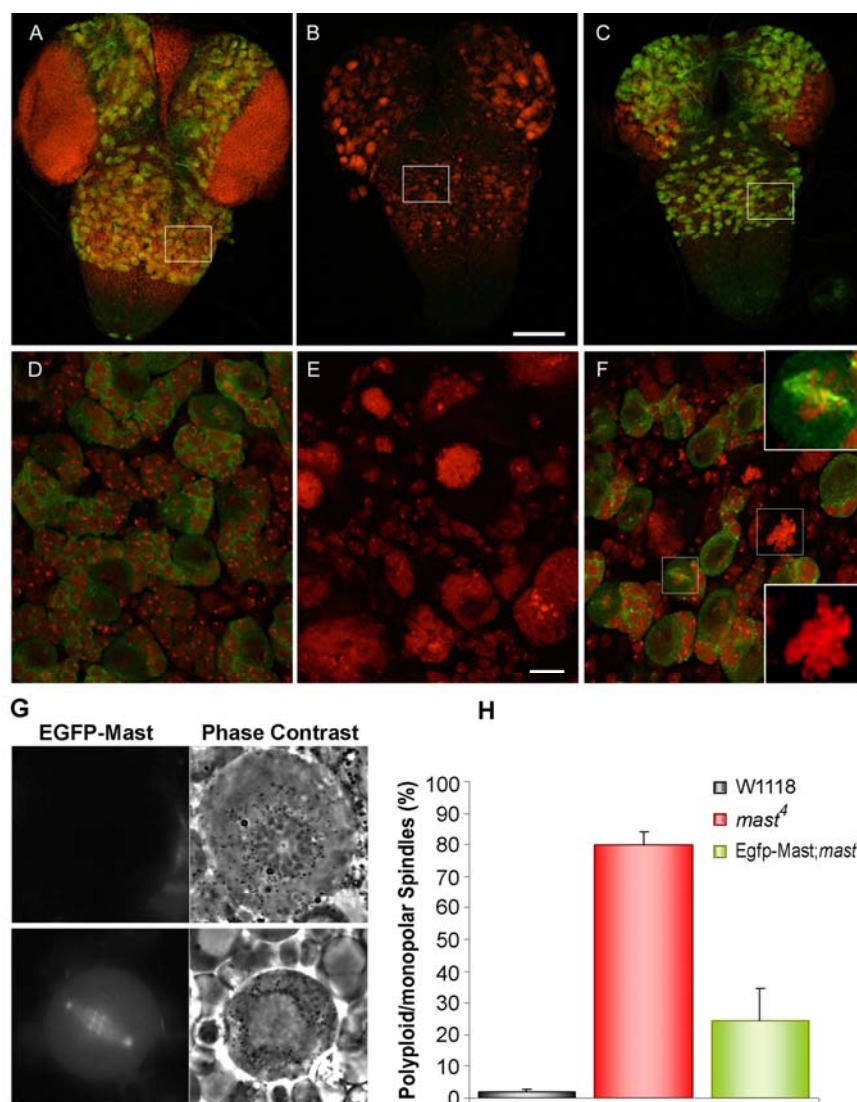


Figure 3.2. Functional analysis of EGFP-Mast transgenes. (A) Brain from a control larva expressing EGFP-Mast on a wild type background. (B-C) Brains from *mast*⁴ larvae (B) that does not express EGFP-Mast or (C) expressing EGFP-Mast (green). (D-F) High magnifications of the regions highlighted in A-C. (G) Neuroblasts from (C), showing the phenotype of cells without EGFP-Mast and with EGFP-Mast. (H) Quantification of polyloid/monopolar spindles present in brains from W1118 (control), *mast*⁴ mutants (w; UAS-EGFP-Mast/Cyo; *mast*⁴/*mast*⁴) or flies *mast*⁴ expressing ubiquitously EGFP-Mast (w; UAS-EGFP-Mast/Act-Gal4; *mast*⁴/*mast*⁴) (n= 5 brains for each strain). Scale bars correspond to 100μm (B) and 10μm (E).

We then used laser-confocal time-lapse imaging to analyze the behavior of both EGFP-Mast and the EGFP-Mast- Kt2 deletion construct during mitotic progression in syncytial embryos (Fig. 3.3 and Movies 3.1 and 3.2, respectively). Still images from the movies show that both the full-length protein (EGFP-Mast) and the C-terminal fragment alone (EGFP-Mast-Kt2) accumulate strongly at kinetochores from prometaphase until metaphase when the signal starts to decrease (Fig. 3.3A). Quantification of fluorescence intensity of EGFP-Mast and EGFP-Mast-Kt2 at kinetochores from prophase to anaphase, confirmed a highly dynamic pattern of accumulation with a decrease of the kinetochore signal starting at metaphase and continuing during anaphase (Fig. 3.3B and C), as seen also with kymograph analyses. These results suggest that as cells undergo metaphase-to-anaphase transition, the levels of Mast at kinetochores are significantly reduced.

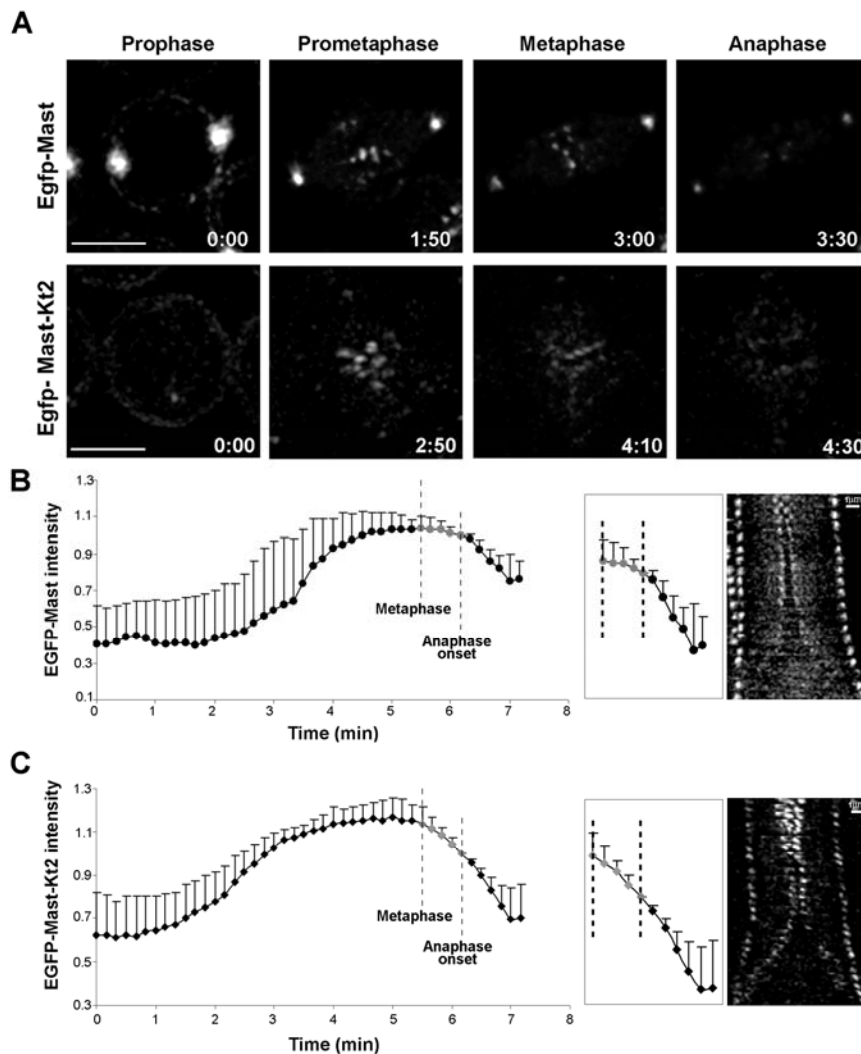


Figure 3.3. Dynamic behavior of Mast during mitosis. (A) Still images from time-lapse movies of syncytial embryos expressing either EGFP-Mast or EGFP-Mast-Kt2. Time is in min:sec. Quantification of (B) kinetochore intensity of EGFP-Mast and (C) EGFP-Mast-Kt2 during syncytial divisions (in each case, three nuclei per embryo of ten embryos at cycle 13 were analyzed). Panels on the right show a higher magnification of EGFP-Mast and EGFP-Mast-Kt2 intensity at the metaphase-anaphase transition and the corresponding kymographs. Scale bars: 5 μ m (A); 1 μ m (B,C).

2.2 Mast shows poleward streaming during metaphase and anaphase

We showed that the accumulation of Mast at kinetochores decreases during metaphase and anaphase. To further characterize this dynamic behavior, we first expressed either the full-length EGFP-Mast or the shorter version with the kinetochore targeting domain EGFP-Mast-Kt2 in *Drosophila* lines simultaneously expressing the centromere marker mRFP-Cid (Fig. 3.4A and Movie 3.3). The results indicate that EGFP-Mast-Kt2 localizes externally to the centromere marker from NEBD until anaphase, as expected. The full-length EGFP-Mast showed an identical localization pattern (data not shown). Moreover, analysis of the time-lapse images revealed that at metaphase and anaphase, both the complete EGFP-Mast and EGFP-Mast-Kt2 (which does not contain the microtubule binding domain) could be seen streaming poleward along spindle microtubules (Fig. 3.4B and Movies 3.4 and 3.2, respectively).

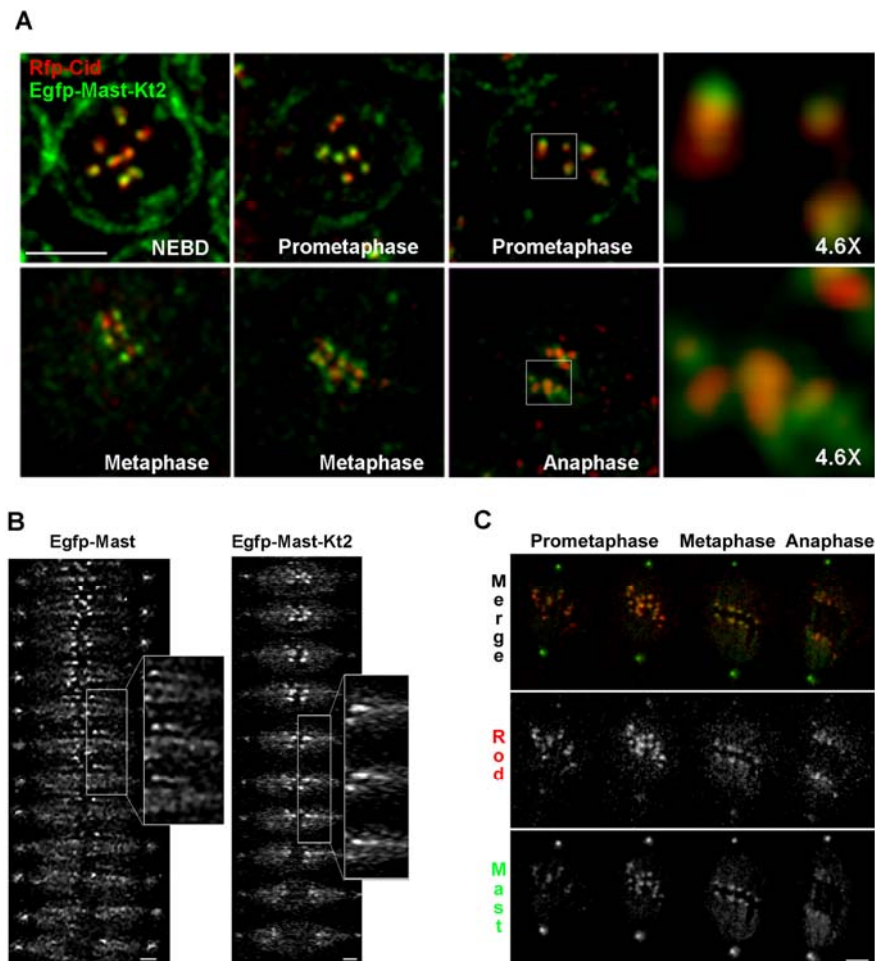


Figure 3.4. Streaming of Mast from kinetochores during metaphase and anaphase. (A) High magnification image of a nucleus from a syncytial embryo expressing EGFP-Mast-Kt2 (green) and mRFP-Cid to label centromeres (red). High magnification images are shown on the right. Scale bar: 5 μ m. (B) Kymographs from time lapse movies of embryos expressing either EGFP-Mast or EGFP-Mast-Kt2. High magnification images show details of the poleward streaming of EGFP-tagged particles during metaphase and anaphase. Scale bars: 2 μ m. (C) High magnification images of nuclei from a syncytial embryo expressing EGFP-Mast (green) and mRFP-Rod (red). Scale bar: 2 μ m.

