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MICROTUBULE-ASSOCIATED PROTEINS IN KINETOCHORE FUNCTION AND SPINDLE ASSEMBLY

Instituto de Ciências Biomédicas de Abel Salazar

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MICROTUBULE-ASSOCIATED PROTEINS IN KINETOCHORE FUNCTION AND SPINDLE ASSEMBLY

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Dedicated to my wife and parents for

their love and encouraging,

to Claudio and Bill for giving me the opportunity

to learn about cells,

and to the memory of Theodor Boveri and

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ABSTRACT

Chromosome segregation during mitosis is mediated by a complex and dynamic microtubule-based machine termed the mitotic spindle. Some of the basic principles underlying the mechanisms of spindle assembly and function have been elucidated through the discovery and characterization of numerous microtubule-associated proteins. Most of these proteins can be classified as either motors, which generate force and movement along the surface of microtubules, or conventional microtubule-associated proteins (MAPs), many of which are thought to stabilize microtubules. While motors are known to participate in numerous mitotic events, the mitotic functions of MAPs remain far more elusive.

Our investigation of this problem began with the study of mutations in the gene *mast* (*multiple asters*) of *Drosophila melanogaster*. Cytological analysis of *mast* mutant cells revealed that they accumulate in prometaphase with highly condensed chromosomes organised in circles and also with severe polyploidy, suggesting a role in spindle function and mitotic exit. We took advantage of a P-element insertion in *mast*[1] allele to clone the gene and found that *mast* encodes a novel microtubule-associated protein that is widely conserved from yeast to humans. In order to determine the function of MAST in mitosis, we performed RNAi in *Drosophila* S2 tissue culture cells and found that they were unable to form a metaphase plate and instead assembled monopolar spindles with chromosomes localized close to the center of the aster. In these cells, kinetochores either failed to achieve end-on attachment or were associated with short microtubules. Remarkably, when microtubule dynamics was suppressed in MAST-depleted cells, chromosomes localized at the periphery of the monopolar aster associated to the plus-ends of well-defined microtubule bundles. Moreover, in these cells Dynein and Zw10 accumulated at kinetochores and failed to transfer to microtubules. Ultimately, MAST-deficient cells ended-up forming highly polyploid cells and exit mitosis by a rather abnormal pathway independent of sister-chromatid separation, spindle-checkpoint inactivation and Cyclin B degradation.

From this study we identified several isoforms of the human homologues of MAST, CLASP1 and CLASP2, and showed that CLASPs localize at centrosomes, spindle and kinetochores throughout mitosis and accumulate at the central spindle and midbody during late mitotic stages and cytokinesis. Moreover, we found that CLASP1 is localized preferentially near the plus-ends of growing microtubules at prometaphase *in vivo* and is also a component of a novel outer kinetochore domain that we have termed "the outer corona". Functional mapping of CLASP1 demonstrated that this localization is directed by two distinct domains and is sensitive to dynamic microtubules. A truncated form of CLASP1 lacking the kinetochore-binding domain behaved as a

dominant-negative leading to the formation of unique single or double asters comprised of radial arrays of microtubule bundles whose plus ends were associated with EB1, but not with kinetochores. Cells microinjected with antibodies specific to CLASP1 showed deficiencies in chromosome congression and originated monopolar spindles that resulted from spindle collapse with kinetochores not associated with the microtubule-plus-ends at the periphery of the aster. Suppression of microtubule dynamics in injected cells rescued the kinetochore association with the microtubule plus ends. We propose that CLASP1 is a microtubule-plus-end-associated protein whose mitotic function is to either regulate microtubule dynamics at the kinetochore, or to allow kinetochores to attach to dynamic microtubules while maintaining spindle functional integrity. Additional evidence from the dominant-negative form of CLASP1 has indicated that CLASPs may act as microtubule stabilizers responsible for the correct targeting of other microtubule-plus-end-associated proteins.

Finally, we have studied a distinct class of microtubule-associated proteins known as chromosomal passengers. We started by analysing the dynamic behaviour of these proteins in living cells. Then, in order to investigate their function during mitosis, we performed RNAi in *Drosophila* tissue culture cells. We found that the *Drosophila* INCENP and Aurora B behave as canonical chromosomal passengers and interference with their functions led to deficient metaphase chromosome congression and to abnormal kinetochore disjunction during chromosome segregation. Together, the results presented in this thesis provide new insights into the roles of two conserved families of MAPs during mitosis, namely in spindle assembly and kinetochore function.

RESUMO

A segregação cromossómica durante a mitose é mediada por uma maquinaria dinâmica e complexa que tem como base microtúbulos e que se designa por fuso mitótico. Alguns dos princípios básicos por detrás dos mecanismos responsáveis pela formação e função do fuso têm vindo a ser esclarecidos através da descoberta e caracterização de numerosas proteínas associadas aos microtúbulos. A maioria destas proteínas podem ser classificadas como motoras, que geram força e movimento através da superfície dos microtúbulos, ou como proteínas associadas aos microtúbulos convencionais (MAPs), muitas das quais se pensa estabilizarem os microtúbulos. Enquanto é sabido que as proteínas motoras participam em numerosos eventos mitóticos, as funções mitóticas das MAPs permanecem amplamente desconhecidas.

A nossa investigação deste problema foi iniciada pelo estudo de mutações no gene *mast* (*multiple asters*) em *Drosophila melanogaster*. A análise citológica de células mutantes para o gene *mast* revelou que estas se acumulam em prometafase com cromossomas altamente condensados organizados em forma de círculos e elevada poliploidia, sugerindo um papel na formação de um fuso funcional e na saída de mitose. Tirou-se partido de uma inserção de um elemento P no allelo *mast*^{1} para a clonagem do gene e descobriu-se que codificava para uma nova proteína associada aos microtúbulos extremamente conservada desde as leveduras até aos humanos. De forma a determinar a função da MAST durante a mitose, realizou-se uma técnica de interferência com ARNs de cadeia dupla em células de cultura S2 de *Drosophila* e descobriu-se que, na ausência da MAST, estas são incapazes de formar uma placa metafásica e em alternativa formam fusos monopolares com os cromossomas localizados perto do centro astral. Nestas células, os cinetocóros não se ligam nas extremidades ou estão associados com microtúbulos muito curtos. Notavelmente, quando a dinâmica dos microtúbulos é suprimida em células deficientes para a MAST, os cromossomas localizam-se na periferia do fuso monopolar e encontram-se associados com as extremidades "mais" em feixes bem definidos de microtúbulos. Adicionalmente, nestas células a dineína e o Zw10 acumulam-se nos cinetocóros, não se transferindo para os microtúbulos. Por último, células deficientes em MAST acabam por se tornar altamente poliplóides e saem de mitose por uma via altamente anormal que é independente da separação das cromatidas filhas, da inactivação do sistema de controlo de qualidade sobre a formação do fuso e da degradação da Ciclina B.

Deste estudo foram identificadas diversas isoformas de proteínas humanas homólogas da MAST, conhecidas por CLASP1 e CLASP2, que se mostrou localizarem nos centrossomas, no fuso e cinetocóros ao longo da mitose e se acumulam no fuso central e corpo médio durante os últimos estadios mitóticos e citocinése.

Adicionalmente, foi descoberto que a CLASP1 se localiza preferencialmente perto das extremidades "mais" dos microtúbulos em crescimento durante prometafase *in vivo* e é também uma componente de um novo domínio do cinetocóro que designamos de "coroa exterior". O mapeamento funcional da CLASP1 demonstrou que esta localização é governada por dois domínios distintos e que é sensível à dinâmica dos microtúbulos. Uma forma truncada da CLASP1 sem o domínio de ligação ao cinetocóro comporta-se como um dominante negativo levando à formação de centros mono- ou bi-astrais compostos por arranjos radiais de feixes de microtúbulos cujas extremidades "mais" se encontram associadas com a EB1, mas não com os cinetocóros, e cuja descrição não tem precedentes. Células micro-injectadas com anticorpos específicos para a CLASP1 mostram deficiências na congressão dos cromossomas e originam fusos monopolares que resultam do colapso do fuso, com os cinetocóros não associados com as extremidades "mais" dos microtúbulos na periferia do centro astral. A supressão da dinâmica dos microtúbulos em células injectadas reestabelece a associação dos cinetocóros com as extremidades "mais" dos microtúbulos. Nós propomos que a MAST/CLASP1 é uma proteína associada as extremidades "mais" dos microtúbulos cuja função mitótica é regular a dinâmica dos microtúbulos no cinetocóro, ou permitir que os cinetocóros se liguem a microtúbulos dinâmicos enquanto mantêm a integridade funcional do fuso. Resultados adicionais do dominante negativo da CLASP1 indicaram que as CLASPs deverão actuar como estabilizadores dos microtúbulos, responsáveis pela correcta localização de outras proteínas associadas às extremidades "mais" dos microtúbulos.

Por último, foi estudada uma classe distinta de proteínas associadas aos microtúbulos conhecidas por proteínas passageiras dos cromosomas. Começou-se por analisar o comportamento dinâmico destas proteínas em células vivas. Seguidamente, de forma a investigar a sua função durante a mitose, realizou-se um RNAi em células de cultura de *Drosophila*. Descobriu-se que as proteínas INCENP e Aurora B de *Drosophila* se comportam como passageiras dos cromossomas de uma forma canónica e a interferência das suas funções origina uma congressão metafásica dos cromossomas deficiente e uma disjunção dos cinetocóros anormal durante a segregação cromossómica. No seu conjunto, os resultados apresentados nesta tese trazem novos avanços acerca da importância durante a mitose de duas famílias conservadas de MAPs, nomeadamente ao nível da formação do fuso e na função dos cinetocóros.

RÉSUMÉ

La ségrégation des chromosomes lors de la mitose est médiée par une machinerie de microtubles complexe et dynamique, appelée fuseau mitotique. Certains des principes de base qui déterminent les mécanismes d'assemblage et la fonction du fuseau mitotique ont été élucidés grâce à la découverte et la caractérisation d'un grand nombre de protéines associées aux microtubules (MAPs). La majorité de ces protéines peut être divisée en deux catégories: les protéines motrices qui génèrent les forces et le mouvement le long des microtubules, et les protéines conventionnelles associées aux microtubules dont la plupart stabiliserait ces derniers. Bien que le rôle joué par les protéines motrices dans les nombreux événements mitotiques est bien connu, celui des MAPs est encore mal compris.

Afin d'étudier cette problématique nous avons commencé par analyser certaines mutations du gène *mast* (*multiple asters*) de *Drosophila melanogaster*. L'analyse cytologique de cellules mutantes pour mast a révélé une accumulation en prométaphase avec des chromosomes hautement condensés organisés en cercles ainsi qu'une sévère polyploïdie. Ces observations suggèrent un rôle de *mast* dans le fonctionnement du fuseau mitotique et la sortie de mitose. Nous avons utilisé à notre avantage l'existence d'un élément P inséré dans l'allèle du gène *mast1* qui nous a permis de le cloner et de montrer qu'il code une nouvelle protéine associée aux microtubules très largement conservée de la levure à l'homme. Afin de déterminer la fonction de MAST en mitose nous avons utilisé la technique d'interférence aux ARNs sur des cellules S2 de *Drosophila*. Nous avons montré que ces cellules étaient incapables de former une plaque métaphasique et qu'elles assemblaient des fuseaux monopolaires avec les chromosomes localisés à proximité du centre de l'aster. Les kinétochores ne semblent pas parvenir à capter les microtubules, cependant certains sont trouvés associés avec de courts microtubules. De façon remarquable, lorsque les microtubules ne sont plus dynamiques dans les cellules déplétées de MAST, les chromosomes sont localisés à la périphérie de l'aster monopolaire et associés aux pôles positifs de microtubules organisés en fagot. De plus, dans ces cellules, la dynéine et ZW10 s'accumulent aux kinétochores et ne sont pas transférés sur les microtubules. Finalement, les cellules déplétées de MAST deviennent fortement polyploides et sortent de mitose par une voie indépendante de la séparation des chromatides sœurs, de l'inactivation du point de contrôle lié au fuseau et de la dégradation de la Cycline B.

Cette étude nous a également permis d'identifier plusieurs isoformes des homologues humains de MAST, CLASP1 et CLASP2. Nous avons montré que les CLASPs étaient localisés aux centrosomes, au fuseau et aux kinétochores au cours de la mitose et s'accumulent sur le fuseau central et au niveau du pont cytoplasmique lors

des dernières phases de la mitose et cytocinèse. De plus, *in vivo*, nous avons montré que CLASP1 était préférentiellement localisé à proximité des pôles positifs des microtubules en croissance lors de la prométaphase. Nous avons également démontré que CLASP1 est un composant d'un nouveau domaine externe du kinétochore que nous avons appelé "la couronne externe". Une cartographie fonctionnelle de CLASP1 a démontré que sa localisation dépendait de deux domaines distincts et était sensible à la dynamique des microtubules. CLASP1 délété du domaine de liaison aux kinétochores a un effet dominant-négatif et induit la formation d'asters, simples ou doubles, qui comprennent des faisceaux radiaux de microtubules dont les pôles positifs sont associés à EB1 mais pas aux kinétochores. Les cellules micro-injectées avec des anticorps spécifiques de CLASP1 montrent des anomalies dans la congrégation des chromosomes et forment des fuseaux monopolaires. Ceci induit un effondrement du fuseau avec les kinétochores dissociés des pôles des microtubules à la périphérie de l'aster. La suppression de la dynamique des microtubules dans les cellules injectées restaure l'association des kinétochores avec le pôle positif des microtubules. Nous avons postulé que CLASP1 est une protéine associée au pôle positif des microtubules dont la fonction en mitose serait de réguler la dynamique des microtubules aux kinétochores ou, de permettre aux kinétochores de s'ancrer aux microtubules dynamiques tout en maintenant l'intégrité fonctionnelle du fuseau mitotique. Des données obtenues à l'aide des formes dominantes négatives de CLASP1 ont montré que les CLASPs pourraient agir en tant que protéines stabilisatrices des microtubules permettant un positionnement correct d'autres protéines associées au pôle positif des microtubules.

Finalement, nous avons étudié une autre classe de proteines associées aux microtubules connues sous le nom de passagers chromosomiques. Nous avons tout d'abord analysé la dynamique de ces protéines dans des cellules vivantes. Puis, dans le but d'étudier leurs fonctions pendant la mitose, nous avons utilisé la technique d'intérference aux ARNs dans une lignée de cellules de *Drosophila*. Nous avons montré que les protéines INCENP et Aurora B de *Drosophila* se comportent comme des passagers chromosomiques canoniques. Le dysfonctionnement de ces protéines induit une déficience dans la congrégation métaphasique des chromosomes et une disjonction anormale des kinétochores lors de la congrégation des chromosomes. Les résultats présentés ici apportent de nouveaux éléments à la compréhension des rôles de deux familles conservées de MAPs lors de la mitose dans le processus d'assemblage du fuseau mitotique et la fonction du kinétochore.

I. General Introduction

" There are many paths in the advancement of science, but the giant leaps in our Science of the Cell have been made by seeing. First we see and then we interpret and only then do we pursue mechanisms and theories. The earliest microscopes discovered the cell and with that came sensible thought about the nature of living things. How profound a generalization! -- that all the immense variety of life can be comprehended in cells which have so much in common with each other. A century ago, the microscope answered a number of great questions about how life goes on: fertilization, mitosis and the basis of growth, chromosomes as the carriers of heredity, development and social behavior of cells. Now you will be seeing recent technical advances in imaging, learning how the fine points of the physics and chemistry of cells are revealed by microscopes. Some new equipment is fancy and expensive, but you can deal with major questions about chromosomal genetics and cell organization with simpler traditional microscopes. The gifts of the microscopes to our understanding of cells and organisms are so profound that one has to ask: What are the gifts of the microscopist? Here is my opinion. The gift of the great microscopist is the ability to THINK WITH THE EYES AND SEE WITH THE BRAIN. Deep revelations into the nature of living things continue to travel on beams of light."

Daniel Mazia, written in January, 1996.

1. The Cell Cycle

The cell cycle can be generally described as the process by which cells reproduce and ensure the fidelity of transmission of their genome. Since the discovery that all organisms are made of cells (Schleiden, 1838; Schwann, 1839) and in 1855 Rudolf Virchow (reviewed by Wilson, 1925) realized that all cells derived from pre-existing cells (the famous aphorism "*ominis cellula e cellula*"), the cell cycle has been one of the most important areas of investigation in cell biology. It is a highly orderly and regulated process in which the most important events involve the replication of the chromosomal DNA during S phase and the segregation of the replicated chromosomes during mitosis (or M phase) (Fig. 1). Chromosome replication occurs during interphase, the interval between two consecutive mitosis. The gap between mitosis and the onset of DNA replication is called G_1, and the gap between S and M phases is known as G_2. In some situations there is a phase known as G_0 where cells can exit the cell cycle from G1 and remain in a quiescent state and when certain conditions are met to return to G1 and proceed through the cell cycle.

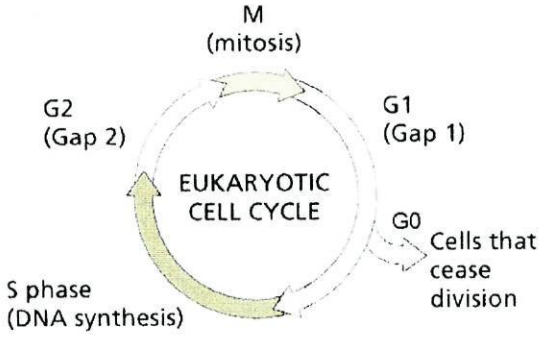


Figure 1. Schematic representation of the eukaryotic cell cycle. DNA synthesis (S phase) and mitosis (M phase) are separated by two gap phases (G1 and G2). Under special conditions cells can cease division and stay out of cycle (G0).

2. Cell Cycle Regulation

The onset of cell cycle events such as S phase and mitosis is regulated by controls that ensure the correct sequence of events, their coordination with cellular growth and correct for errors in their execution (Reviewed by Nurse, 2000). Accordingly, later events were often found to be dependent upon the successful completion of earlier events and it was reasoned that one way to accomplish that was by mean of signal transduction pathways that could link events separated either in space within the cell or in time between different phases of the cell cycle. This idea was developed further to generate the concept of checkpoint control (Hartwell and Weinert, 1989) and proposes that at different points in the cell cycle, the cell "checks" if an earlier event has been properly

executed before proceeding to a later event. Checkpoints are thought to recognize errors in the execution of a cell cycle event inducing an arrest of the cell in that particular stage until the damage is repaired. Two of these checkpoints are the DNA damage and replication controls, which arrest cells during interphase before entering mitosis when DNA is damaged or DNA replication is incomplete (Reviewed by Zhou and Elledge, 2000). However, cells not only monitor DNA structure but also chromosome segregation during mitosis. The spindle checkpoint arrests mitotic progression if the spindle is not assembled properly, or if one or more chromosomes are not properly attached to the spindle. Checkpoint controls are essential for maintaining genomic stability, since failure of these mechanisms allows cells to divide when DNA is damaged or incompletely replicated, or when chromosomes are incorrectly attached to the spindle, resulting in genetic damage that is likely to be crucial for the generation of cancer (reviewed by Elledge, 1996).

A second important concept is that certain steps in the cell cycle can be rate limiting for cell cycle progression (Nurse, 1975). Factors required for cell cycle progression were good candidates as rate-limiting steps in the cell cycle, and were identified genetically in fission yeast by mutants which accelerated cell division (Nurse and Thuriaux, 1980), and by the purification of the Maturation-promoting factor (MPF) in amphibian eggs (Wasserman and Masui, 1976). A core network of genes composed by the Cdc2p protein kinase, activated by the Cdc25p protein phosphatase and inhibited by the Wee1p protein kinase was identified in yeast and shown to regulate the onset of mitosis (Nurse, 1990). MPF was identified in *Rana* oocytes induced to enter M phase as part of the egg maturation process by injection with cytoplasm derived from eggs in M phase (Masui and Market, 1971). MPF was subsequently purified from *Xenopus* (Lohka et al., 1988) and shown to be composed of two proteins, one of 34 kDa that cross reacted with antibodies raised against the yeast Cdc2p protein kinase, and the other was shown to be a Cyclin. Cyclins were originally discovered in a search for proteins that fluctuated in level through the cell cycle of cleaving see urchin eggs (Evans et al., 1983). Cyclins and their partners, termed as Cyclin-dependent kinases (CDKs), form complexes that exhibit protein kinase activity and were shown to act as universal cell cycle regulators from yeast to mammals in various stages of the cell cycle (Lee and Nurse, 1987).

Another process that also plays an important role in cell cycle progression is proteolysis. Proteolysis not only controls the levels of a number of different Cyclins and thereby regulates the activity of different CDK complexes (reviewed by King et al., 1996) but is also essential for sister-chromatid separation during anaphase (reviewed by Nasmyth, 1999). Therefore, proteolysis is likely to be responsible for the irreversibility of cell cycle transitions.

3. Mitosis

One of the most important events during the cell cycle is chromosome segregation and cell division. Mitosis was first described in the late 1870s (Flemming, 1879; reviewed by Wilson, 1925) and thought to be required for the faithful segregation of the genetic material to daughter cells, as well as the division and distribution of cellular organelles (Reviewed by Earnshaw and Pluta, 1994). Traditionally, mitosis is divided into five stages (Fig. 2): prophase, prometaphase, metaphase, anaphase and telophase (reviewed by Gorbsky, 1992).

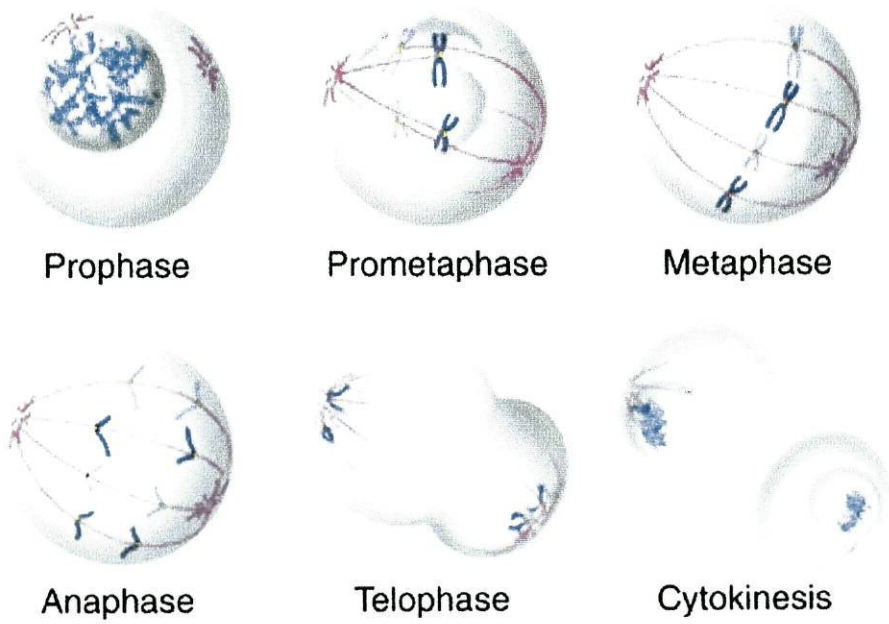


Figure 2. Schematic representation of the mitotic stages and cytokinesis. Chromatin or chromosomes are represented in light and dark blue, MTOCs and spindle microtubules are shown in pink and kinetochores are defined by yellow dots at the centromeric region of chromosomes. Chromatin starts to condense into chromosomes during prophase. The two asters nucleated from the centrosomes split apart and, after nuclear envelope breakdown, the cell is in prometaphase where the chromosomes are captured at their kinetochores by spindle microtubules. After capture by both poles, chromosomes align at the metaphase plate. During anaphase, the cohesion between sister chromatids is lost and they separate into opposite poles. At telophase, chromatids start to decondense and a cleavage furrow is formed in the equatorial region of the cell. The cytoplasm is then divided between two daughter cells during cytokinesis.

These stages were originally defined by early microscopists to reflect the gross structural changes that occur during successive stages of cell division (reviewed by Mitchison and Salmon, 2001). Accordingly, in prophase, chromatin, which owes its name due to the binding of dye molecules, starts to condense so that clearly defined chromosomes are formed. The centrosomes, which are the major microtubule-organizing centres in higher eukaryotes (reviewed by Doxsey, 2001), move to opposite

sides and large organelles are cleared from the region of the nucleus leaving space for the assembly of the mitotic spindle, a bipolar microtubule-based structure responsible for chromosome segregation during mitosis (Reviewed by Wittmann et al., 2001). In higher eukaryotes the end of prophase and the beginning of prometaphase is marked by nuclear envelope breakdown. In the cytoplasm, each chromosome initially orients randomly to one of the mitotic poles, as defined by the centrosomes, which separate further from each other until the formation of a stable bipolar spindle. The mono-oriented chromosomes are then captured by microtubules emanated from the opposite pole becoming bi-oriented, and then move towards the centre of the cell and align at the spindle equator at metaphase. Upon formation of the metaphase plate, the chromosomes show short oscillatory movements and the spindle gets shorter and broader. Subsequently, during anaphase, sister chromatids separate and migrate towards opposite poles of the spindle. This stage can be subdivided in anaphase A and B. During anaphase A, the two chromatids that comprise each chromosome loose the cohesion, split apart and move towards opposite poles while during anaphase B the spindle elongates and the two poles increase their distance from each other. Finally, during telophase each set of chromatids decondense to form two daughter nuclei and a cleavage furrow starts to form between the nuclei. This cleavage furrow then contracts and eventually gives rise to the midbody where it participates in the division of the cytoplasm and the formation of the two daughter cells during cytokinesis.

4. Regulation of Mitosis

Although still useful, the terminology described above often leads to ambiguity, especially when comparing different systems. In order to reflect more accurately the molecular pathways that underlie the key transitions during mitosis, a different and more actual nomenclature has been proposed (Pines and Rieder, 2001). These authors propose that mitosis can be subdivided into five transitional phases characterized by the activity of defined cell-cycle regulators (Fig. 3). Accordingly, during transition 1, the term "antephase" has been used to describe a short period in late G_2 just before the beginning of chromosome condensation. During antephase, chromosome condensation can be inhibited, and even reversed, by various treatments (Rieder and Cole, 1998). Once a cell has passed this stage, entry into mitosis is irreversible and can no longer be prevented. In vertebrates, events that occur in preparation for mitosis during antephase seem to be mediated by Cyclin A-CDK activity. Other kinases may also be important at this time, including the Polo-like kinases (Plks) (for reviews see Donaldson et al., 2001;

Nigg, 1998; Glover et al., 1998) and the Aurora kinase family (for reviews see Giet and Prigent, 1999; Adams et al., 2001a). Transition 2, which represents the events occurring during traditional late prophase, can be seen as the commitment to mitosis and substantial evidence indicates that this is mediated by rapid activation of Cyclin B-CDK1 (CDK1=cdc2). Once in this stage, the cell has lost its ability to inactivate Cyclin B-CDK1 and is then committed to proceed through mitosis. Transition 3 requires the satisfaction of the criteria to exit mitosis. Once committed to mitosis, pathways exist that prohibit the cell from escaping mitosis until defined criteria are met. The spindle formation, chromosome capture and alignment constitute pathways that are monitored by a cell cycle checkpoint, which prevents mitotic exit in case either of these processes do not occur properly. This checkpoint is known as spindle assembly checkpoint and monitors attachment of the kinetochore to the spindle that ultimately prevents the anaphase-promoting complex (APC/cyclosome, a multisubunit enzymatic complex involved in ubiquitin-dependent proteolysis; reviewed by Morgan, 1999), from targeting specific proteins required to hold sister chromatids together for degradation. These proteins must be destroyed for the cell to disjoin its chromatids, initiate cytokinesis and exit mitosis. Transition 3 is equivalent to the traditional stage of prometaphase and is characterized by the presence of active forms of Cyclin B-CDK1 and probably an inactive APC/C. Transition 4 comprises the mechanisms required for exiting from mitosis. Once all of the kinetochores are attached to microtubules, the spindle checkpoint is satisfied and the APC/C is fully activated after binding its activator Cdc20. Two pathways are then initiated, one that leads to the destruction of Securin, inducing the replicated chromosomes to disjoin (reviewed by Nasmyth et al., 2000) – an event that traditionally signals the start of anaphase - and another that leads to the destruction of Cyclin B, which results in the hallmark changes of telophase. In summary, this transition is defined as the stage at which APC-Cdc20 is active and the activity of Cyclin B-CDK1 is in rapid decline due to Cyclin B degradation. Finally, Transition 5, can be defined as the return to interphase. Once Cyclin B has been destroyed and CDK1 inactivated, a new nuclear envelope is assembled around the two groups of separated chromosomes, a midbody forms between the two new daughter nuclei, and the cell initiates the process of cytokinesis that ultimately separates the two daughter cells. In somatic cells, Cdc20 is degraded and replaced by the Cdh1 protein (Dawson et al., 1995; Visintin et al., 1997; Kramer et al., 2000), expanding the range of substrates recognized by the APC/C and helping to coordinate late mitotic events. This leads the cell into interphase that persists until it becomes committed to another round of DNA replication during S phase.

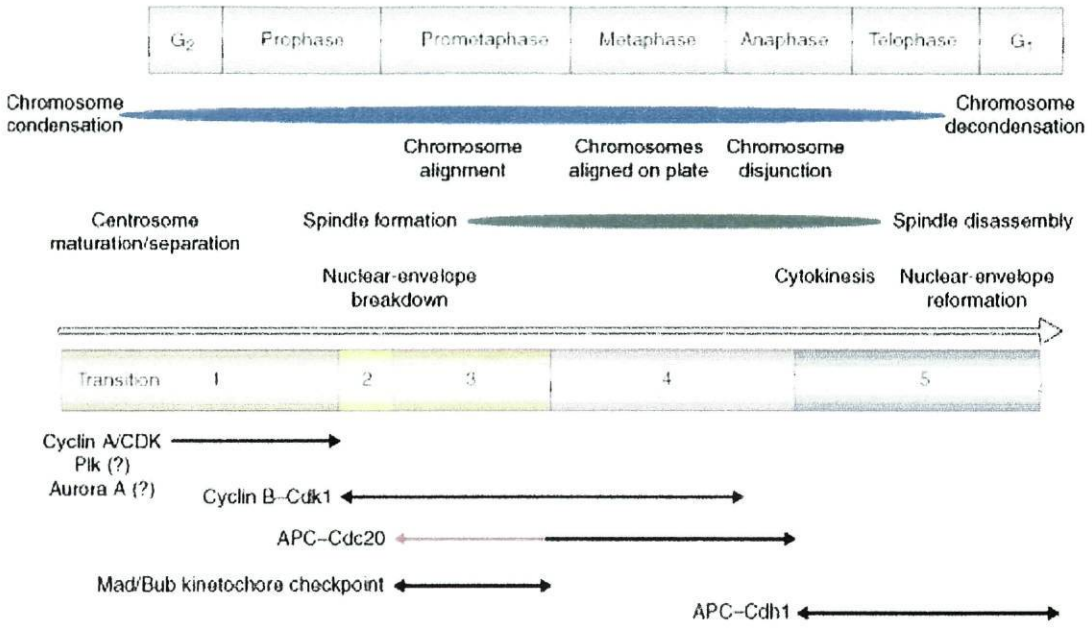


Figure 3. Comparison of the classical mitotic transitions (top) with transitions defined according to regulatory molecular events (Bottom) (adapted from Pines and Rieder, 2001).

5. The Mitotic Apparatus

Experimental work carried over many years has revealed that for chromosome segregation to take place during mitosis, the cell has to build a highly complex molecular machine, the mitotic apparatus, which plays a fundamental role in maintaining genomic stability. This machine is composed of centrosomes that nucleate microtubules forming the mitotic spindle. The assembly and maintenance of the mitotic spindle is ensured by the action of several microtubule-associated proteins and motor proteins and in its all is responsible for the kinetochore capture and subsequent movement of chromosomes during mitosis.

5.1. The Centrosome

5.1.1. Structure and Composition

The term 'centrosome' was originally coined by Bovery in 1888 to describe an autonomous permanent organ of the cell, the dynamic centre of the cell and the coordinator of nuclear and cytoplasmic division (Boveri, 1888; reviewed by Mazia, 1984). The centrosome is the major microtubule-organizing centre (MTOC) in animal cells and thereby determines the number and the distribution of microtubules (reviewed

by Kellogg et al., 1994). The functional equivalent organelle in yeast is called the spindle pole body (for reviews see Winey and Byers, 1993; Francis and Davis, 2000; Hagan and Petersen, 2000). The centrosome had been defined as a small focus of phase-dense material, surrounded by a larger region of less phase density. It is now known that the densely staining structures represent the two centrioles, open-ended cylinders comprised of nine sets of triplet microtubules linked together (Fig. 4), and the undifferentiated cytoplasm is the surrounding pericentriolar material (reviewed by Doxsey, 2001). Regardless of its apparent beauty and simplicity, the centrosome is an extremely complex organelle constituted by over 100 different proteins (reviewed by Bornens, 2002).

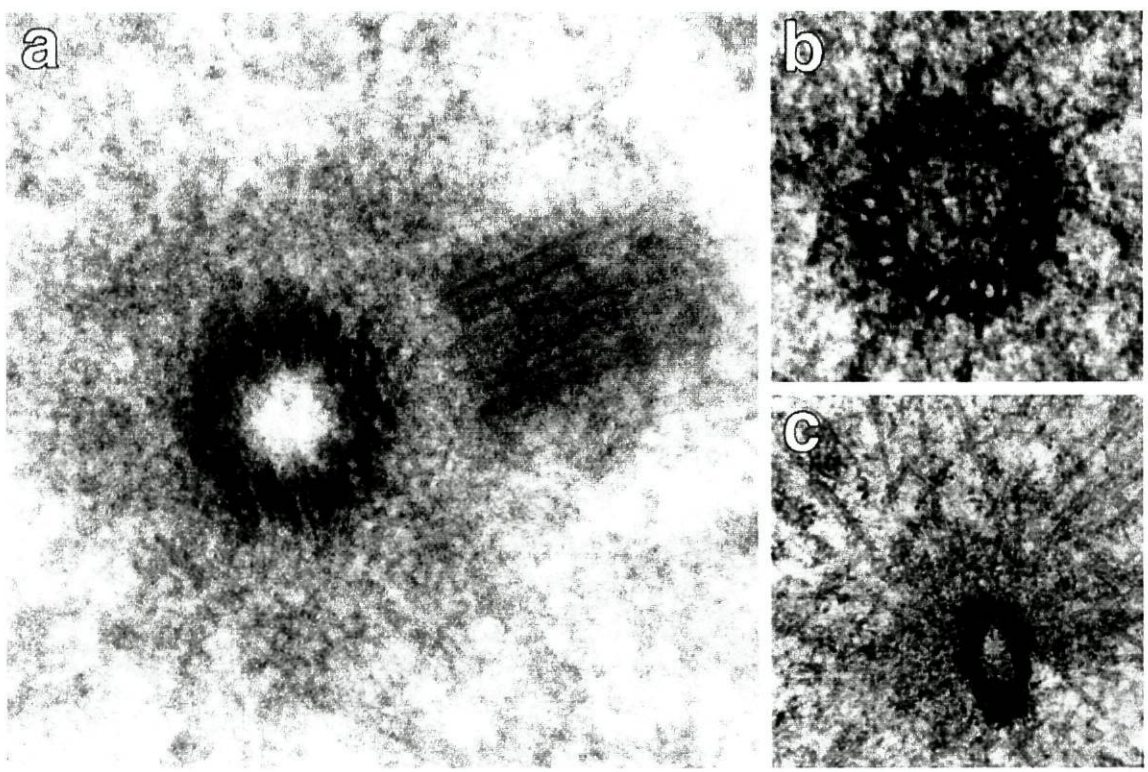


Figure 4. Ultra-structure of the centrosome. **(A)** The centrosome is composed of two perpendicular centrioles embedded in a phase dense pericentriolar mass. **(B)** Each centriole is formed by a circular arrange of nine triplets of microtubules. **(C)** Microtubule nucleation in the pericentriolar region.

5.1.2. The Centrosome Cycle

The centrosome grows and replicates every cell cycle, so that each new daughter cell will inherit a complete centrosome as a result of its association with the mitotic spindle (reviewed by Sluder, 1989). The centrosome cycle (Fig. 5) starts with the initial separation of the two parent centrioles late in G_1 (Kuriyama and Borisy, 1981). At the onset of DNA synthesis short daughter centrioles, called procentrioles, are first seen at

right angles to the proximal ends of the parent centrioles (Robbins et al., 1968; Kuriyama and Borisy, 1981). These procentrioles elongate during S and G_2 phase reaching their mature length during mitosis (Kuriyama and Borisy, 1981). Complete separation of mother/daughter centrioles and the splitting of the centrosome takes place during G_2 (Aubin et al., 1980). The G_2/M transition is accompanied by two changes in centrosome organization: centrosome maturation and separation, two processes highly regulated by protein phosphorylation (reviewed by Fry et al., 2000). Centrosome maturation is characterized by an increase in volume as a result of protein recruitment and microtubule nucleation potential (reviewed by Palazzo et al., 2000), which is thought to be mediated by the action of three distinct protein kinases: CDK1 (Blangy et al., 1995), NIMA (Wu et al., 1998) and polo-like kinases (Plks) (reviewed by Donaldson et al., 2001; Nigg, 1998; Glover et al., 1998). Centrosome separation per se has been the centre of some controversy. In vertebrate cells the centrosomes most often initiate their separation during late G2 and before nuclear envelope breakdown at prophase. Previously it was thought that centrosome separation involved pushing forces generated between interacting microtubules of opposite polarity, derived from opposing centrosomes (reviewed by Mazia, 1987). However, recent evidence suggests that the force-producing mechanism for centrosome separation during spindle formation is intrinsic to each aster and not mediated by microtubule-microtubule interactions between opposing asters (Waters et al., 1993). In addition, there is increasing evidence from different model systems that motor proteins such as Dynein and Kinesins are implicated in this process (Heck et al., 1993; Blangy et al., 1995; Sharp et al., 1999; Sharp et al., 2000a). Centrosome separation is also thought to be regulated by protein phosphorylation. Good candidates as regulators are the Aurora A-related kinase protein family (reviewed by Goepfert and Brinkley, 2000) and the NIMA-related protein kinase Nek2, which phosphorylates the centrosomal protein C-Nap1, thereby causing the dissolution of a putative linker structure that holds mother and daughter centrioles together (Fry et al., 1998; reviewed by Nigg, 2001). The phosphorylation state of C-Nap1 is likely to be counterbalanced by the action of PP1 phosphatase (Helps et al., 2000).

How centrosome duplication is regulated remained an intriguing mystery for more than a century. Cell-cycle progression from G_1 to S phase requires the activity of the centrosome-associated kinase CDK2 and its regulatory subunit Cyclin E. Recent studies in *Xenopus* embryos and egg extracts revealed that CDK2 regulates the splitting of centrioles, one of the earliest stages in centrosome duplication (Hinchcliffe et al., 1999; Lacey et al., 1999). CDK2 is also required for centrosome duplication in mammalian cells but Cyclin A activity seems to predominate over Cyclin E (Matsumoto et al., 1999;

Meraldi et al., 1999). One of the substrates for CDK2-Cyclin E was found to be a previously identified component of the nucleolus: nucleophosmin (Schmidt-Zachmann et al., 1987), which can also be found in un-duplicated centrosomes but not after duplication (Okuda et al., 2000). Later, during mitosis, nucleophosmin is again found at the centrosomes (Zatsepina et al., 1999).

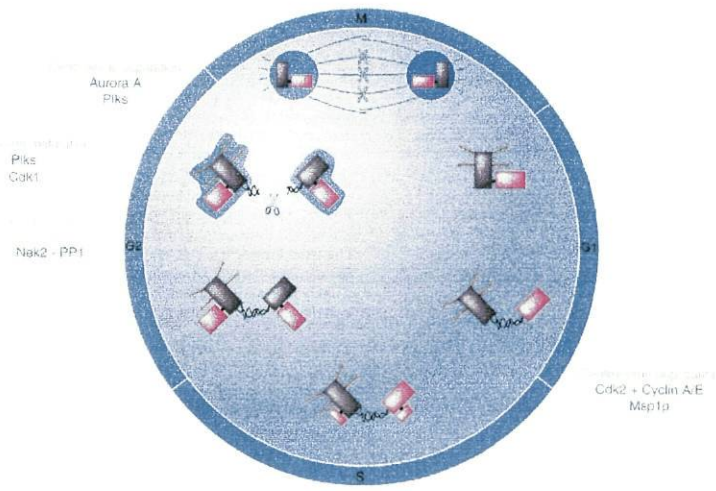


Figure 5. The centrosome cycle. Mother (violet) and daughter (pink) centrioles duplicate in late G1 and during S phase originating 2 pairs of centrosomes. During G2, eventual linkage between centrosomes is disrupted and the maturation process begins while separating apart in late G2. The centrosomes continue to separate during prophase of mitosis to form a bipolar spindle (adapted from Lange, 2002).

CDK2 kinase activity is also required for mMps1p-dependent centrosome duplication (Fisk and Winey, 2001). mMps-1p is the mouse ortholog of the yeast kinase Mps1p, originally identified as an essential protein kinase involved in the duplication of the spindle pole body (Winey et al., 1991), whose activity seem to regulate centrosome duplication in mammals. However, experiments in human cells argue that human Mps1 is not required for centrosome duplication (Stucke et al., 2002). Of course, it is possible that centrosome duplication only requires trace amounts of Mps1 activity, or that individual mammalian species and cell types differ with regard to their requirements for centrosome duplication. More recently, a third kinase called ZYG-1 was identified in *C. elegans* by the use of a genetic approach and is thought to be required for the formation of new centrioles (O'Connell et al., 2001). *zyg-1* mutants form monopolar spindles with single centrosomes containing only one centriole.

5.1.3. Microtubule Nucleation

Centrosomes are the main site of microtubule nucleation in animal cells. Microtubule nucleation occurs in the pericentriolar material and requires a ring-shaped multiprotein complex containing γ-tubulin, a protein related to the α- and β-tubulins (Oakley and Oakley, 1989; Moritz et al., 1995; Zheng et al., 1995; reviewed by Gunawardane et al., 2000). These γ-tubulin ring complexes (γTuRCs) form a lattice structure by association of γ-tubulin with pericentrin, a component of the pericentriolar material (Doxsey et al.,

1994; Dictenberg et al., 1998). Recent studies on the detailed structure and function of the γTuRCs provided experimental evidence for a model where several small γ-tubulin sub-complexes are organized into a ring by other members of the ring complex, and may function as a microtubule minus-ends cap (Wiese and Zheng, 2000; Keating and Borisy, 2000; Moritz et al., 2000) (Fig. 6A). This model postulates that α- and β-tubulin heterodimers bind longitudinally to γTuRCs, creating a layer where additional dimmers bind and a microtubule can form. It also explains how microtubule polarity is established by the centrosome, and assumes that α-tubulin would be the subunit at the minus end and β-tubulin would be at the plus-end, which was later confirmed experimentally (Nogales et al., 1999). Other model for γ-tubulin nucleation postulates that lateral interactions between γ-tubulin and both α- and β-tubulin serve to initiate the assembly of a protofilament where other tubulin dimmers would interact laterally until eventually form a microtubule (Erickson and Stoffer, 1996; Erickson, 2000) (Fig. 1.6B).

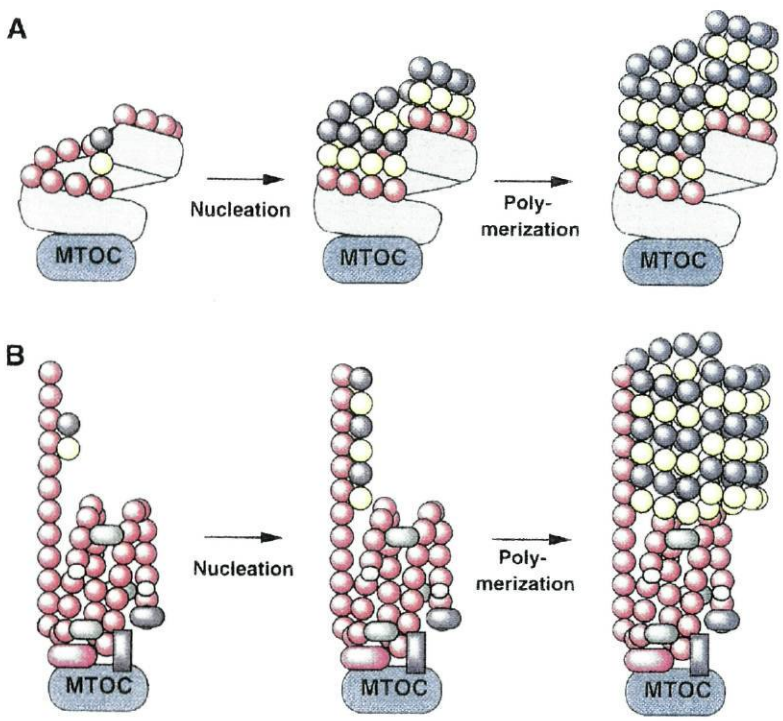


Figure 6. Models for the nucleation of microtubules by the γ-tubulin ring complex. **(A)** In this model, proteins in the γ-tubulin complex provide a scaffold (green) on which γ-tubulin subunits (red) are aligned. α-tubulin (yellow) interacts directly with γ-tubulin, thereby determining the polarity of microtubules. β-tubulin is shown as blue circles and at least one of the scaffold proteins interacts with other components of the MTOC. **(B)** In this model, γ-tubulin forms a protofilament terminating in a helical structure. The polarity of the γ-tubuln helix (red circles) may be determined by accessory proteins (green, brown, pink and blue rectangles), which may also provide contact points with other proteins of the MTOC. γ-tubulin interacts with α- and β-tubulin (yellow and blue circles, respectively). The γ-tubulin protofilament functions as stable seeds upon which additional tubulin subunits assemble to form a microtubule. (Adapted from Pereira and Schiebel, 1997).

5.2. Microtubules

5.2.1. Structure

Microtubules are dynamic polar polymers of α- and β-tubulin heterodimer subunits that normally organize into thirteen linear protofilaments to form a 25 nm diameter cylindrical structure (reviewed by Wade and Hyman, 1997). Microtubule subunits were first purified by their affinity to colchicine, a natural drug that arrests cells in mitosis (Weisenberg et al., 1968) and later β-tubulin was shown to hydrolyse GTP (Weisenberg et al., 1976), which is required for the dynamic behaviour of microtubules.

5.2.2. Dynamics

The study of microtubule dynamics *in vitro* (reviewed by Desai and Mitchison, 1997) indicated that at the steady state (when microtubule length is constant) there is continuous incorporation of tubulin subunits into microtubules preferentially at one end and loss of subunits at the other. Thus, a unidirectional flux of tubulin subunits from one end of the microtubule to the other, or treadmilling, can occur, giving microtubules intrinsic mechanical properties (Margolis and Wilson, 1978; reviewed by Margolis and Wilson, 1981). More recently, it was established that treadmilling is also a major *in vivo* mechanism underlying the dynamics of microtubules (Rodionov and Borisy, 1997; for reviews see Waterman-Storer and Salmon, 1997; Margolis and Wilson, 1998).

Later on, the study of individual microtubule growth from purified centrosomes led to a model in which microtubules were proposed to show dynamic instability. It was postulated that a single microtubule never reaches a steady state length, but interconverts infrequently between polymerisation and depolymerisation states driven by ATP hydrolysis (Mitchison and Kirschner, 1984a,b; Horio and Hotani, 1986). In other words, individual microtubules switch stochastically between phases of slow and fast shrinkage so that in a microtubule population some will be growing and some shrinking (reviewed by Hyman and Karsenty, 1996)

As a consequence of their polarity, microtubules have a faster growing end, referred to as the plus ends and a slower growing end referred as the minus end (Allen and Borisy, 1974). A GTP-tubulin dimmer is incorporated at polymerising microtubule plus ends, the bound GTP is hydrolysed in an exchangeable GTP binding site during or soon after polymerisation (Mitchison, 1993), and P_i is subsequently released (Fig. 7A). This makes the microtubule lattice to be predominantly composed of GDP-tubulin dimmers. The transition to depolymerisation is often referred as 'catastrophe' (Fig. 7A, B) and is characterized by the rapid loss of GDP-tubulin subunits and oligomeres from the microtubule end (Walker et al., 1988). Depolymerising microtubules can also

infrequently transit back to the polymerisation phase, which is termed as 'rescue' (Walker et al., 1988).

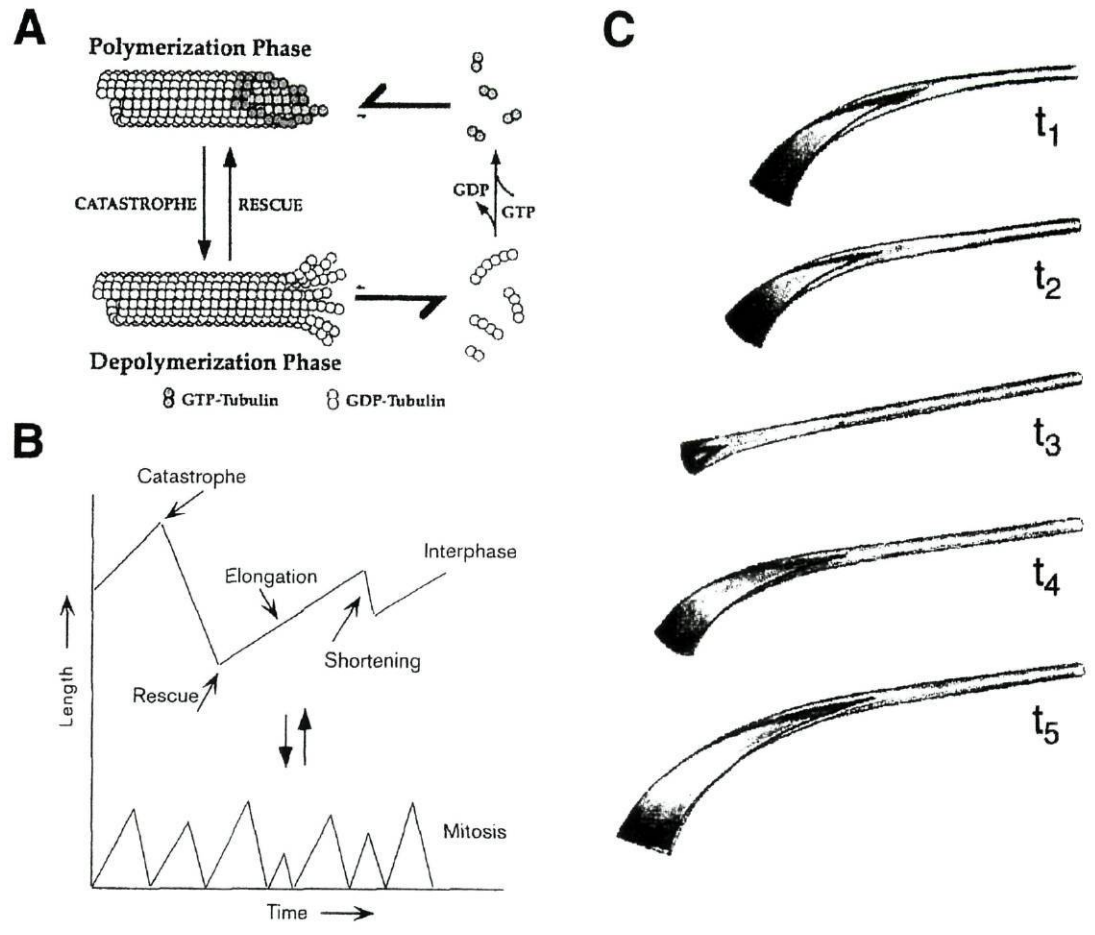


Figure 7. Microtubule polymerisation dynamics. **(A)** Polymerising microtubules incorporate GTP-tubulin subunits, which is hydrolysed during or soon after polymerisation and infrequently transit to the depolymerisation phase (catastrophe). It is represented the notion of a GTP cap acting as a stabilizing structure at polymerising ends. Depolymerisation is characterized by the very rapid loss of GDP-tubulin subunits and oligomers from the MT end. Depolymerising microtubules can also infrequently transit back to the polymerisation phase (rescue) (adapted from Desai and Mitchison, 1997). **(B)** Length changes by dynamic instability for typical interphase and mitotic microtubules (adapted from Cassimeris, 1999). **(C)** Representation of the structure of microtubule plus-ends during polymerisation (adapted from Andersen, 2000).