Poleward streaming started at metaphase and continued during anaphase and is highly reminiscent of the dynamic behavior of Rod or ZW10, two members of the RZZ complex, as they are shed from kinetochores (Williams et al., 1992; Williams et al., 2003). To determine whether the kinetochore streaming of Mast was similar to that of the RZZ complex, we co-expressed EGFP-Mast with mRFP-Rod and followed their dynamic behavior during late syncytial divisions (Fig. 3.4C). Analysis of these double-expressing embryos showed that Mast and Rod follow an identical kinetochore accumulation pattern early in mitosis that is followed by streaming along microtubules during metaphase and anaphase. These observations suggest that Mast streams polewards either bound to the RZZ complex or using a similar mechanism. Quantification of poleward streaming of EGFP-Mast showed that these particles move at a velocity of $14.6 \pm 3.6 \mu\text{m}/\text{min}$ ($n=76$ particles from thirty-one spindles of seven syncytial embryos at cycles 11 or 12), a rate very similar to the dynein-mediated transport observed for either Rod or Spindly (Basto et al., 2004; Griffis et al., 2007). These results show that during the metaphase-anaphase transition Mast and Rod have very similar dynamic behavior and therefore their poleward streaming could be due to a similar molecular mechanism.

2.3 Dynein is required to remove Mast from kinetochores

Rod is part of the RZZ complex (Rod, ZW10 and Zwilch), which is known to leave the kinetochore in a dynein-dependent manner (Wojcik et al., 2001). Since Mast shows a similar poleward streaming pattern, we sought to determine whether Mast also depends on dynein for its removal from kinetochores. To test this hypothesis, we depleted either ZW10 or dynein from S2 cells using RNAi and then analyzed the localization of endogenous Mast by immunostaining using a specific anti-Mast antibody (Fig. 3.4). Both ZW10 and dynein were efficiently depleted (92% and 88%, respectively) from S2 cells after 120 hours of treatment (Fig. 3.5A). Then we determined the level of Mast at kinetochores in metaphase of control, dynein- or ZW10-depleted cells treated with the proteasome inhibitor MG132 to prevent control cells from exiting mitosis (Fig. 3.5B). The level of Mast at kinetochores was quantified by measuring the fluorescence intensity ratio of Mast relative to the constitutive centromere marker Cid (Fig. 3.5C). The results indicate that in the absence of either dynein or ZW10, kinetochores show significant differences on Mast intensity ($P < 0.001$) exhibiting higher peak levels when compared with control cells, suggesting that removal of Mast from kinetochores depends largely on dynein activity. In agreement, a recent study in mammalian cells showed significant kinetochore retention of CLASP1 after expression of the dynein tail that displaces the motor-containing dynein

heavy chain from kinetochores (Varma et al., 2008). Given that dynein requires microtubules for the removal of kinetochore associated proteins (Holzbaur and Vallee, 1994; Howell et al., 2001), the level of Mast at kinetochores in cells lacking microtubules should be independent of dynein. To test this, control cells and cells depleted of either ZW10 or dynein were incubated with colchicine to remove microtubules and the fluorescent intensity ratio between Mast and Cid was determined (Fig. 3.5D). The results show that indeed there are no significant differences in the peak intensities between Mast levels at kinetochores of cells lacking either ZW10 or dynein and control cells, indicating that in the absence of microtubules, accumulation of Mast at kinetochores is independent of these two proteins. Thus, as for other known dynein cargo proteins, Mast removal from kinetochores appears to require microtubules. Taken together, these results indicate that removal of Mast from kinetochores depends upon microtubule attachment and dynein motor activity.

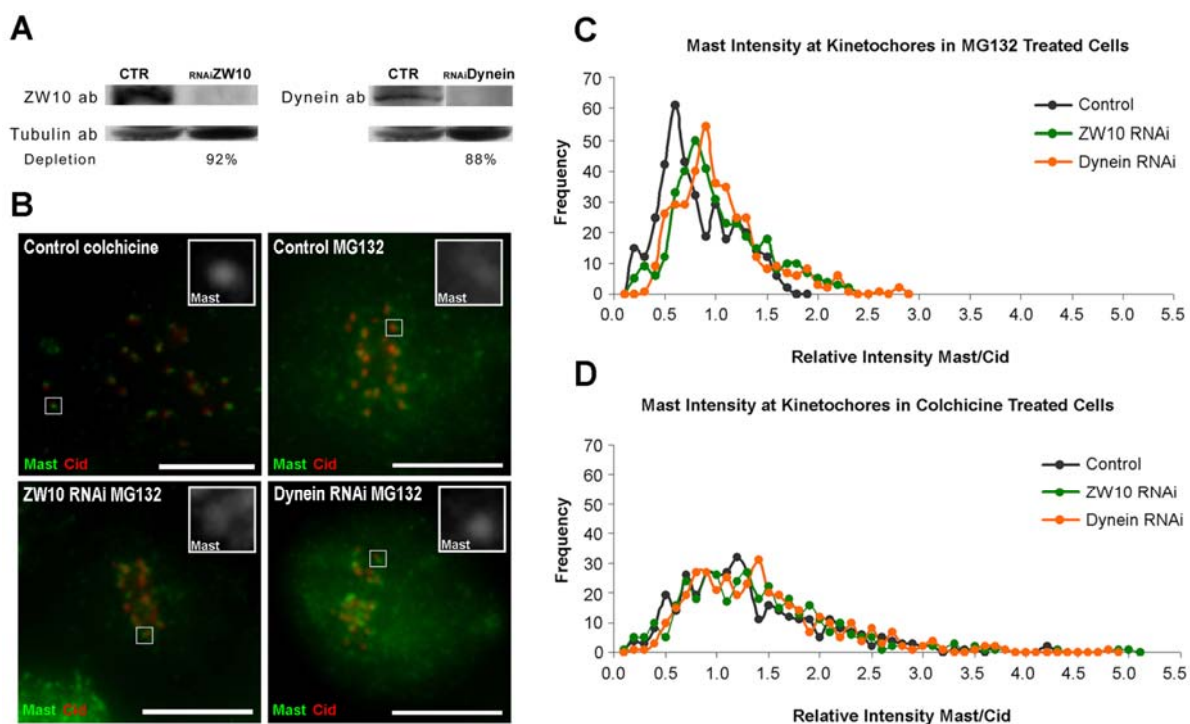


Figure 3.5. Role of dynein in removal of Mast from kinetochores. (A) Western blot of total protein extracts from control and from cells treated with ZW10 or dynein siRNAs for 120 hours. Tubulin was used as a loading control. (B) Immunofluorescence of S2 cells labelled for Mast (green) and for the centromere marker Cid (red) showing higher levels of Mast accumulation at kinetochores in cells treated with ZW10 and dynein siRNAs and also stronger accumulation in control cells treated with colchicine to remove microtubules (see insets for higher magnification images). (C) Quantification of Mast intensity at kinetochores of S2 cells arrested in metaphase with MG132 (D) or in prometaphase with colchicine. Relative Mast:Cid intensity was measured in kinetochores of control cells (black) and in ZW10 (green) or dynein (orange) siRNA-treated cells (n=375 or n=388 kinetochores in each case, for cells treated with MG132 or colchicine, respectively).

2.4 Organization of the metaphase spindle in the absence of Mast and dynein

Previous studies show that the collapse of the spindle after depletion of Mast is due to the inability to incorporate subunits at the microtubule plus-ends, and the constant removal at the minus-end by kinesin 13 depolymerases (Rogers et al., 2004; Maiato et al., 2005; Buster et al., 2007). In this study, we show that dynein is required to regulate Mast levels at kinetochores during the metaphase-anaphase transition. These observations raise the possibility that dynein might affect Mast activity at kinetochores. To test this, we performed depletion of Mast alone or together with dynein or ZW10 (Fig. 3.6) and analyzed the effect upon spindle organization (Fig. 3.7). Analysis of spindle morphology in the different RNAi treatments revealed three major phenotypes, which we classified as monopolar spindles (with microtubules emanating from a single MTOC), bipolar spindles (with a typical fusiform shape) or multipolar spindles (with more than two MTOCs) (Fig. 3.7A). Quantification of these phenotypes showed that the most frequent spindle shape in control and in dynein-depleted cells was bipolar ($93\pm4.5\%$ and $95.7\pm1.1\%$, respectively) whereas depletion of Mast alone caused mostly monopolar spindles ($62.3\pm24.5\%$) (Fig. 3.7B). However, after simultaneous depletion of Mast with either dynein or ZW10, we observed a significant rescue of spindle bipolarity from $31.3\pm22.6\%$ in Mast alone to $77.2\pm8.8\%$ and $75.1\pm11\%$ in cells depleted of Mast and dynein or Mast and ZW10, respectively. More significantly, the frequency of monopolar spindles, the most common phenotype observed after depletion of Mast, was significantly reduced ($62.3\pm24.5\%$) when compared with cells in which dynein or ZW10 are also depleted ($19.3\pm8.8\%$ or $9.4\pm7.8\%$) (Fig. 3.7B).

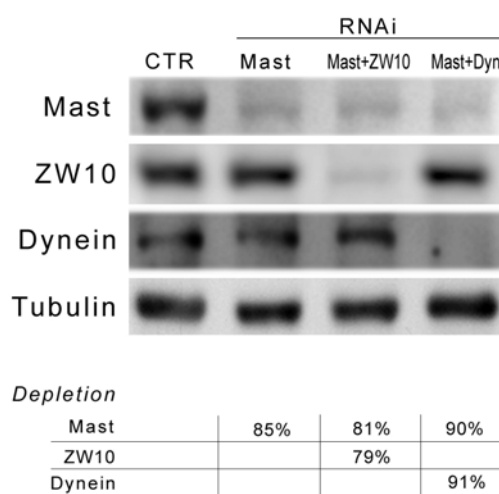


Figure 3.6. Western blots of cell extracts after depletion of Mast alone or Mast+Dynein and Mast+ZW10. Level of protein depletion after 120 hours of treatment is indicated on the table below. Tubulin was used as loading control.

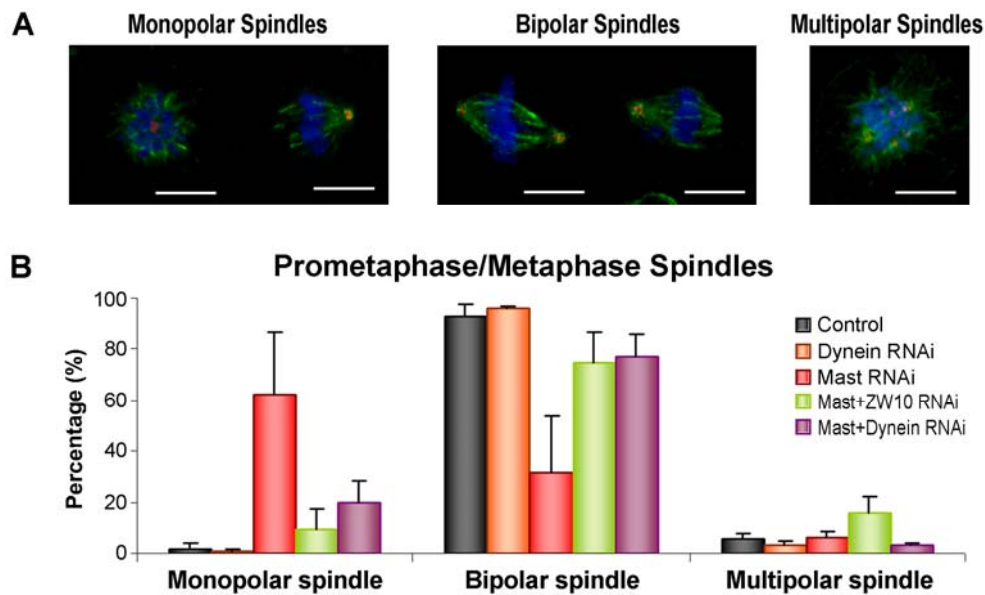


Figure 3.7. Spindle morphology in cells lacking both Dynein and Mast. (A) S2 cells labeled for γ -tubulin (red), α -tubulin (green) and DNA (blue) showing the three major spindle phenotypes found in cells depleted for Mast alone or in combination either with dynein or ZW10. The spindles were morphologically classified as monopolar, bipolar or multipolar. (B) Frequency of the three spindle phenotypes found in control cells (black), and in cells depleted of dynein (orange), Mast (red) and double-depleted Mast+ZW10 (green) or Mast+dynein (violet). At least 400 mitotic cells were quantified for each treatment. Scale bars: 5 μ m.

Given that in the absence of ZW10 or dynein there is abnormal accumulation of Mast at kinetochores (see above), we then analyzed the bipolar spindles of cells depleted of Mast and dynein in more detail, by comparing the length of the metaphase spindle with that of control cells and cells depleted of either dynein or ZW10 alone (Fig. 3.8). Since centrosomes are usually detached from the spindle in dynein depleted cells (Robinson et al., 1999), the length of all the spindles was determined by measuring the distance between the opposite kinetochore microtubule minus-ends. The results indicated that after depletion of ZW10 or dynein, cells consistently exhibited metaphase spindles (10 μ m or 11 μ m, respectively) that were significantly longer than in control cells (8 μ m, $P < 0.001$) or in cells depleted of both Mast and dynein, which exhibited significantly shorter spindles (6 μ m, $P < 0.001$) (Fig. 3.8).

Spindle bipolarity can be rescued either by causing the reformation of the bipolar spindle after it has collapsed, or it can be a true rescue in which the spindle never collapses and is therefore capable of maintaining bipolarity. To determine how spindle bipolarity is rescued in double-depleted cells, we depleted both Mast and dynein from cells expressing GFP-tubulin and analyzed the organization of the spindles over long periods of time (Fig. 3.9). The results indicate that these spindles first reached a normal spindle length (8 μ m), which then over time reduced to almost 6 μ m (Fig. 3.5 and Movie

3.5). After the spindle had reduced in size, it remained at that constant length for a long period and some of these cells were able to exit mitosis into anaphase where the spindle elongated and only rarely collapsed (Fig. 3.9 and Movies 3.6 and 3.7).

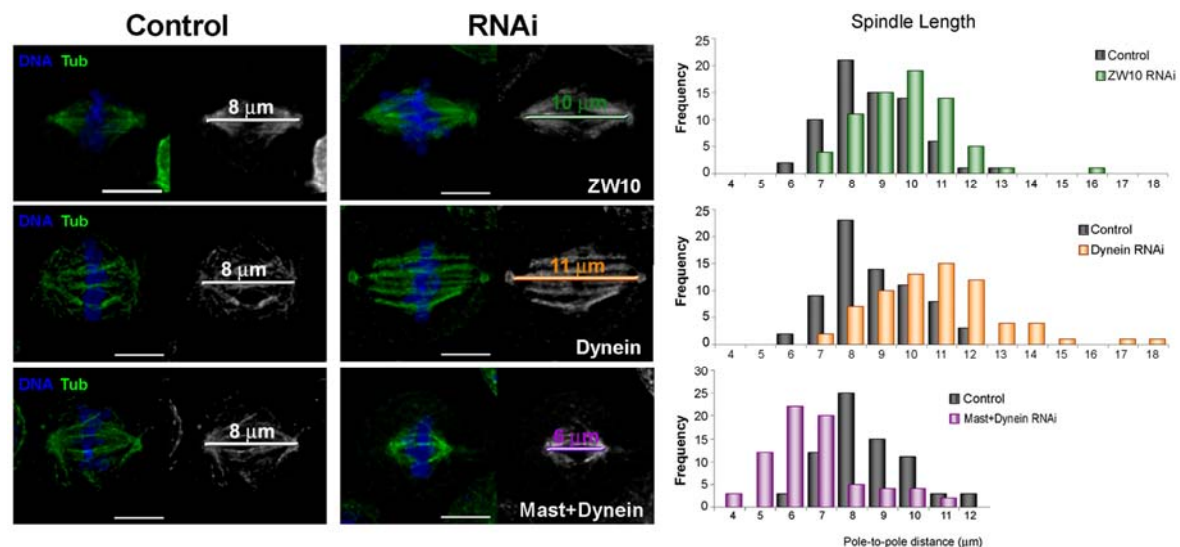


Figure 3.8. Effect of Mast in spindle length. Metaphase spindles of control or siRNA-treated S2 cells from asynchronous cultures labelled for tubulin (green) and DNA (blue). The lines represent pole-to-pole distances. Quantification of the frequency distribution of spindle length for each siRNA treatment is shown on the right (n=72 cells in each case). Scale bars: 5 μ m.

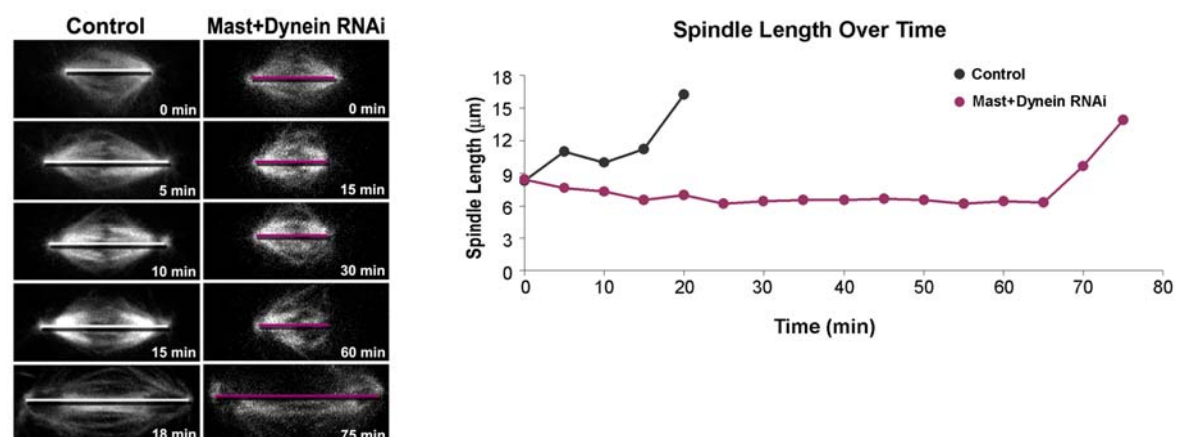


Figure 3.9. Spindle dynamics in cells lacking both, Mast and Dynein. Still images from representative movies of control and dynein+Mast-depleted S2 cells expressing GFP-tubulin. Images were acquired every 30 seconds. Time zero was defined as the time the bipolar spindle is formed. Note that after simultaneous depletion of both Mast and dynein, the spindle remains short for long periods of time. Quantification of spindle length over time.

Taken together, these observations indicate that in the absence of dynein, Mast-depleted cells can form bipolar spindles, and although they have a reduced length, they are able to maintain bipolarity over long periods and do not collapse. Moreover, these results suggest first, that maintenance of spindle bipolarity relies on antagonistic activities

between Mast and dynein, and second, that inappropriate accumulation of Mast at kinetochores might be responsible for the elongated metaphase spindles observed when ZW10 or dynein are absent.

2.5 Depletion of dynein or Mast and dynein simultaneously does not affect functional microtubule-kinetochore attachment

Mast is involved in microtubule plus-end polymerization and therefore, in its absence, the spindle is thought to collapse because of continued depolymerization at the minus-end while the kinetochore remains firmly attached to microtubule bundles. However, the absence of dynein is thought to compromise microtubule-kinetochore interactions, resulting in a decrease in the inter-kinetochore distance and a consequent delay in exiting mitosis because of loss of tension across sister kinetochores (Varma et al., 2008; Yang et al., 2007). Thus, we analyzed the stability of microtubule-kinetochore attachment and its effects upon interkinetochore distances in the absence of dynein or dynein and Mast (Fig. 3.10 and Fig. 3.11). Control or siRNA-treated cells were subjected to cold treatment and the interaction between microtubule bundles and kinetochores was quantified (Fig. 3.10A and B). The results indicated that there is no significant difference between control and siRNA-treated cells and therefore, loss of dynein or dynein and Mast appeared to have no significant effect upon microtubule- kinetochore attachment.

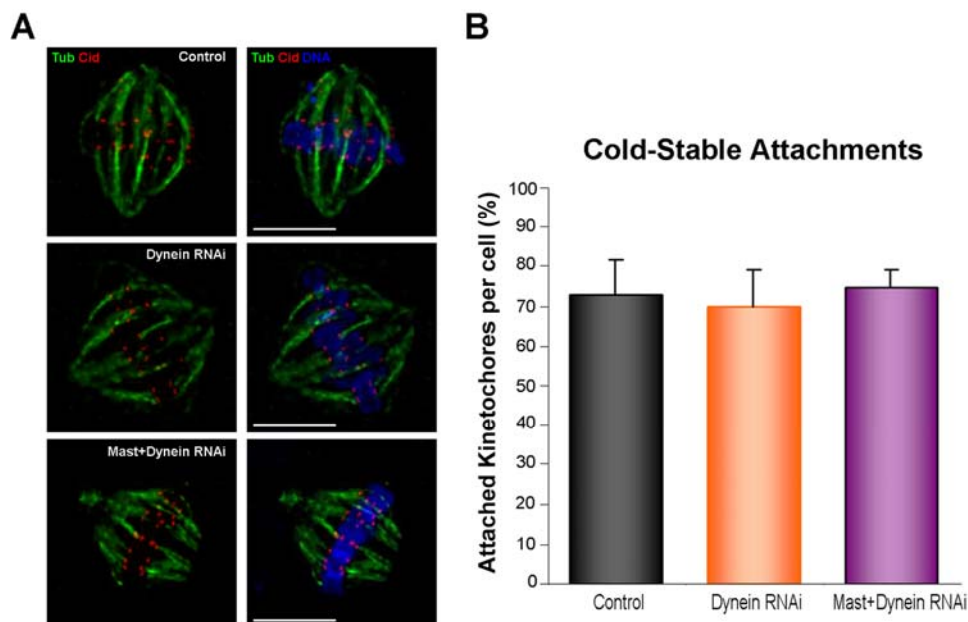


Figure 3.10. Role of dynein and Mast on the stability of kinetochore-microtubule attachments. (A) Control or siRNA-treated cells subjected to cold treatment, fixed and stained to reveal cid (red), α -tubulin (green) and DNA (blue). (B) Quantification of microtubule-kinetochore stability shown as a percentage of attached kinetochores per cell. All the kinetochores in ten cells were analyzed for each condition. Results are means $\pm$ s.d. Scale bars: 5 μ m.

To analyze the impact of these two proteins in generating tension across sister kinetochores, we then measured the inter-kinetochore distance of metaphase cells lacking dynein, or both Mast and dynein, and compared these with both untreated control cells and control cells treated with colchicine (lack of tension) (Fig. 3.11). The results revealed that in cells treated with dynein RNAi and Mast+dynein RNAi, the inter-kinetochore distance was similar to untreated control cells ($1.15 \pm 0.24 \mu\text{m}$, $n=93$; $1.21 \pm 0.21 \mu\text{m}$, $n=126$ and $1.02 \pm 0.16 \mu\text{m}$, $n=137$, respectively) and only statistically different from colchicine-treated cells when microtubules are not present ($0.70 \pm 0.15 \mu\text{m}$, $n=142$, $P < 0.001$) (Fig. 3.11B). Indeed, previous studies also show that loss of dynein at kinetochores after depletion of Spindly, have no effect upon the inter-kinetochore distance in S2 cells (Griffis et al., 2007).

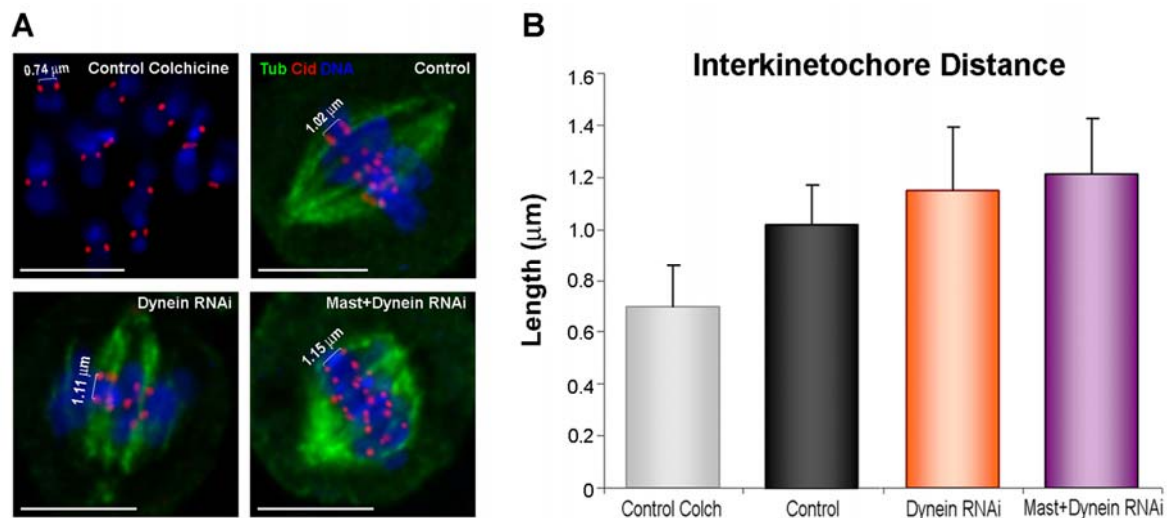


Figure 3.11. Role of dynein and Mast on tension generation across sister kinetochores. (A) Control and siRNA-treated cells were fixed and stained to revealed cid (red), α -tubulin (green) and DNA (blue). As a control for loss of tension across sister kinetochores, control cells were treated with colchicine to depolymerize microtubule before fixation. (B) Quantification of inter-kinetochore distance in control cells treated with colchicine, in untreated cells, and in cells treated with dynein RNAi and Mast+dynein RNAi. Results are means $\pm$ s.d. Scale bars: $5 \mu\text{m}$.