One way to ascertain the role of GTP hydrolysis in microtubule dynamic instability was using non- or slowly-hydrolysable GTP analogues (Hyman et al., 1992; Caplow, 1992). These studies led to the conclusion that GTP hydrolysis occurs very rapidly during polymerisation and that GDP-tubulin makes a very unstable lattice. Concomitantly, there is increasing evidence that polymerising microtubules are stabilized by some special structure at their ends, originally postulated to be a cap of GTP or GDP-P_i-tubulin (Hill and Carlier, 1983; Carlier et al., 1984; Hill and Chen, 1984;

Mitchison and Kirschner, 1984a). The infrequent loss of such a GTP-cap would result in a catastrophe, whereas its regain by a depolymerising end would result in rescue. Attempts to measure the size of the minimum GTP-cap required to stabilize a polymerising microtubule have established that only a few GTP containing subunits corresponding to one to three layers of the lattice are sufficient to stabilize a growing microtubule (Drechsel and Kirschner, 1994; Caplow and Shanks, 1996).

In order to better understand the behaviour of the ends of growing and shrinking microtubules it was necessary to reveal their structure. With the advent of cryoelectron microscopy (cryoEM) work from different laboratories have made possible to study the kinetic intermediates by rapid freezing of the microtubules and image them directly without staining (Mandelkow and Mandelkow, 1986). The model proposes that microtubules elongate by formation of an open curved tubulin sheet (Fig. 7C), which, triggered by GTP hydrolysis, closes at a variable rate to form a microtubule cylinder (Chrétien et al., 1995). In addition, the sheet length fluctuates as the result of rapid closing (which triggers catastrophe) and opening of the cylinder (when rescue starts). Furthermore, the effects of GTP hydrolysis on microtubule structure have also been confirmed by cryoEM by the use of the same slowly hydrolysable GTP analogues (Hyman et al., 1995; Müller-Reichert et al., 1998). More recently, using *Xenopus* egg extracts, it was shown that the structural transitions at microtubule ends correlate with their dynamic properties (Arnal et al., 2000).

All the microtubule properties described above were mostly investigated using *in vitro* systems based on the intrinsic behaviour of purified tubulin. Nevertheless, a growing body of evidence from experiments performed *in vivo* mainly by DIC in flat cells or by microinjection of rhodamine-labeled tubulin into cells, allowed the observation of physiological microtubule dynamics and values for the different parameters have been obtained (Cassimeris et al., 1988; Sammak and Borisy, 1988; Schulze and Kirschner, 1988; Shelden and Wadsworth, 1993). With the advent of green-fluorescent protein (GFP) fusions, the study of microtubule dynamics in living cells was much simplified, reducing the manipulation required and providing a simple and easily manipulated system (Rusan et al., 2001).

Nowadays, the current models of microtubule dynamics generally accepts that treadmilling and dynamic instability are two processes that are likely to coexist in cells, and may account for the execution of different processes such as kinetochore capture during early mitosis and passive transport of associated organelles, including chromosomes (Farrell et al., 1987; Hotani and Horio, 1988; for reviews see Margolis and Wilson, 1998; Waterman-Storer and Salmon, 1997).

5.3. MAPs and Molecular Motors

5.3.1. General Properties of MAPs

MAPs, by definition, are proteins that bind to the microtubule lattice and co-purify with tubulin *in vitro*. A great deal of characterization has been performed on the classical MAPs: MAP1, MAP2, and tau in neurons, and MAP4 in non-neuronal cells (for reviews see Hirokawa, 1994; Mandelkow and Mandelkow, 1995). These proteins bind to, stabilize, and promote microtubule assembly. MAP4 is evolutionary conserved from *Drosophila* to humans and, unlike the neuronal MAPs, it promotes MT assembly by strongly enhancing the rescue frequency without decreasing the catastrophe frequency (Ookata et al., 1995), in a process regulated by phosphorylation through CDK1 (Ookata et al., 1997). However, this MAP is not essential for cell viability perhaps due to functional redundancy with other MAPs (Pereira et al., 1992; Wang et al., 1996).

5.3.2. General Properties of Molecular Motors

Molecular motors are also microtubule-associated proteins but with the special capability to convert chemical energy in the form of ATP into force and movement (reviewed by Barton and Goldstein, 1996). As a consequence of microtubule polarity, these motors can be classified as either minus-end directed, with an activity capable of moving cargo from the plus-end to the minus-end of the microtubule, or plus-end directed, with an activity capable of moving cargo from the minus-end to the plus-end of the microtubule. Mitotic processes known to be mediated by molecular motors include: separation of spindle poles during prophase and the maintenance of centrosome separation throughout mitosis; movement of chromosomes toward and away from the poles during prometaphase, metaphase and anaphase; and last, movement of the spindle poles apart during anaphase B. Protein sequence analysis has indicated that molecular motors fall into two families known as the Dyneins and the Kinesins. These two classes differ mostly in their size and direction of movement along microtubules. Dynein was first discovered in cilia and flagella, where it powers sliding displacement forces in the axoneme by generating minus-end directed microtubule movement (Gibbons and Rowe, 1965). Cytoplasmic Dynein, discovered more recently (Paschal et al., 1987; Lye et al., 1987), is involved in diverse activities, including intracellular transport, nuclear migration, and the orientation of the mitotic spindle (reviewed by Karki and Holzbaur,1999). Cytoplasmic Dynein is a massive multisubunit complex that consists of two heavy chains (>400 KDa), each of which fold to form two heads of the motor domain that binds to microtubules and hydrolyses ATP, three or four intermediate chains (~74 KDa) and four light chains (~55 KDa), which are thought to bind cargo (for

reviews see Schroer, 1994; Karki and Holzbaur, 1999). On the other hand, Kinesin was first found in neural tissue, where it appears to generate plus-end-directed movements needed for axonal transport (Vale et al., 1985). Structurally, conventional Kinesins contain a distinct motor domain, which refers to the force-producing element and often correspond to the microtubule-binding domain (reviewed by Vale and Fletterick, 1997). Additionally, the tail of these proteins is usually separated from the motor domain by a coiled-coil region termed stalk and is thought to target the motor domain to a particular cargo within the cell, thereby constituting the cargo domain. Subsequent studies have shown that other relatives in the Kinesin superfamily have essential functions during cell division. These Kinesin-like proteins (KLPs) share a common core motor domain attached to different tail domains, which are thought to confer diverse associations with other proteins or cellular structures (reviewed by Vale and Fletterick, 1997). Moreover, these proteins can also diverge from conventional Kinesins in the direction of movement within the cell, i.e., some show minus-end directed movement or are bipolar, and in the localization of the motor domain.

5.3.3. Role in the Regulation of Microtubule Dynamics

Another important role for MAPs is in the regulation of microtubule dynamics (reviewed by Cassimeris, 1999). The widely conserved Dis1-TOG family of MAPs have been shown to localize mainly to the centrosomes and spindle microtubules during mitosis (reviewed by Ohkura et al., 2001). Biochemical studies have shown that Dis1-TOG proteins promote microtubule stability by stimulating growth at the plus ends (Tournebize et al., 2000; Dionne et al., 2000; Popov et al., 2001). Genetic analysis indicated that they are required for spindle organization and might regulate the balance of forces during the metaphase-anaphase transition (Nabeshima et al., 1995; Wang and Huffaker, 1997; Matthews et al., 1998; Cullen et al., 1999; Nabeshima et al., 1998). XMAP215, identified in *Xenopus* eggs (Gard and Kirschner, 1987) is the counterpart of the human TOG protein (Charrasse et al., 1998). It was shown to strongly increase the polymerization rate of pure tubulin at the plus ends and to increase the rate of rapid depolymerisation as well as the rescue frequency, thereby increasing microtubule turnover.

The high frequency of microtubule catastrophe observed *in vivo* suggested the existence of factors that can induce catastrophe (for reviews see McNally, 1999; Walczak, 2000). Such factors would oppose the action of MAPs and destabilize microtubules by reducing net tubulin assembly. Op18/stathmin, a protein that is overexpressed in some tumours, was purified as a microtubule-destabilizing factor (Belmont and Mitchison, 1996; reviewed by Cassimeris, 2002). Op18 is regulated

negatively by phosphorylation (Marklund et al., 1996) that is likely to be mediated by Polo-like kinase (Budde et al., 2001) and reversed by the phosphatase PP2A (Tournebize et al., 1997).

A second class of proteins implicated in microtubule destabilization comprises certain members of the Kinesin superfamily (Endow et al., 1994; Lombillo et al., 1995a). XKCM1, the *Xenopus* orthologue of the mammalian MCAK Kinesin protein, represents another microtubule-destabilizing factor (Walczak et al., 1996). Inhibition of XKCM1 function caused a dramatic increase in MT length mostly due to a decrease in the catastrophe frequency of microtubules. XKCM1 was suggested to act as a catastrophe factor by affecting either the structural or chemical properties of special stabilizing structures at the microtubule plus ends (Desai and Mitchison, 1997).

More recently, the use of *Xenopus* egg extracts as a system to investigate the mechanisms that regulate microtubule dynamics have revealed that XMAP215 strongly modulates the catastrophe frequency of microtubules by opposing the microtubule-destabilizing activity of XKCM1 suggesting that the basic parameters of microtubule dynamics might be defined by the activity of MAPs (Tournebize et al., 2000; For reviews see Heald, 2000; Andersen, 2000). More interesting was the ability to reconstitute the essential features of physiological microtubule dynamics by mixing three purified components: tubulin, XMAP215 and XKCM1 (Kinoshita et al., 2001). This strongly suggests that XMAP215 and XKCM1 may be major regulators of microtubule dynamics *in vivo*.

5.4. The Mitotic Spindle

5.4.1. Structure

The interphase cytoskeleton is a highly complex matrix composed, among others, by an array of polar microtubules that is disassembled just before prophase and replaced by a very different and more dynamic structure called the mitotic spindle (for classical reviews see Schrader, 1944; Mazia, 1961). This highly dynamic structure composed of centrosome-nucleated microtubules and microtubule-associated proteins appear to mediate all the events that occur during mitosis. The microtubules that make up the spindle can be classified into three different classes (Fig. 8) (reviewed in Sharp et al., 2000b). The first class are called astral microtubules and are nucleated with their plus ends growing away from the cell centre so that they can make contact to the cell cortex and are thought to contribute to spindle pole separation and positioning within the cell. A second class of microtubules, termed as interpolar microtubules, grow from the

centrosomes towards the cell centre and interdigitate at the equator. They appear to confer spindle stability and are capable of moving spindle poles relative to one another. Finally, the third class of microtubules is constituted by those that grow from the centrosome towards the cell centre and interact with the chromosomes at a specialized structure denominated the kinetochore, and thus are called kinetochore microtubules.

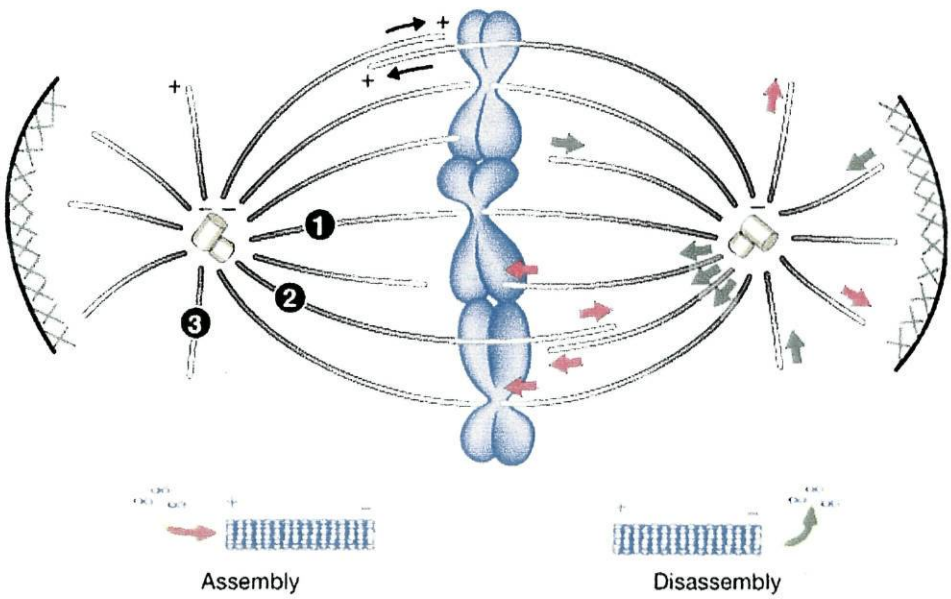


Figure 8. Schematic representation of the mitotic spindle. Kinetochore microtubules (1) connect the centrosomes to the chromosomes. Interpolar microtubules (2) are those that interact with microtubules nucleated from opposite poles. Astral microtubules (3) are responsible for the attachment with the cell cortex. Red arrows mark the microtubules that are growing due to subunit assembly while green arrows mark those that are shrinking due to subunit disassembly (adapted from Wittman et al., 2001).

5.4.2. Role of MAPs and Molecular Motors in Spindle Assembly

Temporal and spatial co-ordination of spindle-associated proteins regulates many aspects of the mitotic apparatus including centrosome separation, formation and maintenance of bipolarity, and the alignment and segregation of the chromosomes (for reviews see Sharp et al., 2000b; Wittman et al., 2001; Karsenti and Vernos, 2001).

The role of multiple microtubule motors in spindle assembly has been the subject of intense investigation (for reviews see Hoyt and Geiser, 1996; Vernos and Karsenti, 1996). Pioneering work in budding yeast showed that disruption of the balance of the Kinesin motors Cin8, Kip1p and Kar3p resulted in rapid collapse of the spindle (Saunders and Hoyt, 1992; Saunders et al., 1997; reviewed by Fuller and Wilson, 1992). Investigations on mitotic spindles assembled *in vitro* using DNA-coated beads incubated in the presence of *Xenopus* egg extracts have led into a model where multiple

microtubule-based motor proteins, such as XKLP1 (Vernos et al., 1995); Dynein; Eg5, the vertebrate homologue of *Drosophila* KLP61F (Le Guellec et al., 1991; Heck et al., 1993); and XCTK2, the *Xenopus* counterpart of *Drosophila* Ncd (Endow et al., 1990; McDonald et al., 1990), are required for establishing spindle bipolarity (Walczak et al., 1998). Subsequent studies in living *Drosophila* embryos have confirmed that spindle assembly, maintenance and elongation depend upon the coordinated activity of motors including the bipolar Kinesin KLP61F, C-terminal Kinesin Ncd and cytoplasmic Dynein (Sharp et al., 1999, 2000c).

KLP61F and Eg5 are members of the conserved *bimC* Kinesin family (reviewed by Kashina et al., 1997) shown to be required for bipolar spindle formation (Sawin et al., 1992; reviewed by Gelfand and Scholey, 1992). Eg5 binds to dynactin in a Cdk1 phosphorylation dependent manner (Blangy et al., 1995; Blangy et al., 1997). Furthermore, Eg5 has also been shown to associate and be phosphorylated *in vivo* by Eg2, the *Xenopus* orthologue of Aurora A kinase (Giet et al., 1999), which is required for the formation of a bipolar spindle (Glover et al., 1995). Indeed, specific inhibition of Eg5 with the drug monastrol arrests cells in mitosis with monoastral spindles (Mayer et al., 1999; reviewed by Compton 1999). Interestingly, chromosomes in monastrol-treated cells frequently have both sister kinetochores attached to microtubules extending to the center of the monoaster, i.e. syntelic oriented (Kapoor et al., 2000). The action of Eg5 and other Kinesins like XKLP2 (Boleti et al., 1996) is thought to generate the pushing forces required for centrosome separation, and the activity of Eg5 is counteracted by that of HSET (Mountain et al., 1999), a human minus end-directed Kinesin-like protein related to Kar3p (Ando et al., 1994; Kuriyama et al., 1995).

Cytoplasmic Dynein and dynactin are localized to astral microtubules and at cortical sites in mitotic epithelial cells (Busson et al., 1997), and are thought to anchor astral microtubules to the cell cortex and, during separation, have been shown to bind the nuclear envelope (Robinson et al., 1999; Gönczy et al., 1999). Through its minus-end-directed motor activity Dynein may provide the pulling forces that maintain spindle pole positioning and promote spindle elongation (Vaisberg et al., 1993). Additionally, the minus-end directed activity of Dynein/dynactin is responsible for accumulation of XKLP2 and NuMA (Wittman et al., 1998; Merdes et al., 2000), a large protein that is present in the nucleus of interphase cells and concentrates in the polar regions of the spindle during mitosis (Lyderson and Pettijohn, 1980; reviewed by Compton and Cleveland, 1994).

NuMA was the first non-motor microtubule-associated protein shown to be required for the establishing and maintenance of spindle bipolarity (Yang and Snyder, 1992). It was proposed that NuMA, together with Dynein/dynactin, are necessary to focus

microtubules into spindle poles and to control the size of mitotic spindles (Merdes et al., 1996; Quintyne et al., 1999; for reviews see Merdes and Cleveland, 1997; Compton, 1998).

5.4.3. Spindle Assembly without Centrosomes

Centrosomes have been considered to be essential for spindle assembly due to their association with spindle poles and their microtubule-nucleating activity. However, higher plants, as well as some *Drosophila* stable cell lines, lack canonical centrioles but can still organize normal spindles and undergo cell division (Smirnova and Bajer, 1992; Debec et al., 1995). Furthermore, in animal cells, for example during *Drosophila* female meiosis, no centrosomes are found at the spindle pole (reviewed by González et al., 1998). These observations suggested that there might be alternative mechanisms to ensure spindle bipolarity. Studies in *Xenopus* extracts supported these observations, since addition of chromatin coated beads to these extracts is sufficient to generate apparently normal looking bipolar spindles in the absence of centrosomes (Heald et al., 1996). Recent studies have shown that such alternative pathways also exist in somatic cells from *Drosophila* to humans (Bonaccorsi et al., 2000; Khodjakov et al., 2000). These observations strongly suggest that in vertebrates and other higher animals, the role of the centrosome as a MTOC is more important for the proper development and maintenance of the organism than for cell viability and that the centrosome has at least one vital function in the cell that is independent of its role as a MTOC, which may be related with cell cycle progression (Reviewed by Rieder et al., 2001). Experimental evidence for this thesis came from studies in vertebrate cells that linked the MTOC-independent centrosome activity to the completion of cytokinesis and progression from G_1 to S phase of the cell cycle. If centrosomes are surgically removed from interphase cells with a microneedle (Hinchcliffe et al., 2001) or by laser ablation from mitotic cells (Khodjakov and Rieder, 2001), mitotic cells can still assemble spindles but cytokinesis is seriously compromised. Regardless of the detailed mechanism by which centrosomes affect cytokinesis, one immediate consequence for cell cycle progression is to ensure that dividing animal cells receive the appropriate number of functional centrosomes. It is still unclear whether centrosomes directly trigger a checkpoint that monitors the completion of mitosis, but some recent results suggest that the final events of cell cleavage during cytokinesis are correlated with the movement of the maternal centriole to the intercellular bridge (Piel et al., 2001).

5.5. The Kinetochore

5.5.1. Structure

The kinetochore is a protein-based structure located on the surface of the chromosomes that interacts directly with spindle microtubules and is responsible for chromosome movements (Brinkley and Stubblefield, 1966; Jokelainen, 1967; Rieder, 1982; Craig et al., 1999). At the ultra-structural level, the mammalian kinetochore has been shown to be composed of a trilaminar disk associated to the primary constriction of chromosomes: the centromere (McEwen et al., 1998). Mammalian kinetochores have four morphologically distinct layers (Fig. 9), although only three of these can be detected in the presence of microtubules (Earnshaw, 1994). The innermost layer is a disk of densely packed material called the inner plate and is continuous with the surface of the centromeric heterochromatin underneath the kinetochore. This region is thought to be responsible for the assembly and size determination of a robust kinetochore capable of withstanding the stresses imposed by the spindle. The most consistent feature of the kinetochore is the outer plate, a dense structure that is typically 0.5 μm in diameter and 30-40 nm thick. The outer plate forms the primary and predominant point of attachment for spindle microtubules. Between the two plates, there is a third layer, more appropriately considered as an interstitial space. This space is a region of 15-35 nm across that is not stained under standard protocols for electron microscopy and is traversed by numerous fibres that appear to connect the inner and outer regions of the kinetochore (McEwen et al., 1993). The outer surface of the kinetochore is associated with a poorly defined 10-20 nm filamentous meshwork known as the fibrous corona that can only be seen in kinetochores that do not have large numbers of associated microtubules.

5.5.2. Molecular Composition

The pioneer identification of mammalian centromere/kinetochore proteins became possible with the discovery of anticentromere antibodies (ACA) in sera derived mostly from patients that developed autoimmune diseases like calcinosis/Raynaud's phenomenon/esophageal dysmotility/sclerodactyly/telangiectasia (CREST) variant of scleroderma (reviewed by Pluta et al., 1990). The first specific centromere proteins to be identified with ACA were a group of three autoantigens designated CENP-A (*CEN*tromere *P*rotein A), CENP-B and CENP-C (Earnshaw and Rothfield, 1985; Earnshaw et al., 1986). The identification of a fourth antigen, CENP-D, has also been described (Kingwell and Rattner, 1987).

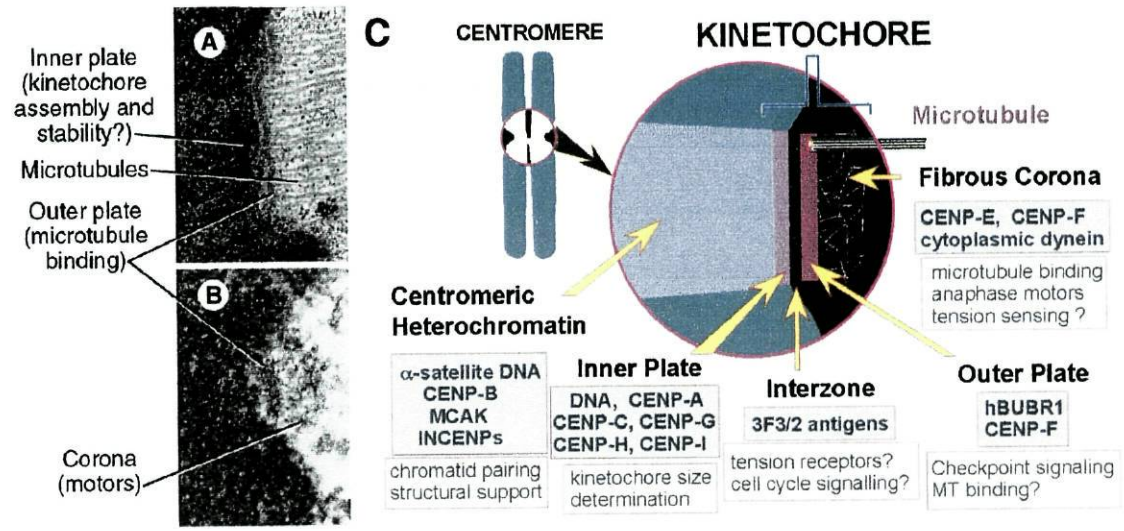


Figure 9. Kinetochore structure and composition. **(A)** Ultra-structure of the microtubule attached kinetochore. **(B)** Ultra-structure of the colcemid treated kinetochore reveal the fibrous corona region. **(C)** Schematic representation of the vertebrate kinetochore and its components. (Adapted from the original kindly provided by Bill Earnshaw).

CENP-A is a highly divergent variant of histone H3 that is found only in centromeres (Palmer and Margolis, 1987; Sullivan et al., 1994) and is probably involved in the specific packaging of the centromeric chromatin. CENP-A has been localized at the light microscopy level underneath the inner kinetochore plate (Warburton et al., 1997) and seems to be important for initiating centromere formation and recruiting other centromere/kinetochore components (Howman et al., 2000; Blower and Karpen, 2001; Oegema et al., 2001). CENP-A is a highly conserved protein and homologues have been found in a wide variety of organisms (Meluh et al., 1998; Takahashi et al., 2000; Buchwitz et al., 1999; Henikoff et al., 2000). Namely, the *Drosophila* homologue of CENP-A, CID (for centromere identifier), was also shown to be required for cell cycle progression (Blower and Karpen, 2001).

CENP-B is an acidic protein that is distributed throughout the centromeric heterochromatin beneath the kinetochore (Earnshaw et al., 1987; Cooke et al., 1990). This distribution is explained by the observation that CENP-B binds to a 17 bp sequence (the CENP-B box) in α-satellite DNA (Masumoto et al., 1989). Studies in knockout mice null for CENP-B have found that this protein is not essential for growth and development (Hudson et al., 1998; Kapoor et al., 1998; Perez-Castro et al., 1998). Although initial studies have suggested a role for CENP-B in interacting directly with microtubules (Balczon and Brinkley, 1987) those studies in mice, together with the observation that CENP-B is found at the inactive centromeres of two stable dicentric chromosomes

(Earnshaw et al., 1989) strongly argues against a direct involvement of CENP-B for microtubule binding *in vivo* and its function remains highly elusive.

CENP-C is a basic protein that is highly concentrated in the region of the inner kinetochore plate, apparently extending outward to the inner surface of the outer plate (Saitoh et al., 1992) and is only present at the active centromere on a dicentric human chromosome 13 (Earnshaw et al., 1989). As predicted from antibody microinjection experiments (Tomkiel et al., 1994), CENP-C is essential both in mouse (Kalitsis et al., 1998) and in chicken cells (Fukagawa and Brown, 1997; Fukagawa et al., 2001b), and seems to be required for kinetochore size determination, timely transition to anaphase and progression through G_1 phase of the cell cycle.

Highly unexpected was the result that came from the purification and sequencing of CENP-D identified by anticentromere autoantibodies (reviewed by Earnshaw and Tomkiel, 1992), which indicated that it corresponds to RCC1 (Ohtsubo et al., 1987; Bischoff et al., 1990). Both genetic and biochemical studies have shown that RCC1 interacts with a nuclear *ras*-like GTP-binding protein, termed *ran* (Bischoff and Ponstingl, 1991; Matsumoto and Beach, 1991). This is surprising since localization studies for RCC1 did not show its localization at centromeres (Ohtsubo et al., 1987). The best explanation to this paradox appears to be that the autoantibodies recognize a posttranslational modification that is specific for a small pool of RCC1 concentrated at the centromeres (Earnshaw, 1994).

The search for novel components of the centromere/kinetochore involved the development of monoclonal antibodies to chromosome scaffolds (Earnshaw et al., 1984). One of these antigens, called CENP-E, has been cloned and extensively characterized (Yen et al., 1991; Yen et al., 1992). Ultra-structural localization studies of this protein during mitosis have shown that it concentrates to the fibrous corona and outer plate of mammalian kinetochores from prometaphase through anaphase (Cooke et al., 1997; Yao et al., 1997). During the final stages of mitosis, CENP-E is also found associated with the overlapping polar microtubules of the central spindle and mid-body (Yen et al., 1991; Cooke et al., 1997). CENP-E is one of the largest members (312 kDa) of the Kinesin superfamily (Yen et al., 1992; reviewed by Vale and Fletterick, 1997). Surprisingly, a CENP-E-associated minus-end directed motor activity was purified from HeLa cells (Thrower et al., 1995). However, expression of a recombinant fragment of CENP-E was subsequently shown to have a plus-end directed motor activity (Wood et al., 1997) making this a controversial issue. One possible interpretation is that the minus-end-directed motor activity might have arisen from another motor protein associated with CENP-E or, alternatively, CENP-E has both plus- and minus-end-directed motor activity, depending on its association with regulatory factors (reviewed by

Grancell and Sorger, 1998). Antibody microinjection experiments (Schaar et al., 1997) and studies of spindle assembly *in vitro* (Wood et al., 1997) revealed that CENP-E is required to complete chromosome alignment at metaphase. However, after depletion of CENP-E most chromosomes can still align indicating, first, that there are redundant mechanisms, and secondly that CENP-E might have a role ensuring reliable bioriented spindle attachment in a specific situation (McEwan et al., 2001).

In a more traditional approach using human autoimmune serum, a novel ~400 kDa cell-cycle dependent kinetochore associated protein, termed CENP-F, was identified and cloned (Rattner et al., 1993; Liao et al., 1995). Its localization pattern during mitosis resembles CENP-E, but is specifically associated to the outer surface of the outer kinetochore plate (Rattner et al., 1993). However, the precise function of CENP-F remains obscure (but see Hussein and Taylor, 2002).

More recently, cytoplasmic Dynein (reviewed by Vallee and Gee, 1998) was shown to accumulate at the kinetochore (Pfarr et al., 1990; Steuer et al., 1990). Latter, it was shown by immunoelectron microscopy that Dynein localization is confined to the fibrous corona of the kinetochore (Wordeman et al., 1991). Other motor proteins have also been shown to localize to the centromere/kinetochore such as dynamitin, Arp1 and $p150^{Glued}$, which are components of the dynactin complex (Faulkner et al., 1998), and the Kinesin-related protein MCAK (Wordeman and Mitchison, 1995). Although still controversial, these proteins are likely to be associated with kinetochores rather than the centromeric heterochromatin, because they are all present at active, but not inactive, centromeres on stable dicentric chromosomes (Faulkner et al., 1998).

Subsequently, CENP-G, a constitutive centromeric protein was identified as an auto antigen using serum from a patient with gastric antral vascular ectasia disease (He et al., 1998). This protein was detected at the centromeric region throughout the cell cycle and was found to be restricted to the kinetochore inner plate (He et al., 1998). CENP-G appears to be preferentially associated with the CENP-B box-containing α-1 subclass of α-satellite DNA suggesting that CENP-G may play a role in kinetochore organization and function (He et al., 1998). However, a recent study has reported that CENP-G is also present in neocentromeres and in the human Y chromosome which lack alphoid DNA sequences suggesting that other DNA sequences are able to bind CENP-G (Gimelli et al., 2000).

Another constitutive component of the mouse and human centromere was recently identified and named, following the same nomenclature, CENP-H (Sugata et al., 1999; Sugata et al., 2000). Confocal microscopic analyses of HeLa cells with anti-human CENP-H-specific antibody demonstrated that CENP-H co-localizes with inner kinetochore plate proteins CENP-A and CENP-C in both interphase and metaphase

(Sugata et al., 2000). CENP-H was present outside centromeric heterochromatin, where CENP-B is localized, and inside the kinetochore corona, where CENP-E is localized during prometaphase. Furthermore, CENP-H was detected at neocentromeres, but not at inactive centromeres in stable dicentric chromosomes suggesting a role in organization and function of the active human centromere-kinetochore complex. (Sugata et al., 2000). *In vitro* binding assays of human CENP-H with centromere-kinetochore proteins suggest that CENP-H binds to itself and MCAK, but not to CENP-A, CENP-B or CENP-C (Sugata et al., 2000). More recently, a conditional loss-of function CENP-H mutant was generated in the chicken DT40 cell line (Fukagawa et al., 2001a) and shown that, in the absence of CENP-H, there is a prometaphase arrest with unattached chromosomes that in general look morphologically abnormal, consistent with loss of centromere function. Furthermore, CENP-C, but not CENP-A, failed to localize to the centromere in CENP-H mutant cells.

To date, only one more constitutive component of the vertebrate centromere was identified and named CENP-I (Nishihashi et al., 2002). CENP-I is an essential centromere protein that co-localizes with CENP-A, -C, and –H throughout the cell cycle. A conditional loss-of-function DT40 mutant for CENP-I shows a prometaphase arrest with misaligned chromosomes and, apparently, cells exited mitosis without undergoing cytokinesis. As in the case of CENP-H mutant cells, loss of CENP-I, affects the localisation of CENP-C but not of CENP-A. Taken together, either CENP-H or CENP-I appear to be essential for kinetochore assembly.

6. Microtubule-Kinetochore Attachment

6.1. Properties of the Kinetochore-Associated Microtubules

As a result of a pioneering work in cell biology, Daniel Mazia proposed that the kinetochore is the point of attachment of the chromosome to the mitotic apparatus or the anchor point for a spindle fiber. Chromosomes without kinetochores are incapable of mitotic movements, and chromosomes that have lost portions of their arms but retain their kinetochores perform normally (reviewed by Mazia, 1961). Thus, the kinetochore was considered to be the only essential part of the chromosome during mitosis. Indeed, kinetochores that were experimentally detached from mammalian centromeres can still interact with spindle microtubules and complete all the mitotic movements in the absence of other chromosomal components (Brinkley et al., 1988). These results further

supported the view that "*the role in mitosis of the chromosome arms, which carry most of the genetic material, may be compared with that of a corpse at a funeral: they provide the reason for the proceedings but do not take an active part in them*" (Mazia, 1961). While this view has changed considerably in recent years, several aspects of kinetochore function are dependent on the behavior of the dynamic microtubule ends to which it is attached (reviewed by Mitchison, 1988).

The first event concerning microtubule-kinetochore attachment is the initial interaction of microtubules with the kinetochore. As cells enter mitosis centrosomes nucleate a large number of highly dynamic aster microtubules (Mitchison et al., 1986). It is thought that their plus ends are continuously probing the cytoplasmic space, in what has been described as a search and capture mechanism (Hill, 1985; reviewed by Kirschner and Mitchison, 1986). This mechanism proposed that those microtubules that accidentally contact a kinetochore would become capped, while those that do not would soon depolymerise. In support of this idea, it was initially shown that the kinetochores of isolated chromosomes can stabilize the ends of microtubules *in vitro* (Mitchison and Kirschner, 1985). Furthermore, kinetochore microtubules turn over less rapidly than astral microtubules *in vivo* (Mitchison et al., 1986) and detachment of the chromosome from the kinetochore fiber (k-fiber, corresponds to the kinetochore associated spindle microtubules; see Witt et al., 1981) with a microneedle leads to rapid microtubule depolymerisation (Nicklas and Kubai, 1985). However, plus ends captured by the kinetochore can still depolymerise at the kinetochore while remaining attached (Koshland et al., 1988; Huitorel and Kirschner, 1988; Hunt and McIntosh, 1998), but at altered transition rates (Hyman and Mitchison, 1990). Additionally, it was found that kinetochores increase the rate at which microtubule ends transit from growing to shrinking (Hyman and Mitchison, 1990). Thus, a captured microtubule is stabilized at a kinetochore not because it cannot undergo catastrophe, but because it cannot detach (reviewed by Hyman and Karsenti, 1996).

6.2. Chromosome Capture

Several observations have suggested that chromosomes initially attach laterally to microtubules before translocating to the plus ends *in vitro* (Mitchison and Kirschner, 1985; Huitorel and Kirschner, 1988). Later work by Rieder and co-workers has elucidated precisely the initial attachment of kinetochores to astral microtubules (Rieder and Borisy, 1981). They found that the attachment and subsequent poleward movement of a chromosome correlates with the lateral association of a single long microtubule with

one of the kinetochores of the chromosome (Rieder and Alexander, 1990). Once this association is established, the kinetochore is rapidly transported poleward along the lateral surface of the microtubule by a mechanism that is not dependent on microtubule depolymerisation (Fig. 10). Furthermore, their work and work by others have demonstrated that chromosome attachment results from an interaction between astral microtubules and the kinetochore so that k-fibers are simply those astral microtubules that come into contact with chromosomes (Hayden et al., 1990; Merdes and De Mey, 1990; Mitchison, 1990; reviewed by Rieder, 1990).

6.3. Chromosome Congression and Polar Ejection Forces

When chromosomes in prometaphase are attached by a single kinetochore to a single pole, i.e., monooriented, they oscillate toward and away from that pole indicating that they are subject to both pulling and pushing forces (Bajer, 1982; Rieder et al., 1986) (Fig. 10). During metaphase the spindle is at steady state and can maintain the same average structure for extended periods. Nevertheless, chromosomes still constantly oscillate on the spindle axis (Roos, 1976; Bajer, 1982), and the net poleward force on a chromosome depends on the number of kinetochore microtubules (Hays and Salmon, 1990). It seems likely that microtubule polymerization occurs at the elongating k-fiber and that this is balanced by depolymerisation of the shortening fiber. The process that is responsible for the arrangement of chromosomes equidistant from the two poles at metaphase is known as congression (reviewed by Rieder and Salmon, 1994).

As the chromosome moves poleward after initial lateral attachment to a single microtubule, the kinetochore captures the plus ends of polar microtubules and chromosome motion slows down. *In vitro* studies have shown that this tethering to the microtubules plus ends can be explained in part by the preferential binding of kinetochores to GTP- rather than GDP-microtubules, and to the plus ends preferentially over the lattice (Severin et al., 1997). The outer kinetochore plate ultimately becomes saturated with polar microtubule plus ends and consequently, neighboring polar microtubules bundle with kinetochore microtubules to form the stabilized kinetochore fiber. Congression is initiated for each chromosome after becoming bioriented (Fig. 10). However, a single kinetochore of a bioriented chromosome possesses the capability of congressing to the spindle equator (Khodjakov et al., 1997). During poleward motion the centromere is typically stretched poleward, while during motion away from the pole it becomes flattened or indented without exerting a significant pushing force on the chromosome (Skibbens et al., 1993; Khodjakov and Rieder, 1996). For bioriented

chromosomes, the centromere becomes maximally stretched when both sister kinetochores are moving poleward, and maximally compressed when both are moving towards the spindle equator. This characteristic tendency of a kinetochore to autonomously and abruptly switch between these phases was termed directional instability (Skibbens et al., 1993).

Assembly of spindle polar microtubule arrays in prometaphase also involves pushing the chromosome arms away from the spindle poles (Rieder et al., 1986; Leslie, 1992; Cassimeris et al., 1994; for reviews see Rieder and Salmon, 1994; Ault and Rieder, 1994; Inoué and Salmon, 1995). These "polar ejection forces" or "polar winds" on the arms are thought to contribute to the congression of chromosomes to the metaphase plate (Salmon, 1988; 1989).

All movement of kinetochores away from the pole is coupled to assembly of kinetochore microtubules at the kinetochore (Wadsworth and Salmon, 1986; Cassimeris and Salmon, 1991; Centonze and Borisy, 1991). Subsequent results have led to a mechanism based on a tension-sensitive, time-delayed switch, participation of polar ejection forces, and kinetochore microtubule number, to explain how congression can occur (Ault and Nicklas, 1989; Skibbens et al., 1995; Skibbens and Salmon, 1997; McEwen et al., 1997; Waters et al., 1996b; King and Nicklas, 2000; for reviews see Inoué and Salmon, 1995; Inoué, 1997).

6.4. Kinetochore Motion at the Met/Anaphase Transition

Once all of the chromosomes have congressed and sister kinetochores oscillate between poleward and away from the pole phases of motion, the cell is considered to be at metaphase (Fig. 10). In contrast, after anaphase onset, the separated sister kinetochores mostly persist in poleward motion moving progressively closer to the pole (Fig. 10) (reviewed by Rieder and Salmon, 1994). Most kinetochore poleward movement during anaphase A is coupled to disassembly of kinetochore microtubules at the kinetochores, and a slow poleward flux of kinetochore microtubules as they disassemble at their minus ends at the spindle poles continues to take place (Gorbsky et al, 1987, 1988; Koshland et al., 1988; Mitchison and Salmon, 1992; Coue et al., 1991; Zhai et al., 1995; Waters et al., 1996a; reviewed by Inoué and Salmon, 1995). In late anaphase (anaphase B), the microtubules are no longer disassembled at the kinetochore, but the chromosomes continue to move poleward due to poleward flux of the kinetochore microtubules (Mitchison and Salmon, 1992).

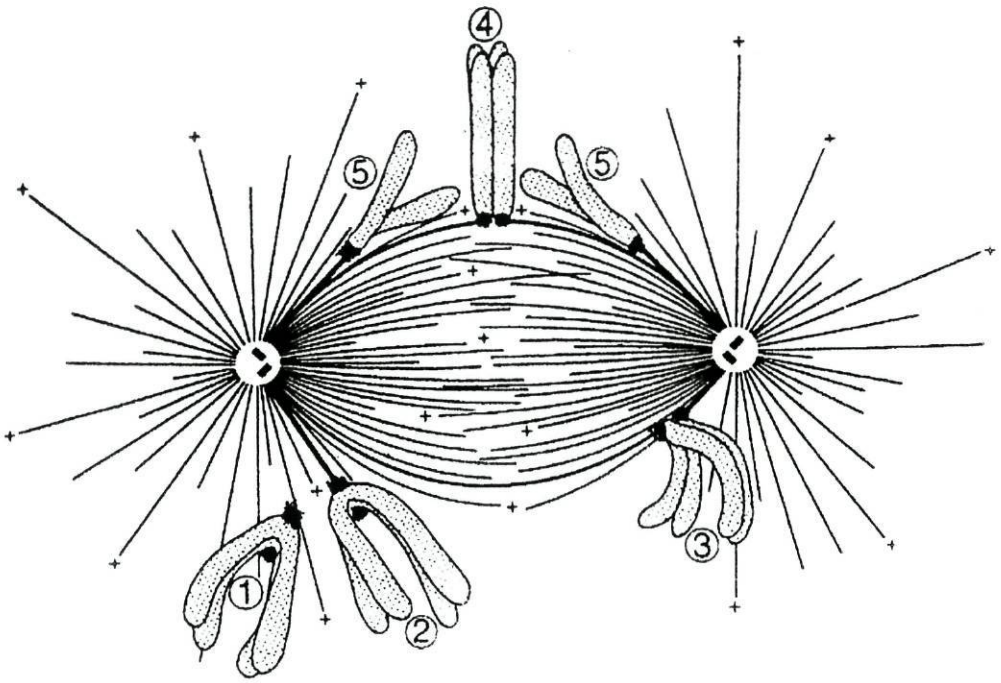


Figure 10 – Chromosome behaviour during mitosis. (1) An attaching chromosome in which one kinetochore is exhibiting rapid poleward movement along the surface of a single astral microtubule. (2) A monooriented chromosome oscillating between poleward and away from the pole phase of motion. (3) A bioriented chromosome initiating congression. (4) a fully congressed metaphase chromosome that is oscillating in the spindle equator. (5) Chromatids in anaphase undergoing poleward movement. (Adapted from Rieder and Salmon, 1994).

6.5. Role of Molecular Motors

The finding that a kinetochore rapidly moves poleward along the lateral surface of a single microtubule that extends beyond the chromosome and that the leading kinetochore does not need to be directly oriented toward the centrosome for this movement to occur (Rieder and Alexander, 1990), led to the proposal that the motors located in the fibrous corona of the kinetochore, on the surface of the astral microtubules, or both could be responsible for this particular movement (reviewed by Sluder, 1990). An obvious candidate to drive kinetochore-to-pole movement in both prometaphase and anaphase is Dynein (Wordeman et al., 1991; reviewed by Banks and Heald, 2001). Cytoplasmic Dynein is thought to function exclusively in conjunction with dynactin, an activating multisubunit complex (for reviews see Schroer, 1994; Hirokawa et al., 1998; Vallee and Gee, 1998; Karki and Holzbaur, 1999). Localization of Dynein at the kinetochores is maximal during prometaphase and decreases substantially at metaphase (King et al., 2000b; Wojcik et al., 2001). Moreover, Dynein leaves the kinetochore very early in mitosis, soon after the kinetochores begin to attach to microtubules. Overexpression of the 50 KDa subunit of dynactin, p50-dynamitin, or

microinjection of antibodies against Dynein have revealed a role in poleward chromosome motility throughout mitosis (Vaisberg et al., 1993; Echeverri et al., 1996; Sharp et al., 2000c). Zw10 and Rough deal (Rod) (Williams et al., 1996; Basto et al., 2000; Chan et al., 2000), have been shown to be involved in targeting the Dynein complex to kinetochores and also be required for poleward chromosome motion (Starr et al., 1998; Savoian et al., 2000; Wojcik et al., 2001).

Directional instability of kinetochores during prometaphase is likely to be the result of the action of antagonistic motors. In this context, microtubules spontaneously oscillate back and forth on surfaces coated with both Dynein and Kinesin (Vale et al., 1992). Additionally, kinetochores on isolated chromosomes always move microtubules over their surface poleward when unphosphorylated and away from the pole when phosphorylated (Hyman and Mitchison, 1991).

After initial poleward movement, the chromosomes then move away from the pole where motor proteins bound to chromosome arms may contribute to the polar ejection force during chromosome congression. In this context, several Kinesin-related motor proteins have been found associated with the chromosome arms: nod in *Drosophila* (Afshar et al., 1995), Xklp1 in *Xenopus* oocytes (Vernos et al., 1995), and chromoKinesin in vertebrates (Wang and Adler, 1995; Tokai et al., 1996; Antonio et al., 2000; Funabiki and Murray, 2000; Levesque and Compton, 2001).

Additional motor proteins with plus-end directed motility seem to be required for chromosome congression during prometaphase. CENP-E, was initially proposed, based on *in vitro* motility assays, to be the molecule responsible for attachment of kinetochores to shortening microtubules (Lombillo et al., 1995a, 1995b; reviewed by Hyman, 1995). However, the observation that disrupting CENP-E function *in vivo* does not affect kinetochore motion, or the velocity of movements towards and away from the pole (Schaar et al., 1997) raises questions about the significance of the results obtained *in vitro* (reviewed by Rieder and Salmon, 1998). Nevertheless, chromosomes lacking CENP-E at their kinetochores show defects in alignment and some remain monooriented and are unable to establish bipolar microtubule connections with the opposite pole (Schaar et al., 1997; Wood et al., 1997; reviewed by Grancell and Sorger, 1998). Thus, in the absence of CENP-E the initial attachment of a single kinetochore, as well as its ability to hold on to shortening microtubule ends is not compromised *in vivo*. Together, the available data suggests that CENP-E could have a critical role in chromosome position and biorientation within the spindle (McEwen et al., 2001).

Other possible candidate for microtubule attachment proteins is the Kinesin MCAK/XKCM1, also known to localize to centromeres near the kinetochore (Wordeman and Mitchison, 1995; Walczak et al., 1996; reviewed by Desai and Mitchison, 1995).

Unlike other motors, MCAK/XKCM1 does not move along microtubules, but instead couples ATP hydrolysis with the depolymerisation of microtubule ends.

6.6. Role of MAPs

Inoué and Salmon (1995) have suggested that the attachment molecules need not be active motor proteins. The idea that non-motor proteins may provide the attachment and stabilization of the kinetochore-microtubule interface, while motor proteins move cargo (i.e. chromosomes) along or in close association with dynamic microtubule behaviour, found strong support after it was shown that CLIP-170 localises strongly to kinetochores of prometaphase but only weakly to that of metaphase chromosomes (Dujardin et al., 1998). Also, dynactin as well as cytoplasmic Dynein were shown to co-localize extensively with CLIP-170 at the distal ends of dynamic microtubules concentrated in the cell periphery during interphase raising the possibility that they may also cooperate at the kinetochores (Vaughan et al., 1999; Valetti et al., 1999). The functional interaction of CLIP-170 and Dynein/dynactin at the kinetochores was recently established and shown to be mediated by LIS1, a protein responsible for causing type I lisencephaly (Tai et al., 2002; Coquelle et al., 2002).

A molecular analysis of microtubule-kinetochore attachment was recently carried out in budding yeast (He et al., 2001; reviewed by Van Hooser and Heald, 2001). This work clearly pointed to a critical role for non-motor MAPs in this process. Among these MAPs, the budding yeast homologue of CLIP-170, Bik1p, is thought to play a central role in kinetochore attachment (Lin et al., 2001). Another protein found in the same study to have a role in kinetochore-microtubule attachment is Stu2 (He et al., 2001). This is the *S.* cerevisiae member of the Dis1-TOG family of MAPs (reviewed by Ohkura et al., 2001). A direct role in the stabilization of microtubule-kinetochore interactions had already been proposed for the *S. pombe* homologue Dis1 (Nakaseko et al., 2001). Either Dis1 or Stu2p associate transiently with kinetochores during mitosis, however, only Dis1 appears to bind kinetochores independently of microtubules. Accordingly, the failure of sister chromatid separation observed in *dis1* and *STU2* mutant cells has been associated with defects in formation/stabilization of kinetochore microtubules. Fission yeast contains a second highly related protein, Alp14, that unlike Dis1, is required not only for overall microtubule assembly but also for the spindle-assembly checkpoint function (Garcia et al., 2001; see section below).

7. Spindle Assembly Checkpoint

7.1. Checkpoint Activation

During prometaphase, when the initial interaction between spindle microtubules and the kinetochores takes place, it is crucial that the cell does not initiate anaphase until all chromosomes are properly bioriented and aligned at the metaphase plate. In 1970, Zirkle proposed that in order to prevent exit from mitosis before all chromosomes had congressed to the metaphase plate there might be a signal emanating from the chromosomes or spindle that prolonged prometaphase until both kinetochores of every chromosome became attached to microtubules (Zirkle, 1970; reviewed by Pluta et al., 1995). Subsequently, the role of maloriented chromosomes in the generation of a non-diffusible inhibitor of anaphase onset was demonstrated (Sluder et al., 1994), and direct evidence for the existence of a checkpoint that monitors sister kinetochore attachment to the spindle was obtained (Rieder et al., 1994). These results were also consistent with previous observations indicating that tension exerted on the chromosomes, presumably through their microtubule attachments, is monitored during mitosis (Nicklas and Koch, 1969). Later, it was shown that in the absence of tension produced by a stable bipolar attachment, kinetochores transmit an inhibitory signal that delays the metaphase-anaphase transition (Li and Nicklas, 1995; for reviews see Murray, 1995a; Nicklas, 1997). In this study carried out using insect spermatocytes, it was shown that a single mono-oriented chromosome resulted in an extended block to anaphase entry. However, if such a chromosome is placed under gentle tension by pulling it away from the pole with a microneedle, the cell overcome its metaphase block and proceed into anaphase.

In a parallel study using cultured vertebrate cells, it was found that molecules in or near the unattached kinetochore of a mono-oriented chromosome are able to inhibit the metaphase-anaphase transition (Rieder et al., 1995). Thus, if the unattached kinetochore on the last mono-oriented chromosome present in a mitotic cell is selectively destroyed with a laser micro beam the cell initiates anaphase without congressing the irradiated chromosome.