These results suggest that the absence of these two proteins, alone or simultaneously, has no significant impact either on microtubule-kinetochore attachment or tension generation in *Drosophila* cells.

2.6 Rates of microtubule flux are normal in cells depleted of Mast and dynein

Previous results have shown that Mast has an essential role in the incorporation of tubulin heterodimers into the plus-ends of mature K-fibers (Maiato et al., 2005) and that the collapse of the spindle in Mast-depleted cells requires the activity of KLP10A, a microtubule minus-end depolymerase (Buster et al., 2007; Laycock et al., 2006). In *Xenopus* extracts, kinesin-13 depolymerases are no longer targeted to spindle poles in the absence of dynein (Gaetz and Kapoor, 2004) and it is therefore possible that the rescue of spindle bipolarity in S2 cells depleted of Mast and dynein is also due to KLP10A mislocalization. However, after simultaneous depletion of Mast and dynein, we found that KLP10A localization to spindle poles was unaltered (Fig. 3.12). In agreement, Kif2A also remained concentrated at the minus-ends of K-fibers after dynein-dynactin inhibition in mammalian PtK1 cells (Cameron et al., 2006).

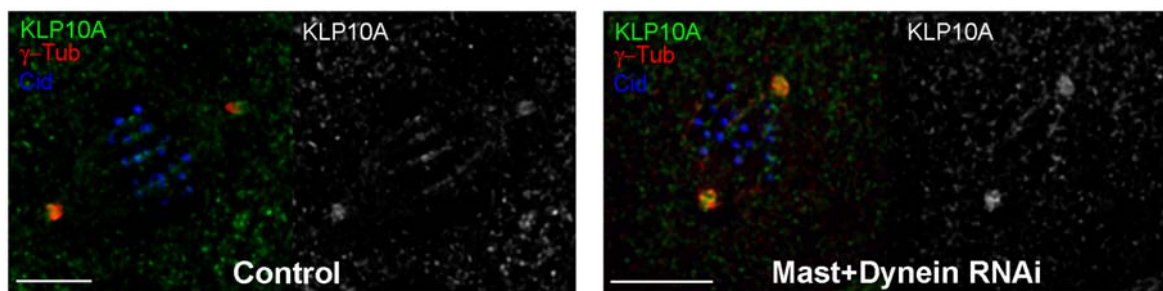


Figure 3.12. Zw10 localization in cells lacking both Mast and Dynein. S2 cells labeled for γ -tubulin (red), KLP10A (green) and Cid (blue) showing KLP10A localization in control cells and cells lacking both Mast and Dynein. Scale bar: 5 μ m.

Since microtubule minus-end depolymerases are normally localized, an alternative possibility is that spindles are bipolar in cells lacking Mast and dynein, because plus-end microtubule polymerization was restored. To test this possibility, we analyzed the microtubule plus-end polymerization rate on K-fibers after depletion of either Mast alone or in combination with dynein by fluorescence speckle microscopy in S2 cells stably expressing low levels of GFP-tubulin and mCherry-Cid (Fig. 3.13). The kymographs were aligned using the mCherry-Cid signal at kinetochores as a reference, allowing us to determine the rate of plus-end microtubule polymerization (Fig. 3.13B). The results confirm that after depletion of Mast alone, microtubule plus-end polymerization was indeed significantly reduced (0.50 ± 0.41 μ m/minute, $n=10$ cells) when compared with either control or dynein-depleted cells (1.49 ± 0.10 μ m/minute, $n=35$ cells or 1.53 ± 0.10 μ m/minute, $n=26$ cells, respectively), as previously shown (Buster et al., 2007; Maiato et

al., 2005). However, after codepleting Mast and dynein, we found that microtubule plus-end polymerization fully recovered, with values that were not significantly different from that of control cells (1.35 ± 0.10 $\mu\text{m}/\text{minute}$, $n=32$ cells).

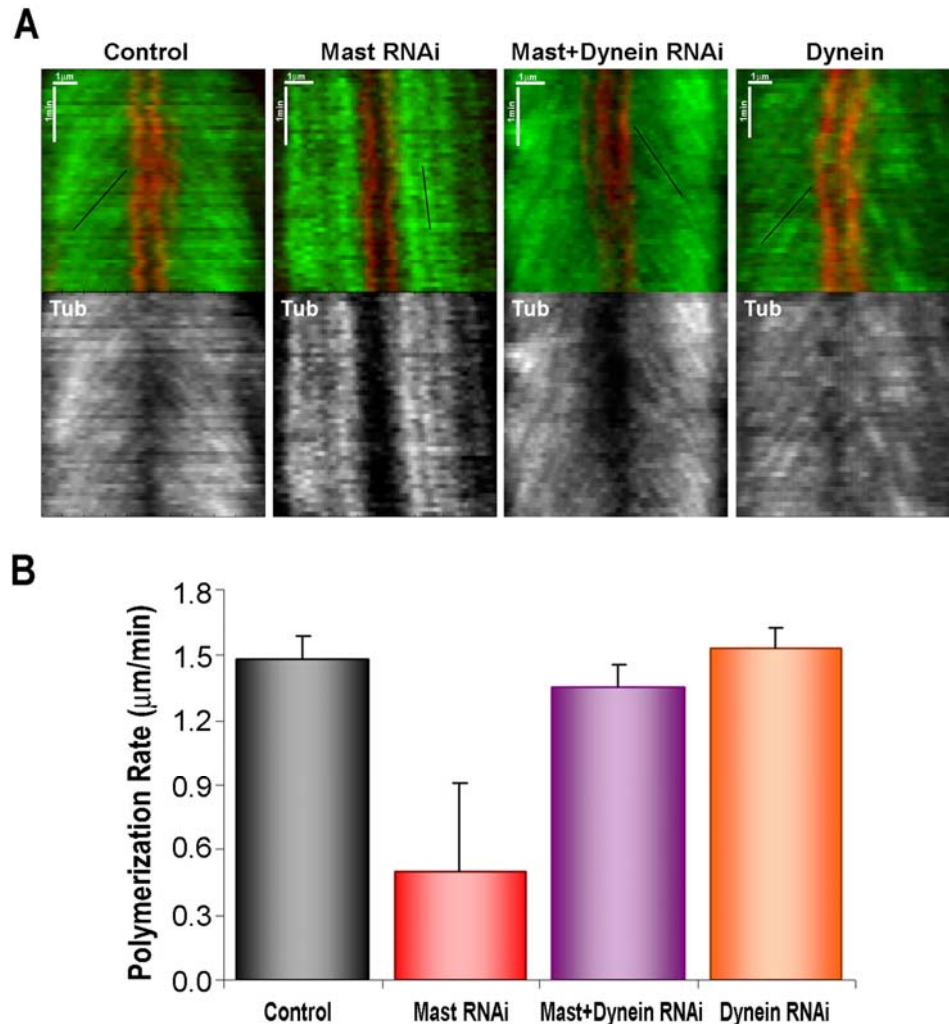


Figure 3.13. Effect of Mast and dynein on microtubule plus-end polymerization rate. (A) Kymographs of control cells and cells depleted for Mast and dynein, individually or simultaneously. These kymographs were obtained from movies of cells expressing low levels of GFP-tubulin and coexpressing mCherry-Cid used to align the kymographs. Black lines represent movement of tubulin speckles relative to kinetochores. Scale bars: 1 μm (horizontal); 1 minute (vertical). (B) Quantification of polymerization rates (mean $\pm$ s.d.) in control, and in cells treated with Mast RNAi, Mast+dynein RNAi and dynein RNAi.

These data further support the hypothesis that the rescue of spindle bipolarity is not due to the absence of KLP10A at the spindle poles, because it was shown that cells lacking KLP10A or Mast and KLP10A have significantly reduced flux rates (Buster et al., 2007; Ganem et al., 2005; Rogers et al., 2004). These results suggest that during metaphase Mast has an important role in facilitating microtubule plus-end polymerization when dynein is present.

3. DISCUSSION

3.1 Mast localizes at kinetochores in a microtubule-independent manner and its removal requires dynein activity

Our data show that Mast localizes to the external region of the kinetochore and that, like CLASP1 (Maiato et al., 2003a), Mast has a dynamic pattern of localization at kinetochores during mitosis and accumulates at high levels during prometaphase but then starts to rapidly decrease during metaphase and anaphase. From the analysis of transgenic flies expressing EGFP-Mast, we show that like many other proteins that localize transiently to the kinetochore, including Rod (Basto et al., 2004), Zw10 (Williams et al., 1996), dynein (King et al., 2000) and CLIP-190 (Dzhindzhev et al., 2005), a significant fraction of Mast leaves the kinetochores by streaming along microtubules.

To date no studies have shown that Mast and dynein interact directly; however, several studies have described interactions between CLIP-190 or CLIP-170 (mammalian orthologue) and dynein (Dujardin et al., 1998; Dzhindzhev et al., 2005; Lansbergen et al., 2004; Tanenbaum et al., 2006). Indeed, it was observed that, in addition to its role in localizing CLIP-190 to kinetochores, dynein also seems to be responsible for its removal from kinetochores (Dzhindzhev et al., 2005). The same kind of dynein dependence was described for mammalian CLIP-170 (Dujardin et al., 1998; Tanenbaum et al., 2006). It was observed that in addition to dynein, CLIP-190 also binds Mast (Mathe et al., 2003). Since CLASPs are CLIP-associated-proteins that bind to CLIP-170 through its conserved C-terminal domain (the same domain that targets Mast to kinetochores) (Akhmanova et al., 2001), it was proposed that CLIP-170 might be a linker for dynein cargo (Dujardin et al., 1998). Therefore, it is very likely that Mast/CLASP indirectly leave the kinetochore in a dynein-dependent manner via CLIP190.

3.2 Mast and dynein regulate spindle organization

MAPs have been involved in the regulation of spindle length given their capacity to change the balance of dynamic behavior and forces of spindle microtubules. Previously, it was demonstrated in S2 cells that the absence of microtubule stabilizers results in shorter spindles, and the absence of microtubule depolymerases results in elongated spindles (Goshima et al., 2005). In particular, regulators present at kinetochores appear to affect spindle length more severely than those located at the poles (Goshima et al., 2005). As Mast is required for microtubule plus-end incorporation in mature K-fibers, we analyzed its role in the overall organization of the spindle after depletion of either ZW10 or dynein that results in abnormally high levels of Mast at kinetochores. We hypothesized that higher

levels of Mast at kinetochores might allow more incorporation of subunits at K-fibers, which would result in an abnormal spindle length. Indeed, we observed that in the absence of either dynein or ZW10, the spindle length increases significantly. However, the polymerization rate at kinetochores does not increase significantly in the absence of dynein, suggesting that higher levels of Mast at kinetochores during metaphase do not affect spindle length directly. Interestingly, recent studies have identified Spindly, a new regulator of dynein recruitment to kinetochores that is essential for congression, timely mitotic exit and maintenance of correct spindle length (Griffis et al., 2007; Gassmann et al., 2008). However, although the molecular mechanism involved has not yet been identified, we can speculate that the absence of kinetochore dynein might be responsible for this effect.

The observation that co-depletion of Mast and dynein results in cells that are able to form bipolar spindles is surprising and suggest that these two proteins have opposite effects upon spindle organization. Moreover, the rescue of spindle bipolarity in these double-depleted cells is probably due to the absence of dynein specifically at kinetochores, because in cells in which Mast and ZW10 are depleted, where only the kinetochore dynein is displaced, the results are identical. Interestingly, analysis of mutants in the Mast orthologue of *Arabidopsis*, an organism that lacks dynein (Ambrose et al., 2005), show mostly normal cell division in which chromosomes align and segregate with no apparent defects; however, the spindles are significantly shorter (Ambrose et al., 2007). Similarly, our results show that in the absence of Mast and dynein the spindles although bipolar are shorter than in control cells and are maintained for long periods. These short spindles resemble those found in EB1-depleted cells, a protein known to be essential for the interaction of astral microtubules with the cell cortex (Goshima et al., 2005; Rogers et al., 2002). Therefore, it is possible that the spindles from cells lacking Mast and dynein are shorter because of the absence of any interaction between astral microtubules and the cell cortex.

3.3 Mast facilitates kinetochore-fiber subunit incorporation in the presence of dynein

Studies in S2 cells have demonstrated that after depletion of Mast, tubulin poleward flux of K-fibers is strongly reduced because the incorporation of tubulin subunits at the microtubule plus-ends of mature K-fibers is shut down (Buster et al., 2007; Maiato et al., 2005). Although collapse of the spindle is prevented by codepleting Mast and the minus-end microtubule-destabilizing kinesin KLP10A, this does not restore K-fiber flux (Buster et al., 2007; Laycock et al., 2006), suggesting that antagonizing activities regulate bipolarity independently of flux and that Mast is indeed an essential protein for K-fiber flux.

However, our results demonstrate that Mast is not involved in the direct incorporation of tubulin at the plus-ends of kinetochore microtubules, but rather regulates this process. Indeed, recent analysis of microtubule flux fully supports these results. Depletion of Mast and centromere-associated microtubule depolymerases KLP67A or KLP59C, also results in restoration of microtubule flux (Buster et al., 2007). However, although the microtubule-depolymerising nature of either KLP67A or KLP59C could in principle explain the rescue of flux when they are co-depleted with Mast, dynein has never been shown to promote microtubule depolymerization at kinetochores. Therefore, our results suggest that dynein affects flux indirectly, for instance by affecting the kinetochore function and/or targeting of a depolymerising enzyme. Indeed, theoretical work supports the idea that dynein located at the kinetochore assists microtubule depolymerases in suppressing rescue events by inserting the plus-ends of kinetochore microtubules into the kinetochore structure (Civelekoglu-Scholey et al., 2006; Gadde and Heald, 2004).

Dynein is also known to be required to target CLIP-190/CLIP-170 to the kinetochore (Dujardin et al., 1998; Dzhindzhev et al., 2005). Although the absence of CLIP-170/CLIP-190 does not affect microtubule dynamics, CLIP-170 appears to facilitate the initial formation of kinetochore-microtubule attachments, possibly through direct capture of microtubules at the kinetochore (Tanenbaum et al., 2006). Accordingly, in mammalian cells the absence of dynein or CLIP-170 causes cells to exhibit spindles with a substantially reduced number of kinetochore microtubules when subjected to cold treatment (Li et al., 2007; Yang et al., 2007), and cells lacking CLIP-170 also display high levels of kinetochore Mad1 (Tanenbaum et al., 2006). However, contrary to mammalian cells, our results suggest that S2 cells lacking dynein have relatively stable K-fibers because they were able to resist cold treatment and to generate normal inter-kinetochore tension. We therefore propose that the main function of dynein and Mast is to regulate kinetochore-microtubule dynamics and not attachment. Accordingly, our results suggest that dynein is essential, not for kinetochore-microtubule attachment per se, but to generate the proper interactions between the plus-end of microtubules and the kinetochore proteins, namely depolymerases. Recent studies in mammalian cells and *C. elegans* suggest that this motor has an important role in pushing the plus-ends of microtubules into the kinetochore structure allowing the maturation of the attachments (Civril and Musacchio, 2008; Gassmann et al., 2008; Varma et al., 2008). Since Mast is required for tubulin incorporation only in mature K-fibers (Maiato et al., 2005), microtubule plus-end incorporation in the absence of both proteins (Mast and dynein) might be restored because microtubules are no longer pushed into the kinetochore structure and expose their plus-ends to the action of depolymerases.

From our data, we propose that at metaphase, dynein controls the level or function of Mast and other microtubule regulators, potentially microtubule depolymerases, to allow tubulin incorporation and flux on K-fibers. This dynein-mediated regulation might be important for the switch from polymerizing to depolymerising state of kinetochore microtubules that occurs during anaphase.

PART III

GENERAL DISCUSSION

Cell division is the basis of the development of multicellular organisms and of its subsequent maintenance as adults by continuous cell renewal and regeneration. Mitosis is an essential, highly regulated process that allows the generation of two identical daughter cells with the same genetic material as the parental cell. The key events of mitosis are the condensation of chromatin into discrete mitotic chromosomes and the dismantling and conversion of the interphase microtubule network into a bipolar spindle with microtubules capable of capturing, align and segregating the sister chromatids to opposite poles of the dividing cell.

At the onset of mitosis, there are extraordinary changes in the dynamic behaviour of microtubules, in which long and stable interphasic tubulin polymers are replaced for shorter and highly dynamic microtubules. During mitosis, microtubule turnover and catastrophe frequency increases, as the frequency of microtubule rescues and time spent in pause decreases (Rusan et al., 2001; Saxton et al., 1984).

Several studies have contributed to unveil a myriad of proteins involved in regulation of microtubule dynamics and, in many cases these proteins were shown to participate in more than one functional activity. In *Drosophila melanogaster*, motor and non-motor MAPs were described to have an important contribution; KLP61F, seems to be involved in microtubule crosslinking and outward antiparallel sliding; Kinesin-13, contributes to microtubule depolymerization by stimulating catastrophes through KLP10A (flux promoter) or by suppressing rescue events via KLP59C (pacman promoter) (Buster et al., 2007; Mennella et al., 2005); KLP67A, seems to be involved in regulating microtubule length also by stimulating microtubule disassembly, at kinetochores (flux inhibitor) (Buster et al., 2007); Dynein has been implicated in microtubule minus-ends transport of both, microtubules and molecules, contributing to pole organization, maintenance of centrosome attachment to the spindle pole, generation of cortical pulling forces and, at kinetochores, in promoting chromosome capture and proper kinetochore-microtubule binding; amongst others (Akhmanova and Steinmetz, 2008; Gadde and Heald, 2004; Loughlin et al., 2008).

In addition, a specific class of MAPs, the +TIPs, were also shown to be of major importance in microtubule behaviour. Due to its plus-end accumulation, +TIPs have the ideal localization to regulate microtubule plus-ends dynamics, attachment of microtubules to the cell cortex or kinetochores and can be involved in force generation. Msps (flux promoter) (Buster et al., 2007) and EB1 seem to promote plus-end microtubule dynamics acting as anti-pause factors (Brittle and Ohkura, 2005; Rogers et al., 2002), while CLIP190 (mammalian CLIP170) promotes rescue events. On the other hand, Mast (flux promoter), was the first protein described to promote microtubule pause, among others (Akhmanova and Steinmetz, 2008; Manning and Compton, 2008a). While most of the

+TIPs act through stabilizing microtubules, another class of non-motor MAPs, the severing proteins, regulates microtubule dynamics by destabilizing it. Microtubule-severing proteins are thought to influence microtubule dynamics by induce depolymerization, for instance, Spastin and Fidgetin stimulate microtubule minus-end depolymerization (flux) and Katanin stimulates plus-end depolymerization (pacman) (Zhang et al., 2007). In order to reach a successful division, a delicate balance between these and other proteins involved in microtubule dynamics must be achieved. Although much is known about individual contributions of several proteins towards microtubule dynamics, the interaction with other proteins and their mechanisms of action are still poorly understood. In this project, we sought to unravel the role of Mast at the plus-ends of microtubules and kinetochores, and its involvement in kinetochore-microtubule interaction.

The work presented in this thesis characterizes Mast functional domains, explores Mast behaviour along the cell cycle and it contributes to a better understanding of Mast interaction with other proteins and its involvement in microtubule dynamics.

By dissecting Mast through expression of different domains of the protein we found that, the microtubule-binding domain of Mast, like in CLASPs, is located at the central basic region of the protein (Maiato et al., 2003a). Also, we showed that the targeting of Mast to the plus-ends of microtubules depends on a small region, located outside of the microtubule-binding domain, that contains the SxIP motif known to be responsible for microtubule plus-end localization of diverse +TIPs, including CLASPs, in an EB1 dependent manner (Honnappa et al., 2009). Therefore, we propose that Mast might reach the microtubule plus-tips by hitchhiking on EB1 and/or CLIP-190. Since it was described that CLIP-190 localizes to microtubule plus-ends in an EB1-dependent manner (Dzhindzhev et al., 2005), it would be interesting analyze Mast plus-end targeting in cells lacking EB1. In addition, we propose that Mast is not required for an efficient kinetochore-microtubule attachment but rather to a normal microtubule polymerization. Moreover, as in humans, we map Mast kinetochore-binding domain to the C-terminal region of the protein and found that Mast targets to kinetochores in a microtubule independent manner.

Through *in vivo* studies of interphase cells expressing EGFP-Mast and EGFP-Mast-fragments, we showed that Mast exhibits a classic +TIP behaviour, binding preferentially at the plus-ends of microtubules when present at normal levels, and along the entire length of microtubule when overexpressed, similarly to what was shown for other +TIPS (Carvalho et al., 2003). In addition, we demonstrated that Mast moves towards the cell periphery with a velocity very close to microtubule polymerizing rate, suggesting that similarly to other +TIPs, Mast dynamic properties are virtually identical to the dynamic properties of growing microtubules. Furthermore, by analysis of the dynamic behaviour of

some EGFP-Mast-fragments we determined that although Mast does not require the full-length sequence to target microtubule plus-ends as was shown for CLASPs (Mimori-Kiyosue et al., 2005) a small central region is sufficient, Mast requires the full protein sequence to display its normal dynamic behaviour. Detailed quantitative analysis also revealed that although Mast almost always behaves as in association with the growing microtubule ends, it can also pause, a behaviour already seen in other +TIPs like mammalian EB1, APC or budding yeast Bik1 (Clip-170 homolog) (Carvalho et al., 2004; Green et al., 2005; Kita et al., 2006). In order to distinguish if Mast binds to pausing microtubules or if it is Mast itself that pauses, it would be important to analyze the localization of the protein in cells incubated with low doses of taxol to abolish the dynamic behavior of microtubules.

Moreover, our results also suggest that the N-terminal region of the protein has an important role in regulating the dynamic behaviour of Mast since the lack of this region results in the ability of this truncated fragment to undergo retrograde movement. More studies must be done to investigate if the retrograde movement of this fragment appears from association with depolymerising microtubules ends or if Mast itself moves toward the minus ends. From the available data, we favor the last hypothesis since the retrograde movement ($5.08 \pm 1.66 \mu\text{m}/\text{min}$) is slower than the depolymerising rate described for S2 cells ($9.33 \pm 2.22 \mu\text{m}/\text{min}$) (Sousa et al., 2007). However, we need to analyze cells expressing both, RFP-tubulin and EGFP-Mast⁴⁴⁶⁻¹⁴⁹¹ to determine the retrograde rate and depolymerising rate in the same microtubules, and visualize their relative locations. In addition, it would also be important to analyze the behavior of EGFP-Mast⁴⁴⁶⁻¹⁴⁹¹ with low doses of taxol, to abolish depolymerization. From our results, we speculate that the N-region of Mast is required for the release from depolymerising microtubule ends or from proteins that exhibit retrograde movement.

Finally, in the last part of our work we give special attention to the function of Mast at kinetochores. We demonstrated that, like CLASP1 (Maiato et al., 2003a), Mast has a dynamic localization at kinetochores, accumulating at high levels during prometaphase and rapidly decreasing at metaphase and anaphase. We propose that the release of Mast from kinetochores depends on dynein and possibly through CLIP190. In addition, our results also suggest that Mast and Dynein play antagonistic roles on kinetochore-microtubules and that this contributes to spindle bipolarity. We found that in the absence of dynein, Mast-depleted cells could form bipolar spindles without collapse, moreover we show that this rescue of spindle bipolarity is correlated with a rescue of microtubule polymerizing rate, which is lost upon depletion of Mast. These data suggest that Mast is not involved in the direct incorporation of tubulin at the kinetochore-microtubule plus-ends, but rather regulates this process. Moreover, our data also suggest that dynein is essential,

not for kinetochore-microtubule attachment *per se*, but to generate proper interactions between the plus-ends of microtubules and the kinetochore proteins, namely depolymerases. Overall, we propose that tubulin incorporation at the microtubule plus-ends of mature k-fibers in the absence of both, Mast and dynein, might be restored because microtubules are no longer pushed into the kinetochore structure by dynein and their plus-ends are free from the full action of depolymerases.