The first biochemical evidence for a link between kinetochore attachment and checkpoint function was provided by the phosphoepitope-specific monoclonal antibody 3F3/2 (Gorbsky and Ricketts, 1993). This antibody preferentially stains kinetochores that are not under tension, and microinjection of 3F3/2 antibody into cells delays the metaphase-anaphase transition without affecting chromosome movements (Campbell

and Gorbsky, 1995). Moreover, it was shown that tension alters the phosphorylation of kinetochore proteins as determined by 3F3/2 staining suggesting that kinetochore protein dephosphorylation caused by tension constitutes the signal for anaphase onset (Nicklas et al., 1995; Li and Nicklas, 1997; Nicklas et al., 1998).

In order to determine whether the inhibitory activity associated with unattached kinetochores that prevents cells from entering anaphase diffuses freely in the cytoplasm or whether it is confined to the chromosomes and the mitotic spindle, cells in mitosis containing two independent chromosome complements and associated spindles in a common cytoplasm were studied (Rieder et al., 1997). It was found that unattached kinetochores on one spindle did not block anaphase onset in a neighbouring mature metaphase spindle that lacked unattached kinetochores. These findings demonstrated that in vertebrate cells the inhibitory activity associated with an unattached kinetochore is not diffusible but acts locally within the spindle containing the unattached kinetochore. Furthermore, these experiments also revealed that once a mature spindle entered anaphase the neighbouring spindle immediately followed and enter anaphase regardless of the presence of monooriented chromosomes. Thus, anaphase onset in the mature spindle triggers anaphase in the adjacent spindle overriding the inhibitory signal produced by unattached kinetochores.

7.2. Molecular Components

Based on the observation that the spindle checkpoint is continuously active in cells treated with drugs that disrupt spindle microtubules (for reviews see Rieder and Palazzo, 1992; Jordan and Wilson, 1998), a genetic screen was designed in yeast to isolate mutations that made cells insensitive to spindle damage. As a result of these screens the first components of the spindle assembly checkpoint were identified (Li and Murray, 1991; Hoyt *et al.*, 1991; reviewed by Murray, 1992,1995b). These studies identified six genes, *BUB1-BUB3* (for "budding uninhibited by benzimidazole") and *MAD1-MAD3* (for "mitotic arrest-deficient") whose products are required for cell cycle arrest in response to microtubule poisons that induce depolymerisation. Our knowledge on the function of these proteins has advanced significantly and new members of the checkpoint have also been discovered (for reviews see Rudner and Murray, 1996; Skibbens and Hieter, 1998; Amon, 1999). These include Mps1 a protein kinase that is required for both normal spindle pole body duplication (Winey *et al.*, 1991) and for delaying mitotic exit in cells with spindle pole body defects or in the presence of nocodazole (Weiss and Winey, 1996). Also, Cdc55 (Minshull *et al.*, 1996; Wang and

Burke 1997), a regulatory subunit of protein phosphatase 2A (PP2A) that has been shown to be involved in regulating exit from mitosis (Healy *et al.*, 1991; Gomes *et al.*, 1993). Further studies in *Xenopus* egg extracts showed that p34-ERK2, a member of the Mitogen-activated protein kinase family, is activated after spindle depolymerisation, leading to a cell cycle arrest in mitosis, and is inactivated by a specific phosphatase that overcomes the mitotic arrest (Minshull *et al.*, 1994; Wang et al., 1997).

Mad1 is a phosphoprotein that can bind and recruit Mad2 to unattached kinetochores (Chen et al., 1998, 1999) and is hyperphosphorylated in response to spindle damage (Hardwick and Murray, 1995) by the protein kinase Mps1 (Hardwick et al., 1996). Bub1 is a protein kinase that can bind and phosphorylate Bub3 (Roberts et al., 1994; Farr and Hoyt, 1998), and this interaction is essential for Bub1 to localize to kinetochores (Taylor et al., 1998). Mad1 also associates with Bub1 and Bub3 when the spindle checkpoint is active (Brady and Hardwick, 2000), and in *Xenopus* egg extracts, Bub1 is required for kinetochore localization of Mad1, Mad2, Bub3, and CENP-E, independently of its kinase activity (Sharp-Baker and Chen, 2001). Yeast also contains a protein called Mad3, that shares conserved NH_2-termini with Bub1. Both proteins also contain Cdc20- and Bub3-binding domains (Hardwick et al., 2000). Multicellular animals however, do not have a Mad3 homologue but a Bub1-Related kinase that has been called BubR1. BubR1, like Bub1, has a Mad3 N-terminal domain containing Cdc20- and Bub3-binding sites, and a C-terminal protein kinase domain (Taylor et al., 1998; Jablonski et al., 1998). BubR1 was also independently found as an interactor of CENP-E at the kinetochores (Chan et al., 1998). Furthermore, both Bub1 and BubR1 were found to be mutated in some colorectal cancers suggesting that inactivation of the spindle checkpoint might play a role in tumour formation (Cahill et al., 1998).

7.3. Checkpoint Mechanism and Kinetochore Function

The checkpoint components are widely conserved among organisms, and homologues of genes involved in checkpoint response have been found in many high eukaryotes (Li and Benezra, 1996; Chen et al., 1996; Taylor and McKeon, 1997; Taylor et al., 1998; Martinez-Exposito et al., 1999; Cahill et al., 1998; Basu et al., 1998; Basu et al., 1999; Kitagawa and Rose, 1999; Sharp-Baker and Chen, 2001; Jin et al., 1998; Dobles et al., 2000; Michel et al., 2001; Abrieu et al., 2001; Fisk and Winey, 2001; Stucke et al., 2002).

Mad2 was one of the first checkpoint proteins to attract special interest due to its highly dynamic distribution during early stages of mitosis. Mad2 was found to localise to

unattached kinetochores in prometaphase and to rapidly disappear from kinetochores at metaphase suggesting that it might play an important role in the inactivation of the checkpoint (Chen et al., 1996; Li and Benezra, 1996; reviewed by Shah and Cleveland, 2000). In contrast to 3F3/2 phosphoepitopes that are present on kinetochores in the absence of tension, loss of tension is insufficient to induce Mad2 to accumulate on kinetochores, whereas unattached kinetochores consistently showed kinetochore-associated Mad2 (Waters et al., 1998). This distinct behaviour of Mad2 seems to be regulated by phosphorylation of kinetochore proteins independently of Bub1 kinase activity (Waters et al., 1999; Sharp-Baker and Chen, 2001). Thus, a kinetochore-microtubule attachment mechanism that is sensitive to kinetochore phosphorylation inhibits Mad2 accumulation at kinetochores and should allow anaphase onset. Accordingly, microinjection of antibodies to Mad2 into mammalian cells induces premature anaphase (Gorbsky et al., 1998).

Like Mad2, also Bub1, BubR1 and Bub3 localise to unattached kinetochores and were hypothesized to monitor and/or generate the inhibitory signal that delays the onset of anaphase (Taylor and McKeon, 1997; Taylor et al., 1998; Martinez-Exposito et al., 1999; Jablonski et al., 1998). However, while Mad2 seems to be monitoring spindle attachment to the kinetochores, other proteins like Bub1, BubR1 and Bub3 seem to sense tension at the kinetochores (Skoufias et al., 2001; Tang et al., 2001; Taylor et al., 1998; for reviews see Gillett and Sorger, 2001; Hoyt, 2001). Furthermore, BubR1 interacts directly with the Kinesin-like motor CENP-E, which was shown to be required for the normal activity of the spindle checkpoint *in vitro* (Chan et al., 1998; Abrieu et al., 2000). This interaction may be part of a force-sensing mechanism that responds to tension (Chan et al., 1999; Yao et al., 2000). However, the role of these proteins in the spindle checkpoint sensing mechanism is not yet resolved since recent reports have shown that loss of tension does not change the amounts of either Mad2 or BubR1 at the kinetochores (Hoffman et al., 2001; Shannon et al., 2002).

Of the checkpoint proteins analysed until now, Mad2 is the protein that shows the most significant reduction in its level of accumulation at metaphase kinetochores. This behaviour appears to be due to the inability to accumulate more Mad2 as microtubules saturate kinetochore binding sites, coupled to the rapid movement of Mad2 along K-fibbers toward spindle poles (Howell et al., 2000), a translocation that seems to be mediated by the motor activity of cytoplasmic Dynein/dynactin (Howell et al., 2001). This observation led to the proposal that Dynein/dynactin has a role in inactivation of the spindle checkpoint by transporting kinetochore proteins, such as Mad2, into the poles. In support of this prediction, *Drosophila* embryos with mutations in cytoplasmic Dynein have recently been shown to accumulate Zw10 at metaphase kinetochores (Wojcik et

al., 2001). Zw10 and Rod are required for the metaphase checkpoint in flies and humans (Basto et al., 2000; Chan et al., 2000). They were the first checkpoint components to be identified that do not have obvious homologues in lower eukaryotes. This suggests that metazoans may require an elaborate spindle checkpoint to monitor complex kinetochore functions. The authors proposed that Zw10 and Rod might be monitoring chromosome congression whereas the classical checkpoint components monitor kinetochore attachment to the spindle.

7.4. Checkpoint Control of Anaphase Onset

Cell cycle arrest by the spindle checkpoint is due to inhibition of the anaphase-promoting complex APC/cyclosome (APC/C) an ubiquitin ligase complex composed of at least 11 subunits, that mediates proteolysis of B-type cyclins and regulates sister-chromatid separation (King et al., 1995; Sudakin et al., 1995; Irniger et al., 1995; for reviews see Zachariae and Nasmyth, 1999; Peters, 2002). In mitosis, APC/C is activated by binding to Cdc20 and this is dependent on high Cdk1 activity. Cyclin B degradation begins in metaphase and starts at the centrosomes, spreads to the spindle, and continues during anaphase while sister chromatids segregate (Clute and Pines, 1999; Huang and Raff, 1999; Wakefield et al., 2000). In most species, Cdc20 is also degraded during this period and replaced by Cdh1, which keeps the APC/C active until the end of the subsequent G1 phase by means of a phosphorylation-regulated mechanism (reviewed by Peters, 2002). The relation between the spindle checkpoint and the APC/C was established after finding that Mad2 associates and inhibits APC/C activity (Li et al., 1997). However the link was only established in yeast where it was shown that Mad2 can bind to Cdc20 (Hwang et al., 1998; Kim et al., 1998). The association of Mad2 with Cdc20 was later confirmed in vertebrates and shown to involve a complex containing Cdc27 and maybe Cdc16 (Fang et al., 1998a,b; Wassmann and Benezra, 1998; Kallio et al., 1998). More recently, a complex isolated from HeLa cells, named mitotic checkpoint complex (MCC), containing BubR1, Bub3, Mad2 and Cdc20 in near equal stoichiometry was found to inhibit the APC/C much more effectively than purified Mad2 alone (Sudakin et al., 2001; reviewed by Hoyt, 2001). Similarly, an *in vitro* reconstitution study showed that purified recombinant BubR1 inhibits APC/C activity much more effectively than Mad2 and independently of its kinase activity (Tang et al., 2001; reviewed by Gillett and Sorger, 2001).

7.5. Sister-Chromatid Separation

Sister chromatids are held together by Cohesins, a multi-subunit protein complex that localises to chromosome arms and centromeres (Michaelis et al., 1997; Guacci et al., 1997; reviewed by Heck, 1997). Sister chromatid separation at anaphase onset is triggered by cleavage of SCC1, one of the subunits of cohesin complex, by the protease "Separase" (Ciosk et al., 1998; Uhlmann et al., 1999; Waizenegger et al., 2000; reviewed by Nasmyth, 2001). The spindle checkpoint inhibits exit from mitosis by regulating the activity of Separase (Fig. 11). As cells enter and progress through early stages of mitosis, Separase is normally bound to a protein called "Securin", which blocks its protease activity. However, once all chromosomes become properly attached to the mitotic spindle and the spindle checkpoint is inactivated, the activity of the APC/C increases. Securin is one of the substrates of the APC/C that is degraded during the metaphase-anaphase transition, leading to separase activation and subsequent release of sister chromatid cohesion (Yamamoto et al., 1996a,b; Cohen-Fix et al., 1996; Funabiki et al., 1996; Cohen-Fix and Koshland, 1999; Tinker-Kulbert and Morgan, 1999; reviewed by Biggins and Murray, 1999). Moreover, Securin inhibits the activation by phosphorylation of Cdh1 preventing protein degradation by the APC/C. In summary, degradation of Cyclin B and Securin at the beginning of metaphase is initiated by the APC/C-Cdc20 only once all chromosomes have attached to both poles of the mitotic spindle. Securin release from Separase is essential for cleavage of the Cohesin complex that will trigger sister chromatid separation.

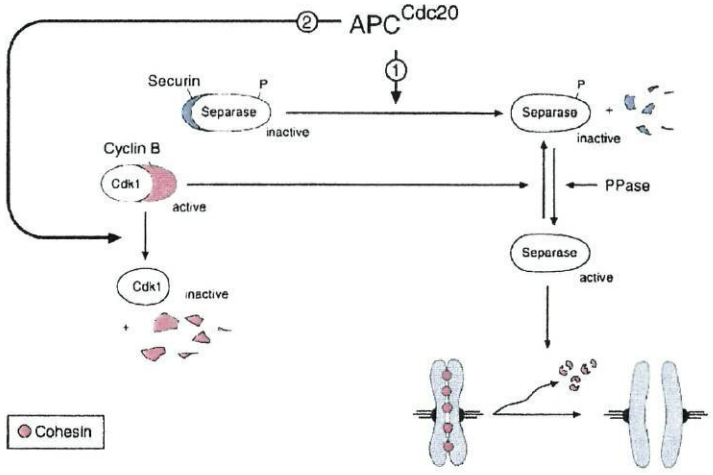


Figure 11. Regulation of Anaphase by APC^{Cdc20}. APC^{Cdc20} initiates anaphase in *Xenopus* embryos through at least two distinct mechanisms. (1) APC^{Cdc20} enables activation of Separase by mediating the degradation of Securin. Active Separase separates sister chromatids from each other by cleaving Cohesin complexes. (2) APC^{Cdc20} also helps to activate Separase by initiating the degradation of Cyclin B and other mitotic Cyclins. Cyclin degradation inactivates CDK1, which in turn allows the removal of inhibitory phosphate residues from Separase by protein phosphatases (PPase). (Adapted from Peters, 2002).

7.6. Control of Spindle Position and Mitotic Exit

A separate branch of the spindle checkpoint is mediated by the *BUB2* gene, which modulates Cdh1 activity and the exit from mitosis (reviewed by Burke, 2000). Bub2 shows few genetic or biochemical interactions with other members of the spindle checkpoint. It localizes at the spindle pole body throughout the cell cycle and activates the mitotic checkpoint via a different pathway from Mad2 that is likely to be triggered by Mps1 (Fraschini et al., 1999; Li, 1999; Fesquet et al., 1999; Alexandru et al., 1999). Bub2 genetically interact with *BFA1*, a gene required for the spindle checkpoint arrest in response to spindle damage that localizes at the spindle pole body and arrests the cell cycle in anaphase when overexpressed (Li, 1999). One of the targets of Bub2 seems to be Dbf2p, a cell cycle regulated protein kinase whose activity peaks in metaphase-anaphase (Fesquet et al., 1999). While the Mad2 dependent pathway may prevent premature disjunction of sister chromatids by monitoring microtubule attachment/tension on kinetochores, the Bub2 dependent pathway seems to prevent exit from mitosis and cytokinesis before completion of chromosome segregation by monitoring the anaphase spindle position (Bloecher et al., 2000; Daum et al., 2000) (Fig. 12).

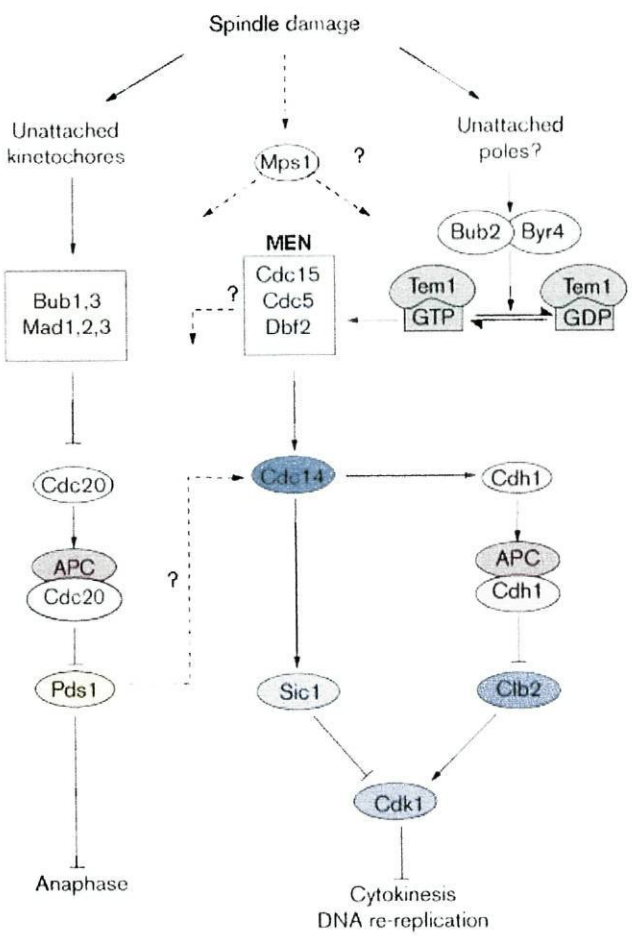


Figure 12. A model showing how spindle damage results in cell cycle arrest in budding yeast. In response to unattached kinetochores, a Mad2-dependent pathway inhibits anaphase by protecting Pds1 (or securing) from degradation mediated by APC–Cdc20. The response to unattached kinetochores also requires Bub1, Bub3, Mad1 and Mad3 proteins. Distinct events, conceivably at the spindle pole, activate a Bub2-dependent pathway that maintains Tem1 in the GDP-bound form. Consequently, components of the mitotic exit network such as Cdc15, Cdc5 and Dbf2 are not activated, and hence Cdc14 remains inactive. Clb2 is therefore protected from degradation mediated by APC–Cdh1, and Sic1 is inactive. Cdk1 activity is therefore sustained, which prevents cytokinesis and DNA re-replication. (Adapted from Taylor, 1999).

Evidence for these conclusions came originally because of sequence similarity between Bub2 and an essential gene called *Cdc16+* from *S. pombe*, which is required for cytokinesis (Gould and Simanis, 1997). Cdc16 binds to Byr4, which in its turn seems to be the putative orthologue of BFA1 from *S. cerevisiae* (Song et al., 1996). Cdc16 and Byr4 activate a GTPase called Spg1/Tem1 that is part of a regulatory network that controls the exit from mitosis (MEN, for mitotic exit network).

The limiting factor for mitotic exit is Cdc14 (Visintin et al., 1998, 1999; Shou et al., 1999) that is activated by the MEN, which in turns activate, among other proteins, Cdh1 which targets proteins for degradation by the APC/C (reviewed by Burke, 2000).

8. Microtubule-Plus-End Tracking Proteins

8.1. CLIP and CLASP Families

Analysis of the dynamic behaviour of microtubules has indicated that interaction of their plus-ends with various factors or cellular structures affects microtubule stability. It has been shown that regional control of microtubule dynamics within cells and local attachment affects microtubule plus end behaviour and plays an important role in the assembly of microtubule-based structures (reviewed by Schuyler and Pellman, 2001). Such factors have been called microtubule-plus-end-tracking proteins and represent a class of highly conserved microtubule-associated proteins that localize to the growing microtubule plus ends.

The first member of this class of proteins to be identified, CLIP-170, was shown to be localized in small patches close to the position of microtubule ends (Rickard and Kreis, 1990), and is required to link endocytic vesicles to microtubules *in vitro* (Pierre et al., 1992). Fluorescence videomicroscopy of living cells showed that CLIP-170 produced as a fusion protein with GFP is localized specifically to growing microtubules, and when microtubules shrink or its dynamics suppressed by drug treatment, GFP-CLIP170 rapidly dissociates from the microtubule end (Perez et al., 1999). Furthermore, it was proposed that GFP-CLIP-170 'treadmills' on the growing microtubule plus end rather than being actively translocated by a motor protein along the elongating tubulin polymer.

CLIP-170 either targets to the microtubule plus-ends by recognition of a specific transient conformation at the growing ends or by co-polymerisation with tubulin. Due to a higher affinity for the GTP-tubulin conformation, CLIP-170 may incorporate into or bind specifically to a cap of GTP-tubulin present at the extremity of polymerising microtubules

(Fig. 13). However, recent evidence from crosslinking and sedimentation velocity experiments favour the second hypothesis, suggesting that the microtubule-plus end targeting of CLIP-170 is closely linked to tubulin polymerisation (Diamantopoulos et al., 1999).

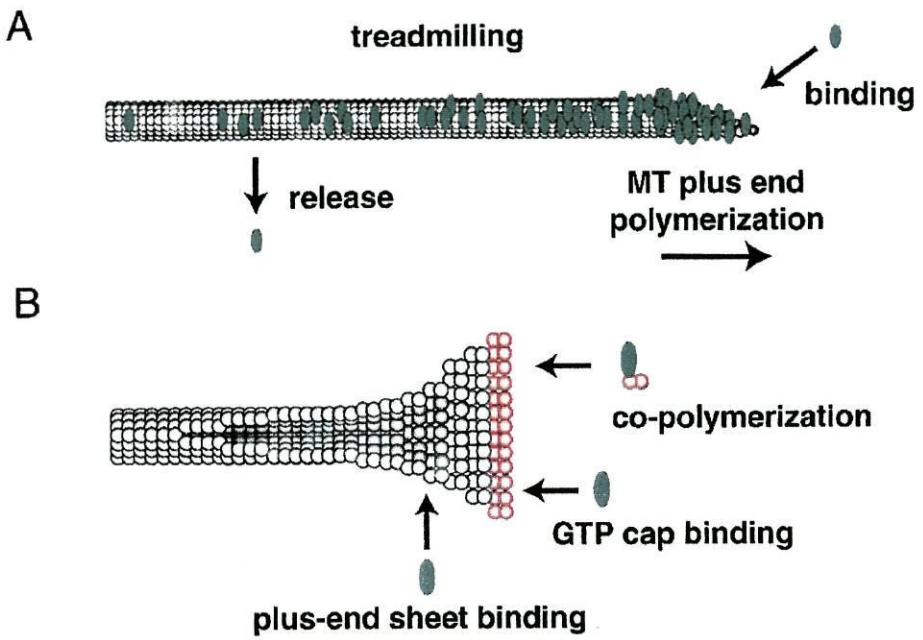


Figure 13. Microtubule-plus-end treadmilling. **(A)** Microtubule-plus-end-tracking proteins are thought to bind the polymerising end of the microtubule and then fall off behind the region of growth. **(B)** Three models exist to explain this treadmilling: the first two are based on the idea that these proteins recognize a specific structural feature of the growing microtubule-plus end, either the GTP-bound tubulin cap or the open sheet of the polymer. One argument against the first model is that the GTP-cap is thought to be small relative to the region decorated by these proteins. However, the true extent of the GTP-cap *in vivo* is not known. The third, model proposes that these proteins bind to free tubulin dimmers and are then co-assembled into the plus-ends during microtubule polymerisation. (Adapted from Schuyler and Pellman, 2001).

Originally it was proposed that CLIP-170 might have a role in regulating microtubule dynamics at the plus-ends (Rickard and Kreis, 1990). While direct evidence for such a role is lacking, experiments in fission yeast have shown that a homologue of CLIP-170, Tip1p, is involved in the regulation of microtubule dynamic instability, by preventing premature microtubule catastrophe until microtubules reach the limit of the cell (Brunner and Nurse, 2000; reviewed by Sawin, 2000).

Fission yeast *tip1* mutants have abnormally short microtubules and fail to target Tea1p to the ends of the cell (Mata and Nurse, 1997; Brunner and Nurse, 2000; reviewed by Hayles and Nurse, 2001). Moreover, short microtubules have also been observed in *Sacharomyces cerevisiae* mutants of *BIK1*, the CLIP-170 homologue in this organism (Berlin et al., 1990).

More recently, other microtubule-plus-end tracking proteins have been identified. From a yeast-two hybrid screen with a conserved region of CLIP-115, a brain microtubule-associated protein that is structurally related to CLIP-170 (De Zeeuw et al., 1997; Hoogenraad et al., 2000), the CLASPs (*CL*IP-*as*sociated *p*roteins) were identified (Akhmanova et al., 2001; reviewed by McNally, 2001). CLASPs are members of the conserved MAST/Orbit family of microtubule-associated proteins (Lemos et al., 2000; Inoue et al., 2000; reviewed by Sharp, 2002). CLASPs were shown to bind microtubules and CLIPs independently (Akhmanova et al., 2001). In polarized fibroblasts, CLASPs localize at the plus ends of microtubules and seem to be responsible for orienting stable microtubule arrays at the leading edge. Thus, like CLIP-170, CLASPs appear to be responsible for at least some local aspects of microtubule dynamics regulation.

Genetic analysis of CLASP homologues in *yeast* and *Drosophila*, have indicated that these proteins might also be required to regulate microtubule behaviour during mitosis. In support for this hypothesis, *STU1*, the putative homologue of MAST/Orbit and CLASPs in *S. cerevisiae* found in a screen for suppressors of cold-sensitive mutants of β-tubulin, was shown to be an essential component of the yeast mitotic spindle (Pasqualone and Huffaker, 1994). Furthermore, mutations in *multiple asters* (*mast*), in *Drosophila melanogaster*, show severe mitotic abnormalities including the formation of mono- and multi-polar spindles organized by clusters of centrosomes (Lemos et al., 2000). Also, during mitosis, MAST, is localized to the mitotic spindle, centrosomes and kinetochores, ending up accumulating in the central-spindle region and ultimately concentrating at the midbody (Lemos et al., 2000).

Another candidate partner for CLIP-170 is Dynein and its regulator dynactin. CLIP-170 co-localizes with Dynein and dynactin subunits both at cortical sites (Vaughan et al., 1999; reviewed by Dujardin and Vallee, 2002) and at the kinetochore, and a role for CLIP-170, Dynein and dynactin in kinetochore function during metaphase chromosome alignment had been proposed (Dujardin et al., 1998; Tai et al., 2002).

8.2. APC and EB1 Families

Cytoplasmic Dynein intermediate chain and dynactin were also shown to interact with EB1, another microtubule-plus-end-tracking protein (Berrueta et al., 1999; reviewed by Tirnauer and Bierer, 2000). EB1 is also part of a conserved family of MAPs that include Bim1p from budding yeast (Schwartz et al., 1997), mal3p from fission yeast (Beinhauer et al., 1997) and dEB1 from *Drosophila* (Lu et al., 2001). EB1 was originally found to bind Adenomatous Polyposis Coli (APC) tumour suppressor protein in a yeast

two-hybrid screen (Su et al., 1995). Subsequently, EB1 has been shown to localize to the centrosome, the mitotic spindle, and the distal tips of cytoplasmic microtubules (Berrueta et al., 1998; Morrison et al., 1998). More recently, the dynamic behaviour of EB1 on the distal ends of microtubules was analysed by expression of a GFP fusion (Mimori-Kiyosue et al., 2000b). The results show that EB1-GFP concentrates at the growing ends of all cytoplasmic microtubules and disappears when microtubules start to shorten.

Until recently it was unclear whether binding of EB1 to APC had any effect upon microtubule dynamics. However, recent experiments showed that EB1 promotes microtubule polymerisation *in vitro* and in permeabilised cells but only in the presence of the C-terminal half of APC, which is required for EB1 binding (Nakamura et al., 2001). Also, it has been shown that this interaction is regulated by phosphorylation and that APC itself promotes microtubule assembly and stability *in vivo* (Zumbrunn et al., 2001). However, immunoprecipitation studies have indicated that the interaction between EB1 and the APC does not appear to occur during mitosis (Askham et al., 2000). This suggests that the EB1-APC interaction only occurs near the ends of microtubules only under restricted conditions.

Like EB1, APC localizes to the microtubule cytoskeleton, as well as to the leading edges of migrating epithelial cells (Nathke et al., 1996). APC is a large multidomain protein of ~300 kDa that is conserved from *Drosophila* to humans (reviewed by Mimori-Kiyosue and Tsukita, 2001). Germline mutations in the *APC* gene are responsible for familial adenomatous polyposis (FAP), an autosomal dominant inherited disease, while somatic mutations in *APC* are frequent in ~80% of sporadic colorectal tumours (reviewed by Fearnhead et al., 2001). FAP is characterized by the appearance of hundreds of adenomatous polyps in the colon and rectum, usually by adolescence or the third decade of life, and when untreated, colorectal cancer invariably develops. The molecular basis of APC function appears to be dependent on its binding to β-catenin, a protein that functions in cell adhesion and Wnt-based signal transduction pathways (Rubinfeld et al., 1993; Su et al., 1993; reviewed by Peifer and Polakis, 2000).

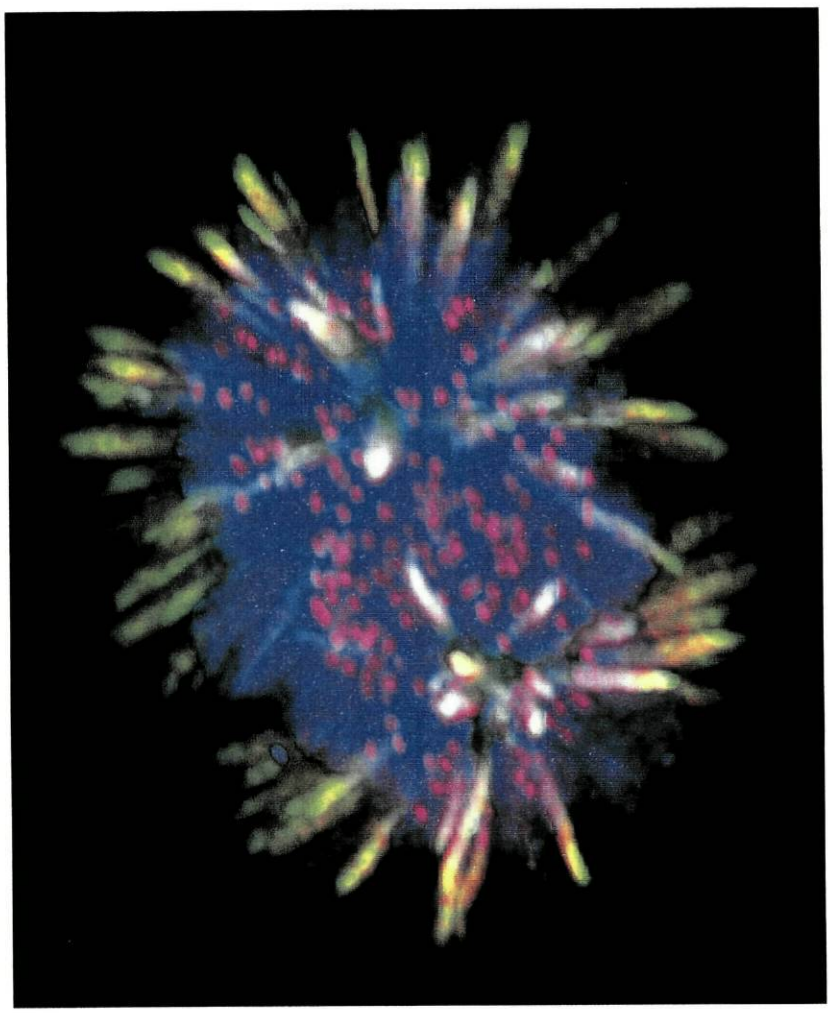
Analysis of GFP-tagged APC in living cells has revealed in detail the peculiar dynamic behaviour of APC (Mimori-Kiyosue et al., 2000a; reviewed by Mimori-Kiyosue and Tsukita, 2001). It was shown that APC-GFP moved continuously along a subset of microtubules toward their distal ends in an ATP-dependent manner and accumulated as granular aggregates at the growing plus ends, and when microtubules begin to shorten, the APC granules drop-off from the microtubule ends.

9. Objectives

How microtubules organize to form a functional spindle during mitosis remains poorly understood. Most advances into this subject have been accomplished through the characterization of several motor proteins that function during several stages of mitosis, from centrosome separation to chromosome movement. In addition to these proteins, several others have been found that also associate with microtubules but do not have motor activity and are collectively known as microtubule-associated proteins or MAPs.

The mitotic role of MAPs in spindle assembly is even more mysterious than the process itself. The aims of this work were to unravel the function of some of these proteins during mitosis. The first part of the work, described in chapter I, involved the genetic and molecular characterization of *mast*, a new *Drosophila* gene that was identified by P-element insertion mutagenesis. In the second part, described in chapters II and III, we aimed to analyse the role of MAST in the process of spindle organization and its involvement in the attachment of spindle microtubules to kinetochores of mitotic chromosomes. Furthermore, we have characterized the process of mitotic exit in the absence of MAST. In the third part, described in chapters IV and V, we set out to identify and analyse the role of the human homologues of MAST, CLASPs, in microtubule stability and spindle function. Finally, in the last part, described in chapter VI, we studied the role of two microtubule-associated proteins that belong to the "chromosomal passenger" family, namely, the *Drosophila* INCENP and Aurora B for their role in mitosis.

II. Experimental Work



CHAPTER I

MAST is a Novel Evolutionary Conserved Protein Essential for Mitosis

1. Introduction

Drosophila melanogaster is one of the most important model organisms in biology. It has been the organism of choice for genetic studies for almost a century and has proven to be invaluable to study essential biological processes like the cell cycle and embryonic development. *Drosophila* has a short life cycle of only two weeks and there are extensive collections of mutants with the most diverse genetic deficiencies. Furthermore, sequence and subsequent analysis of its genome has shown to contain a high degree of conservation with higher eukaryotes including humans.

During the life cycle of *Drosophila* there are two periods in which diploid cell proliferation is essential. The first one takes place during the early embryonic stage and the other during larval development. The early embryonic divisions represent one of the fastest eukaryotic cell cycles found in nature. The first 13 mitotic cycles are nuclear divisions that occur in synchrony in a syncitial embryo. These nuclei oscillate quickly between S and M phase without any gap phase (for reviews see Glover, 1989, Glover et al., 1989; Glover, 1991; Orr-Weaver, 1994). During these early cycles, gene transcription is not required and the embryo relies on the contribution of maternal genetic products (mRNA and proteins) deposited in the egg during oogenesis. After cycle 14, cellularisation takes place, embryonic transcription starts and a clearly defined G2 phase is added to the cell cycle. From this moment on, embryonic cells no longer divide in synchrony but are restricted to mitotic domains. After mitosis 16, for the first time a G1 phase can be detected and the embryo already has differentiated larval tissues, as well as imaginal and nerve cells, which will make part of the adult body. The cell cycle of larval precursor tissues is polytenic while the adult cell cycle is diploid. However, in the third instar larvae, the central nervous system and imaginal cells already have a classic cell cycle with G1, S, G2 and M phases (reviewed by Orr-Weaver, 1994). Polytenisation of larval tissues is a consequence of a modified cell cycle, where an S phase alternates with a gap phase without mitosis, i.e., larval tissues grow only in size but not in the number of cells (reviewed by Ripoll et al., 1987). Due to

the particular *Drosophila* development during larval stages, the only mitotically active tissues are the neuroblasts and the imaginal discs.

Drosophila genome contains approximately 14,000 genes distributed in four pairs of chromosomes (Adams et al., 2000a): sexual chromosomes X or Y, and the autosomes 2, 3 and 4. Before the *Drosophila* genome was sequenced, genes were mapped on polytene chromosomes that form during larval stage due to the characteristic banding pattern. One of the most common approaches used to identify and study genes involved in mitotic progression consists on selecting mutants in which mitotic functions are defective (reviewed by Ripoll et al., 1987). Cytological analysis of cell division of wild type and mutant lines using third instar larval neuroblasts of *Drosophila melanogaster* have allowed the identification of important proteins for the regulation of mitosis (Ripoll et al., 1985; Sunkel and Glover, 1988; Gonzalez et al., 1988; Gatti and Baker, 1989; reviewed by Gatti and Goldberg, 1991):

The powerful methodology for mutagenesis by insertion of P-element transposons offers one of the fastest and direct ways from the identification of a mitotic gene to its molecular cloning (Rubin and Spradling, 1982, Spradling and Rubin, 1982; Cooley et al., 1988b; for reviews see Karess, 1985; Cooley et al., 1988a, Craig, 1990; Rio, 1990; Sentry and Kaiser, 1992). The basis for this methodology involves the mobilization of a genetically marked P element transposon from a pre-existing site of integration in the genome to a new location where the function of a mitotic gene may be disrupted. Genes defined by such mutations can then be cloned by inverse PCR or plasmid rescue (if the P-element contains plasmid sequences), taking advantage of the P-element as a molecular tag. The study of revertents for the induced mutation allows the confirmation and specificity of the phenotype observed in the mutants that have resulted from the insertion of a single P-element, and this can be done reintroducing the 'jumpstarter' element by means of genetic crosses with the appropriate strain. The loss of the phenotype is related to the reestablishment of the marker gene from the 'mutator' element.

In a search for new proteins required for mitosis we have carried out cytological and molecular characterization of several alleles of a novel *Drosophila* mutation that we have termed as *multiple asters* (*mast*). The first *mast* mutant allele (*mast*^{1}) was identified by Omel'yanchuk and colleagues from a collection of P-element insertion mitotic mutants in *Drosophila melanogaster* (Omel'yanchuk *et al.*, 1997). Subsequently, two other P-element induced alleles, *mast*^{2} and *mast*^{3}, were identified from the Berkeley *Drosophila* Genome Project (BDGP) database. A fourth allele, *mast*^{4}, an imprecise excision allele, was obtained after remobilisation of the P-element in *mast*^{1} (Lemos et al., 2000).

2. Results

2.1. Characterisation of the multiple asters (mast) Mutations

mast^{1} and *mast*^{4} cause late larval/pupal lethality when homozygous or hemizygous over Df(3L)31A. *mast*^{3} causes early embryonic lethality of homozygous individuals, suggesting the presence of a second mutation, since *mast*^{3}/Df(3L)31A die during late larval/pupal stages. *mast*^{2} allele is semi-lethal and viable adult homozygous, hemizygous or heterozygous over the other alleles are obtained. These adults are sterile, moreover testis and ovaries of *mast*^{2} and *mast*^{2}/Df(3L)31A adults are rudimentary (Lemos et al., 2000).

In comparison with wild type mitosis (Fig. 1.1A and I), neuroblasts of homozygous or hemizygous larvae carrying *mast* mutant alleles show severe mitotic abnormalities. These include highly condensed chromosomes (Fig. 1.1B-F) that are frequently organised in circular arrangements (Fig. 1.1B-D and M, asterisks) and occasionally scattered (Fig.1.1 C-D, arrows), highly polyploid cells (Fig. 1.1E-H, N and O, asterisks) and few abnormal anaphases (Fig. 1.1J-L). Quantification of mitotic parameters shows that *mast*^{1} and *mast*^{2} do not cause a significant increase in the mitotic index. However, *mast*^{3}/Df(3L)31A shows an elevated mitotic index and *mast*^{4} a severe mitotic arrest (Fig. 1.1P). Quantification of mitotic figures with respect to mitotic progression indicates that all mutant alleles cause a decrease in the number of cells in prophase, a significant increase of cells in prometaphase/metaphase and a decrease in the proportion of cells in anaphase or telophase (Fig. 1.1Q). Quantification of the different types of mitotic abnormalities suggests that most alleles cause either a severe increase in the proportion of polyploid cells, metaphases with a circular chromosome configuration or abnormal anaphases (Fig. 1.1R). The effects on viability, mitotic phenotype and mitotic progression allowed us to order the alleles from least affected to very severe according to the following series: *mast*^{2} < *mast*^{1} < *mast*^{3} < *mast*^{4}. Taken together, these results indicate that mutations in *mast* cause severe abnormalities in chromosome segregation leading cells to arrest at prometaphase/metaphase. However, the arrest can be overcome and cells undergo multiple rounds of proliferation since most of them are polyploid.

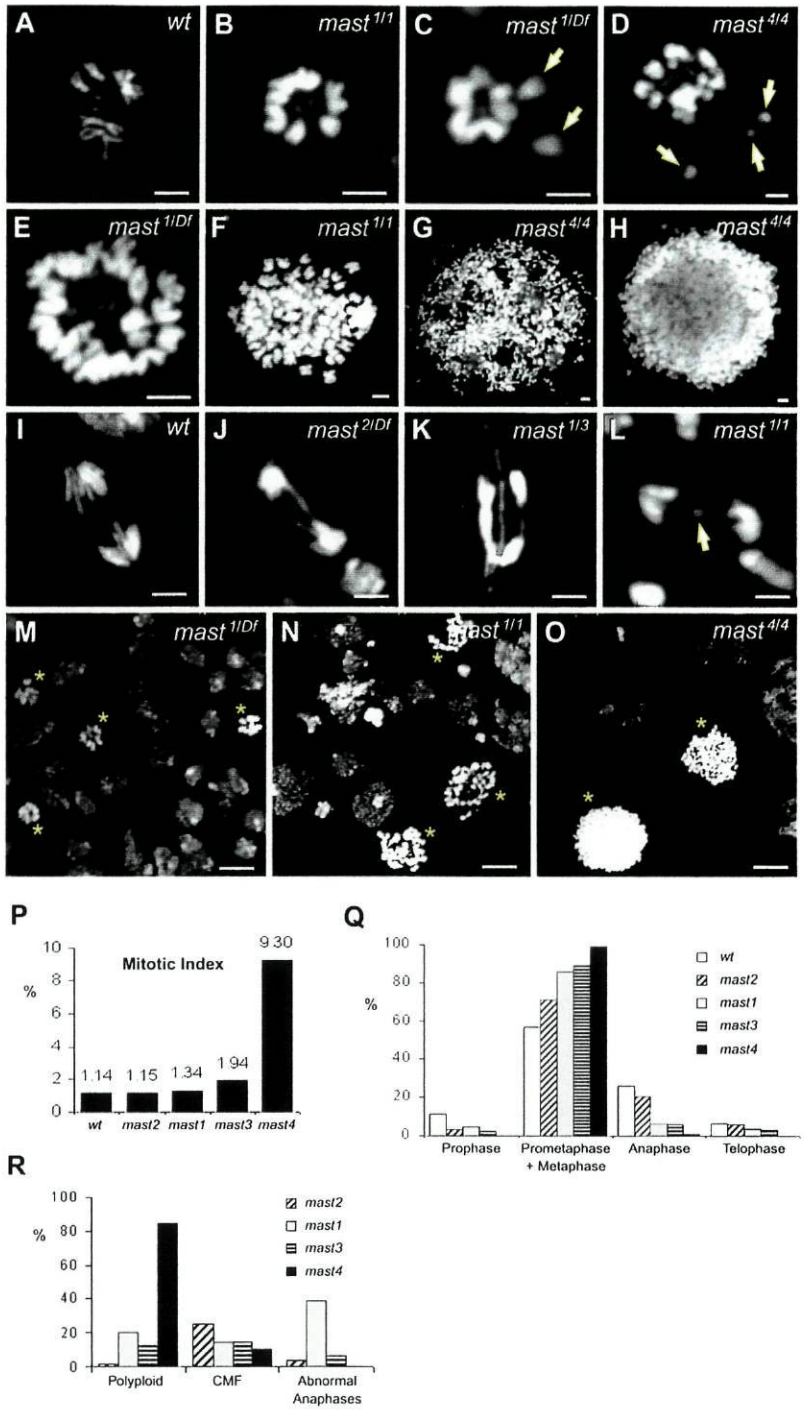


Figure 1.1. Cytological analysis and quantification of mitotic phenotypes in *mast* mutant neuroblasts. Third instar larval brains were dissected from wild type **(A** and **I)** or *mast* mutant **(B-H** and **J-O)** individuals. Wild type cells in metaphase **(A)** or anaphase **(I)** are shown for comparison. *mast* mutant cells show either diploid **(B-C)** or polyploid **(D-E)** circular mitotic figures with chromosomes organised with their centromeres facing a central region where the small fourth chromosomes are located. In addition, scattered chromosomes can occasionally be found **(C-D**, arrows**)**. Most cells show highly condensed chromosomes **(B-F)** and some are hyperpolyploid **(G-H)**. *mast* mutant cells at anaphase **(J-L)** can also be found and occasionally show chromatin bridges and abnormal chromosome segregation. *mast*^{1} show a mitotic phenotype that range from multiple circular mitotic figures **(M)** to polyploidy **(N)**, while in the most severe *mast*^{4} allele, cells show hyperpolyploidy with most chromosomes organised in a sphere-like conformation **(O)**. Asterisks indicate circular mitotic figures in M and polyploid cells in N and O. **(P)** Quantification of mitotic index. **(Q)** Quantification of mitotic cells with respect to different stages of mitosis. **(R)** Quantification of the abnormal mitotic parameters in all alleles. Bar corresponds to 5 μm except in figures M, N and O, that is 50 μm.

2.2. Molecular Cloning of the multiple asters Gene

In order to identify the mutated gene, we characterised the locus at the molecular level. A single P-element insertion in the *mast*^{P1} allele was mapped by *in situ* hybridisation to the 78C1-C2 cytological region on chromosome 3 and both sides of the insertion were cloned by plasmid rescue and inverse PCR (Lemos et al., 2000). DNA sequence analysis with BDGP databases indicated that *mast* corresponded to a new gene and a number of ESTs were identified. We fully sequenced the largest cDNA (LD11488) derived from an embryonic library, and found to be composed of 5938 bp (these sequence data is available at DDBJ/EMBL/GenBank databases under accession number AF250842). A second transcript with 5.2 kb derived from cDNA libraries of adult heads and larva/early pupa was also identified (Lemos et al., 2000). Since *in situ* hybridisation on polytene chromosomes with the largest cDNA hybridises to a single site (Fig.1.2), the simplest interpretation is that tissue-specific alternative splicing produces the two transcripts.

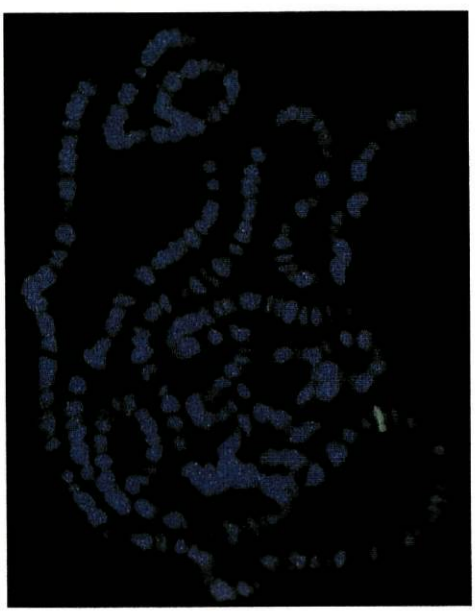


Figure 1.2. Fluorescence *in situ* hybridisation in *Drosophila* polytene chromosomes (blue) using the LD11488 cDNA of *mast* as a probe (green). Mapping of the single *mast* gene is confined to the cytological region 78C1-C2 of chromosome 3.

The 5938 bp cDNA sequence contains a single ORF of 1491 amino acids, encoding for a protein with a predicted molecular mass of 165.5 kDa and a pI of 9.17 (Fig. 1.3). Analysis of the protein sequence showed that MAST contains a 170 amino acid region that shares limited homology with the proline-rich domain of MAP4 (Fig. 1.3), that is thought to be involved in the high efficiency binding to microtubules (Aizawa *et al.*, 1991). It also contains two regions with significant homology to the HEAT repeat (Andrade and Bork, 1995), at positions 169-207 and 1414-145. This motif was identified in HUNTINGTIN, a protein associated to Huntington's disease, is also present in the 65

kDa regulatory subunit of PP2A (Hemmings *et al.*, 1990) and in proteins of the dis1-TOG family (Tournebize *et al.*, 2000). The MAST protein also contains two consensus phosphorylation sites for CDK1 (Kennelly and Krebs, 1991).

Figure 1.3. Predicted amino-acid sequence of MAST. Black boxes represent HEAT repeats and predicted sites of phosphorylation by CDK1 are bold underlined with the putative phosphorylated residues marked with asterisks. The grey region defines the conserved MAP-4 microtubule-binding domain.

2.3. Evolutionary Conservation of MAST

In order to determine whether the MAST protein is evolutionary conserved we performed searches against current databases. MAST shares significant identity with proteins encoded by two human cDNAs, KIAA0622 and KIAA0627 (Ishikawa *et al.*, 1998), three putative proteins in *C. elegans* , C07H6.3, R107.6 and ZC84.3 (Wilson *et al.*, 1994) and also limited identity with Stu1p from *S. cerevisiae* (Pasqualone *et al.*, 1994) and its putative homologue in *S. pombe*, that we have called SpStu1p. Multiple alignment of the MAST sequence with those most closely related from other species showed that all the proteins share identity throughout their sequence, however three regions (CR-1, CR-2 and CR-3) are more highly conserved (Fig. 1.4 and 1.5A). These results suggest that MAST and its homologues define a new evolutionary conserved protein family, that we have named MAST/Orbit due to an independent work by another group that identified the same *Drosophila* gene, which was given the name *orbit* (Inoue et al., 2000).

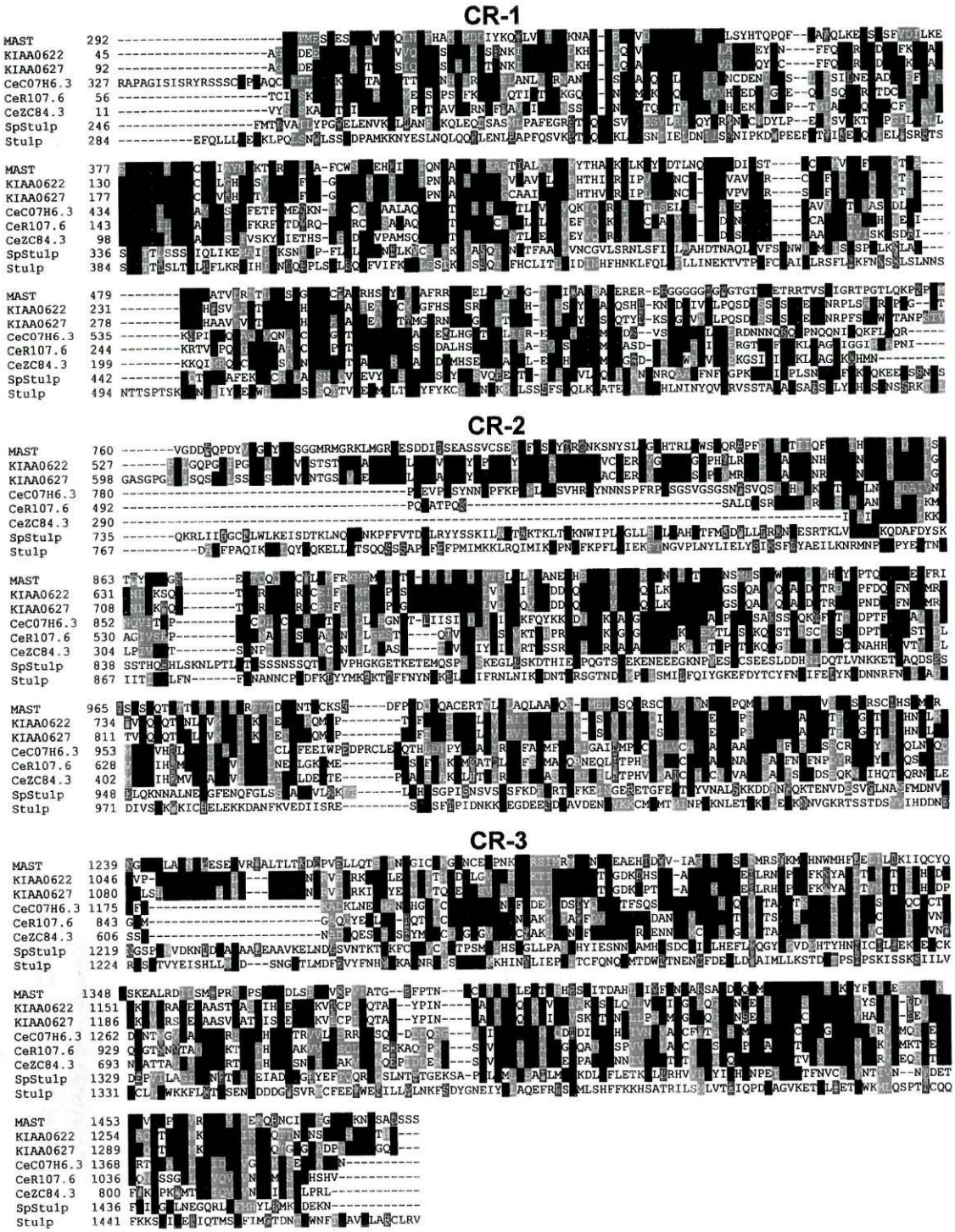


Figure 1.4. Multiple sequence alignment of the predicted protein sequences closely related to MAST, including two human (KIAA0622 and KIAA0627), three *C. elegans* (CeC07H6.3, CeR107.6 and CeZC84.3), one *S. pombe* (SpStu1p) and one *S. cerevisiae* (Stu1p).