This work contributed to a better understanding of the different functional domains of Mast, characterizes Mast as a microtubule plus-ends tracking protein and it helped to elucidate the role of Mast in the vast and complex mechanism of spindle dynamics.

PART IV

MATERIALS AND METHODS

1. Construction of recombinant plasmids

The strategies to construct the recombinant plasmids are described in Appendix 3. Constructs pMT-EGFP-Mast¹⁻¹⁴⁹¹, pMT-EGFP-Mast¹⁻⁹²², pMT-EGFP-Mast¹⁻⁴⁴⁵, pMT-EGFP-Mast²³⁴⁻⁸⁶⁶, pMT-EGFP-Mast⁴⁴⁶⁻¹⁴⁹¹, pMT-EGFP-Mast⁹³¹⁻¹⁴⁹¹ and pUASp-EGFP-Mast, were made by Catarina Lemos and the construction strategy is described in "Catarina Lemos (2004) *Caracterização fenotípica e molecular do mutante mast (v40): um gene envolvido na divisão celular*. Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto" and (Lemos et al., 2000). Constructs pMT-EGFP-Mast¹⁻⁶⁶³ and pMT-EGFP-Mast⁴⁴⁶⁻⁶³² were made by Susana Gouveia and the cloning strategy is at "Susana Montenegro Gouveia (2005) *Functional analysis of microtubule binding domain of Mast*. Universidade de Aveiro".

1.1 Construction of pMT-EGFP-Mast¹⁰³⁵⁻¹⁴⁹¹

The insert Mast¹⁰³⁵⁻¹⁴⁹¹ was amplified by PCR using Mast cDNA (LD11488) as template, the primers *Kt2* - 5' CCTGTATAACCTGAATTCCCGCAAATGACCC 3' (creates a EcoRI site) and *RwMast* - 5' GGTGCTGATGCAGGGATCCTATTAAGGGCTCC 3' (creates a BamHI site), and the enzyme Vent[®] DNA polymerase (New England Biolabs) with proof reading activity, using the following program:

95°C - 5min	}	30 cycles
95°C - 30sec		
55°C - 30sec		
72°C - 1min 30sec		
72°C - 10min		

PCR product was digested with EcoRI and BamHI and subcloned into pMT-EGFPC1 digested with the same restriction enzymes.

1.2 Construction of pMT-EGFP-Mast¹¹³⁹⁻¹⁴⁹¹

The insert Mast¹¹³⁹⁻¹⁴⁹¹ was amplified by PCR using Mast cDNA (LD11488) as template, the primers *Kt3* - 5' GCACAACATTGAGAATTCGTCGGAGGAGATCC 3' (creates a EcoRI site) and *RwMast* - 5' GGTGCTGATGCAGGGATCCTATTAAGGGCTCC 3' (creates a BamHI site), and the enzyme Vent[®] DNA polymerase (New England Biolabs) with proof reading activity, using the following program:

95°C - 5min	}	30 cycles
95°C - 30sec		
55°C - 30sec		
72°C - 1min 30sec		
72°C - 10min		

PCR product was digested with EcoRI and BamHI and subcloned into pMT-EGFPC1 digested with the same restriction enzymes.

1.3 Construction of pMT-EGFP-Mast¹²²⁰⁻¹⁴⁹¹

The insert Mast¹²²⁰⁻¹⁴⁹¹ was amplified by PCR using Mast cDNA (LD11488) as template, the primers *Kt4* - 5' CCAAGTCACCAACTCGAGATGCCAAGGTGATC 3' (creates a XhoI site) and *RwMast* - 5' GGTGCTGATGCAGGGATCCTATTAAGGGCTCC 3' (creates a BamHI site), and the enzyme Vent[®] DNA polymerase (New England Biolabs) with proof reading activity, using the following program:

95°C - 5min	} 30 cycles
95°C - 30sec	
55°C - 30sec	
72°C - 1min 30sec	
72°C - 10min	

PCR product was digested with XhoI and BamHI and subcloned into pMT-EGFPC1 digested with the same restriction enzymes.

2. Subcloning

Digestion of plasmid DNAs or PCR products and DNA modifications were performed according to the manufacturer's instructions. Generally, 1 ug of DNA was digested with 1U of restriction enzyme in a final volume of 20 µl. The Klenow enzyme (Roche Applied Science) was used to generate blunt ends by filling-in DNA 5' overhangs or by removing 3' overhangs. After digestion, linearized vector was 5' dephosphorylated by adding 20U of Alkaline phosphatase (Boehringer) to the restriction reaction. Restriction products were separated by agarose gel and were purified using the QIAquick Gel Extraction Kit (Qiagen). Ligations were performed by T4 DNA ligase (Gibco BRL) using a molar ratio of 1:3 vector:insert, in a final volume of 20 µl, and were incubated overnight at 16 °C. Ligation products were used for transformation of competent E.coli XL1 blue cells.

2.1. Competent cells

One colony of XL1-blue from a fresh LB-agar (see Appendix 2 for recipe) plate containing 12.5 µg/ml of tetracycline was grown ON in 5 ml of LB/tet at 37 °C. This culture was diluted 1:100 in 250 ml of LB and incubated at 37 °C until reach an OD_{600nm} of 0.3 (~3.5 hr). Cells were centrifuged 10 min at 3500 rpm at 4 °C, resuspended in 12.5 ml (1/20 culture volume) of resuspending buffer (10 mM PIPES pH 7, 16 % glycerol, 100 mM CaCl₂) and left on ice for 3 hr. Aliquots were frozen in liquid nitrogen and stored at -80 °C.

2.2 Transformation of competent cells

Half of the ligation reaction (10 μ l) was mixed with 100 μ l of competent cells. Cells were incubated on ice 30 min and then heat shocked at 42 °C for 45 sec and put back on ice for 2 min. 300 μ l of LB were added and cells incubated for 1 hr at 37 °C in an orbital incubator. Cells were centrifuged at 4000 rpm for 3 min and after discard 300 μ l of the supernatant, pellet was resuspended on the 100 μ l left. Cells were plated on LB plates containing the appropriate selective antibiotic, generally 50 μ g/ml of ampicillin, and grown ON at 37 °C. At the day after colonies were picked up and transferred into 4 ml of LB/antibiotic and grown ON at 37 °C for a posterior analysis of positive constructs through a small scale plasmid DNA isolation using the 'boiling' method. These plasmid DNAs were then subjected to digestions with appropriate restriction enzymes to confirm the presence of the correct construct. DNAs from positive colonies were isolated and purified using QIAGEN Plasmid Midi Kit (Quiagen) and confirmed by sequencing.

2.3 Small scale DNA plasmid isolation using the 'boiling' method

Cells from 1 ml bacteria culture were centrifuged 3 min at 13200 rpm. Pellet was resuspended in 200 μ l of STET buffer (see Appendix 2 for recipe) with 4 μ l Lysozyme (50 mg/ml) and boiled 1 min. The samples were centrifuged 10 min at 13200 rpm and the pellet was removed with a toothpick. 200 μ l of isopropanol were added to the supernatant in order to precipitate plasmid DNA and the solution was centrifuged 10 min at 13200 rpm. The pellet was air dried and the precipitated plasmid DNA was resuspended in 20 μ l of ddH₂O.

3. Transient Transfections

Transfections were performed using the calcium-phosphate method. *Drosophila* S2 cells (3 ml of 1×10^6 cells/ml) were grown in Schneider's medium (Sigma) with 10 % foetal bovine serum (FBS), at 25 °C over 24 hr. Cells were then incubated for 16 hr at 25 °C with a transfection mix [19 μ g of plasmid, 36 μ l of CaCl₂ 2 M and 245 μ l of sterile water, and 300 μ l of 2x HEPES-Buffered Saline (see Appendix 2 for recipe)]. The calcium phosphate solution was removed by cell centrifugation (3 min at 8000 rpm) and cells were then washed and re-suspended with complete Schneider's medium. After 12 hr incubation at 25 °C, expression of EGFP-constructs was induced from the metallothionein promoter by adding 1.0 mM CuSO₄ for 16 hr at 25 °C. Cells were analyzed either by immunofluorescence for co-localization studies or by time-lapse microscopy every 2

seconds in a Zeiss Axiovert 200M equipped with a AxioCam MR (Carl Zeiss, Germany), for dynamicity studies.

4. Immunofluorescence of *Drosophila* S2 cells

For immunofluorescence, 2×10^5 cells were centrifuged onto slides at 1000 rpm for 5 min, fixed in 3.7% formaldehyde (Sigma-Aldrich, USA) in PHEM (see Appendix 2 for recipe) for 12 min and detergent-extracted with PBST 0.5% (0.5% Triton X-100 in PBS, see Appendix 2 for recipe) three times for 5 min. In some experiments, cells were fixed in pre-cold methanol for 10 min at -20 °C and re-hydrated three times 5 min with PBST 0.1%. Blocking was performed in PBST 0.1% with 10% of FBS for 30 min at room temperature. Cells were incubated at least 1hr at room temperature (RT) or overnight at 4 °C in primary antibodies diluted in blocking solution. After washing three times with PBST 0.1%, cells were incubated with secondary antibodies according to manufacturer's instructions (Molecular Probes). Slides were washed again three times with PBST 0.1% for 5 min and mounted in Vectashield medium containing DAPI (Vector).

For microtubule depolymerization, cells were incubated 4 hours with 30 μ M colchicine (Sigma-Aldrich) and for proteasome inhibition, 2 hours with 20 μ M MG132 (Calbiochem). Cold treatment was performed by incubating cells 10 minutes on ice. Fluorescence images were acquired on an AxioImager Z1 microscope (Carl Zeiss, Germany) using an AxioCam MR ver.3.0 (Carl Zeiss, Germany), deconvolved with Huygens Essential version 3.0.2p1 (Scientific Volume Imaging, Hilversum, The Netherlands), projected using ImageJ 1.3v software (<http://rsb.info.nih.gov/ij/>) and processed with PhotoShop CS (Adobe Microsystems, CA).

Mast intensity levels at kinetochores of S2 cells treated with either MG132 or colchicine were measured using ImageJ 1.3v software (<http://rsb.info.nih.gov/ij/>). The intensity of Mast and Cid was measured in individual kinetochores for each cell. After subtracting the respective backgrounds, it was expressed as a ratio of Mast/Cid intensity for each kinetochore. Spindle length quantification was obtained by measuring the distance between spindle poles using the software Axiovision 4.5 (Carl Zeiss, Germany).

5. Antibodies

Primary antibodies used for immunofluorescence were 1:5000 anti- α -tubulin mouse B-5-1-2 (Sigma- Aldrich), 1:500 anti- γ -tubulin mouse GTU88 (Sigma-Aldrich), 1:2000 anti-Cid rat (C.E.S. and Sore Steffensen, IBMC, University of Porto, Portugal, unpublished), 1:10 anti-Mast rabbit IP726 (Lemos et al., 2000), 1:150 anti-KLP10A (Rogers et al., 2004).

Secondary antibodies used for immunofluorescence were 1:2000 Alexa 488, Alexa568, Alexa 594, 647 from mouse, rabbit and rat (Molecular Probes)

Primary antibodies used for immunoblotting were 1:8000 anti- α -tubulin mouse DM1A, Sigma-Aldrich), 1:10000 anti-Dynein Heavy Chain mouse P1H4 (McGrail and Hays, 1997), 1:100 anti-Mast rabbit IP726 (Lemos et al., 2000) and 1:1500 anti-ZW10 rabbit (Williams et al., 1992).

Secondary antibodies used for immunoblotting were HRP mouse and rabbit (Vector Laboratories).

6. SDS-PAGE and Western Blot

Protein extracts were obtained by centrifuging 1×10^6 cells, 10 min at maximum velocity at 4 °C, cells were washed with PBS supplemented with protease inhibitor (Complete, Roche), centrifuged again and resuspended in 10 μ l of SDS-PAGE sample buffer (see Appendix 2 for recipe). After boiling for 5 min, samples were loaded on a SDS-polyacrilamide-gel electrophoresis and transferred to a nitrocellulose membrane (Schleicher & Shuel) using a semi-dry system for 1hr at 25V. To analyse transference efficiency, membrane was incubated for 5 min with Ponceau S solution (see Appendix 2 for recipe) and the excess of dye was washed with ddH₂O. After analyse protein transference, Ponceau was washed with TBST and membrane was blocked 2 hr at RT or overnight at 4 °C with 3% BSA and 1% fish skin gelatin-FSG (Sigma) in TBST (see Appendix 2 for recipe). All antibodies were diluted in the same solution as blocking and incubated for 1hr at RT or ON at 4°C. After incubation with primary antibody, membrane was washed three times 10 min with TBST with 1% BSA and incubated for 45 min with secondary antibody. After wash two times 5 min with TBST and once with PBS, blots were developed by ECL method (Enhanced Chemiluminescent) (see Appendix 2 for recipe). The membrane was then used to impress an X-ray film (Fuji Medical X-Ray Film) and the results were obtained by manual or automatic development of the film.

7. Double stranded RNA interference in *Drosophila* S2 cells

The depletion of Mast, Dynein heavy chain and ZW10 from *Drosophila* S2 cells by RNAi was performed as previously described (Maiato et al., 2003b), except that the amount of dsRNA per well was 30 μ g. Double-stranded RNAs (dsRNAs) of ~700 bp were generated from PCR products using specific primers that incorporate a 5' T7 RNA polymerase promoter site (underlined sequence):

Protein	Sequence
Mast	Fw 5' <u>TAATACGACTCACTATAGGG</u> GAAGGACGAATAGACATT 3'
	Rw 5' <u>TAATACGACTCACTATAGGG</u> TCCTGTTTGACCTGGTCG 3'
Dynein	Fw 5' <u>TAATACGACTCACTATAGGG</u> AGTAGCCCGAGGAATGATCC 3'
	Rw 5' <u>TAATACGACTCACTATAGGG</u> CCATTGGGGAGCTAAGTGG 3'
ZW10	Fw 5' <u>TAATACGACTCACTATAGGG</u> TGGCACCTACGTTTCGATT 3'
	Rw 5' <u>TAATACGACTCACTATAGGG</u> ATCATGCAGCGTGGGAAG 3'

The PCR products were purified and used as templates for the transcription reaction using MEGAscript T7 kit (Ambion) according to the manufacturer's instructions. In all RNAi experiments, 30 µg of dsRNA (in double depletion assays were used 30 µg of each dsRNA) was added to 1×10^6 cells in 1 ml of Schneider's medium (Gibco BRL) and incubated at 25 °C for 1 hr, in a six-well-plate. After 1hr, 2 ml of complete medium, Schneider's medium supplemented with 10% of FBS (foetal bovine serum) (Gibco BRL), were added and cells were incubated 120 hrs, at 25 °C. After five days of add dsRNA, cells were collected and processed for western blots, immunofluorescence analysis or in vivo assays. Cells that were subjected to drug treatments were incubated with the drug just before harvest.

8. Time-lapse fluorescence imaging of S2 cells

S2 cells stably expressing GFP-tubulin (kindly provided by Ron Vale) were incubated with dsRNA and plated after 120 hours for at least 30 minutes on glass coverslips previously coated with 0.5 µg/µl concanavalin A (Sigma). When required, coverslips were placed in special incubations chambers (Rose et al., 1958) with Schneider's medium supplemented with 10% of FBS and images were collected every 30 seconds using a spinning disk confocal system Andor Revolution XD (ANDOR Technology).

9. Plus-end dynamics of EGFP-Mast/fragments in S2 cells

S2 cells transiently transfected with each EGFP-fusion protein (described above in 3) were plated in coverslips previously coated with 0.5 µg/µl concanavalin A (Sigma) and mounted in a special system (see point 8). Images were collected every 2 seconds using a Zeiss Axiovert 200M microscope (Carl Zeiss, Germany) with an Axiocam MR (Carl Zeiss, Germany). The movies were assembled using AxioVision 4.3 software (Carl Zeiss, Germany) and the +TIP dynamics were tracked throughout the sequence of images using a mouse based tracking software Retrac version 2.10.05 (<http://mc11.mcri.ac.uk/retrac/>).

10. Quantification of microtubule flux

The flux rate was measured by fluorescent speckle microscopy (FSM) (Waterman- Storer et al., 1998) in S2 cells stably expressing low levels of GFP-tubulin under control of a leaky metallothionein promoter without induction and CID-mCherry (a gift from H. Maiato). By collecting images every 5 seconds at 25 °C, using a spinning disk confocal system (ANDOR Technology) we could follow the movement of the fluorescent speckles within the spindle. This movement was represented on a kymograph, keeping the labelled kinetochores as the reference point. The slope of the lines obtained from individual speckles was used to calculate flux rates on kinetochore microtubules. All kymographs were analyzed using a program from a custom routine written in Matlab (Natick, MA) (Matos et al., 2009).

11. Fly stocks and Embryos time-lapse analysis

As a control strain we used W118 obtained from the Bloomington Stock Center (IN), mRFP-Rod was a gift from Roger Karess (CNRS, Centre de Genetique Moleculaire, Gif sur Yvette, France) and mRFP-Cid a gift from Stefan Heidmann (Bayreuth Center for Molecular Biosciences (BZMB), University of Bayreuth,, Germany). Mast⁴ was previously described (Lemos et al., 2000); UASp-EGPF-Mast and UASp- EGPF-Kt2 transgenic flies were generated by standard P-element-mediated germline transformation. To drive transcription in embryos, the maternal- α -tub VP16 GAL4 driver was used (Bloomington Stock Centre). Embryos at 0-2 hours were collected, manually dechorionated and mounted in oil holocarbon 700 (Sigma). Live images were acquired every 10 seconds for EGFP-Mast or EGFP-Mast-Kt2 kinetochore intensity measurements using a laser-scanning confocal microscope Leica SP2 AOBs SE and processed using Software LCS 2.61 (Leica Microsystems, Wetzlar, Germany). Syncytial embryos expressing UASp-EGPF-Mast or UASp-EGPF-Kt2 undergoing cycle 13 were used for kinetochore fluorescence quantification and the different movies were aligned by the anaphase onset. Quantitative analysis was performed using ImageJ 1.3v software.

12. Quantitative analysis of EGFP-Mast transgene complementation

To test the ability of the EGFP-Mast transgene to complement mutations in the endogenous mast gene, the transgene was expressed in a mutant background (mast⁴/mast⁴) under the control of the Act5C-Gal4 driver (Bloomington) (w; UAS-EGPF-Mast/Act-Gal4; mast⁴/mast⁴), flies from the same cross lacking the driver (w; UAS-EGPF-Mast/Cyo; mast⁴/mast⁴) were used as controls. Third instar larvae brains were dissected in

0.7% NaCl and the attached imaginal discs were removed as much as possible. Individual brains were fixed in acetic acid 45% for ~30 seconds followed by 60% acetic acid, squashed between a slide and a coverslip containing a drop of 60% acetic acid, frozen in liquid nitrogen and stained with DAPI for quantification.

13. Cytological analysis of *Drosophila* neuroblasts

Brains from third instar larvae under control of the neuroblast-specific driver MZ1061 (a gift from Joachim Urban, Institute of Genetics, University of Mainz, Germany), expressing EGFP-Mast on a wild type background, or on a mutated background (MZ1061; UAS-EGFP-Mast; *mast*⁴/*mast*⁴), were dissected in 0.7% NaCl. The attached imaginal discs were removed as much as possible and the brains fixed in 3.7% formaldehyde in 0.7% NaCl for 20 minutes. After 3 washes in 0.7% NaCl it were incubated for 1h with RNase in 0.1% Triton-X, 0.7% NaCl and then DNA stained with ToPro3 diluted 1:11000 in 0.7% NaCl (Molecular Probes) for 2 min. The brains were mounted in VectraShield with ventral ganglia facing the cover slip. Z-stack confocal images were acquired using the Leica Confocal SP2 system (Leica Microsystems, Germany).

14. Statistical analyses

All the statistical analyses were performed using the SPSS version 14.0 (SPSS Inc, Chicago, IL, USA). After analyse the distribution, we use the independent samples t-test (2-tailed) (parametric) when the distribution is simultaneously symmetric and mesokurtic (normal) or Mann-Whitney test (non-parametric) if these two requisites are not satisfied. To determine if a variable is symmetric, we calculate the ratio between the Skewness coefficient and the standard error, if it is between 2 and -2 the distribution is symmetric. To determine if a variable is mesokurtic, the Kurtosis coefficient is divided by the standard error, if it is between 2 and -2 the distribution is mesokurtic. The significance levels used were $P < 0.01$ and $P < 0.001$.

PART V

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PART VI

APPENDIXES

APPENDIX 1

Abbreviations

a.a.: aminoacids
APC: Adenomatous Polyposis Coli
APC/C: Anaphase-Promoting Complex/Cyclosome
Ab: Antibody
BDGP: Berkeley *Drosophila* Genome Project
bp: base pairs
BSA: Bovine Serum Albumin
Bub: budding uninhibited by benzimidazole
C. elegans: *Caenorhabditis elegans*
CAK: Cdk-activating kinase
CCAN: Constitutive Centromere-Associated Network
Cdc: Cell division cycle
Cdk: Cyclin-dependent kinase
CENP: Centromere-associated Protein
CID: Centromere identifier
CKI: Cdk-inhibitor
CLASP: CLIP-Associated Proteins
CLIP: Cytoplasmic Linker Proteins
DAPI: 4',6'-diamidino-2-phenylindole
DNA: deoxyribonucleic acid
dsRNA: double stranded RNA
DTT: dithiothreitol
EBs: End Binding proteins
E. coli: *Escherichia coli*
ECL: Enhanced ChemiLuminescence
EDTA: Ethylenediaminetetracetic acid
EGFP: Enhanced Green Fluorescent Protein
EM: Electron Microscopy
FBS: Fetal Bovine Serum
FSG: fish skin gelatine
g: gram
G1: Gap phase 1
G2: Gap phase 2
GFP: Green Fluorescent Protein
GSK3 β : Glycogen Synthase Kinase-3 β
hr: hours
HRP: Horse redish peroxidase

IF: immunofluorescence
ipMTs: interpolar microtubules
Kb: kilobase
KBD: Kinetochore-Binding Domain
kDa: kiloDalton(s)
K-fiber: kinetochore fiber
Klp: Kinesin-like protein
kMTs: Kinetochore Microtubules
Kt: Kinetochore
L: Liter
LB: Luria-Bertani culture medium
M: Molar
Mad: Mitotic-arrest deficient
MAP: Microtubule Associated Proteins
MBD: Microtubule-Binding Domain
MCAK: Mitotic Centromere Associated Kinesin
min: minutes
MIPs: Microtubule Interacting Proteins
ml: mililiter
mM: milimolar
MPF: Maturation/Mitosis-Promoting Factor
mRFP: monomeric Red Fluorescence Protein
mRNA: messenger RNA
Msps: Mini-Spindles
MT(s): Microtubule(s)
MTOC: Microtubule-Organizing Center
n: number of samples in the study
NEBD: Nuclear Envelope Breakdown
nm: nanometer
OD: Optical density
ORF: Open Reading Frame
PAGE: Polyacrilamide Gel Electrophoresis
PCM: Pericentriolar Material
PBS: Phosphate-buffered saline
RNA: ribonucleic acid
RNAi: RNA interference
rpm: Rotations per minute

RT: room temperature
RZZ: Rod-ZW10-Zwilch
SAC: Spindle Assembly Checkpoint
S phase: DNA synthesis phase
***S. cerevisiae*:** *Saccharomyces cerevisiae*
***S. pombe*:** *Schizosaccharomyces pombe*
S2: *Drosophila* Schneider 2 cell line
SD: standard deviation
SDS: Sodium dodecyl sulphate
SDS-PAGE: Sodium dodecyl sulfate-Polyacrylamide Gel Electrophoresis
sec: seconds
ssDNA: single stranded DNA
ssRNA: single stranded RNA
Tris: Tris(hydroxymethyl)aminomethane
t-test: Student's *t* test
UAS: upstream activating sequence
UV: ultraviolet
wt: wild type
***X. laevis*:** *Xenopus laevis*
 γ -TuRC: γ -Tubulin Ring Complex
 μ g: microgram
 μ l: microliter
 μ m: micrometer
+TIPs: Microtubule Plus-end Tracking Proteins

APPENDIX 2

Recipes

LB Medium:

1 % Tryptone
0.5 % Yeast extract
1 % NaCl

LB Agar:

1.5 % (w/v) Agar in LB medium

Ampicillin plates:

Autoclaved LB Agar was melted and ampicillin added to a final concentration of 50 µg/ml

Tetracycline plates:

Autoclaved LB Agar was melted and tetracycline added to a final concentration of 12.5 µg/ml

STET Buffer:

10 mM Tris-HCl pH 8.0
0.1 M NaCl
1 mM EDTA pH 8.0
5 % (v/v) Triton X-100

2x HEPES-Buffered Saline:

50 mM HEPES
1.5 mM Na₂HPO₄
280 mM NaCl, pH 7.1

PHEM:

60 mM PIPES
25 mM HEPES pH 7.0
10 mM EGTA
4 mM MgSO₄

PBS (Phosphate-Buffered Saline) pH 7.4:

137 mM NaCl
2.7 mM KCl
10 mM Na₂HPO₄
1.8 mM KH₂PO₄

2x SDS-PAGE sample buffer:

100 mM Tris-HCl pH 6.8
4% (w/v) SDS
0.2% (w/v) Bromophenol blue
20% (v/v) Glycerol
200 mM DTT (dithiothreitol)

Protein Electrophoresis:

Stacking gel: 4% acrilamide; 125 mM Tris-HCl, pH 6.8; 0.1% SDS;

Separating gel: 7.5% acrilamide; 375 mM Tris-HCl, pH 8.8; 0.1% SDS;

Running buffer: 25 mM Tris, pH 8.3; 250 mM Glycine; 0.1% SDS

Ponceau S:

0.1% Ponceau

5% acetic acid

TBST:

50 mM Tris-HCl, pH 7.5

150 mM NaCl

0.05% Tween 20

Transfer Buffer:

25 mM Glycine

192 mM Tris pH 8.3

Enhanced Chemiluminescent (ECL):

Solution A: 10 ml Tris 100 mM pH 8.5, 44 µl Cumaric acid (Sigma) 90 mM and 100 µl Luminol (FLUKA) 250 mM;

Solution B: 10 ml Tris 100 mM pH 8.5 and 6 µl H₂O₂ 30% (Merck)

Solution A and B are mixed and incubated with the membrane at the time of ECL detection.