Database searches also indicate that MAST shares identity to proteins from the dis1-TOG family, specially at the N-terminal half of the protein (amino acids 1-494), where they are 20-25% identical and 40-45% similar. Inside this region, there is a small domain of 18 amino acid residues that is highly conserved among these proteins, that

we have termed as TOG-box (Fig. 1.5A). Phylogenetic analysis including the most relevant sequences from the two groups suggested that they are evolutionarily close, but distinct, since they were positioned in different branches of the unrooted tree (Fig. 1.5B).

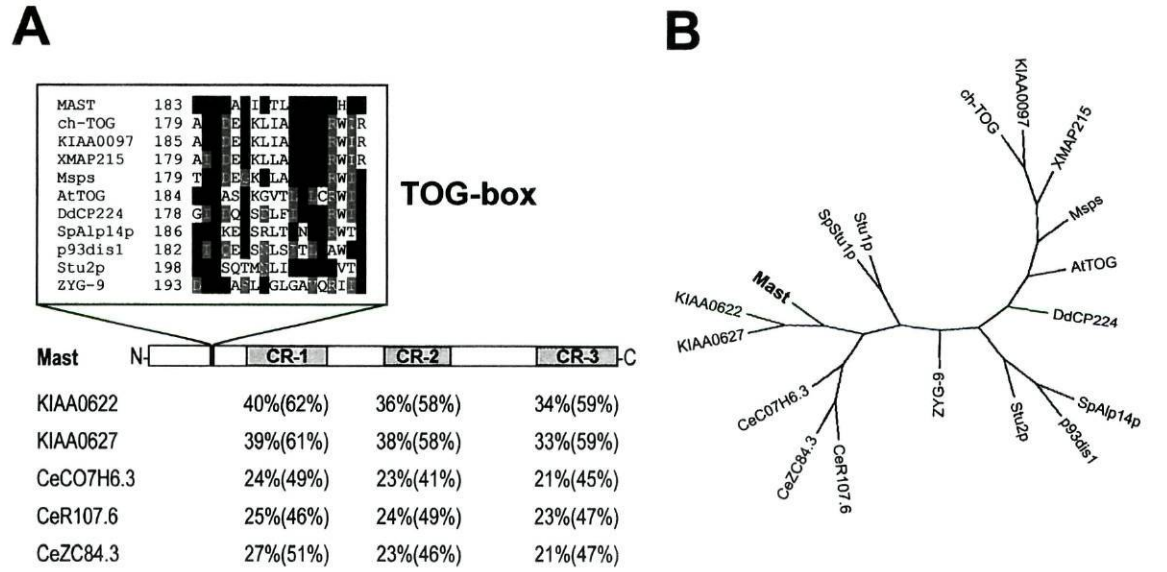


Figure 1.5. MAST is closely related but distinct to Dis1-TOG family of MAPs. **(A)** Conserved regions between MAST family members are represented in grey boxes and percentage identity and similarity (in parenthesis) of the most conserved proteins is indicated below. Additionally, a small domain of 18 amino acid residues that is highly conserved between MAST and members of the Dis1-TOG family is represented as the TOG-box. **(B)** Phylogenetic unrooted tree with proteins that share significant sequence identity with MAST.

3. Discussion

3.1. MAST is Essential for Mitosis in Drosophila

We have found that MAST has an essential role during mitosis. According with the gradual severity of the alleles, mutations in MAST cause an increase in mitotic index and a strong accumulation of cells in prometaphase/metaphase, mostly with chromosomes organized in circles. This particular organization is typical of chromosomes associated with monopolar spindles and resembles the phenotype of other mutations in *Drosophila* like *polo*, *aurora* and *klp61f* (Sunkel and Glover, 1988; Glover et al., 1995; Heck et al., 1993). All these mutations affect proteins involved in bipolar spindle organization. Polo and Aurora A are the founding members of conserved protein kinase families (reviewed by Nigg, 2001) and have been implicated in initial centrosome separation and maturation.

More recent results about Aurora A function point toward a role in the assembly and maintenance of spindle bipolarity (Giet and Prigent, 2000; Hannak et al., 2001; Giet et al., 2002). A similar role has been proposed for KLP61F (Sharp et al., 1999, 2000a), whose homologue in vertebrates, Eg5 was shown to interact and to be phosphorylated by Eg2, the Aurora A homologue in this organism (Giet et al., 1999). Accordingly, the abnormal chromosome distribution in *mast* neuroblasts is likely to be due to an aberrant spindle organization and suggest that MAST might have a role either in centrosome separation or assembly/maintenance of spindle bipolarity.

Additional defects in *mast* mutant neuroblasts were also found, including abnormal anaphases with lagging chromosomes and prometaphase cells with scattered individual chromosomes. Since MAST localizes to the kinetochores independently of microtubules (Lemos et al., 2000), this might reflect a role in the attachment of microtubules to kinetochores, a process that is critical during chromosome congression and segregation.

3.2. MAST and the Control of Mitotic Progression

Despite the increase in mitotic index, which is per se a strong indicative of a functional spindle checkpoint, mutations in *mast* cause the formation of highly polyploid cells. Mutant cells respond to the spindle checkpoint when arrested in prometaphase by incubation with colchicine (data not shown). Furthermore, mutant cells with abnormal spindle morphology and highly condensed chromosomes show strong accumulation of the spindle checkpoint protein BubR1 (Lemos et al., 2000). This staining pattern is similar to that observed in chromosomes of cells arrested in prometaphase after microtubule depolymerisation (Basu *et al.*, 1999), suggesting that in *mast* mutant cells the interactions that occur between microtubules and chromosomes are unable to inactivate the spindle checkpoint, leading to a prolonged prometaphase arrest. However, since highly polyploid cells are formed, these cells must have undergone multiple cell cycles in the absence of chromosome segregation and cytokinesis. Therefore, it is most likely that after some time, these cells either become insensitive or override the spindle checkpoint and progress into a new cycle of proliferation.

3.3. MAST is Part of a New Family of Microtubule-Associated Proteins

Database searches and phylogenetic analysis have shown that MAST is part of a new conserved family of proteins that contains two human, three *C. elegans* and two yeast members. MAST is more closely related to the humans and the three *C. elegans* than either to the *S. pombe* or the *S. cerevisiae* proteins. Indeed, extensive identity is observed between the *Drosophila*, human and *C. elegans* members throughout the protein sequence.

MAST was shown to associate with polymerised microtubules *in vitro* (Lemos et al., 2000). Sequence analysis of the MAST protein revealed that it shares conservation to both bovine and mouse MAP4. The homology is restricted to a domain rich in proline and basic residues thought to be involved in microtubule binding (Aizawa *et al.*, 1991). This domain falls outside the conserved regions CR-1, CR-2 and CR-3. In agreement, Stu1p was also demonstrated to interact with microtubules (Pasqualone and Huffaker, 1994). The presence of two putative CDK1 phosphorylation sites suggests that MAST might undergo specific post-translational modifications during G2/M transition allowing it to reach specific mitotic structures. A number of MAPs are known to be phosphorylated by CDK1 upon entry into mitosis allowing for modifications in microtubule dynamics to take place (Verde *et al.*, 1992).

MAST shares limited homology with members of the dis1-TOG family. However, like MAST, all these proteins localise either to centrosomes and/or spindle microtubules during mitosis (Lemos et al., 2000). In some respects, MAST shows patterns of localisation closer to those of ZYG-9, $p93^{dis1}$ and XMAP215. These three proteins co-localise with interphase microtubules and, during late stages of mitosis, MAST shows strong localisation to the spindle midzone, like Msps and ch-TOG, two other members of the dis1-TOG family (Cullen et al., 1999; Charrasse et al., 1998). MAST and ZYG-9, however, do show some unique features since both proteins remain localised to the centromeres of mitotic chromosomes when cells are incubated in the presence of microtubule depolymerising agents (Lemos et al., 2000, Matthews et al., 1998). The localisation of MAST to microtubules, centrosomes, centromeres and the spindle midzone (Lemos et al., 2000) suggests very strongly that this protein might play a role in the regulation of microtubule behaviour as it has been shown *in vitro* and in cell free extracts for some members of the dis1-TOG family (Tournebize *et al.*, 2000).

4. Materials and Methods

Genetic Variants

mast^{1} was initially described as the *v40*^{1} allele of the gene *v40* (Fedorova *et al.*, 1997). It has the P-element P{1ArB} inserted in position 78C1-C2 (after nucleotide 28 in the cDNA sequence) Two other mutant alleles, *mast*^{2} and *mast*^{3}, that correspond to strains EP(3)3515 and EP(3)3403, respectively, were obtained from *Berkeley Drosophila Genome Project*. *mast*^{4} was obtained by mobilisation of the P-element of *mast*^{1} as described (Lemos *et al.*, 2000). Df(3L)31A, a deficiency for the region 78A-78E, was obtained from *Bloomington Drosophila Stock Centre*. All lines were balanced over TM6B. Oregon-R strain was used as wild-type control and all stocks were grown at 25°C under standard conditions and media.

Cytological Analysis in Drosophila Brain Squashes

Third instar larva were dissected in 0.7% NaCl in order to isolate their brains. These were consecutively incubated first in 45% acetic acid for 30 s and then in 60% acetic acid for 60 s. Each brain was squashed with the thumb between a slide and a cover slip, which was then removed after immersion in liquid nitrogen. After complete drying, the squashed brain was incubated with 10 μg/ml RNase for 10 min. After careful removal of RNase, propidium iodide was added 1:1000 in Vectashield (Vector). Image stacking were collected with a MRC600 confocal microscope (BioRad) and projected into a single plane using COMOS software (BioRad). For quantification of the mitotic parameters, the number of cells present in 50 random optic fields (100x) per brain was counted. Five brains were scored for each allele and all the calculations were based on the sum of total number of cells present in the five brains scored.

Molecular Cloning and Sequencing of mast cDNA

Genomic fragments adjacent to P element insertion of *mast*^{1} were cloned by inverse PCR and sequenced with T7-Sequencing Kit (Pharmacia). Three EST clones were identified by sequence homology in the *Berkeley Drosophila Genome Project* database: LD11488 (0 to 24 hours mixed stage embryonic library), LP08134 (larval-early pupal library) and GH26741 (adult head library). The genomic sequence (AC014071), from Celera Genomics, encodes the complete *mast* gene. The LD11488 cDNA clone was fully sequenced as before. This cDNA is 5938 bp long, with a 769 bp 5' untranslated region and a 3' untranslated region of 679 bp.

Database Searches and Sequence Analysis

BLAST searches (Altschul *et al.,* 1997) were performed in non-redundant GenBank CDS translations, PDB, SwissProt, SPupdate and PIR databases. All the unpublished protein sequences that were used can be found under the following accession numbers: CeC07H6.3 (CE01153), SpStu1p (CAA15921), At-TOG (AAD15450), SpAlp14p (CAA22843). Phylogenetic analysis and unrooted dendrogram elaboration were performed using PHYLIP (Retief, 2000).

Mapping of the Cytological Region of mast by FISH on Polytene Chromosomes

Fluorescence *in situ* hybridisation on *Drosophila* polytene chromosomes was performed using the full-length cDNA of *mast* as described previously (Coelho et al., 1996). Image stackings were collected with a MRC600 confocal microscope (BioRad) and projected into a single plane using COMOS software (BioRad). Final images were processed using Adobe Photoshop 5.0 (Adobe Systems).

CHAPTER II

MAST has a Role in Microtubule-Kinetochore Attachment and is Essential for Chromosome Alignment and Spindle Bipolarity

1. Introduction

In the previous chapter we described the identification of MAST and showed that it is an essential protein for normal mitosis, probably required for centrosome separation and/or spindle assembly. During mitosis, MAST is localized to the mitotic spindle, centrosomes and kinetochores, ending up accumulating in the central-spindle region and ultimately concentrating at the midbody (Lemos et al., 2000). Stu1, the putative homologue of MAST in *S. cerevisiae* was also shown to be required for spindle assembly (Pasqualone and Huffaker, 1994).

While genetic analysis remains one of the most powerful methods to determine the function of a particular protein, this is not always possible or may lead to ambiguous interpretation due to complicated phenotypes. In recent years, alternative ways to inactivate a gene either permanently or transiently have been developed. In many species, introduction of double-stranded RNA (dsRNA) induces potent and specific gene silencing, a phenomenon called RNA interference or RNAi (reviewed by Bosher and Labouesse, 2000). Gene silencing through RNAi was first discovered after the introduction of dsRNA into *C. elegans* and found that gene expression was suppressed in a homology-dependent manner much more efficiently than does either sense or antisense RNA (Fire et al., 1998). RNAi seems to act post-transcriptionally, targeting RNA transcripts for degradation (for reviews see Sharp, 2001; Carthew, 2001). More importantly, RNAi has attracted attention because it is a way of knocking off the activity of specific genes in most model organisms in biology, and with the advent of the genome sequencing has become a quick and powerful tool for the study of gene function (Fraser et al., 2000; Gönczy et al., 2000).

Among many other organisms, RNAi has proven to be highly effective in *Drosophila* embryos (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999). Subsequent studies have demonstrated that RNAi can efficiently knock off specific proteins in several *Drosophila* cell lines including S2 cells (Clemens et al., 2000), highlighting the usefulness of RNAi in the study of cellular processes. Indeed, RNAi in *Drosophila* tissue culture cell lines has been used successfully in the study of mitosis (Giet and Glover,

2001, Adams et al., 2001b) and become a prominent tool in *Drosophila* cell cycle research.

In order to gain further insight into the role of MAST during mitosis, we performed a time-course cell cycle analysis after RNAi in *Drosophila* S2 cells. The main reasons for this approach was because we did not have a null allele of *mast* and the available alleles provided a somewhat heterogeneous phenotype. Also, we wanted to determine what are the initial requirements for this protein during mitosis and how the phenotype may evolve in the complete absence of MAST.

2. Results

2.1. Cell Cycle Progression after MAST RNAi

To analyze the immediate effects of MAST ablation on cell cycle progression, we carried out RNAi in *Drosophila* S2 cells. For this purpose we have synthesized a fragment of ~700 bp of dsRNA from the 5' region of *mast* cDNA covering the ATG encoding the first methionine (Fig. 2.1A, B). Addition of dsRNA into cultured S2 cells did not affect cell viability significantly, as determined by trypan blue staining of dead cells (Fig. 2.1C). Immunoblotting analysis showed that MAST levels decreased significantly within the first 48 h following addition of dsRNA and the protein was barely detectable by 144 h (Fig. 2.2A, left). Serial dilution experiments enabled us to determine that addition of 10 μg/ml of dsRNA to 10^6 exponentially growing S2 cells was sufficient to cause >90% reduction in the levels of MAST after 48 h (Fig. 2.2A, right). In order to assess the effects of RNAi upon the cultured cells, samples were collected every 24 h and cell number, mitotic index and cell viability determined. Compared to the control population within the first 72 h, MAST RNAi caused a slight increase in the cell doubling time from 23.6 h to 27.5 h, however, between 72 and 144 h the doubling time increased significantly from 56.4 h to 93.7 h (Fig. 2.2B). By 72 h of RNAi, the mitotic index increased 5-6 fold (Fig. 2.2C and F-F'). Later, the mitotic index decreased, coincident with a slowing of the growth rate (Fig. 2.2B, C).

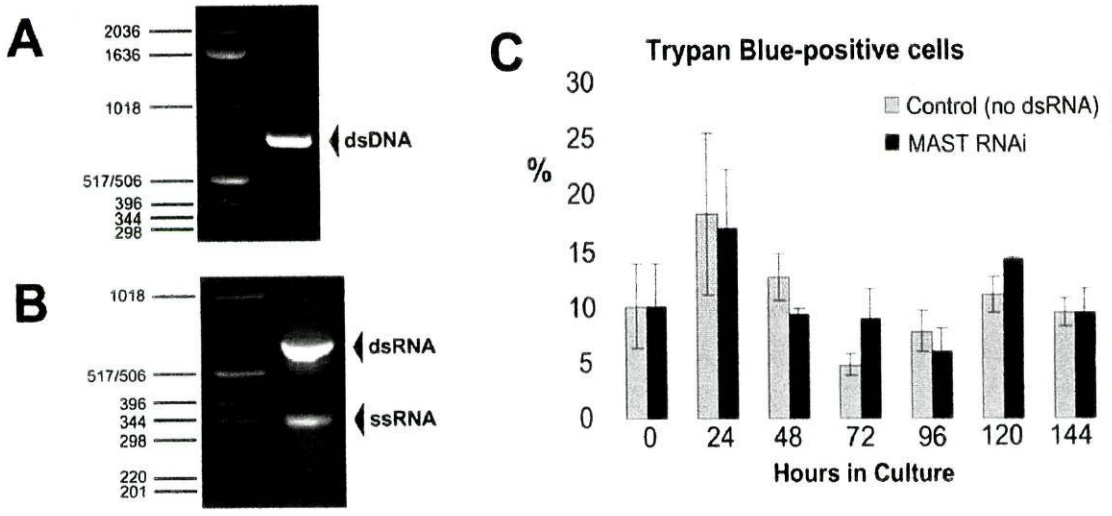


Figure 2.1. Addition of dsRNA to deplete MAST did not affect cell viability. **(A)** Amplification of ~700 bp from *mast* cDNA LD11488 by PCR. **(B)** Preparation of dsRNA by in vitro transcription from the dsDNA obtained by PCR. Low molecular weight band correspond to ssRNA that did not form duplexes. **(C)** Analysis of cell viability upon MAST RNAi by staining of dead cells with trypan blue. Error bars represent standard deviation of the sample.

In order to quantify the mitotic stages after MAST RNAi, we scored MAST negative cells (identified by immunofluorescence) that were also immunostained to visualize α-tubulin (Fig. 2.2D, D'). The results indicate that throughout the course of the RNAi experiment, most mitotic cells (>90 %) were in a prometaphase-like stage displaying either monopolar spindles or spindles that were bipolar with chromosomes that were not aligned at the metaphase plate. Parallel *in vivo* studies of mitosis in *mast*^{5} embryos revealed that the formation of the monopolar spindle is likely to occur post-prometaphase (Maiato et al., 2002). Consistently, after 48 h no cells in metaphase, anaphase or telophase were found (Fig. 2.2D'). Instead, at much later times (120 h) cells in an anaphase-like (Fig. 2.2 D', see also Fig. 2.3H-I) or telophase-like configuration (Fig. 2.2D', see also Fig. 2.3J-J'') containing clustered centrosomes at only one pole became more frequent.

After 72 h of exposure to MAST dsRNA, a significant proportion of the interphase cells had a single nucleus that was substantially larger than that of control cells (Fig. 2.2 E-E'). Staining cells for actin and DNA indicated that after 144 h MAST depleted cells were up to 5 fold larger in diameter when compared with control interphase cells (Fig. 2.2G-G'). This indicates that after long exposure to MAST RNAi, a significant proportion of cells become polyploid. A very similar phenotype was previously described for *mast*^{4} a severe hypomorph allele (Lemos et al, 2000).

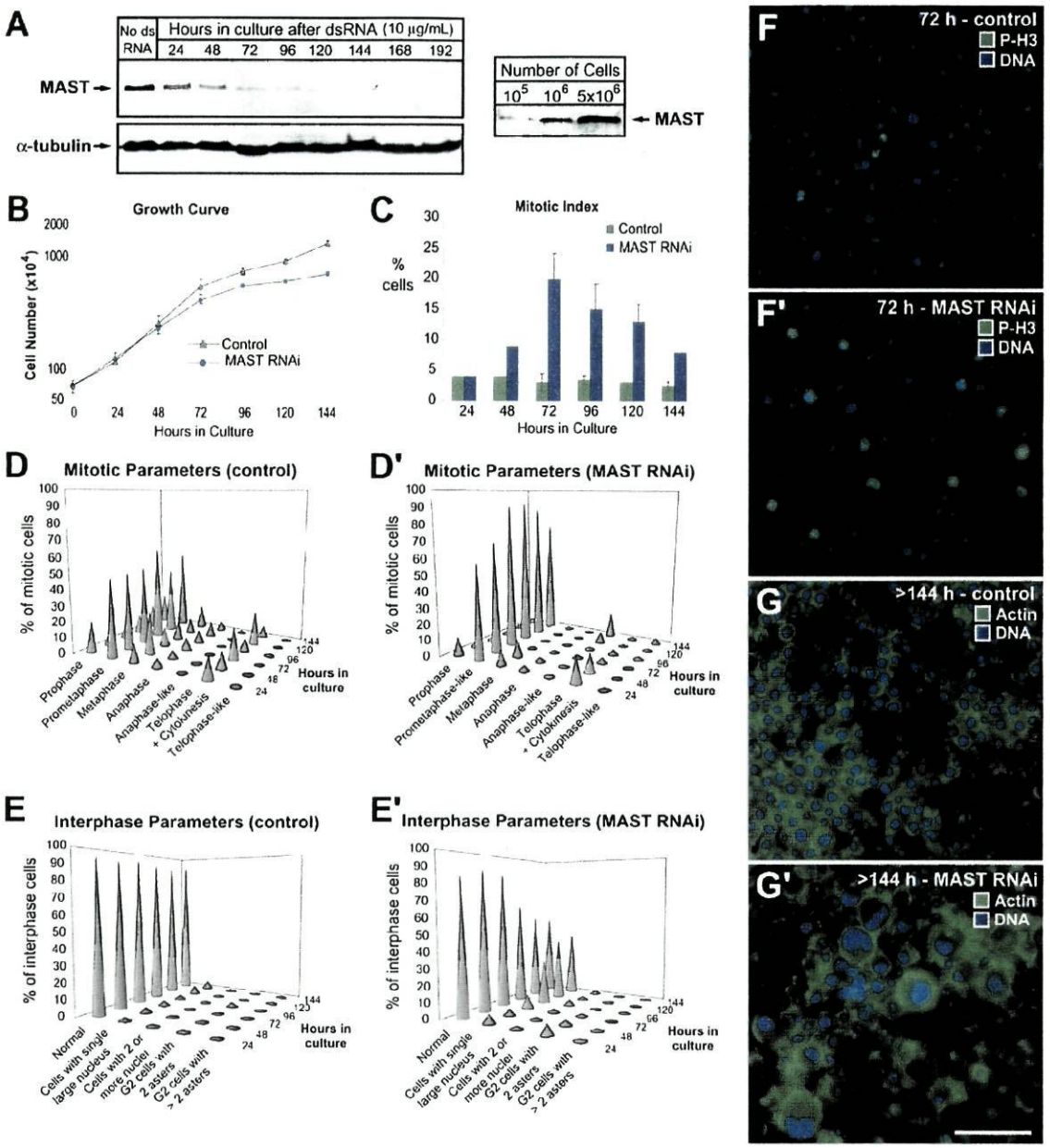
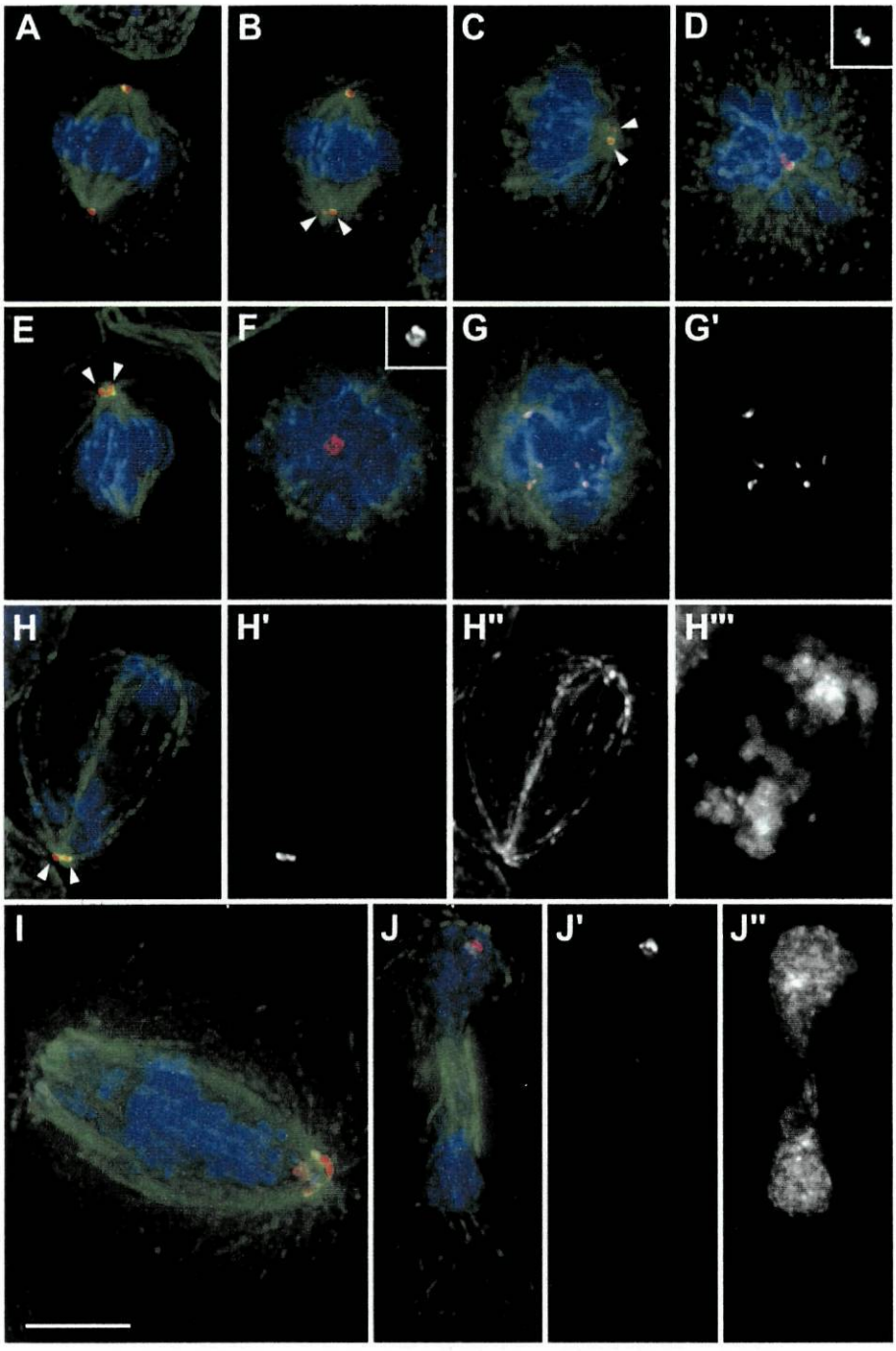


Figure 2.2. Time-course analysis of MAST RNAi in *Drosophila* S2 cells. **(A, left)** MAST inactivation was monitored over time by western blot analysis. 10^6 cells were loaded in each lane and α-tubulin detection was used as a loading control. **(A, right)** Titration of anti-MAST antibody. **(B)** Proliferation analysis of S2 cells after MAST RNAi. **(C)** Mitotic index after MAST RNAi is represented as the average percentage of mitotic cells (n>100) in the total population. **(D-D')** Quantification of the mitotic parameters during the course of the experiment in control and in cells after MAST RNAi, respectively (n>100). **(E-E')** Quantification of interphase parameters during the course of the experiment in control and in cells after MAST RNAi (n>250). **(F-F')** Low magnification views of representative optical fields by 72 h in control and MAST RNAi cells, respectively, stained with an anti-P-Histone 3 antibody to detect cells undergoing mitosis or after 144 h **(G-G')** stained for actin. Note the formation of cells with a very large (4-5 fold) nucleus after MAST RNAi. Scale bar is 50 µm.

2.2. Organization of the Mitotic Apparatus in MAST RNAi Treated Cells

To characterize the organization of the mitotic apparatus after MAST RNAi, cells were collected every 24 h and immunostained to visualize the spindle (α-tubulin), centrosomes (CP190), and chromosomes (DAPI) (Fig. 2.3). In most control cells, the mitotic apparatus was well organized with a single centrosome at each pole (Fig. 2.3A and K). During the course of the experiment, between 5-10% of mitotic cells of both control and RNAi treated S2 cells had asymmetric spindles with irregular centrosome number at each pole suggesting that there is a background level of mitotic abnormalities in this cell line (Fig. 2.3B, K and K', mentioned as bipolar asymmetric). However, after 48 h exposure of cells to dsRNA, there was a large increase in the frequency of cells with monopolar spindles and no or very few cells in metaphase, anaphase or telophase were found (Fig. 2.2D'; Fig 2.3C-D and K'). This phenotype was maximal at 72 h (Fig. 2.3K'). After 96 h, two different prometaphase-like cell populations were observed. Nearly 20% showed a bipolar array of microtubules with centrosomes at only one pole (Fig. 2.3E and K', mentioned as bipolar acentrosomal, but see Wilson et al., 1997) and about half appeared polyploid with a monopolar spindle surrounding a cluster of several centrosomes (≥4) (Fig. 2.4F and K'). Polyploid cells with multiple asters, i.e. showing dispersed centrosomes within the chromosome mass were regularly found throughout the course of the experiment (Fig. 2.4G, G' and K'). Other cells adopted an anaphase-like configuration with a distinct set of chromosomes near each pole (Fig. 2.3H-H''', and after 120 h in Fig. 2.2D'). At even later time-points in the RNAi experiment (144 h in Fig. 2.2D'), a small percentage of cells (3%) were in a telophase-like state with an organized central spindle structure and decondensed chromatin (Fig. 2.3J-J''). Interestingly, the centrosomes in these cells were often found clustered at a single pole.

Figure 2.3. Organisation of the mitotic apparatus after MAST RNAi. Cells after MAST RNAi were stained with an anti-CP190 antibody to reveal the centrosomes (red), anti-α-tubulin antibody to visualise the microtubules (green) and DAPI to counterstain the DNA (blue). **(A)** Normal bipolar spindle in a control cell. **(B)** A Bipolar asymmetric spindle after MAST RNAi. **(C-D)** Lateral and top view of a monopolar spindle with 2 centrosomes respectively observed 72 h after MAST RNAi. **(E)** Bipolar acentrosomal spindle after MAST RNAi with two centrosomes (arrowheads) at only in one pole. **(F)** Polyploid monopolar spindles with at least 4 centrosomes, respectively, observed 96 h after MAST RNAi. Centrosome staining alone can be seen in the insertion on top right of the figures D and F. **(G)** Polyploid cell with multiple asters after MAST RNAi. **(G')** Corresponding centrosome staining alone. **(H)** Anaphase-like cell displaying a bipolar spindle with centrosomes in a single pole and chromosomes distributed in two distinct populations in each side of the spindle. Unmerged images to reveal the centrosomes **(H')**, spindle **(H'')** and chromosomes **(H''')** are shown. **(I)** A cell with a bipolar spindle and multiple centrosomes clustered in a single pole where the chromosomes appear distributed along the spindle. **(J)** Abnormal telophase-like cell showing the formation of a cleavage furrow (arrows), with centrosomes in only one pole and de-condensed chromatin. Separate centrosome and DNA staining can be seen in **(J')** and **(J'')**, respectively. Scale bar is 5 μm. **(K-K')** Quantification of the observed spindle defects in control and MAST-depleted cells, respectively (n>100). (See next page).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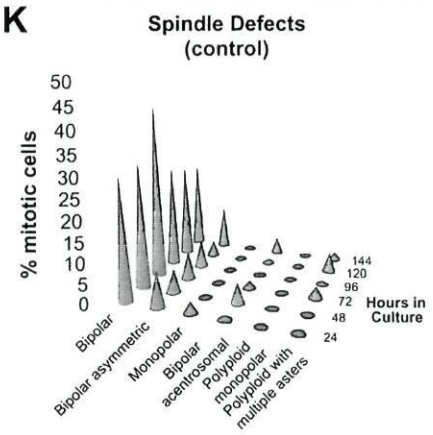
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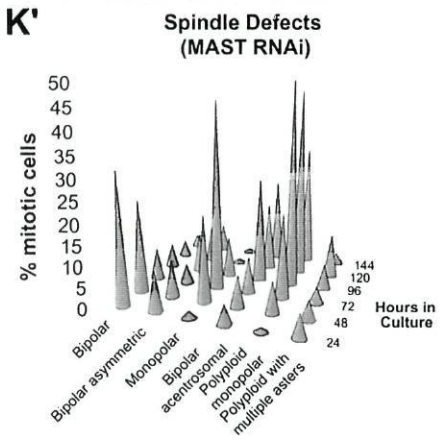
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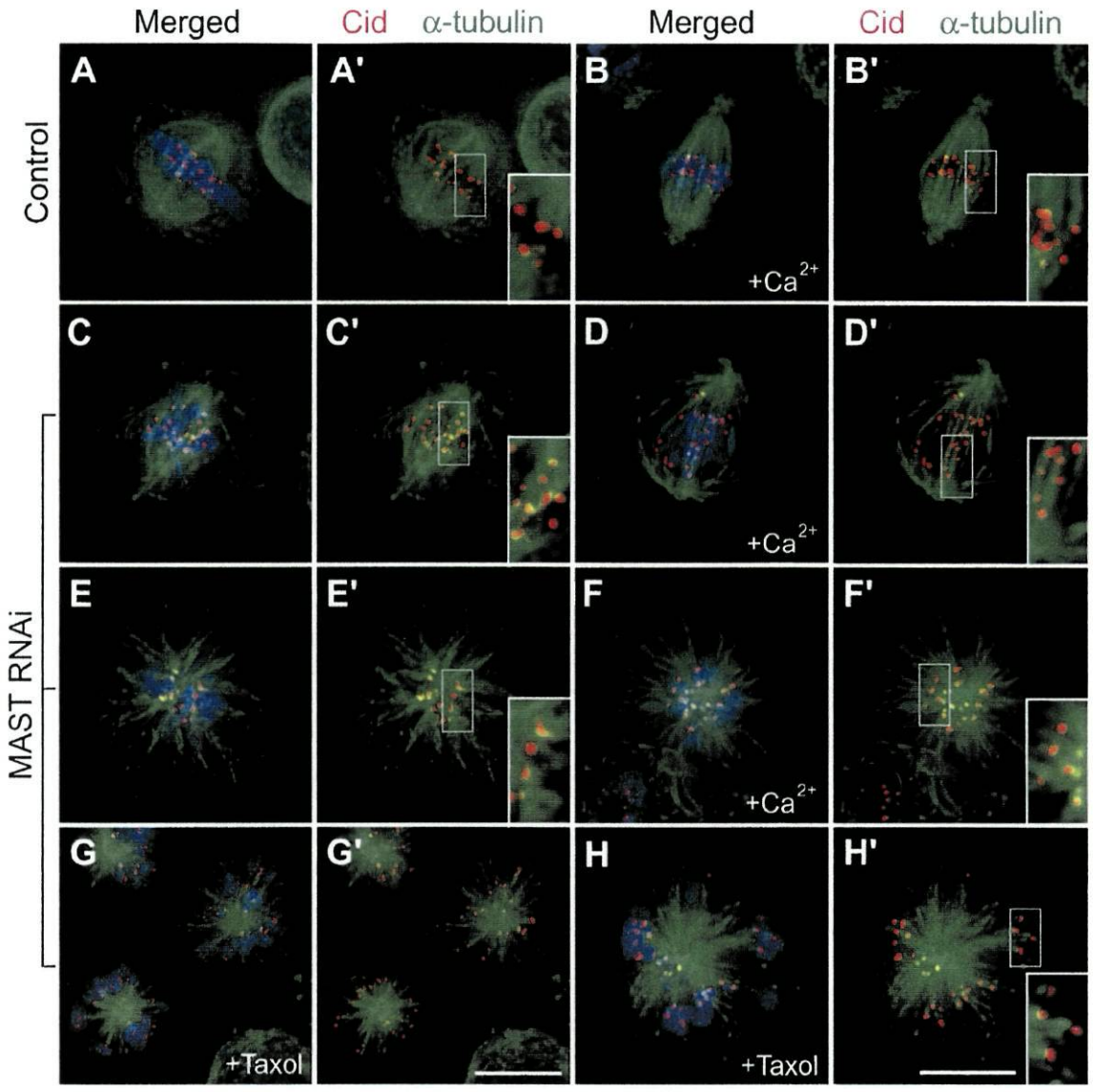
2.3. Characterisation of Microtubule-Kinetochore Attachment after MAST RNAi

In order to determine whether spindle microtubules in MAST depleted cells are able to establish stable end-on interactions with kinetochores, RNAi treated cells were immunostained for tubulin and the kinetochore marker Cid, the *Drosophila* CENP-A homologue (Henikoff et al., 2000), with or without a pre-incubation in a buffer containing calcium to selectively dissociate non-kinetochore microtubules (Mitchison et al., 1986; Kapoor et al., 2000) or with taxol to promote microtubule stabilization (Fig. 2.4). Consistent with published observations, in the presence of calcium most kinetochores were clearly seen to associate specifically with the ends of microtubule bundles (Fig. 2.4 A-B'). However, in MAST-depleted bipolar prometaphase cells kinetochores are distributed along the spindle (Fig. 2.4C-D') and never organize a metaphase plate. In MAST-depleted cells with monopolar spindles, chromosomes were mostly buried within the aster and kinetochores either do not show a clear association with the plus-end of microtubules or appear to bind short microtubules (Fig. 2.4E-F' and I). However, if RNAi treated cells were incubated for 30 min with taxol, 75% of kinetochore pairs are associated with the plus-ends of long microtubule bundles (Fig. 2.4G-H' and I). Furthermore, the distribution of chromosomes relative to the aster was significantly different in the presence of taxol, with chromosomes localized at the periphery with most kinetochores facing the center (compare Fig. 2.4E-F' with G-H'). These data show that if in MAST-depleted cells microtubule dynamics is suppressed long microtubule bundles are formed with kinetochores associated at their plus-ends.

Figure 2.4. Analysis of kinetochore-microtubule attachment in MAST depleted cells. Control cells **(A-B')** as well as MAST depleted cells by RNAi **(C-H')** were stained with antibodies against Cid (red), α-tubulin (green) and counterstained with DAPI to reveal the DNA (blue). **(A-A')** Control cell in metaphase showing that all the kinetochores are end-on attached to microtubules. **(B-B')** Control cell in metaphase that was pre-treated with calcium prior to fixation showing that the kinetochore microtubules were selectively preserved. **(C-C'** and **D-D')** Cells with bipolar spindles after MAST RNAi with misaligned chromosomes with or without a pre-treatment with calcium, respectively. **(E-E'** and **F-F')** Cells with monopolar spindles after MAST RNAi with or without a pre-treatment with calcium, respectively, showing chromosomes buried close to the center of the aster, and the kinetochores can not be found associated with microtubule plus-ends. **(G-H')** Pre-treatment of MAST-depleted cells with taxol leads chromosomes to be organized at the periphery of the aster clearly associated with microtubule plus-ends. Insertions inside panels represent the selected area in the cells that was magnified twice. Scale bar is 5 μm. (See next page).



2.4. Ultra-structural Analysis of MAST-Depleted S2 Cells

In order to analyze in more detail the organization of the mitotic apparatus and the attachment of chromosomes to the spindle after depletion of MAST, RNAi treated cells were processed for transmission electron microscopy (Fig. 2.5). In control mitotic cells we could observe bipolar spindles organized from well-defined centrosomes (Fig. 2.5A and A2). In these cells, bundles of microtubules are visible running from the spindle pole and making contact with individual chromosomes at the kinetochore (Fig. 2.5A1, A1'). However, in MAST depleted cells that show a monopolar organization (Fig. 2.5B, C) we found no evidence of microtubule bundling and chromosomes were located close to the center of the aster where multiple centrioles can be found (Fig. 2.5B). A higher magnification view of a cell with a monopolar spindle shows individual microtubules passing by the chromosomes on the same plane (Fig. 2.5C). These results are

consistent and further support the observations that depletion of MAST perturbs the interaction between kinetochores and the plus ends of spindle microtubules.

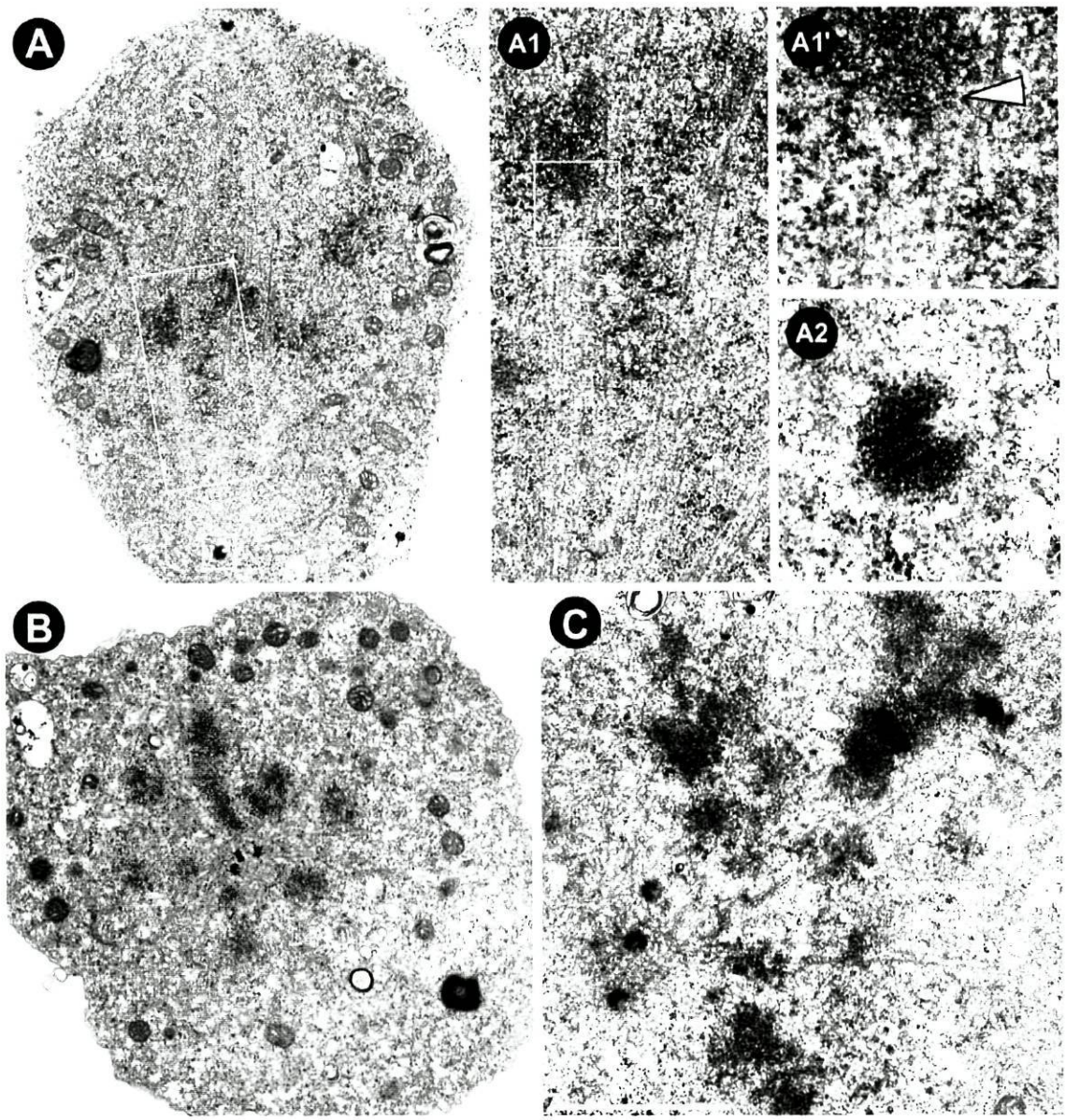


Figure 2.5. Ultra-structural analysis of S2 cells after MAST RNAi. **(A)** Control cell showing a bipolar spindle with bundles of microtubules organized from the centrosomes. **(A1)** Higher magnification of chromosomes being captured by bundles of microtubules in a region that corresponds to the kinetochore (A1', arrowhead). **(A2)** Higher magnification of one of the centrioles. **(B)** Top view of a MAST-depleted cell after RNAi showing a monopolar spindle with chromosomes very close to the center of the aster where 3 centrioles can be seen in this section. **(C)** Side view of another MAST-depleted cell showing a monopolar aster where individual microtubules can be seen on the same plane as the chromosomes. Scale bar is 2 μm.

2.5. Distribution of Zw10, Dynein and D-CLIP-190 in the Absence of MAST

To further ascertain whether the interactions between kinetochores and microtubules is altered following depletion of MAST by RNAi, we analyzed the distribution of Zw10 and Dynein, two proteins that localize to kinetochores early in mitosis and are transferred to spindle microtubules after kinetochore attachment (Williams et al., 1992; King et al., 2000b). The results show that while in control cells (Fig. 2.6A, B) Zw10 was localized to kinetochores of mono-oriented chromosomes during prometaphase and extended along the spindle microtubules at metaphase, after RNAi treatment it was always found associated with the kinetochores in cells with both monopolar and bipolar spindles (Fig. 2.6C, D). Similarly, while in control cells Dynein showed accumulation in kinetochores of mono-oriented chromosomes during prometaphase (Fig. 2.6E) and there was little or no staining at metaphase (Fig. 2.6F), in RNAi treated cells Dynein was always found associated with kinetochores (Fig. 2.6G, G'). We have also analyzed the distribution of D-CLIP-190 (Lantz and Miller, 1998), the *Drosophila* homologue of CLIP-170, a protein that has been shown to bind CLASPs (Akhmanova et al., 2001), the human homologues of MAST. D-CLIP-190 was found associated with kinetochores of prometaphase chromosomes (Fig. 2.6H) but at metaphase we found the staining dispersed over the spindle region (Fig. 2.6I). After RNAi, monopolar cells showed D-CLIP-190 staining mostly associated with kinetochore pairs, although some punctate staining was also visible in the area of microtubules (Fig. 2.6J, J'). These results show that, after depletion of MAST, Zw10 and Dynein were retained at the kinetochores. Furthermore, the data also show that the kinetochore localization of D-CLIP-190 was not dependent upon the presence of MAST.

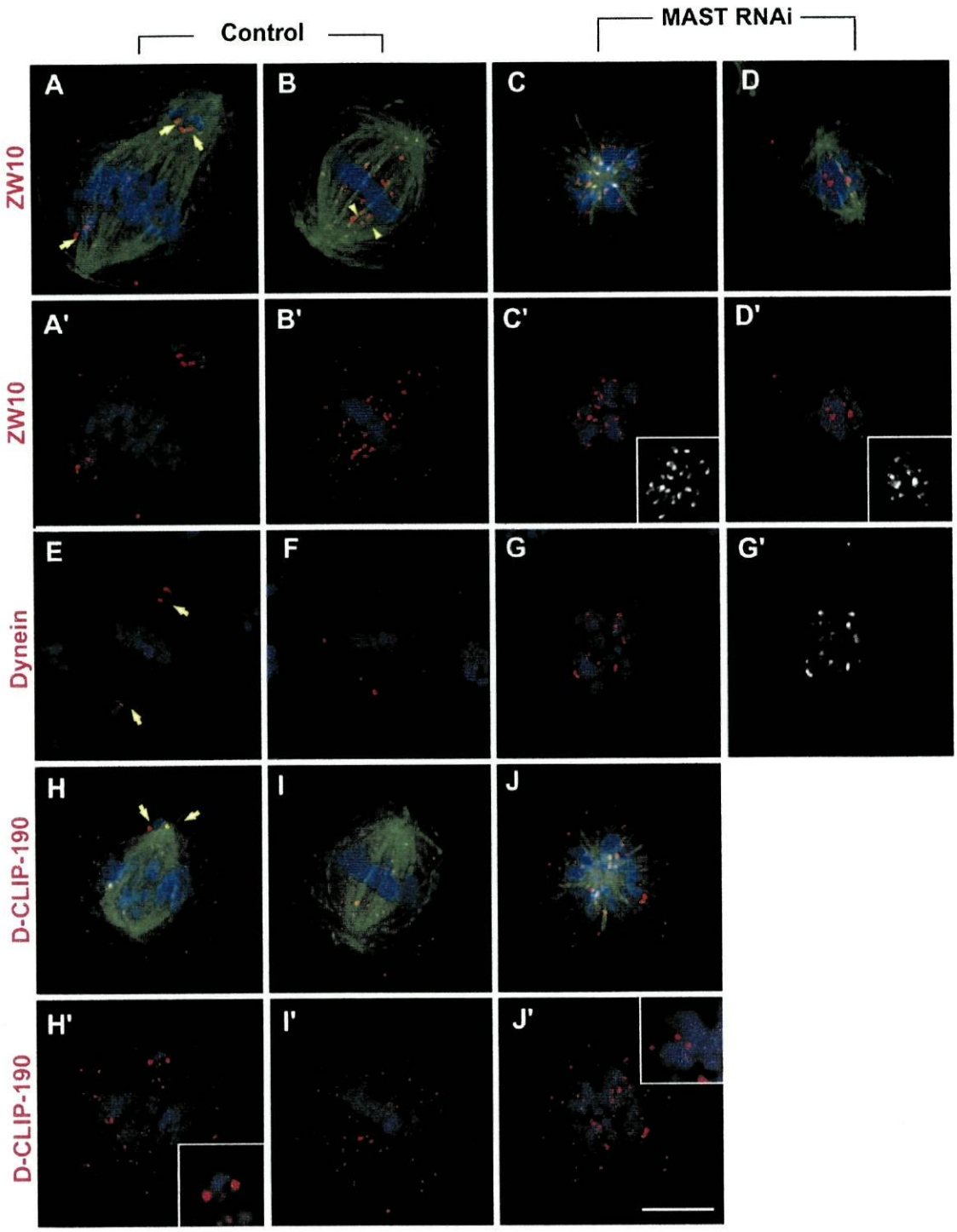


Figure 2.6. Immunolocalisation of kinetochore-associated proteins in the absence of MAST. **(A, B, E, F, H** and **I)** Untreated control cells or **(C, D, G, G', J** and **J')** cells at 72 h after MAST RNAi were stained (red) with anti-Zw10, -Dynein and -D-CLIP-190 antibodies. α-tubulin (green) and DNA (blue) are also shown when possible. **(A, E** and **H)** Cells in prometaphase with mono-oriented chromosomes showing kinetochore-associated Zw10, Dynein and D-CLIP-190 (arrows). **(B, F** and **I)** During metaphase, when all the chromosomes become bi-oriented, Zw10 transfers to the kinetochore microtubules (arrowheads), and Dynein and D-CLIP-190 are barely detectable at the kinetochores. **(C)** Cells with monopolar or **(D)** bipolar spindles with misaligned chromosomes showing strong staining of Zw10 at kinetochores. Insertions in panels C and D show Zw10 staining alone. **(G, G'** and **J, J')** In cells after MAST RNAi, Dynein and D-CLIP-190 respectively, strongly accumulate at kinetochores. Scale bar is 5 μm.