Schneider's Insect Medium:

Schneider's Insect Medium, with L-glutamine and sodium bicarbonate, (Invitrogen) was supplemented with 10% (v/v) FBS (Invitrogen)

Fly Media (3L):

Mixture 1

ddH₂O: 2000 ml

Molasses: 103 ml

Agar: 24 g

Mistura 2

ddH₂O: 1000 ml

Cornmeal: 240 g

Yeast extract: 54 g

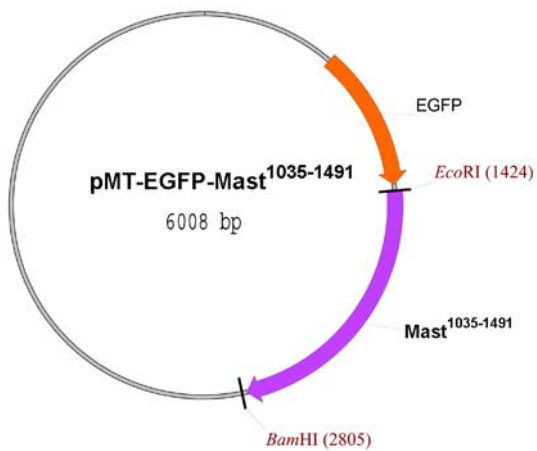
Flour of soy: 30 g

Malte: 60 g

After boiling mixture 1, add mixture 2 and let it boil for 35 minutes. When it cools down to 60 ° C, add 17.4 ml of a solution composed by 500 ml of propionic acid and 32 ml of phosphoric acid 85%.

APPENDIX 3

Plasmids

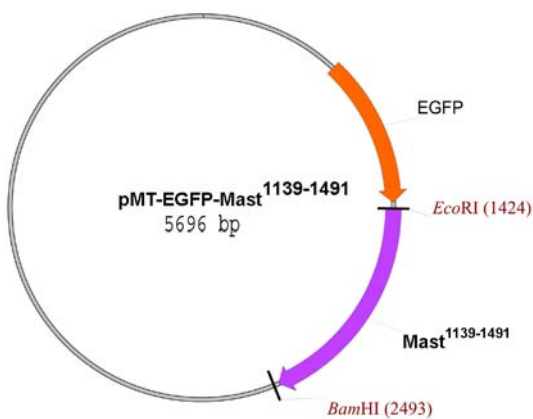
pMT-EGFP-Mast¹⁰³⁵⁻¹⁴⁹¹

Mast¹⁰³⁵⁻¹⁴⁹¹ was amplified by PCR using Mast cDNA (LD11488) as template and the following primers:

Kt2 - 5'CCTGTATAACCT^{EcoRI}GAATTCCCCGCAAATGACCC3'

RwMast - 5'GGTGCTGATGCAGG^{BamHI}GATCCTTATTAAGGGCTCC3'

Digested PCR product was cloned into pMT-EGFPC1 using the same restriction sites (EcoRI and BamHI).

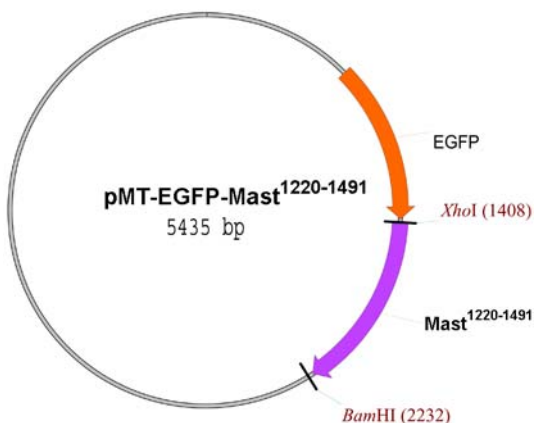
pMT-EGFP-Mast¹¹³⁹⁻¹⁴⁹¹

Mast¹¹³⁹⁻¹⁴⁹¹ was amplified by PCR using Mast cDNA (LD11488) as template and the following primers:

Kt3 - 5'GCACAACATTCAG^{EcoRI}AATTCGTCCGAGGAGATCC3'

RwMast - 5'GGTGCTGATGCAG^{BamHI}GGATCCTTATTAAGGGCTCC3'

Digested PCR product was cloned into pMT-EGFPC1 using the same restriction sites (EcoRI and BamHI).

pMT-EGFP-Mast¹²²⁰⁻¹⁴⁹¹

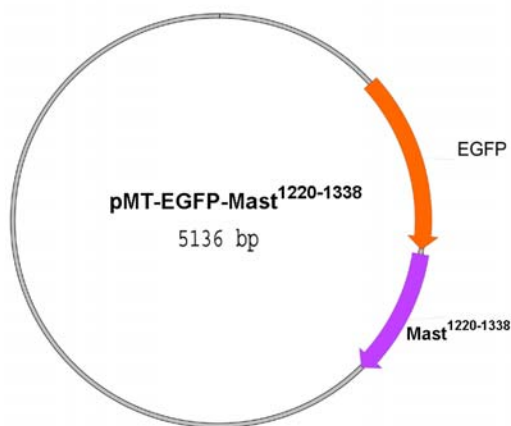
Mast¹²²⁰⁻¹⁴⁹¹ was amplified by PCR using Mast cDNA (LD11488) as template and the following primers:

Kt4 - 5'CCAAGTCACCA^{XhoI}CTCGAGATGCCAAGGTGATC3'

RwMast - 5'GGTGCTGATGCAGG^{BamHI}GATCCTTATTAAGGGCTCC3'

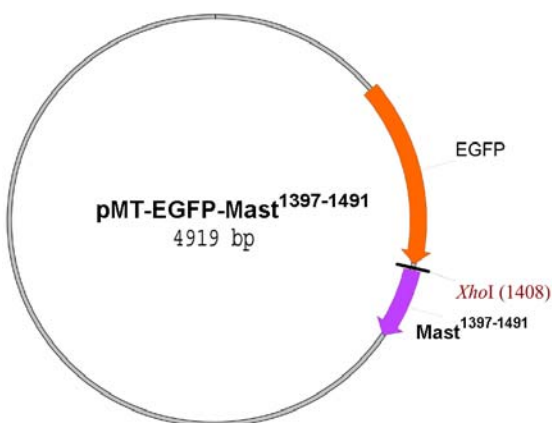
Digested PCR product was cloned into pMT-EGFPC1 using the same restriction sites (XhoI and BamHI).

pMT-EGFP-Mast¹²²⁰⁻¹³³⁸



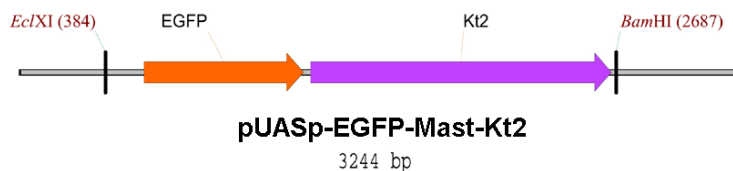
pMT-EGFP-Mast¹²²⁰⁻¹³³⁸ was constructed by digesting pMT-EGFP-Mast¹²²⁰⁻¹⁴⁹¹ with BstEII and BamHI. Plasmid cohesive ends were transformed into blunt ends by Klenow enzyme (Roche Applied Science) and the plasmid was religated.

pMT-EGFP-Mast¹³⁹⁷⁻¹⁴⁹¹



pMT-EGFP-Mast¹³⁹⁷⁻¹⁴⁹¹ was constructed by digesting pMT-EGFP-Mast¹²²⁰⁻¹⁴⁹¹ with XhoI and BstEII. Plasmid cohesive ends were transformed into blunt ends by Klenow enzyme (Roche Applied Science) and the plasmid was religated.

pUASp-EGFP-Mast-Kt2



The EGFP-Mast-Kt2 insert was obtained from the plasmid pMT-EGFP-Mast^{1035-1491(Kt2)} digested with EclXI and BamHI. It was subcloned into the vector pUASp digested with NotI (compatible cohesive end with EclXI) and BamHI.

APPENDIX 4

Movies Legends

Movie 2.1. Plus-end tracking dynamic behaviour of Mast. A S2 cell transiently expressing EGFP-Mast was imaged by fluorescence microscopy every 2 seconds. The accumulation of EGFP-Mast at the plus-ends of microtubules provides the typical comet-like effect of +TIPs.

Movie 2.2. Overexpression of Mast abolishes microtubule dynamics. A S2 cell overexpressing EGFP-Mast was imaged every 2 seconds by fluorescence microscopy. The overexpression of EGFP-Mast induces the formation of stable microtubule bundles with Mast decorating the entire length of the polymer.

Movies 2.3. Dynamic behaviour of the full length EGFP-Mast. A S2 cell transiently expressing EGFP-Mast was imaged by fluorescence microscopy every 2 seconds.

Movies 2.4. Dynamic behaviour of EGFP-Mast¹⁻⁹²². A S2 cell transiently expressing the fragment EGFP-Mast¹⁻⁹²² was imaged by fluorescence microscopy every 2 seconds. This fragment is less dynamic than the full length protein.

Movies 2.5. Dynamic behaviour of EGFP-Mast²³⁴⁻⁸⁶⁶. A S2 cell transiently expressing the fragment EGFP- Mast²³⁴⁻⁸⁶⁶ was imaged by fluorescence microscopy every 2 seconds. This fragment, that in mitosis induces a dominant negative phenotype, exhibits a stable behaviour when compared with the full-length Mast.

Movies 2.6. Dynamic behaviour of EGFP- Mast⁴⁴⁶⁻¹⁴⁹¹. A S2 cell transiently expressing the fragment EGFP- Mast⁴⁴⁶⁻¹⁴⁹¹ was imaged by fluorescence microscopy every 2 seconds. Contrary to the full-length Mast and the other fragments analysed, Mast⁴⁴⁶⁻¹⁴⁹¹ moves in both directions, to the periphery and to the center of the cell.

Movies 3.1. Embryos expressing EGFP-Mast. Laser-confocal time-lapse imaging of syncytial embryos at stage 13 expressing EGFP-Mast. Images were acquired every 10 seconds.

Movies 3.2. Embryos expressing EGFP-Mast-Kt2. Laser-confocal time-lapse imaging of syncytial embryos at stage12 expressing EGFP-Mast-Kt2. Images were acquired every 10 seconds.

Movies 3.3. Embryos expressing EGFP-Mast-Kt2 and mRFP-Cid. Laser-confocal time-lapse imaging of syncytial embryos at stage 11 expressing EGFP-Mast-Kt2 (green) and mRFP-Cid (red). Images were acquired every 10 seconds.

Movies 3.4. Embryos expressing EGFP-Mast. Laser-confocal time-lapse imaging of syncytial embryos at stages 12 expressing EGFP-Mast. Images were acquired every 10 seconds.

Movies 3.5. Dynein+Mast-depleted S2 cell expressing GFP-tubulin. Images were acquired every 5 seconds. The spindle first has a normal length, similar to control, which then is reduced over time.

Movies 3.6. S2 cell expressing GFP-tubulin. Images were acquired every 30 seconds.

Movies 3.7. Dynein+Mast-depleted S2 cell expressing GFP-tubulin. Images were acquired every 30 seconds. A bipolar spindle is formed and maintained over a long period before exit mitosis into anaphase.