3. Discussion

3.1. Possible Roles for MAST in Microtubule-Kinetochore Attachment and Chromosome Congression

The studies reported here provide strong evidence suggesting that MAST is required for microtubule plus-ends to establish a functional attachment to kinetochores. In the absence of MAST, chromosome congression does not take place, kinetochores do not show a clear end-on attachment and others appear to bind through lateral interactions. Most surprising is the monopolar configuration in MAST-depleted cells where we find chromosomes mostly localized closed to the center of the aster. If in MAST depleted cells microtubule-kinetochore interaction is compromised it would be expected that the action of chromokinesins and even passive impacts by elongating microtubules would push the chromosomes to the periphery of the asters (the "polar wind"). This suggests that the localization of chromosomes to the interior of asters is likely to involve an active, rather than a passive process that can be explained by at least three different models. MAST could be required for kinetochores to hold on to dynamic microtubules. If it were true that in cells lacking MAST kinetochores could not hold onto shortening microtubules, then every time a kinetochore fiber would begin to shorten, it would be released by its kinetochore. That kinetochore would then have to make an initial encounter with microtubules all over again through lateral interactions which could be mediated by cytoplasmic Dynein as it is during prometaphase and the chromosome would be expected to exhibit rapid poleward movement (Rieder and Alexander, 1990). Consistent with this model, levels of kinetochore-associated Dynein and Zw10 remained abnormally elevated in the absence of MAST. A second interpretation of these results is that in the absence of MAST, chromosomes attach to microtubules that can shorten but cannot re-grow so that chromosomes would end up mostly in the region close to the center of the aster suggesting that MAST could have a role in promoting microtubule plus-end stability. Indeed, the human homologues CLASPS, have been shown to promote plus-end microtubule stabilization in interphase (Akhmanova et al., 2001). Furthermore, the nature of the physical association with microtubules was recently established for Stu1, which was shown to bind specifically to β-tubulin (Yin et al., 2002). These observations, together with the finding that MAST binds to microtubules in a GTP-dependent manner (Inoue et al., 2000) have strong implications for the role of these proteins in regulating the dynamics of kinetochore attached microtubule-plus ends. However, MAST does not appear to affect the ability of all microtubules to

elongate since in MAST-depleted cells there is no obvious effect upon growth of astral microtubules. Therefore, if MAST has a role in microtubule dynamics, kinetochore microtubules are particularly sensitive. Finally, it is also possible that in MAST-depleted cells kinetochores attach to microtubules but minus-end-directed motility of the kinetochore dominates so that the movement of chromosomes towards the poles prevails over the "polar wind" pushing the chromosomes towards the plus-ends of microtubules. It could be that kinetochore-associated MAST (Lemos et al., 2000) is required for the binding of essential kinesins with plus-end-directed motility. A putative candidate could be CENP-E, a plus-end directed motor (Wood et al., 1997), which has been shown to localize to the fibrous corona of the kinetochore (Cooke et al., 1997; Yao et al., 1997). CENP-E is thought to be required for stable bi-oriented attachment of chromosomes to spindle microtubules (Yao et al., 2000). Surprisingly, depletion of CENP-E by microinjection also causes misaligned chromosomes to be sequestered very close to the spindle poles (McEwen et al., 2001).

Although, at present, the data presented here does not allow us to clearly distinguish amongst these interpretations, we favor the first two models since they could readily explain the results obtained after taxol treatment of MAST-depleted cells. Suppressing microtubule dynamics after depletion of MAST causes the formation of monopolar cells with most kinetochores associated to the plus ends of microtubule bundles. Thus, MAST could be part of a protein complex required to hold on to the plus end of dynamic microtubules. The ability of kinetochores to hold onto the ends of bundles of dynamic microtubules while they are growing and shrinking is one of its most remarkable attributes. This plus-end tethering ability has attracted considerable interest since its discovery (Mitchison and Kirschner, 1985; Koshland et al., 1988; Coue et al., 1991; Hyman and Mitchison, 1991), however the underlying mechanism has proven to be highly elusive. This is likely to be due in part to the redundant nature of kinetochore-microtubule interactions (Hyman and Mitchison, 1991; Kitagawa and Hieter, 2001). Future studies on the role of MAST should provide significant insights into the molecular nature of this process.

3.2. MAST Function is Required for Spindle Bipolarity

It is now well accepted that balance between plus end-directed and minus end-directed motors is essential for the maintenance of a bipolar spindle through metaphase and also for its elongation during anaphase (reviewed by Sharp et al., 2000b). This has been best studied in *S. cerevisiae*, where imbalances between the Cin8p/Kip1p and

Kar3p kinesins cause rapid collapse of the bipolar spindle (Saunders and Hoyt, 1992). STU1p, the putative MAST orthologue in *S. cerevisiae*, is also essential for spindle stability (Pasqualone and Huffaker, 1994). Thus, it is possible that MAST is required for the stable association of one or more kinesins with the microtubules and that in the absence of this kinesin, the balance of power is perturbed at metaphase. Indeed, the collapse of the spindle seen after depletion of MAST is very similar to the effect caused by depletion of the *Drosophila* bipolar kinesin-like KLP61F (Heck et al., 1993, Sharp et al., 2000a). Moreover, it was recently found that a temperature-sensitive *stu1* mutant that shows spindle collapse could be suppressed by doubling the dosage of Cin8p (Yin et al., 2002). The presence of MAST in the spindle mid-zone (Lemos et al., 2000) and the ability for Stu1 to form dimmers (Yin et al., 2002) suggests that MAST may also be acting by cross-linking interpolar microtubules and so stabilizing the spindle.

It is also possible that depletion of MAST perturbs the distribution or function of other microtubule-associated proteins that are required for the formation of a functional bipolar spindle. These could include the chromosomal passengers INCENP and Aurora-B (Adams et al., 2001b; Giet and Glover, 2001). In fact, we did observe aberrant behavior of these proteins in anaphase-like cells following depletion of MAST by RNAi (see Chapter III).

4. Materials and Methods

Scoring of RNAi Phenotypes

Trypan Blue (SIGMA) was used to score non-viable cells. The growth curves were plotted considering viable cells only. Microsoft Excel was used to plot all the quantifications. Error bars represent standard deviation and all the values were plotted as numerical average. To determine the doubling time of the cells for each experiment, we calculated the best fit for the growth curves (semi-log scale) and the doubling time was calculated from the slope of the corresponding exponential equations (x=time/hrs and y=number of cells):

Experiment	Cell line	Exponential Equation	Doubling time/hrs
Control (no $dsRNA_{0\text{-}72h}$)	S2	$y=62.43e^{0.0294x}$	23.6
Control (no $dsRNA_{72\text{-}144h}$)	S2	$y=226.66e^{0.0123x}$	56.4
MAST $RNAi_{0\text{-}72h}$	S2	$y=67.679e^{0.0252x}$	27.5
MAST $RNAi_{72\text{-}144h}$	S2	$y=256.09e^{0.0074x}$	93.7

Only MAST negative cells were scored for mitotic parameters in the RNAi experiments (as determined by immunofluorescence). Mitotic Index was determined as % of mitotic cells in the total population. Mitotic stages were quantified in terms of % of mitotic cells. Spindle defects in

MAST RNAi were scored as percentage of mitotic cells and were confirmed independently in three distinct experiments either by γ-tubulin or CP190 staining in MAST negative cells.

Immunofluorescence in Tissue Culture Cells

Drosophila S2 cells were grown in chamber slides containing Schneider medium at 25 °C, supplemented with 10% FBS and antibiotics. The cells were then cytospun at 4,000 rpm for 15 min at 25 °C and were fixed with 4 % paraformaldehyde. Pre-treatment of S2 cells with Calcium after MAST RNAi was performed as previously described (Kapoor et al., 2000). Fixed cells were followed by a permeabilization step with 0.3-0.5% Triton X-100 in cytoskeleton buffer except for MAST staining that required 1% Triton X-100 and blocked for 30 min with 10 % FBS + 0.1 % Triton X-100 in PBS. Cells were then incubated with primary antibodies (see Table I) diluted in the blocking solution for 45 min, washed 3x5 min with dPBS + 0.1 % Triton X-100 and detected with secondary FITC- and Texas Red-conjugated antibodies (Jackson Immunoresearch). DNA was counterstained with DAPI and the preparations mounted in Vectashield (Vector). FITC-conjugated phalloidin (Molecular Probes, Oregon, USA) was used at 200 nM to detect actin. Quantitative three-dimensional data sets of representative cells were collected using a DeltaVision microscope (Applied Precision, Issaquah, WA), based on an Olympus IX-70 inverted microscope with a Photometrics CH350L cooled CCD camera, and subsequently deconvolved and projected onto a single plane using SoftWorx (Applied Precision), except for low magnification images. Adobe Photoshop 5.5 (Adobe Systems) was used to process all images.

Electron microscopy

S2 cells were grown in 6 well plates and, after pelleting at the bottom of an eppendorf tube, were fixed with 2.5 % glutaraldehyde in PHEM buffer for 2 hrs and washed for 3x10 min with PHEM. Then, they were stained with 2 % OsO_4 at 4 °C for 2.5 h and washed as before. Incubation for 5 min in 0.15 % tannic acid in PHEM buffer was followed, washed once with PHEM and twice with dH_2O. Cells were dehydrated with ethanol series from 20-100%, for 20 min each, 1:1 ethanol/propylene oxide for 10 min, absolute propylene oxide for 3x20 min and left overnight in 1:1 Agar 100/propylene oxide. The mixture was changed to Agar 100 3x (1 uncatalysed, 2 catalysed) for 3 h between changes and cured in 60 °C oven for 2 days. Serial sections were cut on a Leica Ultracut UCT ultra-microtome and collected on 100 mesh copper grids. Sectioned cells were stained with 4% aqueous uranyl acetate for 1 hour in the dark and then left for 10 min in lead citrate in a CO_2 free atmosphere. Samples were observed with a Philips CM 120 Biotwin using Kodak SO 163 Electron Image film.

DsRNA Interference in Drosophila S2 Cells

For RNAi in *Drosophila* S2 cells we have designed primers (V40-i2-F and V40-i3-R) to make a PCR product of ~700 bp from the 5' region of corresponding cDNA covering the ATG encoding the first methionine (see Table II). A T7 RNA polymerase promoter site was incorporated at the 5' end of each primer. The PCR products were cleaned and used as templates for the transcription

reaction using MEGAscript T7 System (Ambion) according to the manufacturer's instructions. The RNA was precipitated with LiCl, washed and re-suspended in nuclease free H_2O and the concentration determined by reading the $Abs_{260/280}$. Then, the RNA was heated at 65 °C for 30 min to denature secondary structures and cooled down slowly to RT to make dsRNA. For MAST RNAi in S2 cells, 10 μg of dsRNA per ml of culture medium were used. For western blot and immunofluorescence analysis cells were treated as previously described (Clemens et al., 2000). Treatment of S2 cells with dsRNA synthesized from random human intronic sequence, to rule out unspecific effects of dsRNA, caused no visible defects and was indistinguishable from the untreated control.

Western Blot Analysis

Serial dilution experiments were performed with different concentrations of protein extracts derived from *Drosophila* S2 cells in order to determine the minimal amount required for detection with anti-MAST antibody. Extracts were prepared by sonication of 10^5-$5x10^6$ cells in Laemli buffer and boiled for 3 min to denature the proteins. The extracts were then loaded in a 10% denaturing polyacrylimide gel and subjected to vertical electrophoresis. Proteins were then transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech) that was blocked overnight at 4 °C with TBST + 5% dried milk. On the next day, the membrane was incubated for 1 h at room temperature with anti-MAST and anti-α-tubulin antibodies for a loading control (see Table I) diluted in TBST + 3% dried milk. After washing with TBST (1x15 min; 2x5 min), the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. The membrane was then washed as before and the antibodies detectected with ECL (Amersham Pharmacia Biotech). Finally, the membrane was exposed with a Kodak film and developed. For the RNAi experiment, 10^6 cells were collected every 24 h after addition of MAST dsRNA and processed as before.

CHAPTER III

Absence of MAST Leads to an Abnormal Mitotic Exit Independent of APC/Cyclosome Function

1. Introduction

There is some evidence indicating that checkpoint induced arrest cannot be maintained indefinitely as a result of persistent cell cycle defects (Sandell and Zakian, 1993; Toczyski et al., 1997). The cell is thought to adapt to the defect and re-initiate cell cycle progression even if the abnormality is not properly resolved. Thus, adaptation can occur without necessarily inactivating the checkpoint (reviewed by Rudner and Murray, 1996). From the point of view of the individual cell, continued activation of the checkpoint and cell cycle arrest are likely to be lethal. Thus, any attempt to proceed with cell division in the face of checkpoint arrest due to a persistent defect may be beneficial because it would allow a chance for survival as opposed to the certainty of death (reviewed by Rieder and Palazzo, 1992). On the other hand, in a multicellular organism, where aneuploidy can initiate cell transformation (Hartwell and Kastan, 1994), apoptosis may replace adaptation. Nevertheless, lack of experimental evidence leaves open the possibility that adaptation may itself originate aneuploidy and trigger tumourigenesis.

An alternative possibility to explain the mechanism of adaptation is that indeed there is a molecular pathway that can be turned on during periods of prolonged cell cycle arrest. Cells with mutations in the budding yeast *CDC55* gene, which encodes a regulatory subunit of protein phophatase 2A (PP2A), separate their sister chromatids but leave mitosis without inducing Cyclin B destruction (Minshull et al., 1996). However, MPF is inactivated by inhibitory phosphorylation of CDK1. Thus, PP2A may be a downstream member of an adaptation pathway that responds to a prolonged activation of the spindle-assembly checkpoint. One may predict that this adaptation pathway would not depend on APC/C funtion, which means that it may be independent of sister chromatid separation and segregation, spindle checkpoint inactivation or Cyclin degradation.

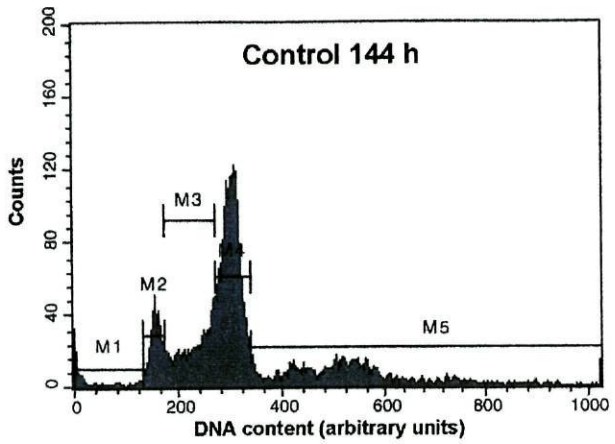
Previous results from the analysis of *mast* mutant neuroblasts led to the observation that some of these cells exhibit a high level of polyploidy that in the case of the more severe allele, *mast*^{4}, can represent more than 80% of the mitotic cells in the brain. Additionally, we found that depletion of MAST by RNAi in S2 initially caused cells to

accumulate in prometaphase mostly with monopolar spindles, however, later on, a significant proportion of cells were very large and highly polyploid. Also, the frequency of the cells contaning monopolar spindles decreases at later times giving rise to several abnormal forms of mitosis that we have termed as anaphase-like and telophase-like. Thus, it seems that after initial mitotic block, MAST-depleted cells exit mitosis straight from prometaphase resulting in at least two distinct forms of mitotic exit: attempt to segregate the chromosomes in the absence of a functional spindle or exit directly to G1 phase of the next cell cycle. To better understand the mechanistic basis of these abnormal forms of mitotic exit observed in the absence of MAST we performed a comparative analysis of S2 cells by FACS and characterized the anaphase- and telophase-like cells by immunofluorescence using antibodies against proteins known to be involved in exit from mitosis.

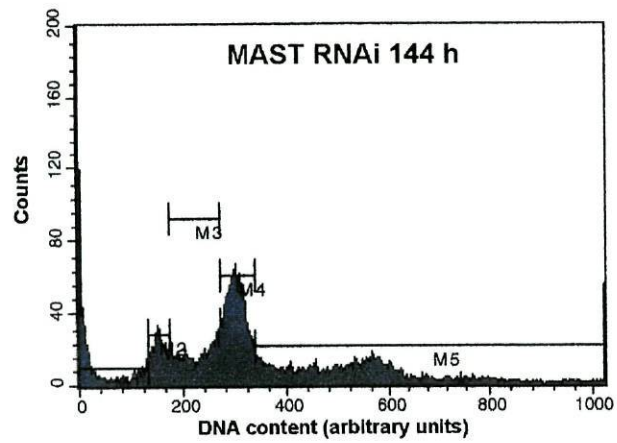
2. Results

2.1. Analysis of Cell Cycle Progression after Prolonged Mitotic Block

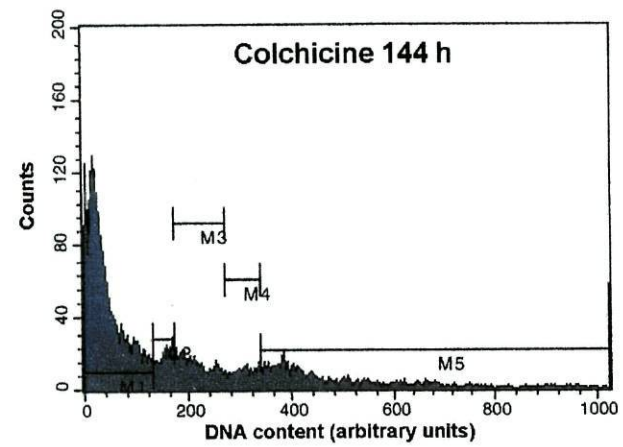
First, we wanted to determine whether the increase in ploidy and size of MAST RNAi treated cells is specifically due to the absence of MAST or just due to prolonged mitotic arrest. For this purpose we followed different S2 cell populations that contained either no dsRNA, MAST dsRNA, or colchicine, which were fixed after 144 h and subsequently analyzed by FACS. We found that in the MAST RNAi experiment there were 20 % less cells with a G2/M DNA content when compared with the control population. On the other hand, there was a 50 % increase in polyploid cells when compared with the background values for the control population, while cells incubated with colchicine undergo massive apoptosis and fail to shown any increase in ploidy (Fig. 3.1).



Marker	Cell Population	% Cells (n=9065)
M1	Apoptotic cells	2.18
M2	Cells in G1/S	9.46
M3	Cells in G1/S or G2/M	20.71
M4	Cells in G2/M	48.39
M5	Polyploid cells	20.19



Marker	Cell Population	% Cells (n=8458)
M1	Apoptotic cells	13.73
M2	Cells in G1/S	8.82
M3	Cells in G1/S or G2/M	16.95
M4	Cells in G2/M	31.04
M5	Polyploid cells	30.28



Marker	Cell Population	% Cells (n=9412)
M1	Apoptotic cells	60.24
M2	Cells in G1/S	6.88
M3	Cells in G1/S or G2/M	11.26
M4	Cells in G2/M	5.38
M5	Polyploid cells	16.72

Figure 3.1. Cell cycle analysis of *Drosophila* S2 cells by FACS after 144 hours in culture. On the left side, the corresponding FACS diagram for each experiment and on the right side the analysis of the different cell populations derived from the FACS diagrams. Top panels correspond to control S2 cells, middle panels correspond S2 cells 144 h after addition of MAST dsRNA, and bottom panels correspond to S2 cells 144 h after addition of colchicine.

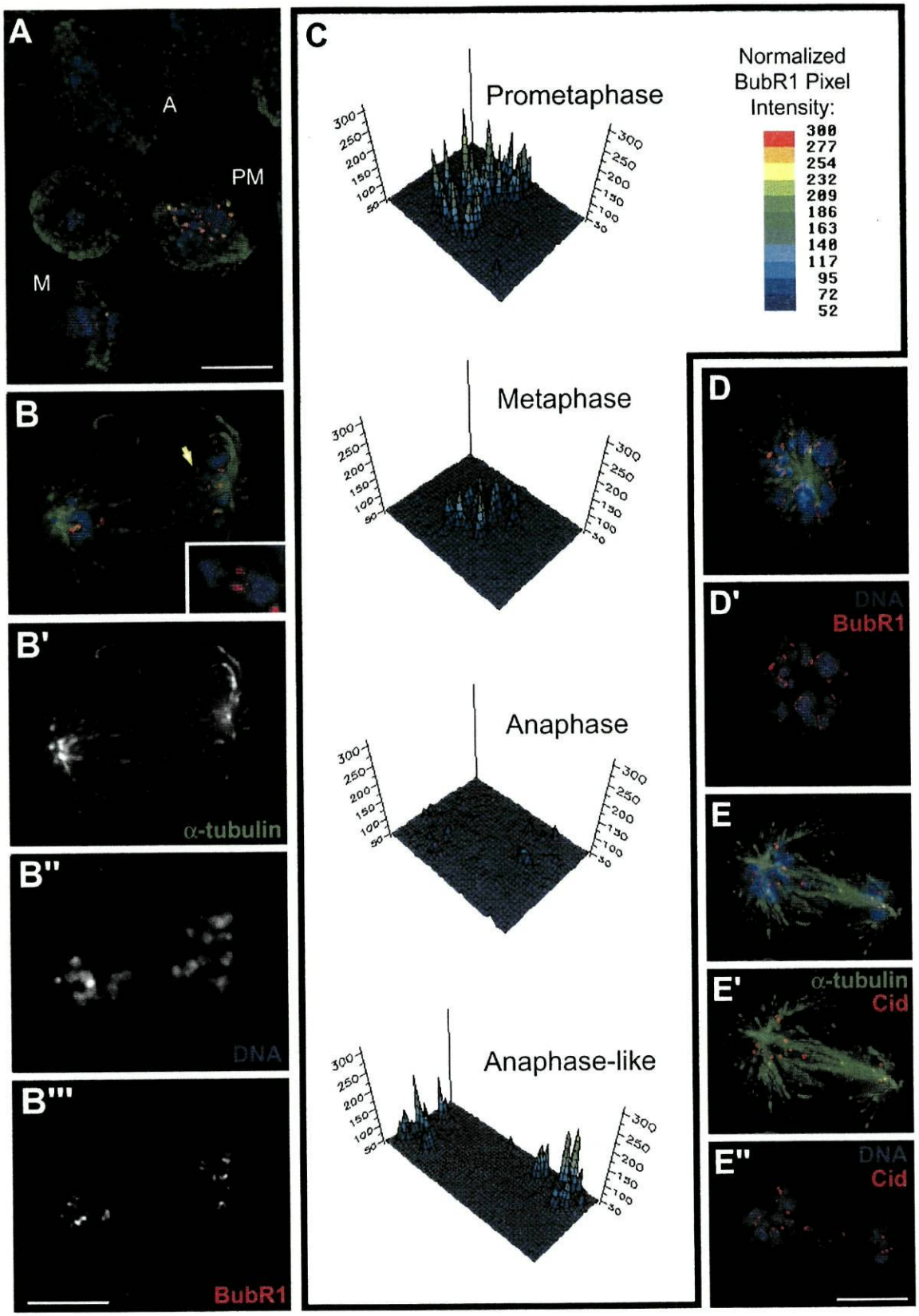
These results demonstrate that the formation of highly polyploidy cells after MAST RNAi is not just due to a non-specific effect caused by prolonged mitotic arrest and therefore might involve the activation of an adaptative pathway.

2.2. Characterisation of Chromosome Behavior after MAST RNAi

In order to characterize the abnormal anaphase-like cells observed after MAST RNAi we determined whether sister chromatids can separate. For this purpose, we performed immunofluorescence analysis to localize the kinetochore markers, BubR1 (Lopes, Bousbaa, Costa and Sunkel, manuscript in preparation) and Cid. In control cells (Fig. 3.2A), BubR1 staining on kinetochores was maximal during prometaphase. As cells progressed into metaphase and the chromosomes aligned at the metaphase plate, BubR1 intensity decreased and after anaphase onset was barely detected (Fig. 3.2A and C). After RNAi, BubR1 was localized on kinetochores of anaphase-like cells (Fig. 3.2B-B'''), as well as in cells with monopolar spindles (Fig. 3.2D-D'). BubR1 was present on all chromosomes and stained kinetochore pairs almost as intensively as in control prometaphase cells, clearly indicating that sister chromatids did not disjoin before migrating to the poles and that the spindle checkpoint is still active and was not inactivated (Fig. 3.2B, C). Identical results were obtained when either anaphase-like or monopolar cells were stained with anti-Cid antibodies (Fig. 3.2E-E''). Finally, scoring of the number of chromosomes or kinetochores (Cid staining) confirmed that, by 120 h after MAST RNAi, 90-95% (n=20) of the anaphase-like cells showed unequal distribution of the chromosomes between the two poles.

These results indicate that chromosomes failed to undergo sister chromatid separation even in cells that displayed an anaphase-like configuration and that they reach this stage with a fully activated spindle checkpoint.

Figure 3.2. Sister chromatid separation in cells after MAST RNAi. **(A-C)** Samples were collected between 96-144 h after MAST RNAi and processed for immunofluorescence analysis with anti-BubR1 and anti-Cid antibodies as markers for kinetochores (red). Microtubules were stained with an anti-α-tubulin antibody (green) and chromosomes were counterstained with DAPI (blue). **(A)** Control cells in the same optical field undergoing prometaphase (pm), metaphase (m) and anaphase (a). **(B)** Anaphase-like cells after MAST RNAi showing very strong BubR1 staining at both kinetochores of each chromosome (arrow, 3x amplified insert) confirming that sister-chromatid separation has not taken place. Unmerged images showing the mitotic spindle **(B')**, chromosomes **(B'')**, and BubR1 **(B''')** can be seen separately. **(C)** Quantification of normalised BubR1 pixel intensity in the control cells shown in A and the anaphase-like cell shown in B. **(D, D')** Cell after MAST RNAi showing the monopolar spindle organization showing very strong BuBR1 staining at kinetochore pairs. **(E-E'')** Cid staining on anaphase-like cells revealed that >90 % of these cells (n=20) show unequal number of chromosomes in each pole. Scale bar is 5 μm. (See next page).


A
A
PM
M
B
B'
α-tubulin
B''
DNA
B'''
BubR1
C
Prometaphase
Metaphase
Anaphase
Anaphase-like
Normalized
BubR1 Pixel
Intensity:
300
277
254
232
209
186
163
140
117
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D
D'
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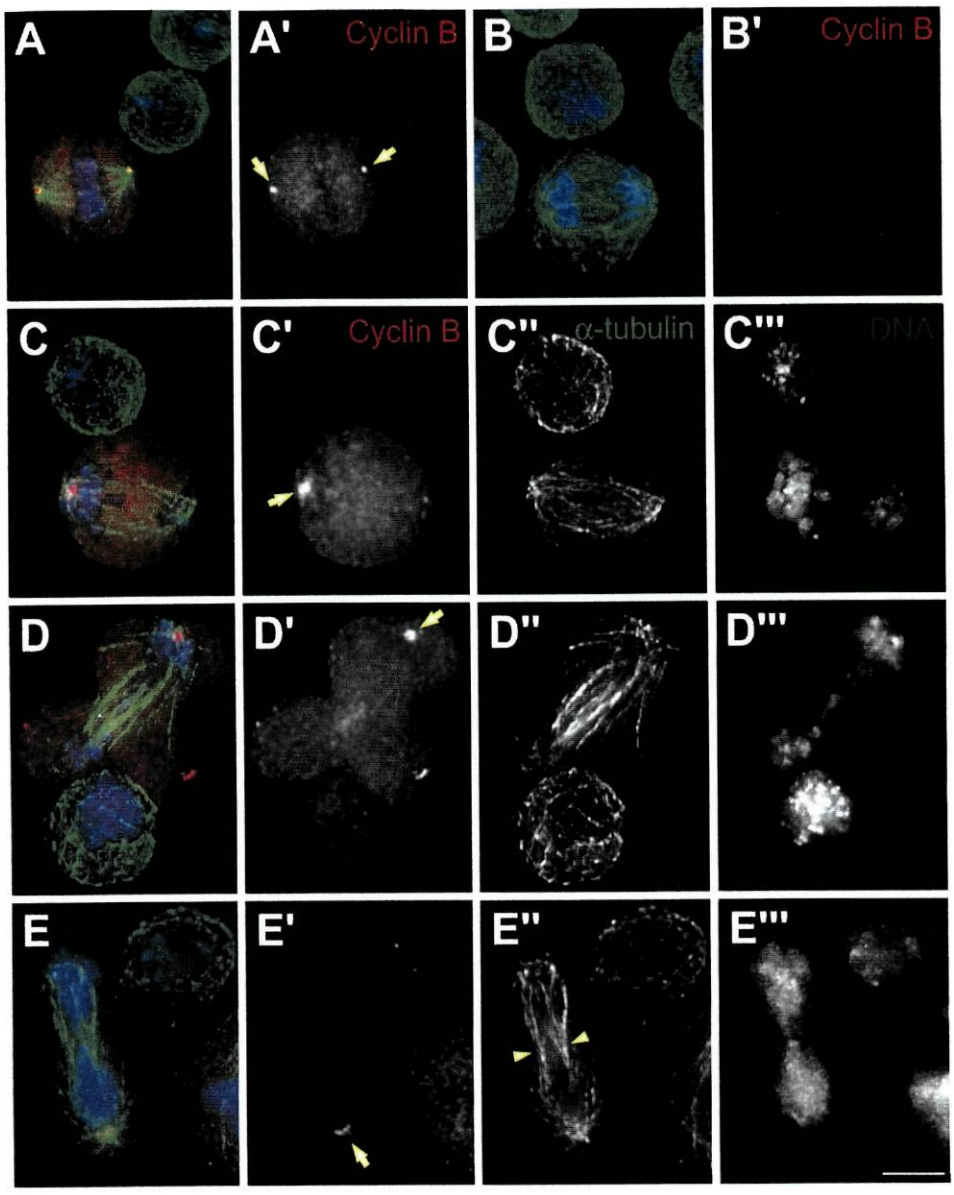
Cid

2.3. Characterisation of the Abnormal Mitotic Exit after MAST RNAi

The presence of kinetochore-associated BubR1 in non-disjoined chromosomes of these anaphase-like cells suggests that an abnormal mitotic exit takes place. During normal mitotic exit, the spindle checkpoint is inactivated at metaphase when all chromosomes are bioriented. Anaphase is initiated as a result of the activation of the APC/C that promotes sister chromatid separation. To determine whether the APC/C was being activated in these anaphase-like cells we immunostained for Cyclin B, a substrate of the APC/C that starts to be degraded at anaphase-onset. In control mitotic cells, cytoplasmic and centrosomal levels of Cyclin B were high until metaphase (Fig. 3.3A) but by anaphase Cyclin B was no longer detected in the cytoplasm or centrosomes (Fig. 3.3B, B'). After MAST RNAi, monopolar cells contained cytoplasmic and centrosomal Cyclin B levels comparable to control prometaphase or metaphase cells (data not shown). Additionally, in the majority of the anaphase-like cells (80-95 %, n=20, between 96 and 144 h), Cyclin B was still highly abundant in the cytoplasm and the centrosomes (Fig. 3.3C-C'''). This not only allowed us to confirm that most anaphase-like cells with high levels of Cyclin B contained centrosomes at only one pole but also strongly suggests that the APC/C was not activated which would account for the absence of sister chromatid separation in these cells. Moreover, even in telophase-like cells (~80 %, n=20) that already showed partially de-condensed chromatin (Fig. 3.3D-D''') and therefore were well advanced into exit from mitosis, Cyclin B levels remained high. Finally, the telophase-like cells that showed fully de-condensed chromatin had lower cytoplasmic but still very abundant levels of Cyclin B at the centrosomes (Fig. 3.3E-E'''). Also, it was possible to observe the formation of what seemed to be a cleavage furrow (Fig. 3.2 E-E'''). By this stage, BubR1 staining was no longer detectable (data not shown).

Figure 3.3. Mitotic exit after MAST RNAi. To determine the level of activity of the APC in anaphase- or telophase-like figures after MAST RNAi, cells were immunostained for Cyclin B. **(A, B)** Control metaphase and anaphase cells, respectively, showing that Cyclin B is degraded after anaphase onset. **(A', B')** Cyclin B staining alone. Note the strong Cyclin B staining at centrosomes during metaphase (arrows). **(C)** Anaphase-like cell showing high levels of Cyclin B. **(C'-C''')** Unmerged images. **(D)** Cell in a telophase-like state with partially decondensed chromatin showing high levels of Cyclin B. **(D'-D''')** Unmerged images. Note the strong Cyclin B staining on the centrosomes in the anaphase- and telophase-like figures (arrows). **(E)** Cell in a telophase-like state with highly de-condensed chromatin showing lower levels of Cyclin B on the cytoplasm but still very concentrated in the centrosomes (arrow). **(E''')** Tubulin staining alone reveals a constriction suggesting that a cleavage furrow (arrowheads) is being formed. **(E'-E''')** Unmerged images. Scale bar is 5 μm. (See next page).



We next wanted to evaluate the condensation state of chromatin in cells that were subjected to MAST RNAi. For this purpose we performed immunofluorescence analysis using an antibody against phosphorylated Histone H3, which is a good marker for mitotic chromosome condensation (Wei et al., 1998; 1999) (Fig. 3.4). On monopolar cells, highly condensed chromosomes were strongly labelled by the phospho-Histone H3 antibody. The same was observed either with the anaphase-like or the early telophase-like cells. However, in telophase cells that showed highly decondensed chromatin forming a bridge with a furrow, Histone H3 was almost all dephosphorylated with the exception of the lagging chromatin bridge.

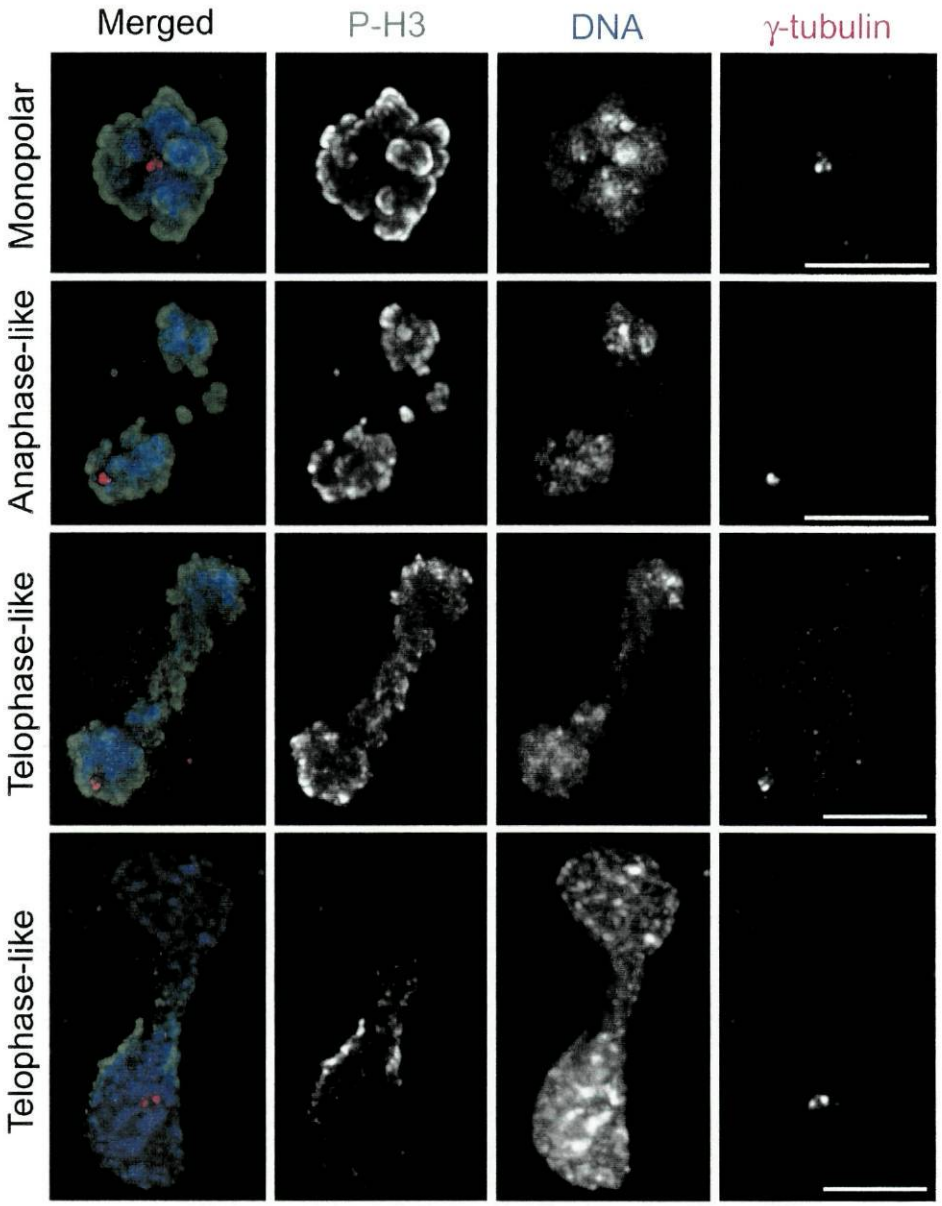


Figure 3.4. Chromosome condensation after MAST RNAi. An anti-phosphorylated Histone H3 antibody (green) was used to determine de condensation of the chromosomes/chromatin (blue) on cells with monopolar spindles, anaphase-like cells, early telophase-like cells and late telophase like cells. An antibody against γ-tubulin (red) was used to monitor centrosome position and number in each cell. Bar is 5 μm.

In order to determine whether a cleavage furrow can be correctly positioned in the telophase-like cells we carried out localisation of the chromosomal passengers Inner Centromere Protein (INCENP) and Aurora-B (Adams et al., 2001b). We observed that INCENP and Aurora-B are associated with the chromosomes in control metaphases and monopolar cells after MAST RNAi (Fig. 3.5A, D and G). Surprisingly, while in control anaphase cells INCENP and Aurora-B transferred to the central spindle (Fig. 3.5B, B' and data not shown), in the anaphase-like cells they remained associated with the chromosomes (Fig. 3.5E, E' and H, H'). Later on, during telophase, control cells contained INCENP and Aurora B strongly accumulated at the mid-body (Fig. 3.5C, C'

and data not shown). However, in the telophase-like cells following RNAi, in spite of the presence of a cleavage furrow suggesting ongoing cytokinesis, INCENP and Aurora B were barely detectable at the mid-body and particularly INCENP was still associated all over the de-condensed chromatin (Fig. 3.45F, F' and I, I'). The reason for this subtle difference between these two chromosomal passengers is likely to be due to antibody stringency.

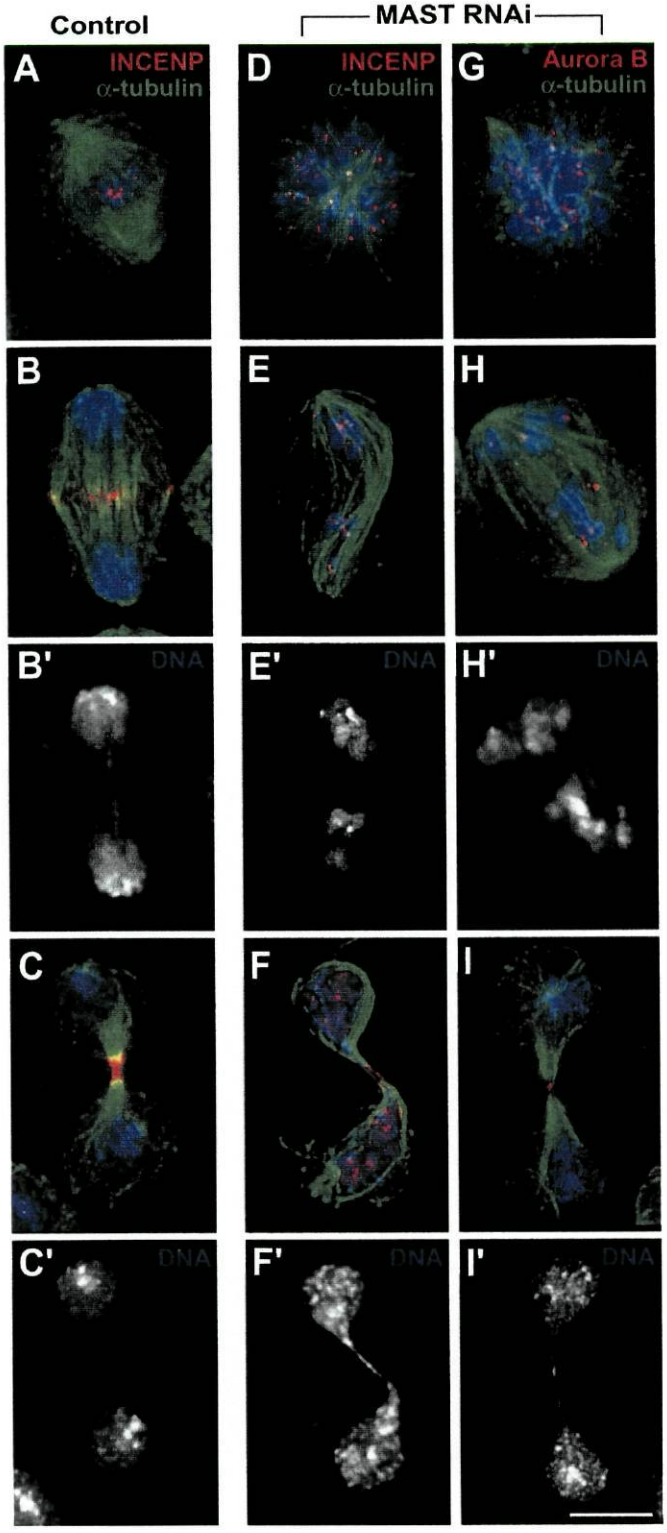


Figure 3.5. Chromosomal-passenger proteins after abnormal mitotic exit in MAST RNAi treated cells. **(A, D** and **G)** INCENP and Aurora B (red) are present on the chromosomes of normal metaphase and MAST deficient monopolar cells, respectively. **(B, E** and **H)** Control anaphase and MAST deficient cells, respectively, show that INCENP and Aurora B didn't transfer to the central spindle in the anaphase-like cells. **(B', E'** and **H')** DNA staining is shown in blue or can be seen alone. **(C, F and I)** Telophase and telophase-like in control and MAST deficient cells, respectively. Note that in the cell after RNAi, INCENP is still strongly associated with chromatin and is only barely detected on the midbody. Aurora B by itself cannot be detected associated with chromatin by this stage and is also barely detected at the midbody. However, in MAST deficient cells, a cleavage furrow is still able to form. **(C', F' and I')** Corresponding DNA staining is also provided. Scale bar is 5 μm.

To determine whether MAST RNAi cells do progress into advanced stages of cytokinesis, cells were fixed and immunostained for actin and Cyclin B. The results show that a proportion of cells do exit mitosis with high Cyclin B levels at the centrosomes (Fig. 3.6). We observed cells that have high Cyclin B at the centrosomes and variable cytoplasmic staining and show well separated telophase nuclei and a properly positioned and formed cleavage furrow (Fig. 3.6A-A"). However, polyploid cells that contain a cluster of centrosomes with high Cyclin B levels also in the cytoplasm, show actin dispersed on the cytoplasm and associated with the cortex (Fig. 3.6B-B"). Finally, we observed cells that are well advanced into telophase but have been unable to fully separate their chromosomes as shown by the phospho-Histone H3 positive chromatin bridges (Fig. 3.6C-C"). These cells show no evidence of cleavage furrow formation.

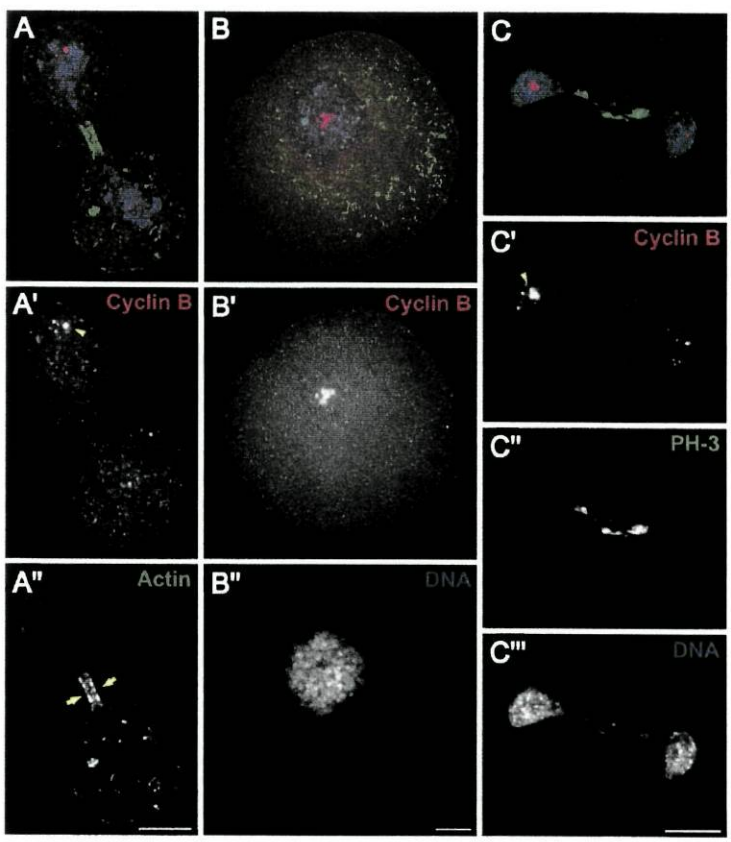


Figure 3.5. Formation of cleavage furrow after abnormal mitotic exit in MAST RNAi treated cells. **(A and B)** Cells immunostained for Cyclin B in red, actin was stained with FITC-falloidin in green, and DNA was stained with DAPI in blue. **(A' and B')** Cyclin B staining alone. **(A")** actin staining alone. **(B")** DNA staining alone. Well organised telophase-like cell undergoing final stages of furrow formation (arrows) with high levels of Cyclin B in the centrosome (arrow heads) and some cytoplasmic staining **(A-A")**. **(B-B")** Polyploid cell showing high levels of Cyclin B both in the centrosome cluster and in the cytoplasm and actin in the cytoplasm and in the cell cortex. **(C-C"')** Highly abnormal telophase-like cell showing poorly decondensed chromatin bridges (phosphorylated-Histone3 positive) and no evidence of cleavage furrow formation by this stage. Bar is 5 μm.

3. Discussion

3.1. MAST-Depleted Cells Exit Mitosis via an APC/C Independent Pathway

During normal exit from mitosis the inactivation of the spindle-checkpoint causes activation of the APC/C, which causes first the release of cohesion that hold sister-chromatids together and secondly promotes the degradation of Cyclin B. However, our results suggest that after depletion of MAST, cells can exit mitosis through a highly abnormal process that does not involve degradation of Cyclin B, sister chromatid separation, spindle checkpoint inactivation or transfer of chromosomal passenger proteins to the central spindle.

Although depletion of MAST function by RNAi causes most cells to arrest in mitosis with monopolar spindles, after long incubation periods a proportion of cells were seen to adopt an anaphase-like configuration with paired sister chromatids migrating to the poles. These cells were Cyclin B-positive, retained BubR1 at the kinetochores and also appeared to undergo cytokinesis with an actin contractile ring. The observed results can readily be explained as direct consequences of abnormal microtubule-kinetochore attachment and deficient metaphase chromosome congression observed in early time points after MAST RNAi. If all chromosomes are not correctly bi-oriented and aligned at the metaphase plate it is not expected that the spindle checkpoint will be inactivated. As a consequence, the APC/C cannot mediate sister chromatid separation and the degradation of cyclins. Therefore, since sister-chromatid separation appears to be a pre-requisite for the correct targeting of chromosomal passengers to the central spindle (see Chapter VI), in MAST-depleted cells chromosomal passengers remain associated with the chromatin. However, since the chromosomal passengers are required only for the final stages of cytokinesis, the residual amount of these proteins associated to decondensed lagging chromatin near the mid-body may be sufficient to allow the

formation of the cleavage furrow, observed in some forms of telophase-like cells after RNAi.

Nevertheless, we cannot exclude an eventual functional interaction between MAST and the chromosomal passenger complex. Indeed, recent studies in *S. cerevisiae* suggest that the functional link may possibly be mediated by the Dam1p/Duo1p complex (Hofmann et al., 1998). This complex is constituted by at least seven proteins (Cheeseman et al., 2001b; Janke et al., 2002) and is thought to be involved in kinetochore function and mitotic spindle integrity (Hofmann et al., 1998; Jones et al., 1999; Enquist-Newman et al., 2001; Cheeseman et al., 2001a). Dam1 shows genetic interactions with Stu1, the homologue of MAST in *S. cerevisiae*, (Jones et al., 1999) and was recently demonstrated to associate directly with Ipl1 and Sli15, the yeast orthologues of the Aurora-INCENP complex, respectively (Kang et al., 2001). Moreover, Ipl1 phosphorylates Dam1 both *in vitro* and *in vivo* and together with sli15 were shown to be required during chromosome segregation. Furthermore, *dam1* mutants carry out anaphase-like events with high levels of Clb2p (the Cyclin B orthologue in yeast) and without spindle checkpoint inactivation. Therefore, *dam1* mutants also exit mitosis by a process that is independent of APC/C.

3.2. MAST-Depleted Cells Become Hyperploid after Initial Mitotic Block

Consistent with our previous observations in *mast* mutant alleles (chapter 1), a large proportion of MAST depleted S2 cells progress into further cycles of DNA replication and become very large and highly polyploid. Natural polyploidy has been recognized in a large variety of both plant and animal cells including *Drosophila melanogaster* (reviewed by Edgar and Orr-Weaver, 2001). This is usually the result of endoreplication a processes that result in successive phases of DNA synthesis without either cell or nuclear division (reviewed by Zimmet and Ravid, 2000). However, other cells like megakaryocytes, initially undergo progression through metaphase, do not complete anaphase or initiate cytokinesis but progress into G1 and become polyploid. Megakaryocytes can reach up to 128N (reviewed by Kaushansky, 1999). It has been demonstrated that 8N ploidy represents a general limit, above which cells cannot execute mitosis and cells with higher ploidy are generally termed as hyperploids (reviewed by Erenpreisa and Cragg, 2001). Since S2 cells have a functional spindle checkpoint as shown by their ability to arrest in mitosis in the presence of colchicine, we

believe that MAST depleted cells by-pass the checkpoint and exit mitosis straight to the next G1 phase via an adaptative pathway.

4. Material and Methods

dsRNA Interference in Drosophila S2 Cells

(Described in Chapter II)

Immunofluorescence in Tissue Culture Cells

(Described in Chapter II)

Quantification of BUBR1 Levels on Immunofluoresce Samples

Levels of the checkpoint protein BubR1 were determined by quantification of pixel intensity on projected series from deconvolved 3-D sets of images using SoftWorx (Applied Precision) after normalization of background fluorescence.

Cell Cycle Analysis of S2 Cells by FACS

For flow cytometric analysis of S2 cells, samples from each experiment were collected in a mini-centrifuge tube, fixed in 70 % ethanol and incubated with RNase (50 µg/ml) for 30 minutes at room temperature. Following two 5 min washes in PBS, the cells were stained with propidium iodide (40 µg/ml final concentration). Samples of 10,000 cells were then analysed on a Becton Dickinson FACSCALIBUR and data was set using CellQuest software.

CHAPTER IV

Molecular and Cellular Characterisation of CLASP1 and CLASP2, Two Human Homologues of MAST

1. Introduction

During the course of database searches using the protein sequence from MAST we identified putative homologues in other species from yeast to humans (Lemos et al., 2000). Among these, two different human cDNAs, namely KIAA0622 and KIAA0627, were obtained by library screening for cDNAS encoding for large proteins in the brain (Ishikawa et al., 1998). The proteins encoded by those cDNAs share significant sequence identity, however, the locus for KIAA0622 is present on chromosome 2 while that for KIAA0627 is present on chromosome 3. Recently, these proteins were designated as CLASP1 and CLASP2, respectively, and shown to associate and stabilize microtubule plus ends during fibroblast motility (Akhamanova et al., 2001). Although no data has been published suggesting a mitotic function for these proteins several results from their homologues in lower organisms suggest that either one or both proteins might be required for mitosis. First, we have shown that the *Drosophila* homologue MAST is essential for mitosis (Lemos et al., 2000). Also, the yeast orthologue of MAST/CLASPs, Stu1, was shown to be essential for spindle assembly (Pasqualone and Huffaker, 1994). Interestingly, CLASPs were identified by their association with CLIP-170, a protein originally identified through its ability to link endocytic vesicles to microtubules (Pierre et al., 1992), and later shown to localise to kinetochores of prometaphase chromosomes (Dujardin et al., 1998). Finally, previous work has shown that CLIP-170 (also known as Restin) is highly expressed in Reed-Sternberg cells, the tumoral cells diagnostic for Hodgkin's disease (Bilbe et al., 1992), which phenotypicaly resembles the giant cells observed in the absence of *Drosophila* MAST (Lemos et al., 2000; Maiato et al., 2002). Therefore, in order to investigate a possible role of CLASPs during mitosis, we have proceeded with their molecular cloning and characterization.

2. Results

2.1. Molecular Cloning of CLASP1 and CLASP2

Using the complete protein sequence of *Drosophila* MAST, homology search in databases allowed us to identify cDNAs encoding for CLASP1 and CLASP2. These cDNAs were called KIAA0622 and KIAA0627, encoding CLASP1 and CLASP2 respectively. They were isolated from Human adult brain libraries and subsequently fully sequenced (Ishikawa et al., 1998). However, analysis of their sequence and subsequent comparison with the human genomic sequence indicated that both cDNAs were incomplete. With the aim of obtaining the full cDNA sequence for both CLASP transcripts, the missing 5' region was amplified by 5'-RACE using the total mRNA from HCT116 cells (Fig. 4.1). Two specific bands with ~750 bp and ~1300 bp corresponding to CLASP1 and one with ~900 bp corresponding to CLASP2 were obtained, which were cloned and subsequently sequenced.

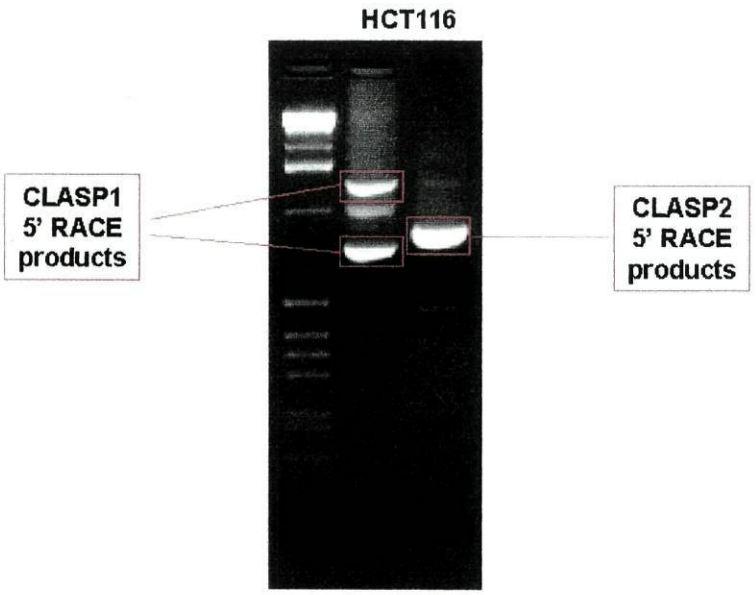


Figure 4.1. Amplification of the 5' region of CLASPs by RACE using total RNA from HCT116 human cell line. Selected bands were cloned and sequenced. On the left side of the gel it is represented the molecular weight marker.

Analysis of the sequence of the two bands obtained for CLASP1 revealed the existence of two cDNAs with different 5' ends. The longest cDNA turned out to be identical to CLASP1α (Akhmanova et al., 2001) and the smaller had an internal deletion of 516 bp encoding a smaller protein that we named CLASP1β (Fig. 4.2). The sequence of the 5' region obtained for CLASP2 encodes a protein whose 5' sequence differs from all known CLASP2 isoforms (Akhmanova et al., 2001) and therefore was called CLASP2δ (Fig. 4.3). Full length CLASP1α was assembled from the combination of the

5'RACE sequence and the sequence of KIAA0622 and subsequently cloned into pEGFP-C1. Further work with CLASP2 was restricted to the use of CLASP2γ isoform (Akhmanova et al., 2001) that corresponds to the sequence of KIAA0627 lacking the first 29 amino acids cloned into pEGFP-C1.

2.2. Sequence Analysis of CLASP1 and CLASP2

Human CLASP1α is composed by 1538 amino acids with a molecular weight of ~170 kDa and a PI of 9.14. The analysis of the CLASP1α sequence revealed the presence of two HEAT repeats, similar to the *Drosophila* MAST protein. A domain conserved with the microtubule-associated protein Tau that lies within a serine-rich region was also identified. Furthermore, 5 putative phosphorylation sites by CDK1 kinase were also found (Fig. 4.2). Human CLASP1β sequence differs from CLASP1α by deletion of amino acids 66 to 238 (which includes the deletion of the first HEAT repeat) resulting in a protein composed by 1365 amino acids with a molecular weight of ~150 kDa and a PI of 9.12.

```
MEPRMESCLAQVLQKDVGKRLQVGQELIDYFSDKQKSADLEHDQTMLDKLVDGLATSWVN  60
SSNYKVVLLGMDILSALVTRLQDRFKAQIGTVLPSLIDRLGDAKDSVREQDQTLLLKIMD 120
QAANPQYVWDRMLGGFKHKNFRTREGICLCLIATLNASGAQTLTLSKIVPHICNLLGDPN 180
SQVRDAAINSLVEIYRHVGERVRADLSKKGLPQSRLNVIFTKFDEVQKSGNMIQSANDKN 240
FDDEDSVDGNRPSSASSTSSKAPPSSRRNVGMGTTRRLGSSTLGSKSSAAKEGAGAVDEE 300
DFIKAFDDVPVVQIYSSRDLEESINKIREILSDDKHDWEQRVNALKKIRSLLLAGAAEYD 360
NFFQHLRLLDGAFKLSAKDLRSQVVREACITLGHLSSVLGNKFDHGAEAIMPTIFNLIPN 420
SAKIMATSGVVAVRLIIRHTHIPRLIPVITSNCTSKSVAVRRRCFEFLDLLLQEWQTHSL 480
ERHISVLAETIKKGIHDADSEARIEARKCYWGFHSHFSREAEHLYHTLESSYQKALQSHL 540
KNSDSIVSLPQSDRSSSSSQESLNRPLSAKRSPTGSTTSRASTVSTKSVSTTGSLQRSRS 600
DIDVNAAASAKSKVSSSSGTTPFSSAAALPPGSYASLGRIRTRRQSSGSATNVASTPDNR 660
GRSRAKVVSQSQRSRSANPAGAGSRSSSPGKLLGSGYGGLTGGSSRGPPVTPSSEKRSKI 720
PRSQGCSRETSPNRIGLARSSRIPRPSMSQGCSRDTSRESSRDTSPARGFPPLDRFGLGQ 780
PGRIPGSVNAMRVLSTSTDLEAAVADALKKPVRRRYEPYGMYSDDDANSDASSVCSERSY 840
GSRNGGIPHYLRQTEDVAEVLNHCASSNWSERKEGLLGLQNLLKSQRTLSRVELKRLCEI 900
FTRMFADPHSKRVFSMFLETLVDFIIIHKDDLQDWLFVLLTQLLKKMGADLLGSVQAKVQ 960
KALDVTRDSFPFDQQFNILMRFIVDQTQTPNLKVKVAILKYIESLARQMDPTDFVNSSET 1020
RLAVSRIITWTTEPKSSDVRKAAQIVLISLFELNTPEFTMLLGALPKTFQDGATKLLHNH 1080
LKNSSNTSVGSPSNTIGRTPSRHTSSRTSPLTSPTNCSHGGLSPSRLWGWSADGLAKHPP 1140
PFSQPNSIPTAPSHKALRRSYSPSMLDYDTENLNSEEIYSSLRGVTEAIEKFSFRSQEDL 1200
NEPIKRDGKKECDIVSRDGGAASPATEGRGGSEVEGGRTALDNKTSLLNTQPPRAFPGPR 1260
ARDYNPYPYSDAINTYDKTALKEAVFDDDMEQLRDVPIDHSDLVADLLKELSNHNERVEE 1320
RKGALLELLKITREDSLGVWEEHFKTILLLLLETLGDKDHSIRALALRVLREILRNQPAR 1380
FKNYAELTIMKTLEAHKDSHKEVVRAAEEAASTLASSIHPEQCIKVLCPIIQTADYPINL 1440
AAIKMQTKVVERIAKESLLQLLVDIIPGLLQGYDNTESSVRKASVFCLVAIYSVIGEDLK 1500
PHLAQLTGSKMKLLNLYIKRAQTTNSNSSSSSDVSTHS
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Figure 4.2. Full-length amino acid sequence of human CLASP1α. Like its *Drosophila* homologue, MAST, human CLASP1α also contains two HEAT-repeat motifs at the N- and C-terminal (red). A central region rich in serine residues (blue) containing a conserved domain of the microtubule-associated protein Tau (highlighted in yellow) were also identified. Detailed analysis revealed the presence of five residues that are putative targets for phosphorylation by CDK1 (bold with asterisks). Human CLASP1β is the product of a deletion between residues 66 to 238 in CLASP1α (bold underlined), resulting in a truncated protein. The non-conserved 22 residues peptide, present only in CLASP1 but not CLASP2, was used for antibody production (grey box).

At the present, CLASP2α has only been identified in mouse (Akhmanova et al., 2001). However, CLASP2β and CLASP2γ were already identified in humans and differ by a distinct short N-terminus sequences that are thought to target the protein to the Golgi apparatus (Akhmanova et al., 2001). Here we describe the identification of human CLASP2δ whose N-terminal is also different from the other two human CLASP2 isoforms (Fig. 4.3). Human CLASP2δ is composed by 1310 amino acids with a molecular weight of ~143 kDa and a PI of 8.71.

CLASP2β: MRRLICKRICD...
CLASP2γ: MAMGD...
CLASP2δ: MIFAKFDEVQSSGGMILSVCK...

Figure 4.3. N-terminal sequence diversity of human CLASP2 isoforms.

Analysis of the protein sequence from CLASP2δ also revealed the presence of a long central serine-rich region and, in contrast with CLASP1α, a single HEAT repeat at the C-terminal (Fig. 4.4). Furthermore, 3 putative phosphorylation sites by CDK1 kinase were also found lying on the serine-rich region.

Figure 4.4. Full-length amino acid sequence of human CLASP2β isolated from a human colon carcinoma cell line. Its N-terminal (bold underlined) is distinct from all other human CLASP2 isoforms. Sequence analysis revealed the presence of a Serine rich region in the middle of the protein (blue) and a single HEAT repeat at the C-terminal (red). Putative phosphorylation sites by Cdk1 are indicated in bold with asterisks. (See next page).

MIFAKFDEVQSSGGMILSVCKDKSFDDEESVDGNRPSSAASAFKVPAPKTSGNPANSARK 60
PGSAGGPKVGGASKEGGAGAVDEDDFIKAFTDVPSIQIYSSRELEETLNKIREILSDDKH 120
DWDQRANALKKIRSLLVAGAAQYDCFFQHLRLLDGALKLSAKDLRSQVVREACITVAHLS 180
TVLGNKFDHGAEAIVPTLFNLVPNSAKVMATSGCAAIRFIIRHTHVPRLIPLITSNCTSK 240
SVPVRRRSFEFLDLLLQEWQTHSLERHAAVLVETIKKGIHDADAEARVEARKTYMGLRNH 300
FPGEAETLYNSLEPSYQKSLQTYLKSSGSVASLPQSDRSSSSSQESLNRPFSSKWSTANP 360
STVAGRVSAGSSKASSLPGSLQRSRSDIDVNAAAGAKAHHAAGQSVRRGRLGAGALNAGS 420
YASLEDTSDKLDGTASEDGRVRAKLSAPLAGMGNAKADSRGRSRTKMVSQSQPGSRSGSP 480
GRVLTTTALSTVSSGVQRVLVNSASAQKRSKIPRSQGCSREASPSRLSVARSSRIPRPSV 540
SQGCSREASRESSRDTSPVRSFQPLASRHHSRSTGALYAPEVYGASGPGYGISQSSRLSS 600
SVSAMRVLNTGSDVEEAVADALKKPARRRYESYGMHSDDDANSDASSACSERSYSSRNGS 660
IPTYMRQTEDVAEVLNRCASSNWSERKEGLLGLQNLLKNQRTLSRVELKRLCEIFTRMFA 720
DPHGKRVFSMFLETLVDFIQVHKDDLQDWLFVLLTQLLKKMGADLLGSVQAKVQKALDVT 780
RESFPNDLQFNILMRFTVDQTQTPSLKVKVAILKYIETLAKQMDPGDFINSSETRLAVSR 840
VITWTTEPKSSDVRKAAQSVLISLFELNTPEFTMLLGALPKTFQDGATKLLHNHLRNTGN 900
GTQSSMGSPLTRPTPRSPANWSSPLTSPTNTSQNTLSPSAFDYDTENMNSEDIYSSLRGV 960
TEAIQNFSFRSQEDMNEPLKRDSKKDDGDSMCGGPGMSDPRAGGDATDSSQTALDNKASL 1020
LHSMPTHSSPRSRDYNPYNYSDSISPFNKSALKEAMFDDDADQFPDDLSLDHSDLVAELL 1080
KELSNHNERVEERKIALYELMKLTQEESFSVWDEHFKTILLLLLETLGDKEPTIRALALK 1140
VLREILRHQPARFKNYAELTVMKTLEAHKDPHKEVVRSAEEAASVLATSISPEQCIKVLC 1200
PIIQTADYPINLAAIKMQTKVIERVSKETLNLLLPEIMPGLIQGYDNSESSVRKACVFCL 1260
VAVHAVIGDELKPHLSQLTGSKMKLLNLYIKRAQTGSGGADPTTDVSGQS

2.3. Expression Profile of CLASP1 and CLASP2 in Tumor Cell Lines

In order to investigate whether both CLASP1 and CLASP2 were expressed in human tumour cell lines, a semi-quantitative RT-PCR was performed. Total RNA was extracted from HeLa cells (Human cervical carcinoma), MCF-7 cells (Human breast carcinoma), HCT116 cells (Human colon carcinoma), U2OS cells (Human osteosarcoma), HL-60 cells (Human leukaemia) and Colo320 DM cells (Human colon carcinoma). Synthetic oligomeres specific to either cDNAs from CLASP1 or CLASP2 were used as primers for the RT-PCR reaction and amplification of β-actin was used as an internal quantitative control (Fig.4.5).

The results show that both genes are expressed in all the cell lines studied. However, more detailed quantification would have to be performed in order to determine whether the levels of the different CLASP1 or CLASP2 isoforms fluctuate in the different tumour cell types.

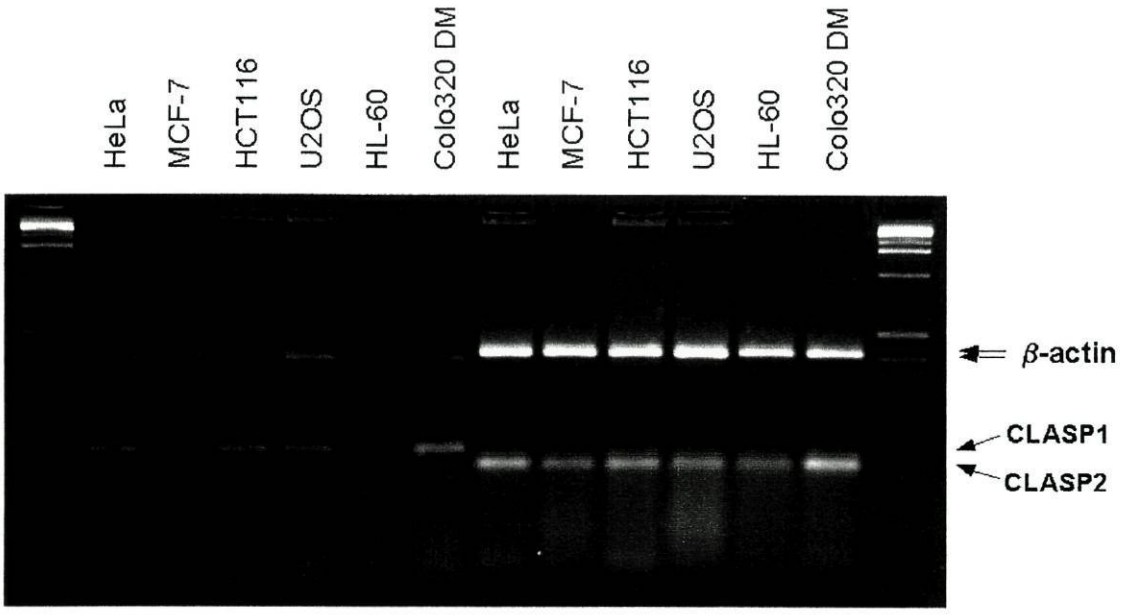


Figure 4.5. RT-PCR analysis of the expression of CLASP1 and CLASP2 in human tumor cell lines. β-actin was used as an internal control for RNA loading. On the sides of the gel the molecular weight markers are represented.

2.4. Cellular Localization of CLASP1 During Mitosis

In order to investigate whether CLASP1 localise to any particular intracellular compartment during mitosis we raised polyclonal antibodies specific for CLASP1 and performed immunofluorescence analysis in mitotic HeLa cells (Fig. 4.6). Preimmune or peptide competition with the immune sera failed to give any specific staining in mitotic cells (data not shown). In parallel, we transiently expressed the full length CLASP1 protein with EGFP fused at its N-terminus in order to confirm the specificity of the immunostaining (Fig. 4.6).

The results indicate that both the endogenous CLASP1, as shown by antibody staining, and the EGFP-CLASP1 protein localized to spindle poles throughout mitosis. Also, already during prophase, CLASP1 accumulates in dots that are coincident with chromosomes. During metaphase it is clear that these dots localise at the end of microtubule bundles where kinetochores should be located. After anaphase onset, CLASP1 was also found at the central spindle, accumulating in the mid-body during telophase and cytokinesis.

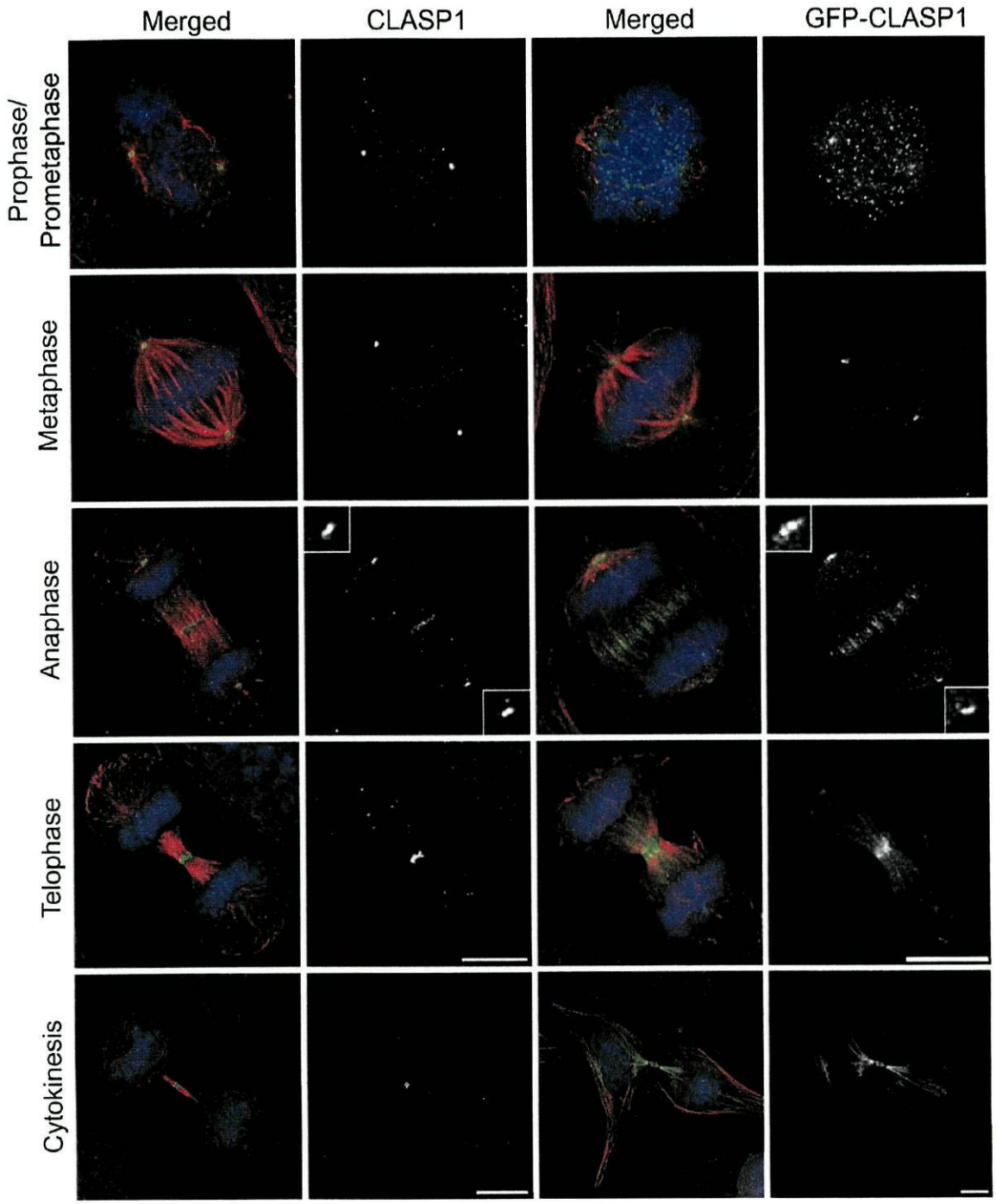


Figure 4.6. Cellular localization of CLASP1 during mitosis in human HeLa cells. The two columns on the left are immunofluorescence images showing localization of endogenous CLASP1 using a specific antibody (green), microtubules (red) and DNA (Blue) during mitosis. The two columns on the right shows HeLA cells during different stages of mitosis that were transfected with EGFP-CLASP1. Insertions in the anaphase pictures are 3X amplifications of the centrosome staining in the corresponding cells. Bar is 10 μm.

2.5. Cellular Localization of CLASP2 During Mitosis

In parallel to the study of CLASP1, we would also like to determine the localization of CLASP2 during mitosis in order to investigate whether these two proteins might

perform similar functions within the cell. For this purpose, we have used a polyclonal antibody that was raised against the C-terminal region of recombinant CLASP2 (Akhmanova et al., 2001). However, since both CLASP proteins are highly homologous through most of their sequence, we also transiently expressed the CLASP2γ isoform fused with the EGFP protein at its N-terminus in HeLa cells (Fig. 4.7).

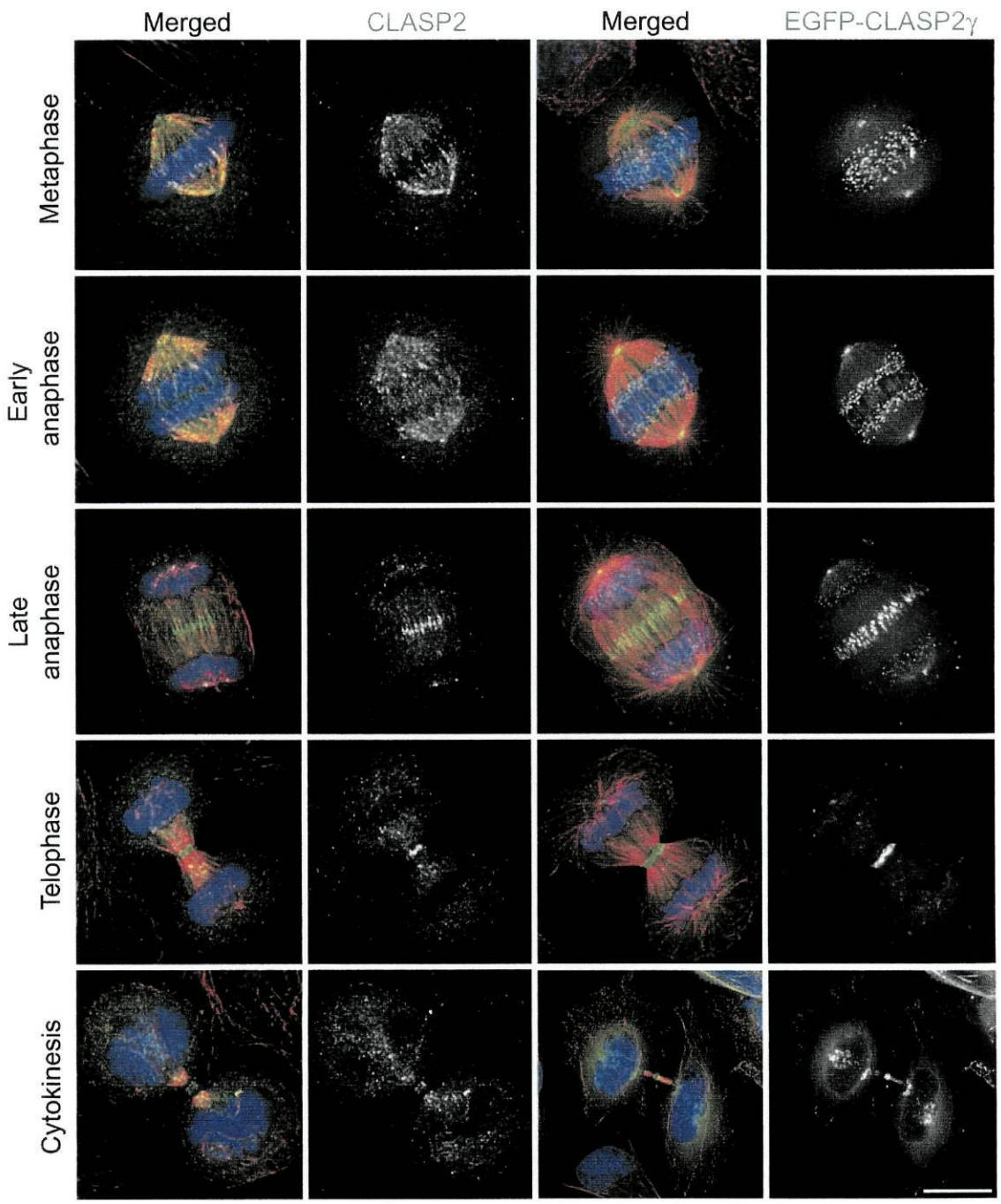


Figure 4.7. Cellular localization of CLASP2 during mitosis in human HeLa cells. The two columns on the left are immunofluorescence images showing localization of endogenous CLASP2 by antibody staining (green), microtubules (red) and DNA (Blue). The two columns on the right show mitotic HeLA cells that were transfected with EGFP-CLASP2γ. Bar is 10 μm.

The results show that CLASP2 has a very similar if not identical pattern of localization to CLASP1 during mitosis. Both the endogenous and the EGFP-CLASP2γ proteins localise to centrosomes throughout mitosis and during early stages some spindle staining is also visible. During metaphase and anaphase CLASP2 accumulates strongly to kinetochores (this is clearer with the EGFP- CLASP2γ), which is no longer evident during telophase. Also, during anaphase CLASP2 and EGFP-CLASP2γ can be found as a broad band in the central spindle and later during telophase and cytokinesis accumulate at the midbody.

3. Discussion

3.1. Human CLASPs Localise to Specific Mitotic Compartments

Here we report the identification and independent cloning of the human homologues of *Drosophila* MAST, which are known as CLASPs (Akhmanova et al., 2001). We showed that both CLASP1 and CLASP2 mRNAs are expressed in a variety of tumour cell lines. Also, we found 2 novel isoforms of CLASPs expressed in a human colon tumour cell line. These new isoforms were named as CLASP1β and CLASP2δ. CLASP1β is encoded by an mRNA with an internal deletion of 516 bp resulting in a truncated protein when compared with CLASP1α (Akhmanova et al., 2001). CLASP2δ is encoded by an mRNA, which has a distinct 5' end resulting in a protein with a distinct N-terminus compared with other CLASP2 isoforms (Akhmanova et al., 2001). Whether these new isoforms represent specific modifications of CLASPs in tumours or may be just novel alternative spliced forms remains to be determined.

Even though CLASP2 has been shown to function during interphase and in contradiction with initial speculations (McNally, 2001), we have found that this protein localises to very specific compartments of the mitotic apparatus and chromosomes during mitosis. Indeed, during mitosis, CLASP2 has a pattern of localization identical to that of CLASP1. It has been proposed that CLASPs function during interphase to promote the stabilization of microtubule-plus ends at the migration front during fibroblast motility. However, our observations suggest that CLASPs might also have mitotic functions. Like MAST, we show that during mitosis, CLASPs localize to the mitotic spindle, centrosomes and kinetochores, ending up accumulating in the central-spindle region and ultimately concentrating at the midbody.

The pattern of localization by CLASPs is reminiscent of the localization of CENP-E, a kinesin-related protein that has been implicated in kinetochore attachment to the mitotic spindle and in checkpoint signalling (Yen et al., 1992; Yao et al., 2000; Abrieu et al., 2000). However, these proteins are unlikely to function in the same pathway, as the phenotypes produced by interference with CENP-E and MAST/CLASP1 function are distinct (Schaar et al., 1997; Wood et al., 1997; Maiato et al., 2002; see below). Furthermore, we have found that perturbation of CENP-E localization by injection of function-blocking antibody or by overexpression of a dominant-negative construct have no effect on the behaviour of CLASP1 in cells (data not shown).

3.2. Possible Role for CLASPs During Mitosis

Detailed sequence analysis revealed a previously unidentified domain that shares homology with the microtubule-associated protein Tau (Aizawa et al., 1988). This domain falls in a serine-rich region where multiple putative phosphorylation sites for CDK1 can be found. These results, together with the conserved localization data between CLASPs and MAST and with the finding that CLASPs bind to microtubules in vitro (Akhmanova et al., 2001) strongly supports a role for CLASPs in spindle assembly and regulation of microtubule dynamics during mitosis. Their co-localization throughout mitosis might suggest that they may function together. Accordingly, there is only one homologue of CLASPs in *Drosophila* and *S. cerevisiae*, which represent essential genes in both organisms (Lemos et al., 2000; Pasqualone and Huffaker, 1994). It is possible that in vertebrates the existence of different members of this family represent a gene duplication event. In support of this hypothesis, we have shown that injection of antibodies specific to CLASP1 is sufficient to cause a CLASP2-independent phenotype (see Chapter V). Nevertheless, since it was found that Stu1 interacts with itself in vivo (Yin et al., 2002), it is possible that CLASPs could function as homo- or hetero-dimmers. If this turns out to be true, it could readily explain why interference of CLASP1 is sufficient to cause a phenotype and why its function is not redundant with CLASP2.

CLASPs were found to associate with the microtubule-plus ends during interphase (Akhmanova et al., 2001). A number of proteins that show this particular localization have also been shown to have a role during mitosis. This would be expected since the microtubule tips assume critical roles in centrosome separation and chromosome movement, namely by interaction with the cell cortex, interaction between interpolar microtubules and kinetochore attachment. Indeed, genetic studies in *Drosophila* have suggested a role for APC-EB1 in spindle orientation during mitosis (McCartney et al.,

1999; Lu et al., 2001; McCartney et al., 2001). Furthermore, *Drosophila* EB1 was recently shown to be important for spindle assembly and dynamics (Rogers et al., 2002). More surprisingly, was the finding that in human tissue culture cells APC is localized at kinetochores, and that APC mutant cells are defective in spindle formation and chromosome segregation causing chromosome instability (Fodde et al., 2001; Kaplan et al., 2001). These findings have led to the hypothesis that APC is required for microtubules to search and capture the kinetochores during mitosis. However, work by others and our own unpublished observations could not confirm the presence of APC at the kinetochores making this a controversial issue demanding further clarification (see review by Bienz, 2002). Furthermore, CLIP-170 was found to be required for chromosome congression during prometaphase (Dujardin et al., 1998). In summary, our results provide strong evidence for a role of CLASPs during mitosis.

4. Materials and Methods

Expression Analysis of CLASP1 and CLASP2 in Tumour Cell Lines by RT-PCR

Total RNA was extracted from human HeLa, U2OS, HL-60, MCF-7, colo320 DM and HCT116 cell lines. Briefly, 10^7 cells were lysed in RNA lysis buffer and 2M sodium acetate pH 4 together with water saturated phenol. Chloroform was added to the mixture that was subsequently centrifuged for 20 min at 6000 rpm at 4 °C. The upper phase was collected and the RNA precipitated with isopropanol at –20 °C for 1 h. After centrifugation at 15000 rpm for 30 min at 4 °C, the pellet was washed with 70 % ethanol and re-suspended in RNase-free water. RNA concentration and purity was determined by measuring the $Abs_{260/280}$. RT-PCR was performed using Access RT-PCR System (Promega) according to the manufacturer's instructions. Specific PCR primers for CLASP1 (primers RT-622-F/RT-622-R) or CLASP2 (RT-627-F/RT-627-R) were designed from the respective cDNA sequence and synthesized (Oswel), and β-actin was used for PCR internal control (see Table II).

Cloning of the Full-Length CLASP1 cDNA and Alternative Splicing Forms of CLASP1 and CLASP2 by 5'RACE

Total RNA from HCT116 cells was prepared as before. For the amplification of the missing 5' end of CLASP1 and identification of alternative splicing forms of either CLASP1 and CLASP2 5'RACE System (Gibco) was used according to the manufacturers instructions. Three specific primers were designed for each gene (CLASP1 primers: RC-622-1/2/3; CLASP2 primers: RC-627-1/2/3) and synthesized (Oswel) (see Table II), and the resultant amplified 5' fragments were gel purified, cloned using pGEM-T Vector System (Promega) according to the manufacturer's

instructions and subsequently sequenced. A human EST cDNA (accession number H49239) that showed 100% identity with the sequence of CLASP1 5'RACE product was obtained from the IMAGE consortium and sequenced. A fragment of ~830 pb corresponding to the missing 5' end of CLASP1 was obtained by high fidelity PCR, cloned into pGEM-T and fully sequenced. Full-length CLASP1 was cloned into pEGFP-C1 vector (Clontech) after joining the cDNA derived from KIAA0622 with the missing 5' PCR product using convenient restriction sites present in KIAA0622 and those incorporated in the primers (BspEI-F and AclI-R primers, see Tables II and III).

Immunofluorescence in Human Tissue Culture Cells

Human HeLa cells were grown in RPMI at 37 °C in the presence of 5 % CO_2 and supplemented with 10% FBS and antibiotics. Cells were grown adherently in poly-lysine treated coverslips and were fixed either with 4 % paraformaldehyde or methanol at –20 °C. For anti-CLASP1 staining the cells were blocked with O.5 % BSA in dPBS containing 0.1 % Tween and then incubated for 40 min with Rb1277 diluted in the same buffer. Cells were washed twice with dPBS and once with dPBS containing 0.1 % Tween between and after incubation with secondary antibodies. Aldehyde fixations were followed by a permeabilization step with 0.3-0.5% Triton X-100 in cytoskeleton buffer. All the other stainings that did not involve Rb1277 were blocked for 30 min with 10 % FBS + 0.1 % Triton X-100 in dPBS. Cells were then incubated with primary antibodies (see Table I) diluted in the blocking solution for 45 min, washed 3x5 min with dPBS + 0.1 % Triton X-100 and detected with secondary FITC- and/or Texas Red-conjugated antibodies (Jackson Immunoresearch). DNA was counterstained with DAPI and the preparations mounted in Vectashield (Vector). Quantitative three-dimensional data sets of representative cells were collected using a DeltaVision microscope (Applied Precision, Issaquah, WA), based on an Olympus IX-70 inverted microscope with a Photometrics CH350L cooled CCD camera, and subsequently deconvolved and projected onto a single plane using SoftWorx (Applied Precision). Adobe Photoshop 5.5 (Adobe Systems) was used to process all images.

Constructs and Transfections

EGFP-CLASP1 resulted from the cloning of full-length CLASP1α into pEGFP-C1 (Clontech). EGFP-CLASP2γ was a gift from Anna Akhmanova and has been described previously (Akhmanova et al., 2001). Transfections were performed using FuGene (Roche) according to the manufacturer's instructions.

Sequence Analysis

Analysis of conserved protein domains present in CLASPs was performed using ProfileScan (ISREC), MOTIF (Kyoto, Japan) and NetPhos 2.0 (CBS).

CHAPTER V

Human CLASP1 Mediates Kinetochore Interactions with Dynamic Microtubule-Plus-Ends and is Required for Mitotic Spindle Integrity

1. Introduction

Analysis of the subcellular distribution of CLASPs during mitosis and results from the study of their homologues in *Drosophila* and budding yeast (Maiato et al., 2002; Yin et al., 2002; reviewed by Sharp, 2002) strongly suggested that CLASPs may play a role in microtubule kinetochore attachment and spindle integrity. Accordingly, we have previously shown that MAST is essential for spindle assembly and function (Lemos et al., 2000). Subsequent RNAi and time-lapse microscopy analysis of MAST mutant embryos revealed that the protein is required for the stability of a bipolar spindle in mitosis (Maiato et al., 2002). Nearly identical results have been reported in a functional analysis of the budding yeast MAST/Orbit homologue Stu1p (Yin et al., 2002). Additionally, the spindle collapse phenotype caused by Stu1p inactivation can be rescued by increasing the dosage of *cin8*, which encodes a homologue of KLP61F. Interestingly, several of the observations suggested that MAST/Orbit also directly influences the interactions between spindles and chromosomes, consistent with its localization to the kinetochores (Lemos et al., 2000). For example, prior to spindle collapse at metaphase, mitosis in *mast* mutant embryos is marked by clear defects in the ability of chromosomes to find stable positions at the spindle equator (Maiato et al., 2002). Moreover, in *Drosophila* S2 cells depleted of MAST/Orbit by RNA inhibition (RNAi), defects in sister chromatid separation arise and cells in metaphase could never be found. In these cells, the normal end-on associations between kinetochores and bundles of microtubules appear either not to have occurred or to be aberrant. Such defects could explain the abnormal behavior of chromosomes following MAST/Orbit inhibition. Surprisingly, we have found that kinetochores do attach to the ends of microtubule bundles in MAST/Orbit deficient cells if microtubule-dynamics were suppressed by treatment with the microtubule-stabilizing drug taxol. Thus, MAST/Orbit may be required to link kinetochores specifically to the ends of dynamic microtubules.

In order to investigate whether CLASPs show a conserved function at the kinetochores and spindle, we proceed with the characterization of human CLASP1. Our

main goal was to provide mechanistic insights on how CLASP1 may influence the attachment of kinetochores to dynamic microtubule-plus ends.

2. Results

2.1. Cellular Localization of CLASP1 In Vivo During Mitosis and Cytokinesis

Analysis of fixed cells either by immunostaining or expression of fluorescently tagged CLASPs indicated that both proteins have a highly dynamic localisation pattern. On the other hand, CLASPs were previously shown to associate with the plus ends of microtubules in interphase (Akhmanova et al., 2001). In order to analyse these patterns of localization in more detail we performed *in vivo* recordings of HeLa cells during mitosis that were transfected with EGFP-CLASP1. Four-dimensional analysis by restoration microscopy in living HeLa cells revealed that EGFP-CLASP1 was preferentially associated near the plus-ends of growing microtubules during prometaphase and confirmed its association at the centromeric region of attached chromosomes (Fig. 5.1 and movie 1). At the metaphase-anaphase transition, EGFP-CLASP1 was re-distributed on the spindle microtubules, accumulating at the central spindle region and midbody at telophase (Fig. 5.1 and movie 2). During cytokinesis, EGFP-CLASP1 is associated with the leading edge of the daughter cells where the cytoplasm expands at the beginning of the G1 phase of the next cell cycle (Fig. 5.1 and movie 3). Additionally, movement of EGFP-CLASP1 molecules was also observed along or with the microtubules in a fashion similar to that described during interphase cells (Akhmanova et al., 2001). This pattern of localization suggests that CLASP1 remains associated with the plus ends of microtubules throughout mitosis and cytokinesis.

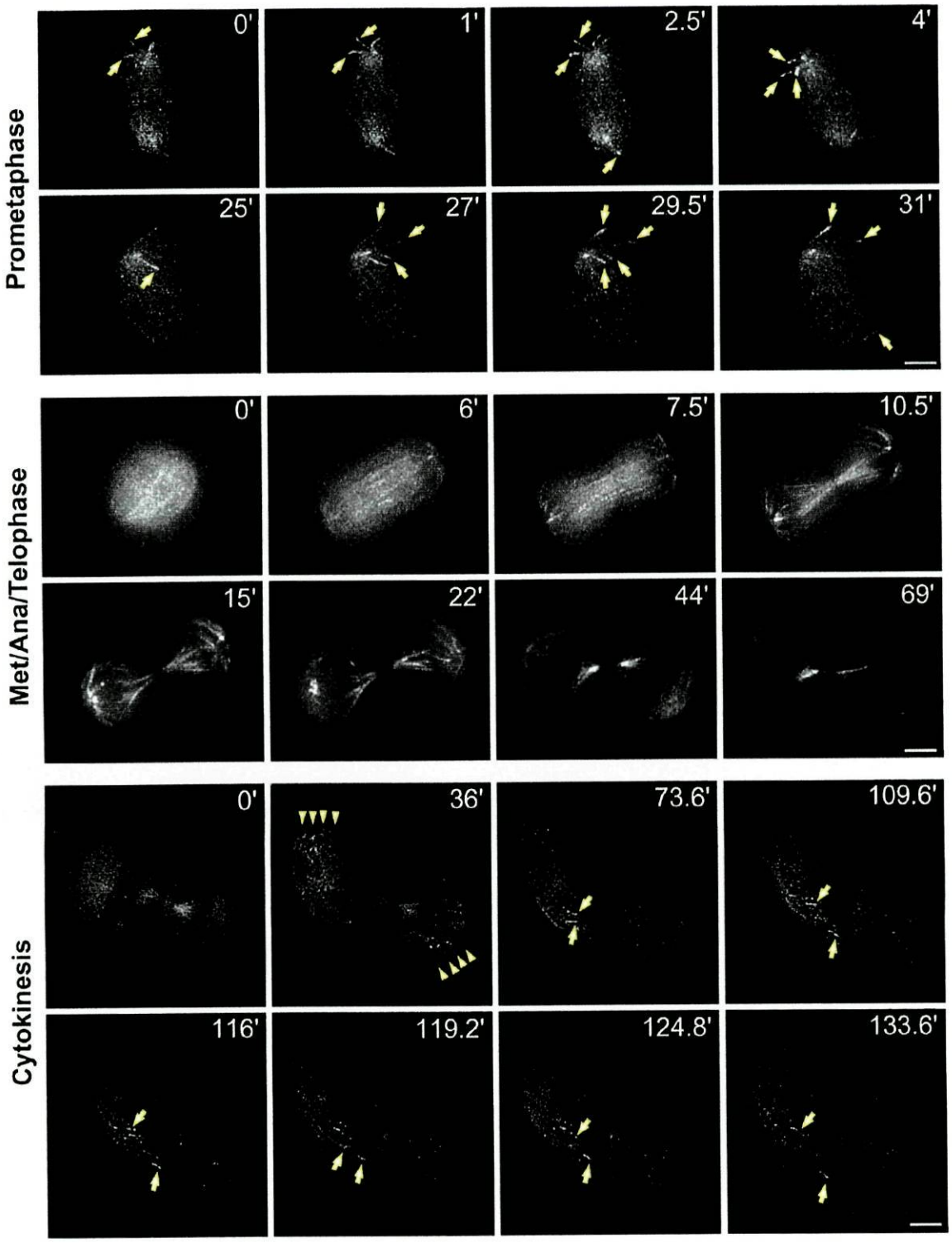


Figure 5.1. Analysis of living HeLa cells expressing EGFP-CLASP1. EGFP-CLASP1 can be seen associated with the spindle poles and with growing microtubule plus-ends (arrows) during prometaphase. In microtubules that have become attached, EGFP-CLASP1 shows some concentration at the kinetochores. During metaphase, EGFP-CLASP1 is localized all over spindle microtubules. After anaphase onset EGFP-CLASP1 concentrates at the central spindle, accumulating at the midbody at telophase. During cytokinesis, EGFP-CLASP1 can be seen flowing towards the migration front of the expanding cytoplasm (arrowheads) and along the microtubule network (arrows). Time-lapse is shown in minutes. Bar is 10 μm.

2.2. CLASP1 Defines a New Outer Kinetochore Domain Sensitive to Microtubule Dynamics

Immunofluorescence studies in fixed cells showed that CLASP1 localises to centromeres from prophase to late anaphase. In order to determine its localisation more precisely, co-localization experiments using kinetochore markers were performed. We observed that EGFP-CLASP1 localizes at the leading kinetochores during anaphase (Fig. 5.2A). In chromosome spreads prepared in the absence of microtubules, CLASP1/EGFP-CLASP1 localization within the kinetochore is distal to the area stained by anticentromere antibodies (ACA), which extends from the centromeric heterochromatin to the inner kinetochore plate as defined by CENP-C localization (Fig. 5.2B, C). More surprisingly, we found that EGFP-CLASP1 was also largely distal to CENP-E, a constituent of the fibrous corona that lies outside the outer kinetochore plate (Cooke et al., 1997; Yao et al., 1997) (Fig. 5.2D). This result is highly unexpected and indicates that CLASP1 appears to define a new region of the kinetochore separated from the outer kinetochore plate by a zone rich in CENP-E, which we have termed the "outer corona".

CLASP1 localization at kinetochores did not require intact microtubules. However, *in vivo* analysis of CLASP1 distribution shows the protein to associate with plus-ends of growing microtubules during mitosis. To determine whether the distribution of the protein on attached kinetochores was dependent upon microtubule dynamics we treated EGFP-CLASP1 expressing cells with taxol to specifically suppress plus end microtubule dynamics for 30 minutes (Jordan and Wilson, 1999). Surprisingly, if microtubule dynamics are suppressed by a brief incubation with taxol, the bulk of the protein was substantially depleted from the kinetochore-proximal microtubule plus ends, and redistributed to regions of microtubules closer to the poles (Fig. 5.2E-F"). Traces of kinetochore-associated CLASP1 could be seen upon more careful examination. This behaviour is remarkably similar to that of CLIP-170 in interphase (Perez et al., 1999). Taxol is known to alter the structure of the tubulin lattice (Arnal and Wade, 1995), and these results suggest that the preferential localization of CLASP1 to the kinetochore-proximal microtubule plus ends may involve recognition of a particular lattice conformation.

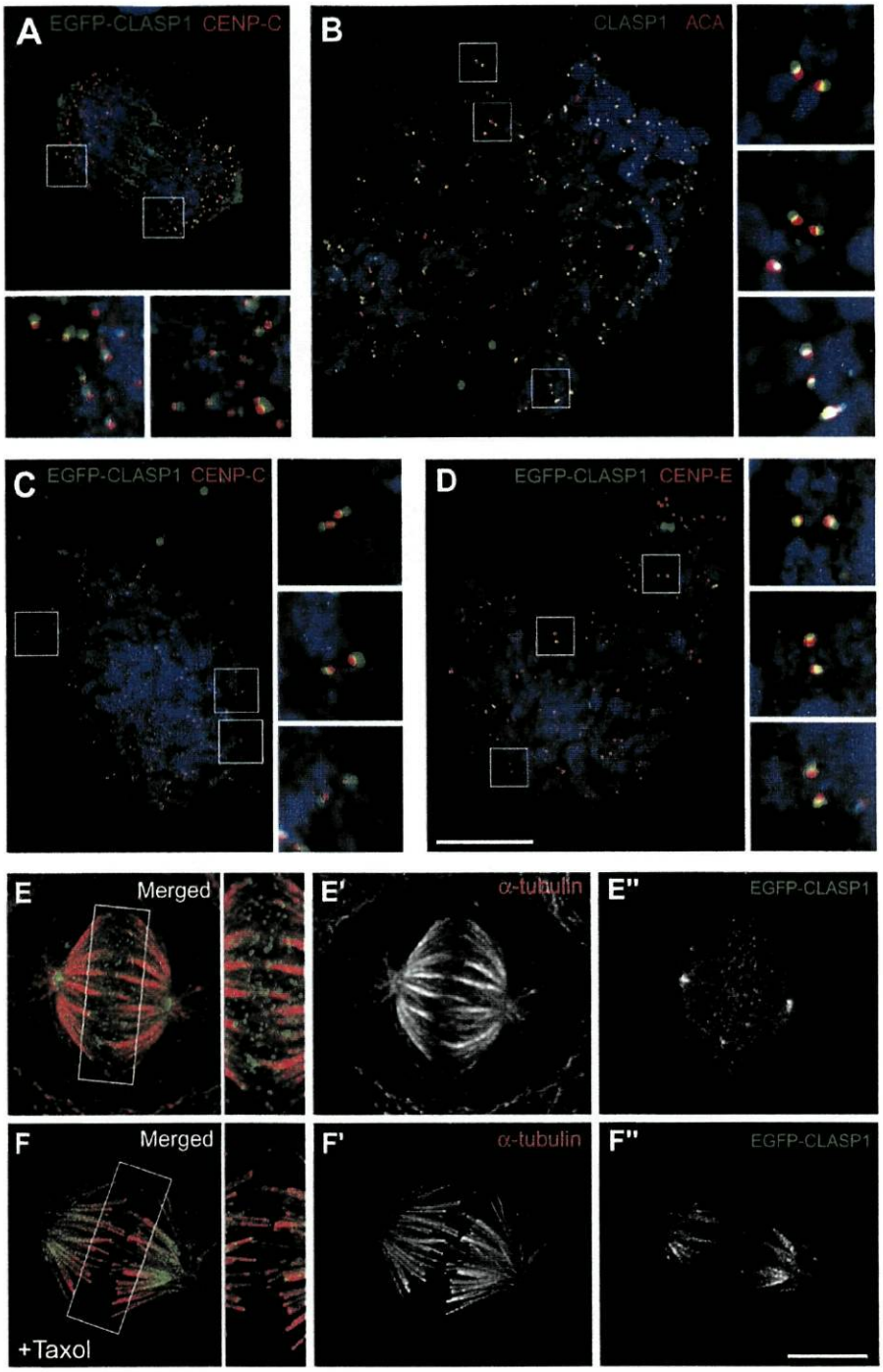


Figure 5.2. Kinetochore localisation of CLASP1. **(A)** Anaphase cell expressing EGFP-CLASP1 (green) at the leading front of kinetochore migration, here shown by CENP-C immunofluoresce (red). **(B)** Chromosome spread showing DNA (blue), endogenous CLASP1 (green) and the kinetochores determined by ACA (red). **(C)** Chromosome spread from a cell expressing EGFP-CLASP1 (green) localized externally to the kinetochore inner plate as shown by CENP-C staining (red). **(D)** Chromosome spread from a cell expressing EGFP-CLASP1 (green) localized at the outer region defined by the fibrous corona determined by CENP-E staining (red). **(E-E'')** HeLa cell expressing EGFP-CLASP1 (green) in metaphase. The kinetochore localization at the plus-ends of the microtubules (red) is shown in detail. **(F-F'')** HeLa cell expressing EGFP-CLASP1 (green) in metaphase after treatment with Taxol for 30 min to suppress microtubule dynamics. Under these conditions, EGFP-CLASP1 is barely detected at the plus-ends of kinetochore microtubules (red). Instead it can be found accumulated at the microtubules near the poles. Bar is 10 μm.

2.3. CLASP1 is an Integral Component of the Centrosome and Associates with the Golgi Apparatus

The localisation of CLASP1 at the centrosome throughout mitosis led to the hypothesis that it might be an integral constituent of this organelle. To test this hypothesis we incubated HeLa cells expressing EGFP-CLASP1 with the microtubule depolimerising drug colcemid for 16 h in order to determine whether CLASP1 requires intact microtubules for centrosome targeting. We observed that the centrosome staining of EGFP-CLASP1, as confirmed by co-localisation with γ-tubulin, does not require the presence of microtubules and thus constitute a novel integral component of the human centrosome (Fig. 5.3A-A"). Furthermore, we observed that during telophase EGFP-CLASP1 accumulates in small areas surrounding the centrosome and also at the opposing edges of the central spindle (Fig. 5.3B-B"). Co-localisation with the Golgi protein p115 has indicated that CLASP1, as previously described for CLASP2 (Akhmanova et al., 2001), also localises to the Golgi apparatus especially during cytokinesis and interphase (Fig. 5.3B-B" and data not shown).

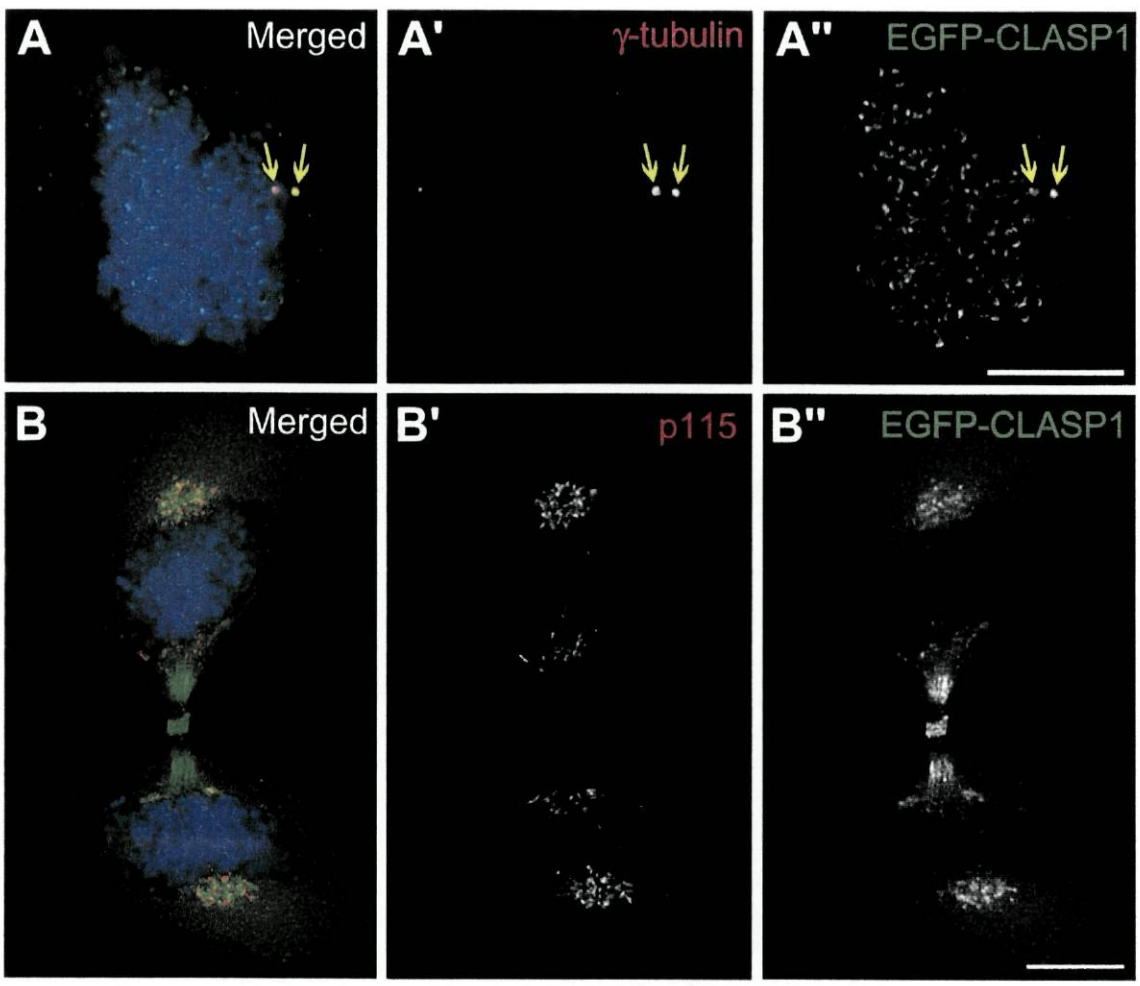


Figure 5.3. CLASP1 does not require microtubules for localization at the kinetochores and centrosomes, and can be found associated with the Golgi apparatus. **(A-A")** Centrosome localization (arrows) of EGFP-CLASP1 (green) was determined by co-localization with γ-tubulin (red) after treatment with the microtubule depolimerysing drug colcemid for 16 h. Also, the kinetochore localization of EGFP-CLASP1 does not seem to be dependent on the presence of microtubules. **(B-B")** Co-localisation of EGFP-CLASP1 (green) with the Golgi-associated protein p115 (red) during cytokinesis. Bar is 10 μm.

2.4. Mapping the Functional Domains of CLASP1

As shown in the previous sections we have found that CLASP1 has a highly dynamic pattern of localization to multiple compartments of the mitotic apparatus and kinetochores. To determine whether different domains of CLASP1 are responsible for its localisation to different compartments of the mitotic apparatus, we constructed a series of vectors expressing different regions of the protein fused with EGFP at the N-terminal and its localisation was determined by transient transfection into HeLa cells. The results are summarized in Fig.5.4 and revealed that the 300 C-terminal amino acids of CLASP1 were necessary and sufficient for proper kinetochore targeting. This required the HEAT repeat motif (Andrade et al., 2001), which might be involved in interactions with other proteins such as CLIP-170 (Akhmanova et al., 2001). A large central region comprising residues 250-943 of CLASP1 was sufficient for association with microtubules.

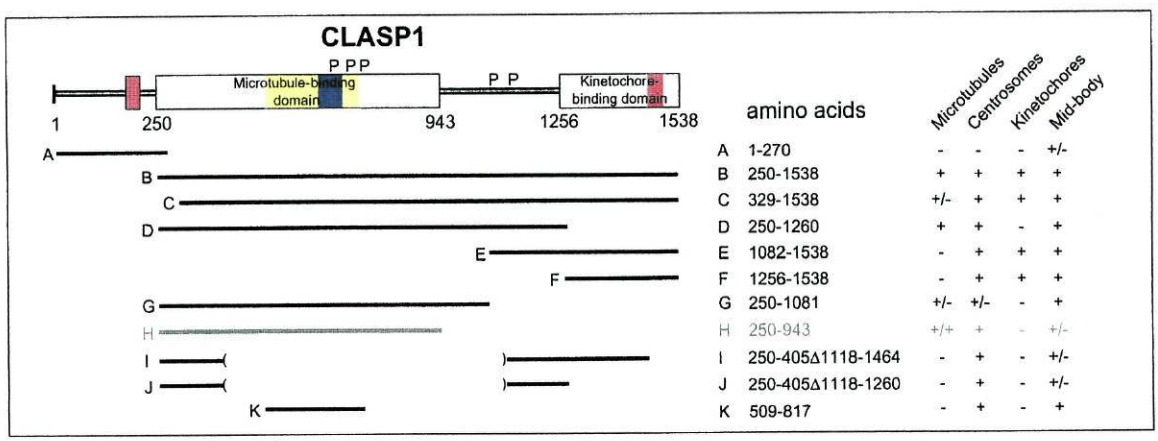


Figure 5.4. Functional analysis of CLASP1 domains and identification of a dominant-negative mutant. Mapping of the microtubule- and kinetochore-binding domains of CLASP1 by transient expression of deletion constructs tagged with EGFP at the N-terminal. Red and blue boxes represent HEAT repeats and a region shared with Tau, respectively. Putative phosphorylation sites "P" by CDK1 correspond to residues S^{688}, S^{731}, S^{757}, T^{1099} and S^{1123}. Localization into different cellular compartments is summarized on the right. +, indicates that the protein targets to the specified compartments; -, indicates that the protein is not present in the specified compartments; +/-, indicates that the protein still accumulates in the specified compartments but in a less extension than normal; +/+, indicates that the accumulation of the protein in the specified compartments is higher then normal.

Interestingly, all fragments of CLASP1 were found to localise to the mid body, although at variable intensity (Figs. 5.4-5.6 and data not shown). This association to the mid body is significant since a number of these fusion proteins do not show association with spindle microtubules. On the other hand, the Golgi association of CLASP1 seems to be highly correlated with the presence of the C-terminal of the protein and is independent of the HEAT repeat. Moreover, in some of the constructs, without any particular correlation of the region of the protein that was expressed, we noted the formation of cytoplasmic aggregates, which may reflect the requirement of particular regions for proper folding of the protein, or posttranslational modifications that make CLASP1 functional during the cell cycle. Additionally, EGFP-CLASP1 (509-817), which seems to retain at least some of the properties required for proper localization during mitosis, was found to accumulate strongly to the nucleus and especially at the nucleolus during interphase. This observation may suggest a possible nucleo-cytoplasmic shuttling mechanism that targets CLASP1 to the cytoskeleton during particular stages of the cell cycle.

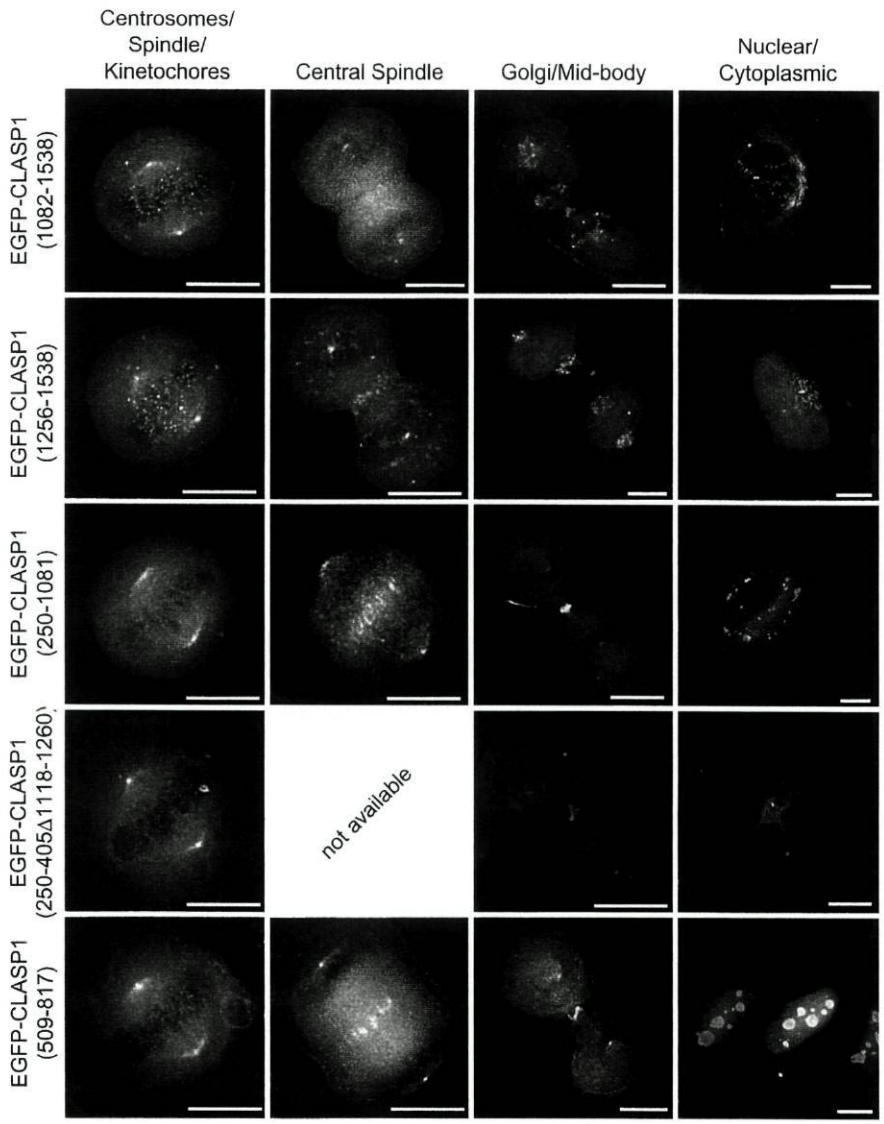


Figure 5.5. Sub-cellular localisation of EGFP-CLASP1 deletion constructs. Expression of c-terminal residues 1082-1538 or 1256-1538 is sufficient for kinetochore targeting of CLASP1. Both truncated proteins can be seen accumulated at the Golgi during cytokinesis and interphase. One difference between these constructs is that deletion of residues 1082-1255 cause accumulation in the nucleus during cytokinesis and interphase. This can be explained due to deletion of two putative CDK1 phosphorylation sites in this region. In general, removal of the 250 first amino acids from CLASP1 did not interfere with normal localization of the protein, while removal of residues 1082-1538 causes mistargeting of CLASP1 to the kinetochores, demonstrating that the C-terminal region of the protein constitutes the kinetochore-binding domain. Deletion of a large central region comprising residues 405-1118 confirms the previous observations and show that small regions are responsible for centrosome targeting. On the other hand, expression of the serine rich region of CLASP1 containing the Tau domain from residues 509-817 was not sufficient for kinetochore or microtubule targeting. However, the centrosome localization was not perturbed, indicating that this particular targeting may involve several regions of CLASP1. Moreover, expression of this region causes accumulation at the nucleus, especially in the nucleolar regions. Bar is 10 μm.

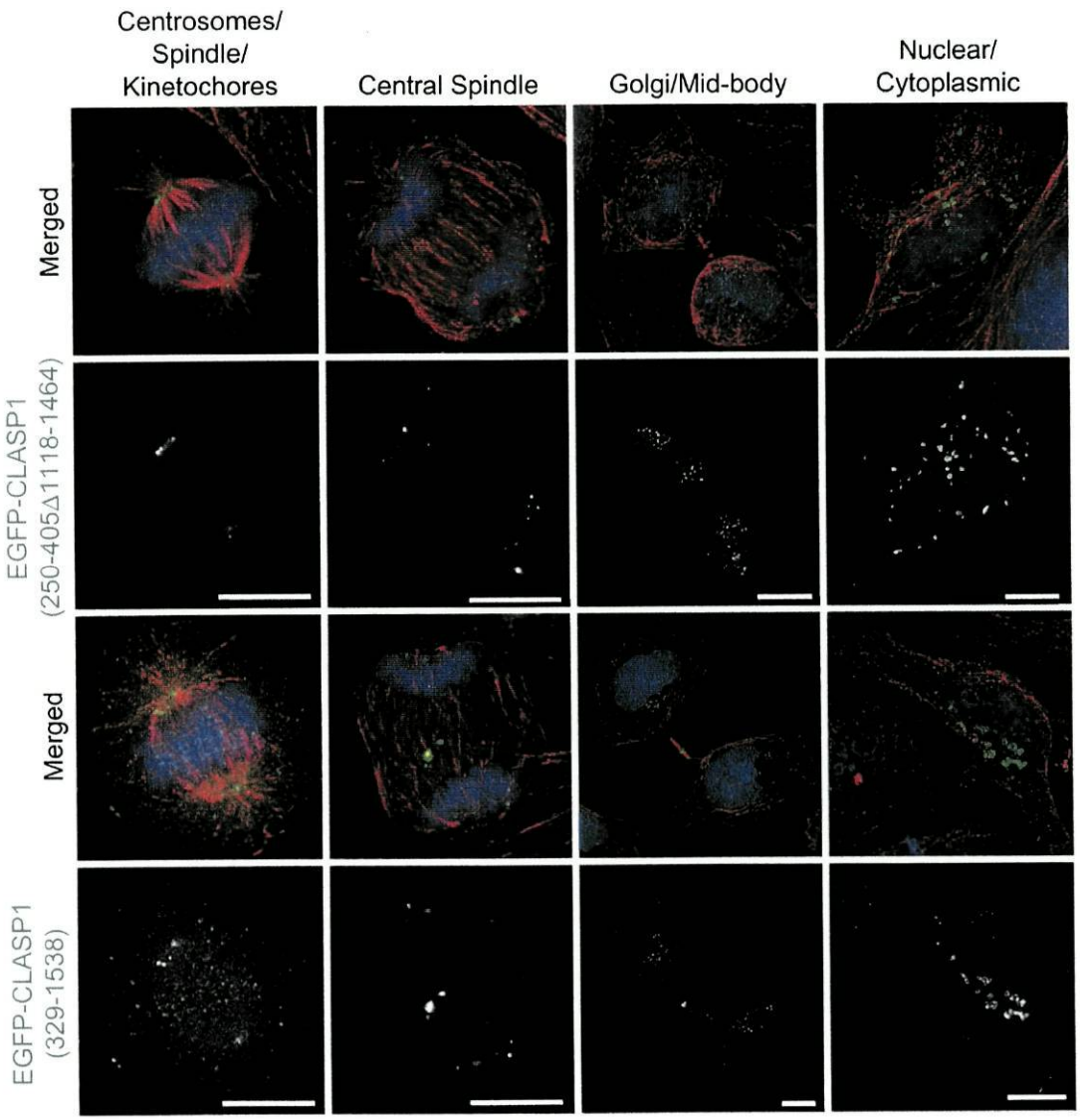
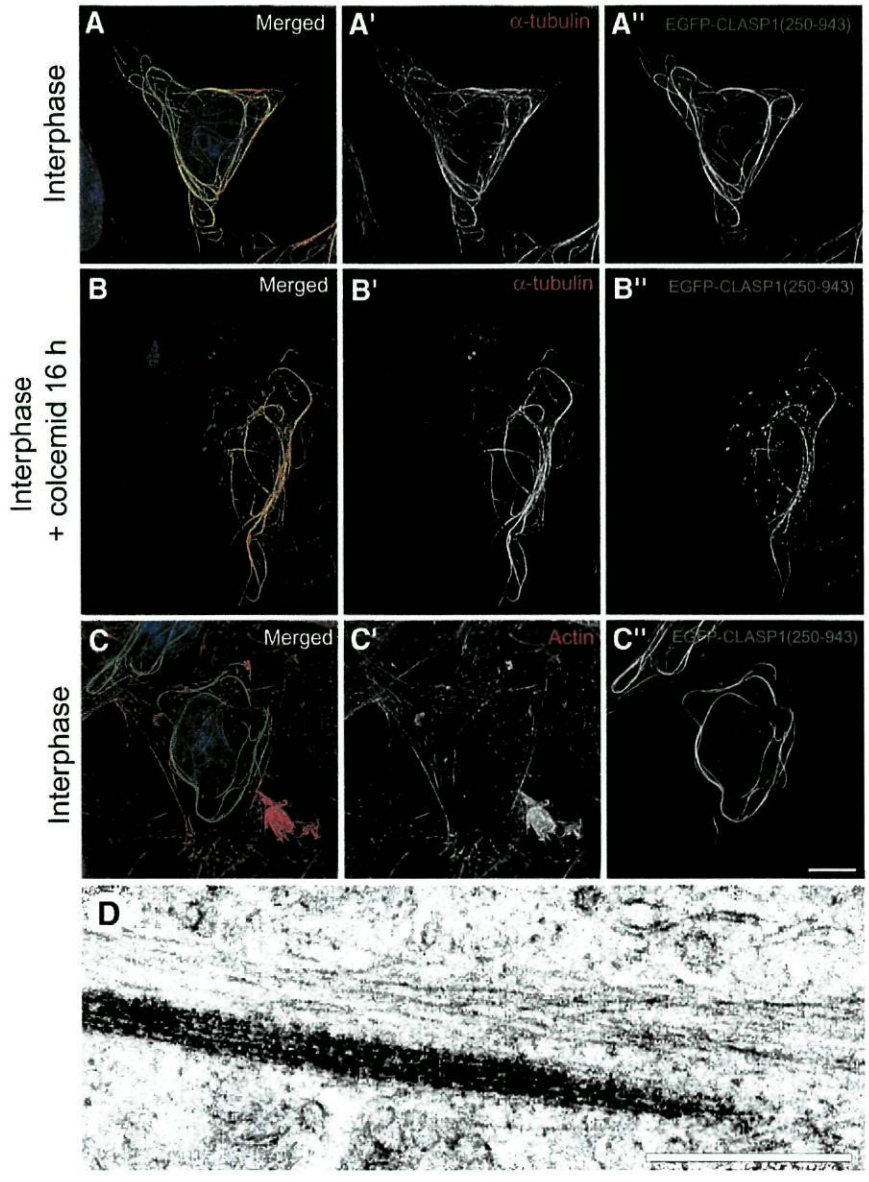


Figure 5.6. Sub-cellular localisation of EGFP-CLASP1 deletion constructs. Deletion of a very large central region from residues 405-1118 causes complete delocalisation of CLASP1 form the microtubules during mitosis or interphase. Instead the protein accumulates in cytoplasmic aggregates and in the Golgi. Curiously, deletion of residues 1-329 of CLASP1 had strong implications for microtubule targeting, indicating that residues 250-329 harbour an essential region that regulates microtubule localization of CLASP1. Bar is 10 μm.

2.5. Expression of the Microtubule Binding Domain of CLASP1 Causes a Dominant-Negative Effect

Analysis of HeLa cells expressing the construct EGFP-CLASP1$_{250-943}$ led to the observation that the morphology of the microtubule network was dramatically changed. In cells transfected with this construct, the microtubules looked curved and bundled (Fig.5.7A-A"). Four-dimensional analysis of cells expressing EGFP-CLASP1$_{250-943}$ indicated that the microtubules were extremely rigid and EGFP-CLASP1$_{250-943}$ did not show the comet-like localization at the tips of the microtubules (see movie 4). In these cells, the bundles of microtubules were highly decorated by EGFP-CLASP1 along all their entire length (Fig.5.7A-A" and movie 4). We reasoned that EGFP-CLASP1$_{250-943}$ might be stabilizing the microtubule network by modifying microtubule dynamics. To test this hypothesis we incubated HeLa cells that had been transfected with EGFP-CLASP1$_{250-943}$ with colcemid for 16 h in order to determine whether microtubules were able to resist depolimerisation. As expected, in cells that were not transfected the microtubule network was completely depolimerised as determined by α-tubulin staining (Fig. 5.7B-B"). However, in transfected cells we could observe intact microtubules even after a long period of incubation with colcemid (Fig. 5.7B-B"). Moreover, the action of EGFP-CLASP1$_{250-943}$ appears to be restricted to microtubules and did not extend to the actin cytoskeleton (Fig. 5.7C-C"). The effect of microtubule bundling was then confirmed by ultra-structural analysis of the microtubules in cells that were transfected with EGFP-CLASP1$_{250-943}$ (Fig.5.7D). In summary, the expression of EGFP-CLASP1$_{250-943}$ had a dominant effect over the endogenous protein and led to the bundling and stabilization of the microtubules.

Figure 5.7. Effect of the expression of dominant negative CLASP1 on the interphase microtubule network. **(A-A")** HeLa cell expressing EGFP-CLASP1$_{250-943}$ (green) results in the formation of curved microtubule bundles (red). **(B-B")** Expression of EGFP-CLASP1$_{250-943}$ (green) in HeLa cells incubated with colcemid for 16 h. Note that microtubules (red) resist depolymerisation. **(C-C")** HeLa cell expressing EGF-CLASP1$_{250-943}$ (green) causes microtubule bundling but did not interfere with the actin cytoskeleton (red). (D) Electron micrograph showing the ultra-structure of a microtubule bundle caused by expression of EGFP-CLASP1$_{250-943}$. Bar is 10 μm except in D that is 200 nm. (See next page).



2.6. Effect of Dominant-Negative CLASP1 over other Microtubule-Plus-End-Tracking Proteins

Expression of EGFP-CLASP1$_{250-943}$ leads to microtubule bundling and reorganization of the microtubule cytoskeleton during interphase. In order to determine whether this has an effect on the localization of other microtubule-plus-end-tracking proteins we performed immunofluorescence with antibodies specific to the APC protein, CLASP2, CLIP-170 and EB1 (Fig. 5.8). We observed that expression of EGFP-CLASP1$_{250-943}$ had no consequence on the localization of APC at the cell periphery, while CLASP2 and CLIP-170 were to some extent sequestered to the microtubule bundles. The strongest effect was observed on EB1 where this protein was found completely co-localized along the entire microtubule bundle network caused by expression of EGFP-CLASP1$_{250-943}$.

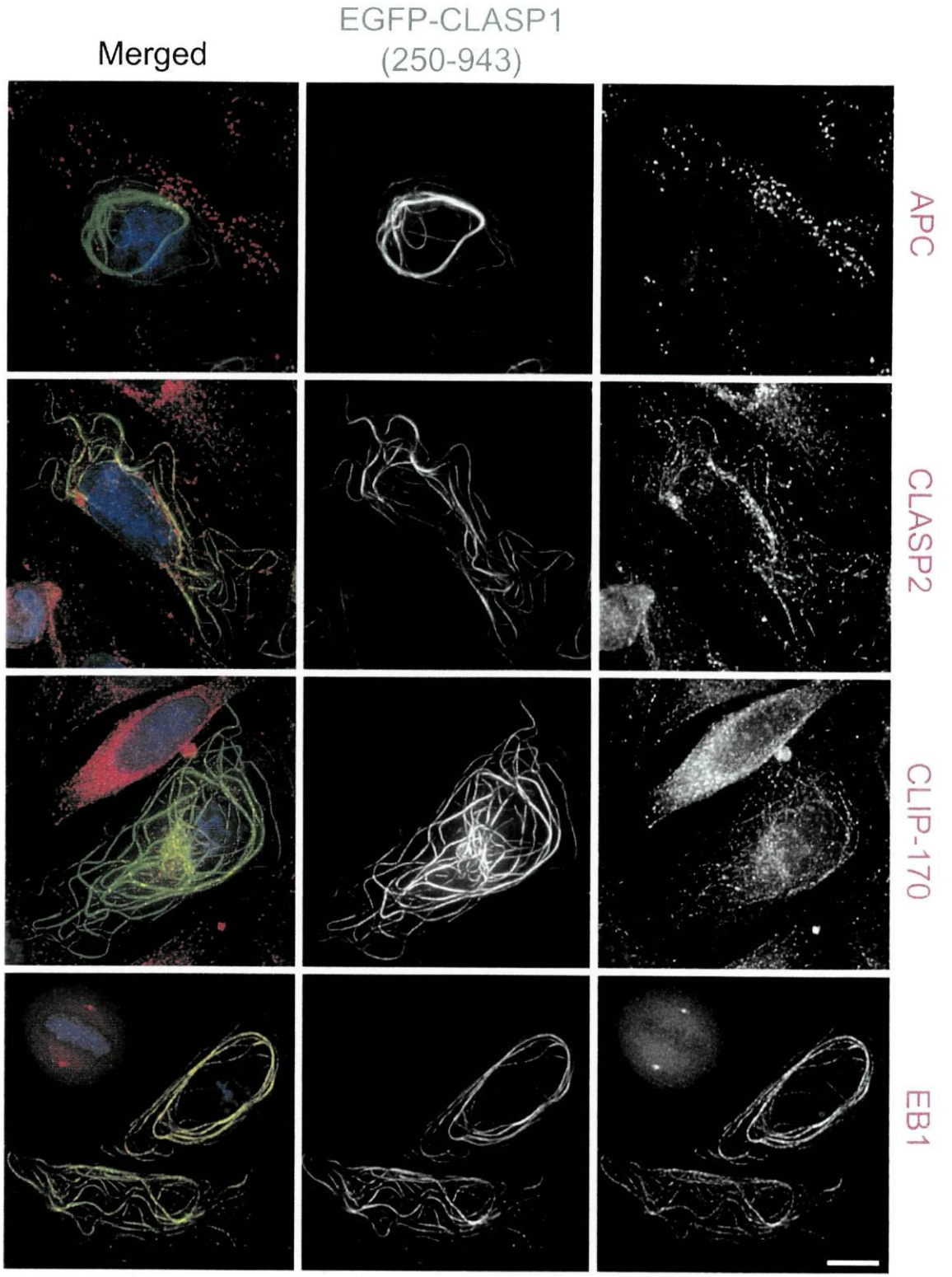


Figure 5.8. Effect of expression of dominant-negative CLASP1 in other microtubule-plus-end-tracking proteins. The microtubule bundling seen in cells expressing EGFP-CLASP1$_{250-943}$ had no consequence in appropriate targeting of the APC protein to the cell periphery. However, this affected in some extent the localization of CLASP2 and CLIP-170 that were partially recruited to bundles. The strongest effect was seen for EB1 that was completely sequestered to the microtubule bundles. Bar is 10 μm.

These observations suggest that CLASP1 may act upstream or together with CLASP2, CLIP-170 and EB1 and may be epistatic of APC in the process that organizes the microtubule plus-end complex, which is known to have important implications for the organization of the microtubule cytoskeleton (Reviewed by Schuyler and Pellman, 2001).

2.7. Overexpression of Dominant-Negative CLASP1 During Mitosis

The transfection of the EGFP-CLASP1$_{250-943}$ construct not only had a significant effect upon the microtubule network during interphase but also had a highly potent dominant negative effect when overexpressed during mitosis (Fig. 5.9A, A'). Over 60% of transfected mitotic cells had single or double asters in which the astral rays consisted of bundles of extremely closely packed microtubules (Fig. 5A-D'). Within the bundles, the center-to-center spacing between adjacent microtubules was 24.3 ± 4.5 nm. Since the diameter of a microtubule is 25 nm, this means that EGFP-CLASP1$_{250-943}$ can bundle microtubules so that there is little or no free space between then. In fact, in images where bundles were visualized in cross section, it was possible to see microtubule profiles that appeared to be close-packed with no space in between (insert in Fig. 5E).

The distal ends of these bundles were enriched for EGFP-CLASP1 and EB1 (Fig. 5.9B-C"), but lacked detectable APC protein (data not shown). Furthermore, the chromosomes and respective kinetochores were not found at the end of the astral rays as normal in a monopolar spindle as revealed by anti-CENP-C immunostaining (Fig. 5D, D') and electron microscopy (Figure 5E). Instead, chromosomes and kinetochores were buried deep within the asters. Although connections between kinetochores and microtubules were not observed by fluorescence microscopy, upon ultra-structural examination, many kinetochores were found to be attached end on to few short microtubules close to the centrosome (Figure 5F, G). This discrepancy probably reflects the large differences in the amount of tubulin in the astral bundles as opposed to the kinetochore fibres. The unusual radial distribution of microtubule bundles induced by EGFP-CLASP1$_{250-943}$ suggests that the protein either induces microtubule-microtubule interactions after growth from the poles has started, or that these bundles persist after the detachment of kinetochores.

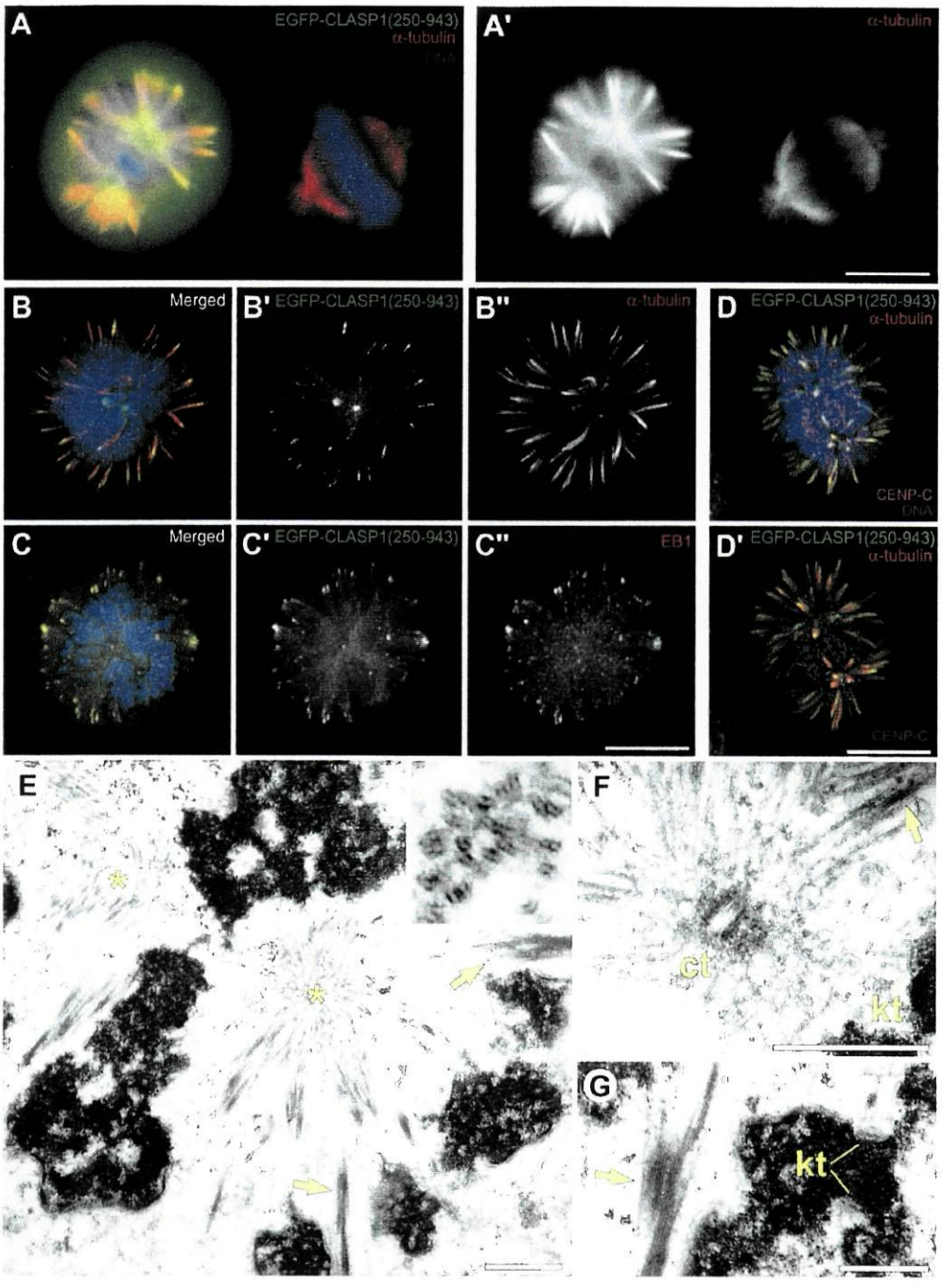


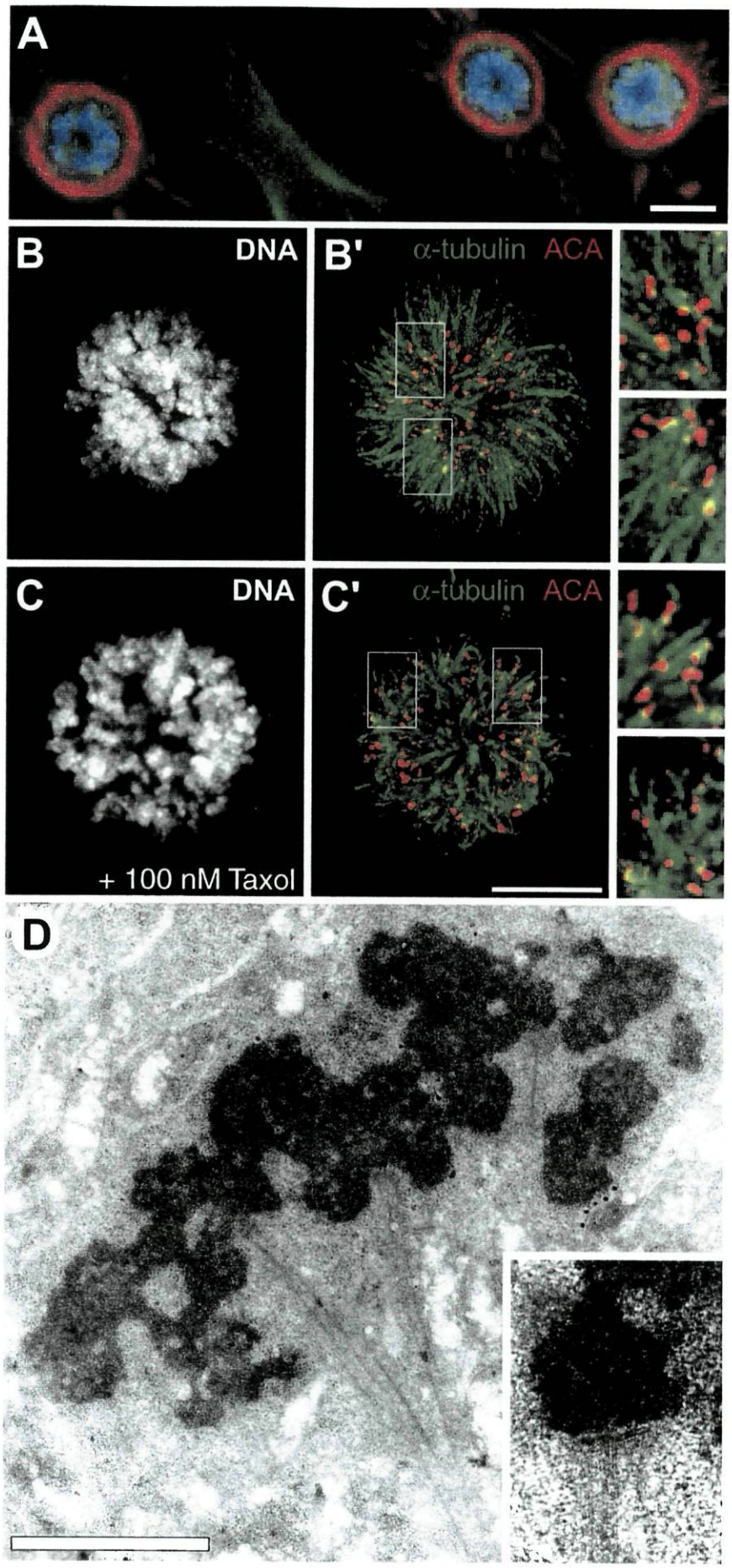
Figure 5.9. Effect of overexpression of dominant negative CLASP1 during mitosis. **(A, A')** A transfected cell expressing EGFP-CLASP1$_{250\text{-}943}$ (green) showing a monopolar spindle formed by bundles of microtubules (red) organized by two asters is shown in the same optical field of a normal untransfected cell in metaphase. **(B-B")** Another cell with a monopolar spindle (red) organized by a single aster with 2 centrosomes that resulted from overexpression of EGFP-CLASP1$_{250\text{-}943}$ which can be seen associated with the microtubule-plus-ends. **(C-C")** Co-localisation of EGFP-CLASP1$_{250\text{-}943}$ (green) with EB1 (red) at the microtubule-plus-ends in a monopolar spindle. **(D, D')** HeLa cell overexpressing EGFP-CLASP1[250-943] (green) showing the kinetochores, as determined by CENP-C staining, not associated with the microtubule-plus-ends from the bundles. Nevertheless, the chromosomes (blue) can be seen in the interior of the aster. (E) Electron micrograph from a serially sectioned mitotic cell overexpressing EGFP-CLASP1$_{250\text{-}943}$ showing the ultra-structure of the monopolar spindle formed by two astral arrays (*) of microtubule bundles (arrows). Insert shows a cross section of closely packed microtubules. **(F)** Deeper section of the same cell showing the ultrastructure of the centrosome (ct) with a bundle of microtubules (arrow) and a kinetochore (kt) where few microtubules can be found. **(G)** Another section from the same cell showing a microtubule bundle (arrow) and a kinetochore (kt) free from microtubules. Bar is 10 μm except in figures E, F and G that is 1 μm.

2.8. Microinjection of Anti-CLASP1 Antibodies Affects the Dynamic Behaviour of Kinetochore Microtubules

To further probe the function of CLASP1 in mitosis, we microinjected cells with anti-CLASP1 or control (pre-immune) antibodies during interphase. Approximately 50 % of cells that reached mitosis 12 h after microinjection with anti-CLASP1 antibodies formed monopolar spindles (Fig. 5.6A), a 10-fold increase over control injections (data not shown). Injection of anti-CLASP1 antibodies caused dispersal of the endogenous protein to the cytoplasm (Fig. 5.6A and 5.7B', C') and the formation of monoasters with chromosomes buried deep within the radiating microtubule bundles. Very few kinetochores were associated with the plus-ends of microtubules around the periphery (Fig. 5.6B, B'). Remarkably, this phenotype observed after a 12h exposure to antibody changed dramatically within 30 min after the addition of low doses of taxol. Kinetochores were now seen to occupy their normal positions at the periphery of the aster in association with the plus ends of astral microtubules (Fig. 5.6C, C'). Similar results were obtained when antibody-injected cells were treated with nanomolar concentrations of nocodazole, which suppresses microtubule dynamics rather than promoting microtubule disassembly (Jordan et al., 1992; Vasquez et al., 1997; and data not shown).

When antibody-injected cells were fixed and subjected to serial section electron microscopy, kinetochores in the interior of the asters were found to have normal end-on attachments to microtubules (Fig. 5.6D). Taken together these results indicate that functional CLASP1 is not required for kinetochores to bind to microtubule plus ends *per se* but may instead be essential for the normal dynamic behaviour of kinetochore-associated microtubules.

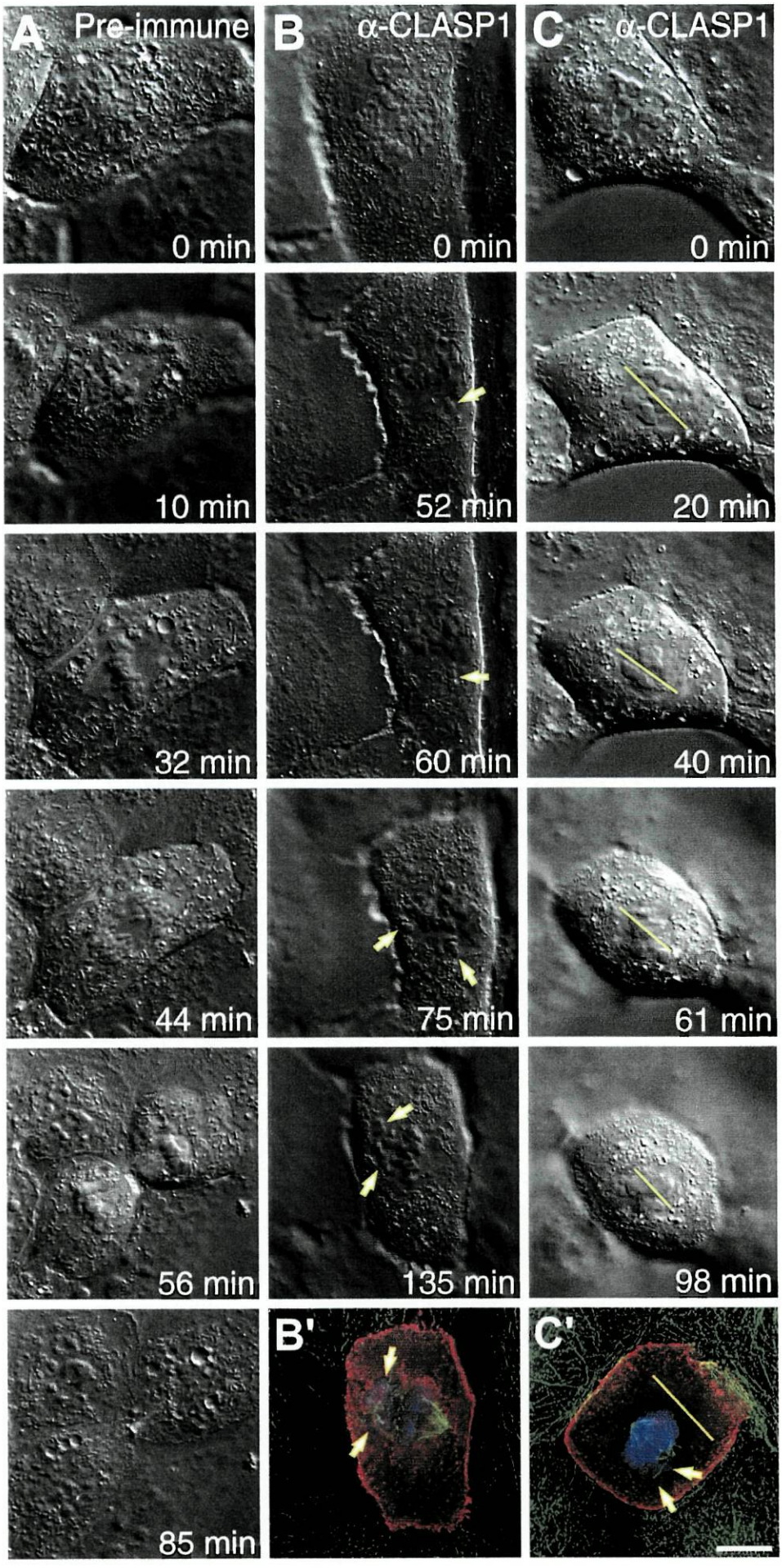
Figure 5.6. Analysis of microtubule kinetochore attachment after injection of antibodies specific for CLASP1. **(A)** Effect of inhibition of CLASP1 12 h after the injection during interphase HeLa cells (anti-CLASP1 injected cells=433; control IgG injected cells=499). **(B, B')** Detailed analysis of the microtubule (green) attachment with the kinetochores (red), as determined by ACA staining in a cell injected with anti-CLASP1 antibodies. Insertions show that most kinetochores are not associated with the microtubules at the periphery of the aster. **(C, C')** The inhibition of microtubule dynamics by a short incubation with ultra-low dose of taxol rescues the association of the kinetochores (red) with the microtubule-plus ends (green) at the periphery of the aster in a cell injected with anti-CLASP1 antibodies. **(D)** Thin section through a monopolar spindle formed after injecting a HeLa cell during G2 with anti-CLASP1-antibody. Two kinetochore fibres are visible near the middle of the image. In these cells many of the kinetochore fibre microtubules terminate in the outer kinetochore plate (insert). Bar is 10 μm except in figure D that corresponds to 0.5 μm. (See next page).


A
B
DNA
B'
α-tubulin ACA
C
DNA
C'
α-tubulin ACA
+ 100 nM Taxol
D

2.9. CLASP1 is Essential for Chromosome Congression and Spindle Integrity

Microinjection of anti-CLASP1 antibodies caused the formation of monopolar spindles. To determine how this phenotype arises, we used video microscopy to follow cells microinjected during late prophase with either control or anti-CLASP1 antibodies (Fig. 5.7). Cells injected with control (pre-immune) antibodies underwent normal mitotic progression (Fig. 5.7A and movie 5). However, cells injected with anti-CLASP1 antibodies showed a lengthy delay in prometaphase: chromosomes failed to oscillate normally and some failed to congress to the metaphase plate (Fig. 5.7B, B' and movie 6). Furthermore, although these cells initially assembled apparently normal bipolar spindles, these suffered a progressive collapse during mitosis (Figure 5.7C, C' and movie 7). As discussed below, all of these phenotypes are consistent with a role for CLASP1 in the regulation of microtubule dynamics at kinetochores.

Figure 5.7. Mitotic progression of cells injected with control or anti-CLASP1 antibodies. Time-lapse series of CF-PAC cells injected with **(A)** pre-immune or with anti-CLASP1 antibodies **(B, C)** analysed by DIC microscopy. **(B)** Time-lapse series of a CF-PAC cell injected with anti-CLASP1 antibody showing abnormal chromosome congression. **(B')** Same cell as in B that was fixed and processed for immunofluorescence immediately after time lapse imaging, showing the spindle (green) and the chromosomes (blue). The cell was identified using anti-rabbitt antibodies (red). **(C)** Time-lapse series of a CF-PAC cell injected with anti-CLASP1 antibody showing the progressive collapse of the spindle indicated by yellow line. **(C')** Same cell as in C that was fixed and processed for immunofluorescence reveal anti-CLASP1 antibodies (red), the spindle (green) and chromosomes (blue). Arrows in B, B' and C' show some of the chromosomes that did not align at the metaphase plate. Time was counted from the moment after breakage of the nuclear envelope in prophase. Bar is 10 μm. (See next page).


A Pre-immune
0 min
B α-CLASP1
0 min
C α-CLASP1
0 min
10 min
52 min
20 min
32 min
60 min
40 min
44 min
75 min
61 min
56 min
135 min
98 min
85 min
B'
C'

3. Discussion

3.1. Human CLASP1 Regulates Microtubule-Plus-End Dynamics at the Kinetochore

Genetic analysis in *Drosophila* first identified the product of a conserved gene, *mast/orbit*, which when mutated, resulted in the production of monopolar spindles and highly polyploid cells (Lemos et al., 2000; Inoue et al., 2000). A subsequent study reported that the two human homologues of this family are associated with the microtubule plus-end tracking proteins CLIP-115 and CLIP-170 in interphase cells (Akhmanova et al., 2001). These CLIP-associated proteins, termed CLASPs were proposed to be involved in regulating microtubule dynamics at the leading edge of motile fibroblasts, however no role for CLASPs in mitosis was proposed. The data presented here provide strong support for the hypothesis that these proteins, which we propose to refer to as CLASPs in all species, have an essential role in the regulation of microtubule dynamics at kinetochores.

The central region of CLASP1 directs the association of the protein with microtubules, and causes a remarkable microtubule bundling phenotype when overexpressed in cells. Given the localization of CLASP1 to the central spindle and midbody in normal mitotic cells, this could mean that the protein has a normal role in bundling and stabilizing microtubules in these regions. However, although defects in microtubule bundling could contribute to the spindle collapse seen when CLASP1 function is inhibited, as discussed below, this phenotype could also result from a perturbation of microtubule dynamics.

The characteristic phenotype produced by interference with CLASP1 function is the production of monopolar spindles with chromosomes buried in the interior. This is seen with the *Drosophila* mutants as well as following RNAi in *Drosophila* tissue culture cells, microinjection of anti-CLASP1 antibody into HeLa cells, and overexpression of the CLASP1 microtubule-binding domain in HeLa cells. The phenotype is highly unusual, as multiple redundant mechanisms exist to expel chromosomes from the interior of asters (the "polar wind") (Rieder and Salmon, 1994). Two hypotheses could explain this phenotype. First, CLASPs could be required for the stable binding of kinetochores to dynamic microtubules. Second, CLASPs could be required for the regulation of the dynamics of kinetochore-associated microtubules.

According to the first hypothesis, disruption of CLASP1 function would cause kinetochores to lose their grip on microtubules exhibiting dynamic behaviour. This would

normally cause chromosomes to be expelled from the aster, but if they were able to reattach laterally and move poleward along the sides of microtubules, as in the initial interactions normally seen during prometaphase (Rieder and Alexander, 1990), then they could accumulate in the interior of the aster. Indeed, our previous analysis of mitotic *Drosophila* cells depleted for MAST supported this hypothesis (Maiato et al., 2002). However, serial section electron microscopy in HeLa cells injected with anti-CLASP1 antibodies or overexpressing the CLASP1 microtubule-binding region has clearly shown kinetochores within the aster to have normal end-on attachments with microtubules. This appears to rule out the first hypothesis.

Overall, our data are more consistent with hypothesis 2, in which CLASP1 is involved in regulation of the dynamics of kinetochore-associated microtubules (Fig. 5.8). Specifically, if CLASP1 were required for regulation of the switching of kinetochore-associated microtubules from a shrinking to a growing phase, then in the absence of CLASP1, any transition to shortening would cause the chromosome involved to be "reeled in" to the centrosome. Under this model, the kinetochores would have almost exclusively end-on attachments with microtubule bundles and the chromosomes would be very close to the centrosomes, as we have observed by electron microscopy. If the dynamics of non-kinetochore microtubules were less affected by the absence of CLASP1, then, the overall astral morphology would be normal, and the chromosomes would be "buried" within the aster.

A number of observations are consistent with a role for CLASP proteins in regulating microtubule dynamics. First, the microtubule-binding region of CLASP1 bundles microtubules and renders them non-dynamic. These bundles were highly resistant to depolymerization induced by colcemid. Second, chromosome oscillations appear to be dampened following the microinjection of anti-CLASP1 antibodies. Chromosome oscillations require dynamic instability of the microtubule bundles attached to the kinetochores (Skibbens et al., 1993). *Drosophila* MAST/Orbit mutants and cells injected with anti-CLASP1 antibodies are defective in chromosome alignment, with chromosomes oscillating aimlessly on the spindle. This could also be explained by defects in the regulation of microtubule dynamics. Third, the distribution of CLASP1 on microtubules during mitosis was radically different following the addition of taxol, a drug classically used to alter microtubule dynamics. Fourth, the abnormal astral structure observed in antibody-injected HeLa cells was largely reversed upon the addition of taxol. Suppression of microtubule dynamics with taxol would be expected to stabilize the kinetochore-attached microtubules, preventing them from further shrinking. Taxol would alter the equilibrium towards net microtubule growth, thereby pushing the chromosomes toward the periphery of the aster, and could account for the observed reorganization of

the asters after addition of the drug. This effect was not solely due to any specific property of taxol, as the relocation of chromosomes from the interior of asters was also observed following treatment of injected cells with low dose nocodazole or vinblastine (data not shown).

One of the key phenotypes observed following perturbation of CLASP function in *Drosophila* and human cells is the accumulation of monopolar spindles in mitosis (Lemos et al., 2000; Inoue et al., 2000; Maiato et al., 2002). Although it was initially thought that this was likely to represent an inability of cells deficient in CLASP function to separate spindle poles, we now know that these cells can initially assemble what appear to be structurally normal mitotic spindles. These spindles subsequently undergo a slow and progressive collapse, ultimately producing the monopolar structures. Importantly, when crane fly spermatocytes are treated with taxol, they assemble bipolar spindles, but these spindles progressively collapse as meiotic division progresses. This provides another link between alterations in microtubule dynamics and the phenotypes seen upon perturbation of CLASP function.

3.2. Model for a Role of CLASP1 in the Dynamic Regulation of Kinetochore-Associated Microtubule-Plus-Ends

Recent EM data have led to a model where the plus ends of growing microtubules are present as open sheets (Arnal et al., 2000) that are postulated to close prior to microtubules entering a shrinking phase. It is possible that the flared ends of kinetochore-associated microtubules seen in cryo-electron microscopy studies correspond to the sheets seen in *in vitro* assembly studies (Mastronarde et al., 1997; McEwen et al., 1998; O'Toole et al., 1999). In any event, the location of CLASP1 in the outer kinetochore corona would place the protein along the sides of the microtubules near the plus ends – possibly near the junctions where the flared tubules adopt a cylindrical profile (Fig. 5.8). In its position near the plus ends at the kinetochore, CLASP1 would be well placed to influence the topology of the microtubules, for example, by promoting the transition of closed tubes to sheets (and thereby favouring growth).

A variety of evidence suggests that the preferential localization of CLASP1 to the kinetochore-proximal microtubule plus ends might at least partly involve recognition of a particular lattice conformation. First, the binding of CLASPs to microtubules is likely to be direct, as the *S. cerevisiae* homologue of CLASP1, Stu1p, binds directly to β-tubulin (Yin et al., 2002). Second, the distribution of CLASP1 on microtubules *in vivo* is sensitive to taxol, which alters the structure of the tubulin lattice (Arnal and Wade, 1995). Third, the

Orbit protein (one name for *Drosophila* CLASP) binds to microtubules in the presence of GTP, but not GTP-γS (Inoue et al., 2000). This could also reflect a sensitivity of CLASPs to the microtubule lattice, as shown for the related poorly hydrolyzable GTP analogue GMPPCP (Hyman et al., 1995). Given the observation that the microtubule lattice is influenced by whether the tubulin is in the GTP or GDP form (Hyman et al., 1995), a preference for binding to one state of the lattice could provide a mechanism by which CLASP1 might influence the structure at the end of the microtubule (Fig. 5.8).

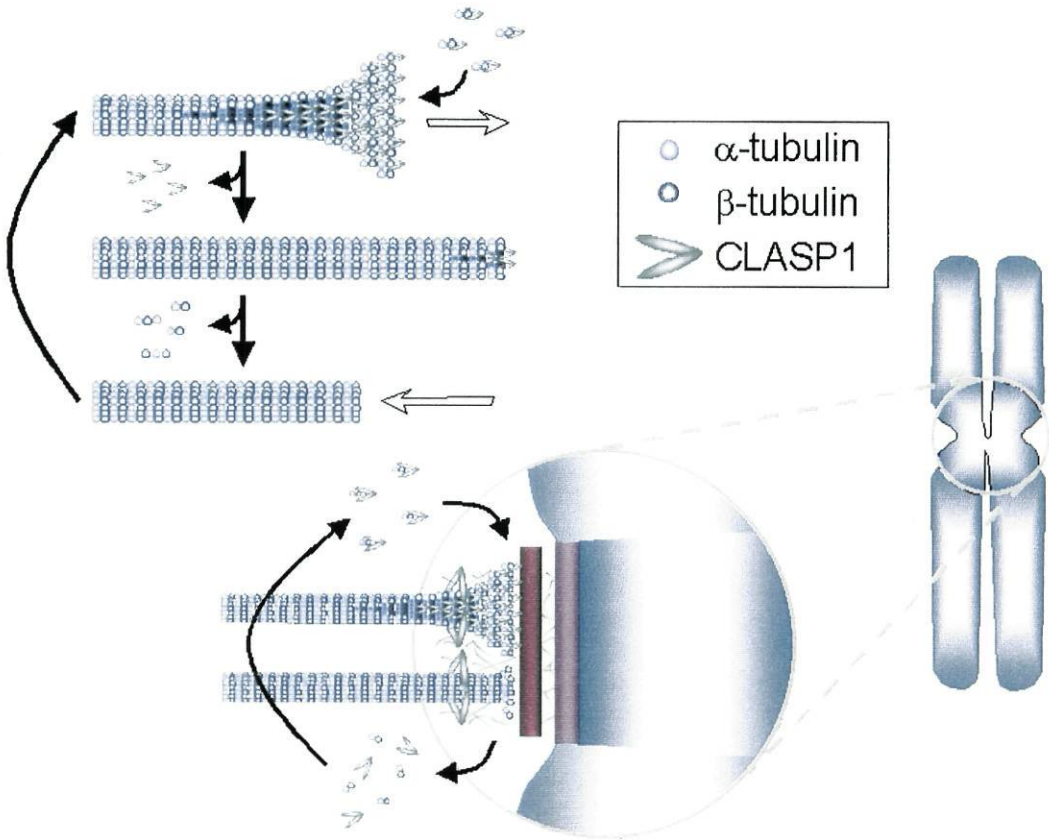


Figure 5.8 - Proposed model for the role of CLASP1 at microtubule-plus ends and kinetochores. CLASP1 may assemble with the polymerising microtubules either by recognition of a particular conformation of the lattice or by co-assembly with tubulin heterodimers. The first possibility might involve preferential binding to open sheets at the plus ends of growing microtubules. The second might involve specific binding to β-tubulin monomers (Yin et al., 2002). If CLASP1 undergoes the treadmilling characteristic of +TIPs (Schuyler and Pellman, 2001), this implies that it must detach behind the region of growth. This could be a consequence of GTP-hydrolysis and result in tube closure, and could correlate with a tendency of the microtubules to depolymerize and shrink. The microtubule-independent anchoring of CLASP1 at kinetochores may promote the addition and loss of tubulin subunits on microtubules that remain attached. Under this model, CLASP1 assumes a key role in the regulation of microtubule dynamics at the kinetochore, essential for proper chromosome positioning and spindle stability.

Although the previous analysis of mutants in MAST/Orbit, the *Drosophila* homologue of CLASP1, suggested that this protein might have a complex role in mitosis (Lemos et al., 2000; Inoue et al., 2000), our present work suggests that CLASP function can be

largely understood in terms of regulation of microtubule dynamics at the kinetochore. Regardless of its detailed mechanism of action, CLASP1 appears to be a key component in enabling kinetochores to perform one of their most distinctive behaviours in mitosis – interaction with microtubules that are constantly adding or loosing subunits at their plus ends.

3.3. CLASP1 in the Context of the Microtubule-Plus-End-Tracking Protein Complex

Very little is known about how the microtubule-plus-end-tracking proteins assemble at the plus ends of the microtubules. One certainty is that these proteins are among the most conserved components of the microtubule cytoskeleton. It is thought that the comet-like streak on the microtubule-plus-ends characteristic of these proteins is due to treadmilling, i.e., they bind to the polymerising end of the microtubule and then fall off behind the region of growth. Recent experiments in fission yeast suggest that at least some of these proteins might be involved in targeting microtubules to particular subcellular locations (such as the kinetochore and the cell cortex) by local effects on microtubule dynamics (Brunner and Nurse, 2000). Three general models for microtubule-plus-end tracking have been proposed (reviewed by Schuyler and Pellman, 2001). The first two models are based on the idea that these proteins recognize a specific structural feature of the growing microtubule-plus end, either the GTP-bound tubulin cap or the open sheet of the polymer. One argument against the first model is that the GTP-cap is thought to be small relative to the region decorated by these proteins. However, the true extent of the GTP-cap *in vivo* is not known. In the second model microtubule-plus-end-tracking protein would have to distinguish between the conformation of tubulin in sheets from tubes. This model also cannot explain why overexpression of these proteins leads to binding along the entire length of the microtubule. The third, and perhaps the most attractive model is that these proteins bind to free tubulin dimmers and then co-assemble into the plus-ends during microtubule polymerisation. Thus, the relative localization between the different microtubule-plus-end-tracking proteins may result from their relative affinity for tubulin dimmers/tubulin polymer in the cell. Here we have shown that overexpression of the microtubule-binding domain of CLASP1 induces the formation of robust microtubule bundles either in interphase or mitotic cells. In these bundles the fragment of CLASP1 is found to be associated along their entire length in interphase and affects the localization of other microtubule-plus-end-tracking proteins. For, instance, a significant fraction of CLASP2

and CLIP-170, and virtually all EB1 were sequestered to the microtubule bundles. However, APC was not recruited to the bundles and maintained its preferential position near the cell cortex. These observations suggest that the recruitment of at least CLASP2, CLIP-170 and EB1 to the microtubule plus-ends may be in some extent mediated by CLASP1. It is possible that overexpression of the microtubule binding domain of CLASP1 causes a change of the microtubule lattice that in turn facilitates binding of other plus-end-tracking proteins. However, APC is not recruited to the bundles suggesting either that this protein assembles to microtubule-plus ends by a mechanism that is upstream of CLASP1 or independent from the microtubule lattice structure. It would be important to investigate further the sequential events by which different proteins localise to the complex, the interdependence between these proteins and their relative localization at the microtubule-plus end.

4. Materials and Methods

Antibodies, Immunofluorescence and Microscopy

Immunofluorescence and microscopy of HeLa or CF-PAC cells were performed as described in Chapter IV. Other antibodies used were against α-tubulin (clone B512, Sigma), CENP-C (rabbit), CENP-E (mouse, gift from Tim Yen), EB1 (mouse, Transduction Labs) and ACA (human) (see Table I).

Constructs and Transfections

Construct EGFP-CLASP1$_{509-817}$ was obtained by PCR using primers CD622XhoI-F and CD+622SacII-R (see Table II). All the other constructs are derivatives of the full-length CLASP1 cloned into pEGFP-C1 (Clontech) that was digested, sub-cloned or re-ligated into the same vector using convenient restriction sites (see Table III). Transfections were performed as described in Chapter IV.

Four-Dimensional Analysis by Restoration Microscopy

HeLa cells were grown in 40 mm coverslips and transfected with EGFP-CLASP1 (full-length) as before. 24 h after transfection, cells were transferred into FCS2 chambers (Bioptechs) and kept at 37°C in the presence of RPMI without phenol red (Gibco) supplemented with 10 mM Hepes. Three-dimensional data sets of representative cells were collected every 30 s and movie frames processed as before using SoftWorx (Applied Precision).

Microinjection and Time-Lapse Video Microscopy

Rb1277 serum was incubated with protein A sepharose beads (BioRad) for 2 h, washed with PBS, and bound IgGs were eluted with 100 mM Glycine (pH 2.5) then neutralized with 1 M Tris-HCl pH 8.O. Antibodies were concentrated to 20 mg/ml and changed to microinjection buffer containing 100 mM KCl and 10 mM KH_2PO_4 (pH 7.4) using 0.5 ml concentration columns (Millipore). For injections, HeLa or CF-PAC cells were grown in glass coverslips and kept at 37°C in an inverted Nikon Diaphot microscope with a Narishige microinjector within a heated chamber. Interphase cells were microinjected in the cytoplasm (anti-CLASP1 injected = 433 cells; control IgG injected = 499 cells), fixed after 12 h and processed for immunofluorescence analysis as before. Injected cells were detected using anti-rabbit conjugated secondary antibodies. For time-lapse video microscopy, prophase cells were injected in the cytoplasm prior to nuclear envelope breakdown and observed in an inverted Nikon Diaphot DIC light microscope. Frames were collected immediately after NEB every 30 s and fixed immediately at the end of the movie for immunofluorescence analysis. The results were consistent between independent experiments.

Drug Treatments

Taxol was used at 10 μM in HeLa cells transfected with EGFP-CLASP1 full length or at 100 nM in injected cells for 30 min prior to fixation as described previously (Jordan and Wilson, 1999). Injected cells were incubated with Nocodazole used at 100 nM for 30 min prior to fixation as described (Vasquez et al., 1997). For analysis of chromosome spreads, HeLa cells were treated for 3 h with 0.03 μg/ml colcemid, incubated for 20 min in a buffer containing 75 mM KCl and then processed as before.

Electron Microscopy

HeLa cells overexpressing EGFP-CLASP1$_{250\text{-}943}$ were grown in 6 well plates and processed for EM 36 h after transfection as described in Chapter II. For ultra-structural analysis after antibody injection, HeLa cells growing in glass coverslips were injected during interphase as described before either with anti-CLASP1 antibodies or control pre-immune IgGs together with rhodamine-labelled dextran (0.25 mg/ml) and kept in culture for 12 h. Drug experiments were performed at this stage (30 min. incubation). Injected cells in mitosis were then detected under the fluorescence microscope, scribed with a diamond, fixed in 2.5% glutaraldehyde in PHEM buffer, and processed flat for ultra-thin serial sectioning (Rieder and Cassels, 1999).

CHAPTER VI

Role of Chromosomal Passenger Proteins During Mitosis

1. Introduction

More than a decade ago, it was suggested that a class of proteins named 'chromosomal passengers' might act as integrators of chromosomal and cytoskeletal functions in mitosis (Earnshaw and Bernat, 1991). These proteins associate with chromosomes along their length during prophase, becoming concentrated at the inner centromere region by metaphase. During the metaphase-anaphase transition, passenger proteins abruptly transfer to the central region of the mitotic spindle and to the cell cortex in the region where the contractile ring will form. Based on this behaviour, it was suggested that these proteins might have chromosomal functions during early mitosis, but then perform essential cytoskeletal functions during anaphase and telophase (Earnshaw and Cook, 1991).

The distribution of the chromosomal passengers, although remarkably similar, should not be confused to the distribution of other proteins such as CENP-E and other kinesin like proteins, due to slightly different locations on the chromosomes. These other proteins associate with kinetochores until late anaphase and are also found associated with the central spindle after anaphase onset (Earnshaw, 2001).

The first chromosomal passenger protein to be described, INCENP (for *In*ner *Cen*tromere *P*rotein), attracted interest as a result of its movements from the centromeres to the spindle and ending up in the cleavage furrow as cells traverse mitosis (Cooke et al., 1987). However the biological role of INCENP remained enigmatic until very recently. The expression of dominant negative mutants suggested that the protein might have a role in chromosomal mechanics and cytokinesis. One mutant, $INCENP_{1-405}$, which lacks the C-terminal half of the protein, caused delocalisation of endogenous INCENP and induced defects in prometaphase chromosome congression, anaphase segregation and the late stages of cytokinesis (Mackay et al., 1998). A second mutant in which INCENP was tethered to centromeres as a fusion protein with CENP-B, interfered with a very late step in the completion of cytokinesis, but had no noticeable effect on chromosome movements (Eckley et al., 1997).

Subsequent isolation of an INCENP-knockout mouse revealed that INCENP is essential for mouse development (Cutts et al., 1999). Homozygous null embryos die at

the 32-64 cell stage, showing gross defects consistent with a disruption of cytokinesis and with highly abnormal bundling of microtubules. Accordingly, INCENP was shown to bind directly to tubulin and to require dynamic microtubules for targeting to the cleavage furrow and thus can be considered as a microtubule-associated protein (Wheatley et al., 2001a).

A second chromosomal passenger protein, Aurora B, is related to a protein kinase first identified in *D. melanogaster* in a search for genes that regulate the structure and function of the mitotic spindle (Glover et al., 1995). Mitosis in *aurora*-mutant cells was characterised by monopolar spindles with duplicated centrosomes. In budding yeast, the single aurora kinase, Ipl-1p, is involved both in chromosomal and spindle events throughout mitosis (Francisco and Chan, 1994; Francisco et al., 1994). In contrast, metazoans have evolved a multigene aurora kinase family in which the original Aurora protein is now termed Aurora A thought to be involved in spindle dynamics and Aurora B involved in chromosomal events and cytokinesis (for reviews see Bischoff and Plowman, 1999; Giet and Prigent, 1999).

Until very recently, the only known target of the budding yeast aurora (Ipl1p) was the essential kinetochore protein Ndc10p (Biggins et al., 1999). Ndc10p is an in vitro substrate for Ipl1, the two proteins interact in two hybrid screens, and mutations in both genes are characterized by defects in chromosome segregation. More recently, the kinesin-related protein Eg5 and histone H3 have also been shown to be aurora substrates (Giet et al., 1999; Hsu et al., 2000).

The third chromosomal passenger protein, Survivin, is a member of a family of proteins discovered first in baculoviruses, and soon thereafter in mammalian cells, as negative regulators of the apoptotic response (for reviews see Miller, 1999; Reed and Bischoff, 2000). These proteins, known as IAPs (inhibitor of apoptosis), are characterized by a zinc-binding motif termed the BIR (baculovirus iap repeat). In metazoans, several IAPs have been shown to bind to caspases and inhibit their activity. Survivin is defined as an IAP because it has a single BIR domain, however whether this protein has a role in apoptotic regulation remains controversial. It was originally reported that human survivin is concentrated at spindle poles, and that it binds to spindle microtubules throughout mitosis (Li et al., 1998). However, recent studies have reported that Survivin actually exhibits a classical chromosomal passenger distribution (Skoufias et al., 2000; Wheatley et al., 2001b). Moreover, analysis of the function of the *C. elegans* BIR1 protein, which resembles Survivin, failed to identify a role for the protein in apoptotic regulation. Instead, RNAi-mediated inactivation of the protein leads to defects in cytokinesis, suggesting that this protein may normally function in cell division rather

than cell death (Fraser et al., 1999). Accordingly, *survivin* knockout mouse dies by 4.5 days due to defects in cell division and not cell death (Uren et al, 2000).

A number of recent studies have shown that INCENP is a specific partner for an Aurora kinase in organisms from budding yeast to human. Data base searches using a sequence near the carboxyl-terminus of vertebrate INCENP revealed similarity to the budding yeast SLI15 protein. SLI15 is both a genetic interactor and physical binding partner of Ipl-1, and was originally identified in a screen for synthetic lethal mutants with Ipl-1 but had not been recognized as being related to INCENP (Kim et al., 1999). Furthermore, mutants in both proteins exhibit a similar phenotype with dramatic defects in chromosome segregation during mitosis. Sli15p is nuclear during interphase, but then becomes associated with the spindle throughout mitosis.

The biological relevance of the INCENP:Aurora complex in metazoans was confirmed when it was shown that INCENP is stockpiled in Xenopus eggs as an 11S complex together with Aurora B kinase (Adams et al., 2000b). This complex also exists in worms and humans (Kaitna et al., 2000). Furthermore, it was found that the $INCENP_{1-405}$ mutant, that displaces endogenous INCENP from its binding sites on chromosomes and the spindle midzone in transfected cells, also caused the Aurora B kinase to loose its specific association with these structures (Adams et al., 2000b). A similar result was obtained in *C. elegans*, where inactivation of ICP-1, one of two INCENP-related gene products, by RNA-mediated interference (RNAi) caused a failure of Aurora-B (AIR-2 protein) to target correctly to chromosomes and the spindle midzone (Kaitna et al., 2000). Thus, INCENP is required for the correct targeting of the Aurora B kinase during mitosis.

Direct evidence for the presence of Survivin in the complex was obtained only recently (Bolton et al., 2002). However, it was already known that inactivation of *C. elegans* Bir1 by RNAi also causes a failure of Aurora B (AIR-2 protein) to target correctly to chromosomes and the spindle midzone (Speliotes et al., 2000). Moreover, direct interaction between Survivin, Aurora B and INCENP was shown *in vitro*, and in cells where endogenous INCENP localization was disrupted by means of transfection with dominant-negative constructs. Survivin remains associated with the chromosomes and no longer concentrates at the centromeres or transfers to the anaphase spindle midzone (Wheatley et al., 2001).

Studies on cytokinesis in tissue culture cells have led to the hypothesis that the signal(s) determining furrow position come from the chromosomes and/or spindle midzone (Wheatley and Wang, 1996; Eckley et al., 1997). In this regard, it has been proposed that the chromosomal passenger proteins are involved in defining the site of cytokinesis (Earnshaw and Bernat, 1991). However, it was shown in grasshopper

spermatocytes that after all chromosomes were removed by micromanipulation normal anaphase and cytokinesis can occur (Zhang and Nicklas, 1996). Moreover, the observation that functional cleavage furrows can form in vertebrate cells between two centrosomes which are not connected by an intervening spindle (Rieder et al., 1997), supports the hypothesis that bundles of interpolar microtubules rather then chromosomes or spindle microtubules play an important role not only in establishing where cytokinesis will occur but also in stabilizing the furrow after its formation (Wheatley and Wang, 1996; Wheatley et al., 1998). Nevertheless, INCENP can be found in ectopic furrows that form between two independent spindles during mitosis in cultured cells (Savoian et al., 1999)

Recent data from the functional inactivation of INCENP and Aurora B by dsRNA interference (RNAi) in *Drosophila* tissue culture cells have confirmed a role of these passenger proteins in Histone H3 phosphorylation and cytokinesis (Adams et al., 2001b; Giet and Glover, 2001).

In order to gain further insight into the role of chromosomal passengers in mitosis, we have analysed the dynamic localisation of these proteins *in vivo* and also studied the role of *Drosophila* INCENP and Aurora B by RNAi.

2. Results

2.1. Four-Dimensional Analysis of the Chromosomal Passengers During Mitosis

Analysis of fixed cells as indicated that chromosomal passenger proteins undergo significant changes in their subcellular distribution during different stages of mitosis. However, so far it has not been possible to visualize how these changes in localization occur in living cells. Therefore, we have used a stable transfected HeLa cell line expressing GFP-Survivin (Ana Carvalho and Sally P. Wheatley, unpublished) to monitor the dynamic localization of the chromosomal passenger complex since it is known that survivin forms a complex with INCENP and Aurora B (Bolton et al., 2002). In order to have an internal cellular marker to reveal the chromosomes, cells were also stained with vital Hoechst. Live cells were then analysed by four-dimensional restoration microscopy (see movie 8) and representative frames are shown in Fig. 6.1. We observed that during prometaphase/metaphase GFP-Survivin was found concentrated at the centromeres of the mitotic chromosomes. At anaphase onset, during the very initial steps of

chromosome movement towards the poles, GFP-Survivin appears to move with the centromeres. However, as chromosomes move further apart GFP-Survivin then leaves the chromosomes and concentrates in a broad region between the separating chromatids. Later during anaphase B the GFP signal is found concentrated in a band across the overlapping microtubules of the central spindle. The cleavage furrow then contracts and GFP-Survivin accumulates in the midbody during telophase and cytokinesis. Our analysis clearly indicates that the chromosomal passenger complex remains associated with centromeres for a short period after sister chromatids have separated at the metaphase-anaphase transition. Only then the complex appears to leave the chromosomes and is transferred to the mid spindle region.

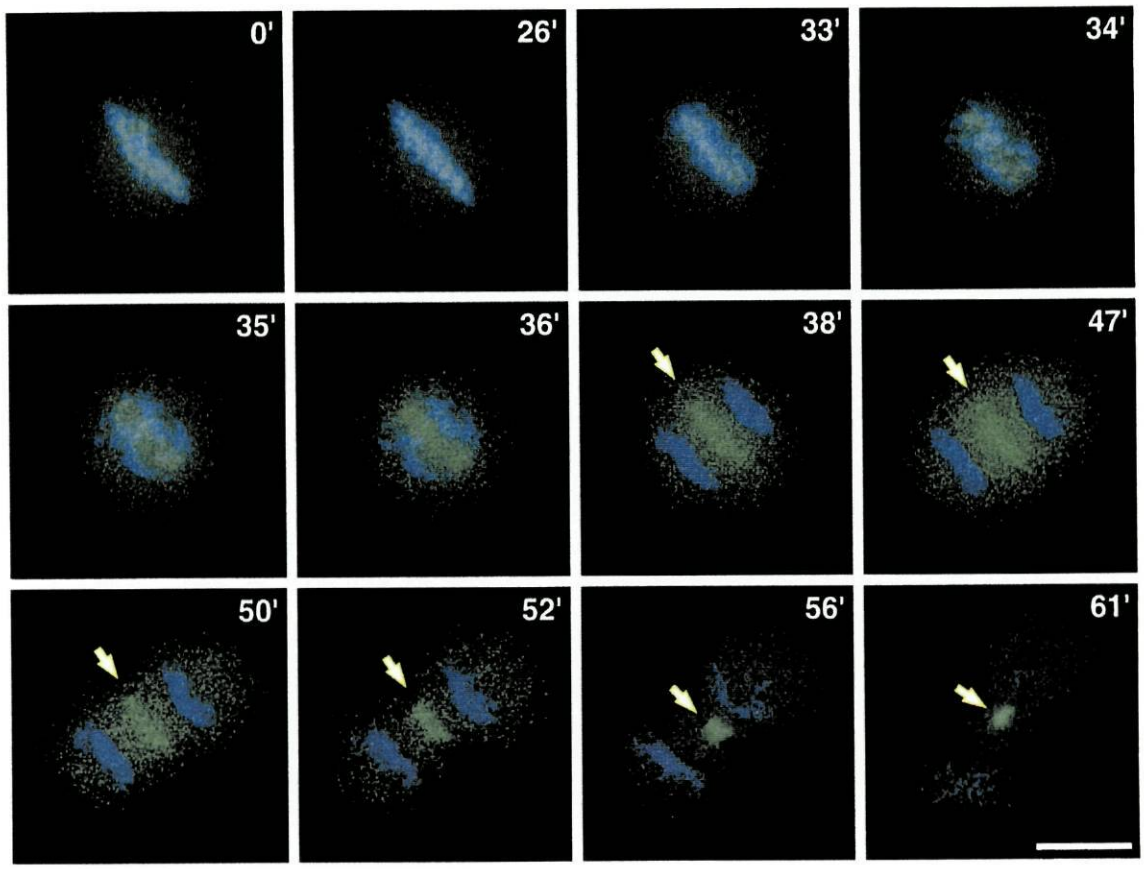


Figure 6.1. Four-dimensional analysis of GFP-Survivin (green) in living HeLa cells. Chromosomes are shown vitally stained with Hoechst (Blue) and the accumulation of GFP-Survivin on the central spindle is indicated by arrows. Bar is 10 μm.

2.2. Drosophila INCENP and Aurora B are Chromosomal Passenger Proteins

A candidate *Drosophila* INCENP gene was recently identified and found to share extensive sequence conservation with the C-terminal of vertebrate INCENP (Adams et al., 2001b). In order to analyse the role of this protein in mitosis we started by determining its subcellular distribution in *Drosophila* tissue culture cells, with an antibody specific to *Drosophila* INCENP (Adams et al., 2001b). We have found that INCENP localises to the centromeric regions of metaphase chromosomes (Fig. 6.2A) and upon entry into anaphase it leaves the chromosomes to form a ring associated with the central spindle (Fig. 6.2B). As telophase progresses, the INCENP ring decreases in diameter until it accumulates at the midbody (Fig. 6.2C).

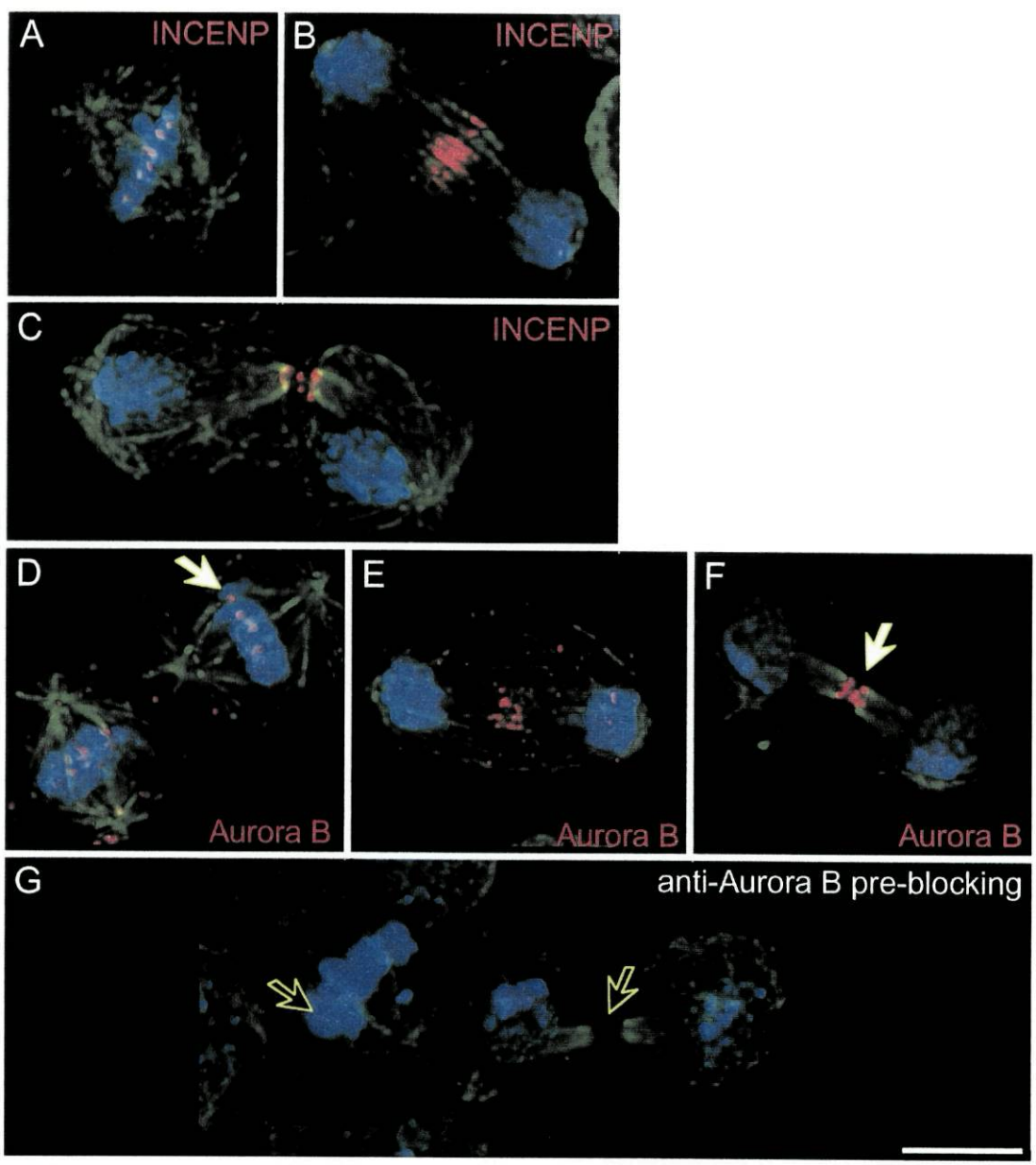


Figure 6.2. Localization of *Drosophila* chromosomal passengers INCENP and Aurora B in mitotic cultured cells. **(A-C)** Dmel2 cells at metaphase, anaphase, and telophase, respectively showing the localization of INCENP (red). **(D-G)** Dmel2 staining at metaphase, anaphase, and telophase, respectively showing the localization of Aurora B (red). White arrows point to centromeric **(D)** and midbody **(F)** staining. **(G)** Immunostaining of Aurora B is prevented by pre-incubation with recombinant Aurora B protein (black arrows show absence of staining). Bars, 5 μm.

Drosophila Aurora B was identified previously (Reich et al., 1999) but its role in mitosis remained to be elucidated. For this purpose, antibodies against recombinant GST- Aurora B fusion protein were raised (Adams et al., 2001b). The affinity purified serum recognized Aurora B in *Drosophila* Dmel2 tissue culture cell lines in a pattern similar to INCENP and to other Aurora B forms in other species (Terada et al., 1998) (Fig. 6.2D-F). In order to ascertain the specificity of the immunostaining, the antibody was pre-incubated with purified recombinant GST-Aurora B and then applied to fixed cells. The results show that pre-incubation of the antibody abolished all specific staining in cultured cells (Fig. 6.2G). We concluded that *Drosophila* INCENP and Aurora B are chromosomal passenger proteins since their distribution during mitosis resembles in all respects their vertebrate counterparts.

2.3. RNAi of INCENP and Aurora B in Drosophila Cells Revealed a Role in Metaphase Chromosome Alignment

In order to investigate the function of *Drosophila* INCENP and Aurora B during mitosis we performed RNAi in *Drosophila* Dmel2 cells. Two negative controls were analyzed in parallel: cells to which no dsRNA had been added and cells to which we added dsRNA synthesized from a human intronic sequence, chosen at random in order to rule out unspecific effects of dsRNA on the cell cycle. The addition of dsRNA for the human sequence caused no defects and was indistinguishable from the untreated control suggesting that dsRNA itself does not have a significant effect on cell proliferation. Analysis of RNAi experiments is complicated by the fact that this technique causes a gradual depletion of the protein under study and that proteins are not necessarily lost from all cells in the population at the same rate. To minimize these complications, we examined the phenotypes described here at various times after the onset of RNAi treatment. In addition, where possible, we limited our phenotypic conclusions to cells that were demonstrably lacking INCENP or Aurora B by indirect immunofluorescence.

Immunofluorescence analysis of cells treated with DmINCENP dsRNA showed that the levels of DmINCENP in the culture decreased significantly by 36–48 h after the addition of dsRNA to the culture (data not shown). The RNAi treatment for aurora B took effect more rapidly, the protein became undetectable by indirect immunofluoresce after 24 h (data not shown). To characterize the effects of RNAi upon cellular proliferation, cultures were harvested at 24, 36, 48, and 72 h after treatment with dsRNA and assessed for the following parameters: cell number, viability, mitotic index, and for the cells in mitosis, the distribution of the various mitotic phases (Fig. 6.3).

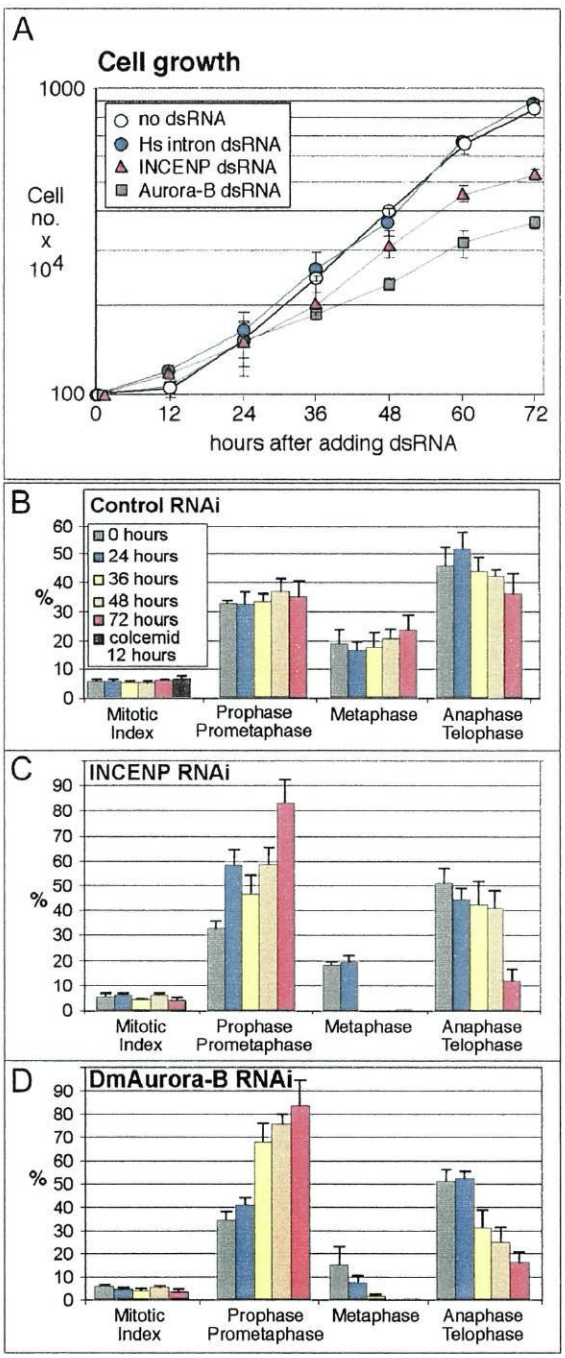


Figure 6.3. Summary of effects of RNAi on cell growth and mitosis. **(A)** Growth curve of control and dsRNA-treated cells. **(B-D)** Histograms showing the percentage of control, INCENP null, or aurora B null mitotic cells at different mitotic stages. For INCENP at t = 0, 24; and aurora B at t = 0, the whole mitotic population was scored due to the scarcity of null cells. For each time point, n = 50.

RNAi treatment of Dmel2 cells caused an increase in the cell doubling time from 21 h (21.6 h in cells after exposure to control dsRNA) to 36.1 and 27.5 h after exposure to either DmAurora B or DmINCENP, respectively (Fig. 6.3A). Strikingly, RNAi of INCENP abolished the ability of cells to achieve a metaphase chromosome alignment (Fig. 6.3, compare B with C). A similar phenotype was observed in the Aurora B RNAi (Fig. 6.3 D). Instead, most mitotic cells showed a prometaphase-like chromosome arrangement. Importantly, this increase in the percentage of prometaphase cells did not reflect an arrest in mitosis, as the mitotic index of the culture remained constant at the control level of ~5% throughout the entire experiment (Fig. 6.3, B–D). As discussed below, we believe that many cells in these cultures must exit mitosis directly from prometaphase without achieving a metaphase chromosome alignment.

2.4. Essential Roles of Drosophila INCENP and Aurora B in Chromosome Structure, Kinetochore Disjunction and Chromosome Segregation

As expected, given the lack of normal metaphase cells, we saw few if any cells that were negative for INCENP or aurora B with a normal anaphase configuration. Instead, the anaphase/telophase cells had a range of abnormalities, including anaphase-like spindles with chromosomes distributed along their length (Fig. 6.4A-B"'), cells in various states of incomplete cytokinesis containing large amounts of amorphous chromatin stretched out between the mitotic spindle (Fig. 6.4C-D"'). Also, highly abnormal cells in which irregularly shaped nuclei were surrounded by a mitotic-like bipolar microtubule array (Fig. 6.4E–G"'). Fig. 6.4E shows two adjacent mitotic cells in the INCENP RNAi, one of which is still expressing INCENP and is at normal metaphase, and the other of which is INCENP negative and is forming an elongated and irregularly shaped nucleus. In cells with chromosomes distributed along the spindle or with irregularly shaped nuclei, centromeres were seen to cluster either near the poles (Fig. 6.4A–D) or at the opposite ends of the elongate nuclei (Fig. 6.4F-G"'). This arrangement of centromeres and chromosomal material strongly suggests that kinetochores had attached to microtubules and that anaphase A movement of chromosomes had occurred. However, in cells that appeared to be in telophase, we often noticed one or more pairs of centromeres that appeared to be stalled midway between the spindle poles (Fig. 6.4.C-D"'). This phenotype would be predicted if the lagging centromeres had successfully become bioriented but were then unable to disjoin at the onset of anaphase. This was never seen in normal anaphases where the centromeres were typically grouped in a tight

cluster at the leading edge of the segregating chromatids (Adams et al., 2001b). Consistent with difficulties in disjunction of sister kinetochores, we also saw numerous paired kinetochores near the spindle poles, as though nondisjoined chromatid pairs had moved together to a single pole (Fig. 6.4A, B and F, double arrows). Together, these observations suggest that DmINCENP and DmAurora B might be essential for a variety of mitotic events, including sister chromatid and kinetochore disjunction, chromosome structure and segregation.

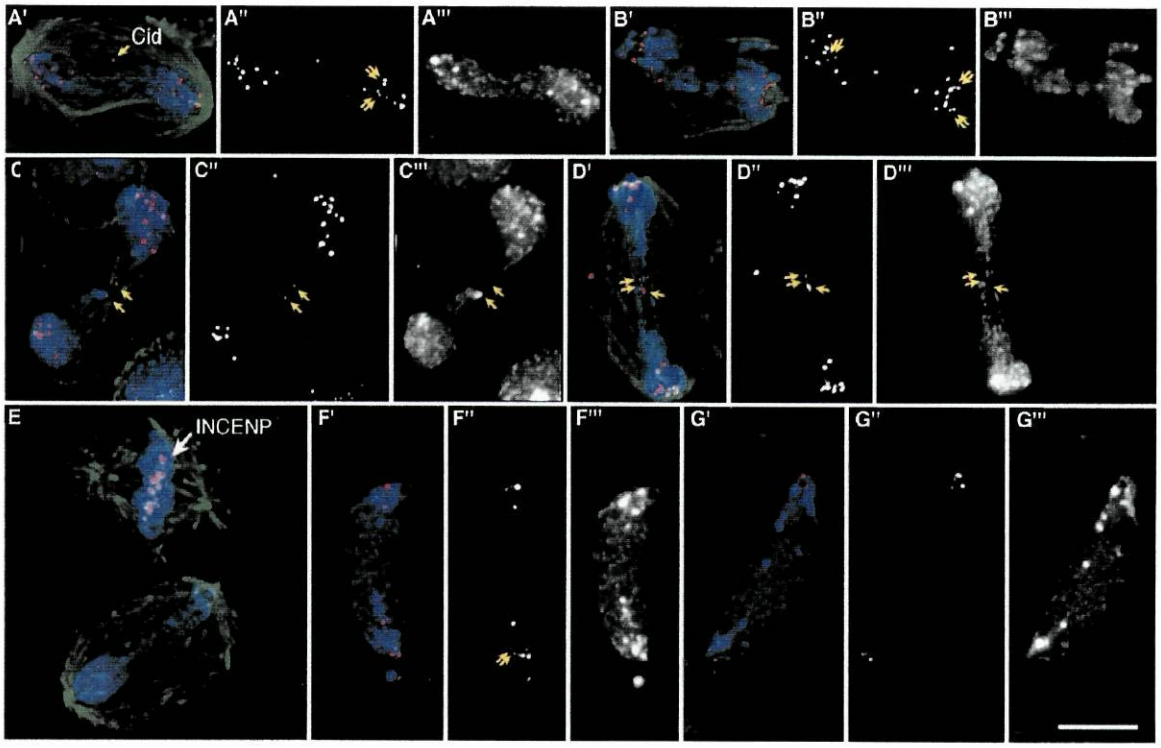


Figure 6.4. Aberrant centromere disjunction and lagging chromatin in the INCENP and Aurora B RNAi. **(A-B''')** Aberrant anaphases with paired CENP-A/Cid spots (red) at poles and lagging chromatin (INCENP RNAi, 36 h). **(C-D''')** Aberrant telophases with Cid spots at the midbody/midzone (INCENP RNAi, 36 h). **(E)** Metaphase and telophase/G1 cell, the latter showing an elongate nucleus with a bipolar microtubule array (INCENP RNAi, 36 h). The metaphase cell is still expressing INCENP, which is not detected in the aberrant telophase/G1 cell. **(F-G''')** Irregularly shaped nuclei similar to those shown in E, showing well-separated clusters of Cid spots joined by a continuum of chromatin (Aurora B RNAi, 36 h). 9, indicates merge (green, a-tubulin); 0, indicates CENP-A/Cid with double arrows showing paired Cid spots; -, indicates DNA (DAPI). Bar is, 5 μm.

3. Discussion

3.1. Targeting of the Human Cromosomal Passenger Complex to the Central Spindle Occurs after Initial Sister Chromatid Separation

The underlying mechanism responsible for the transfer of the chromosomal passenger proteins from the centromeres to the central spindle upon anaphase onset remains highly elusive. Here we show for the first time the dynamic localization of the chromosomal passenger complex in live mitotic HeLa cells by GFP-tagging of human Survivin. Our results indicate that for a short period after sister-chromatids separate at the metaphase-anaphase transition, the passenger proteins remain associated with each centromere pair. Only then, they start to accumulate at the central spindle region where is essential for the organization of the cleavage furrow during later stages of cytokinesis (reviewed by Adams et al., 2001a). Since sister chromatid separation appears to occur before transfer of passenger proteins to the central spindle, it is very likely that dissolution of sister-chromatid cohesion is required for the normal localization of the passengers.

3.2. DmINCENP and DmAurora B Are Chromosomal Passenger Proteins Required for Metaphase Chromosome Alignment

The mitotic distribution of DmINCENP and Aurora B suggest very clearly that they act like classical chromosomal passenger proteins since they accumulate at centromeres early in mitosis and are transferred to the central spindle and the midbody during later stages. Furthermore, depletion of either protein by RNAi revealed that they are required for alignment of chromosomes during metaphase. After addition of dsRNA, we observed a dramatic increase in the percentage of mitotic cells in prometaphase and corresponding decrease in the number of metaphase cells. This was particularly dramatic in the INCENP RNAi, where we failed to observe any INCENP-negative cells in metaphase. Surprisingly, this did not lead to an increase in the mitotic index of the cultures. What is the ultimate fate of these prometaphase cells? We have excluded the possibility that they are removed by cell death since the frequency of dying cells in the

control and RNAi treated culture remained constant at ~5% throughout the experiment (data not shown). An alternative explanation for the lack of an increase in mitotic index would be if the cells transit directly from prometaphase into anaphase or telophase, as is the case for budding yeast cells mutant in the essential kinetochore protein Ndc10p (Goh and Kilmartin, 1993). Consistent with this, we saw a variety of striking abnormalities in cells either undergoing anaphase, or early in the next cell cycle (see below). Although we could observe anaphase/telophase cells with kinetochores at opposite poles of the chromatin mass, the kinetochores were often not clustered as in control cells. This may reflect the initiation of anaphase movement without prior alignment of the chromosomes at a metaphase plate. Why does abrogation of INCENP and/or Aurora B function prevent cells from attaining a stable metaphase chromosome alignment? One obvious possibility is that kinetochore function is impaired, much as when anticentromere antibodies from patients with scleroderma spectrum disease are microinjected into human cultured cells (Bernat et al., 1990) or when cells express the Herpes virus protein ICP0, which causes the destruction of kinetochore proteins CENP-A and CENP-C (Everett et al., 1999). In budding yeast, the Aurora kinase Ipl1p phosphorylates the essential kinetochore component Ndc10p (Biggins et al., 1999). It is therefore possible that, in metazoans, one or more kinetochore components must be phosphorylated by Aurora B in order for kinetochores to function in mitosis. An obvious candidate for this is Cid, the Drosophila homologue of CENP-A. It will be important to determine whether Cid is phosphorylated in an Aurora B kinase–dependent manner. However, our observation that kinetochores assemble correctly, at least as far as Cid binding is concerned, and that kinetochores move to the spindle poles at anaphase/telophase does not support such a role for Aurora B. In fact, this result suggests that after abrogation of INCENP and aurora B function kinetochores are able to bind microtubules and to undergo anaphase A. However, other aspects of kinetochore function, namely the ability to form bipolar spindle attachments and disjoin at anaphase (see below), appear to be defective. How RNAi of INCENP or aurora B leads to defects in chromosome biorientation is unknown, but recent experiments in budding yeast have suggested that Ipl1-Sli15 complex promotes turnover of kinetochore-spindle-pole body connections until traction of sister kinetochores toward opposite poles creates tension in the surrounding chromatin (Tanaka et al., 2002).

3.3. INCENP and/or Aurora B Function are Required for Sister Kinetochore Disjunction and Chromosome Stability in Anaphase

Anaphase/telophase cells after RNAi for INCENP or aurora B exhibited three highly unusual chromosomal phenotypes. First, they often had one or more pairs of sister kinetochores located in the central spindle or flanking the midbody. Second, the foci of CENP-A/Cid staining at or near the spindle poles were often present as pairs, suggesting that sister kinetochores remained paired despite having undergone anaphase A–like poleward movement. Third, separated masses of chromatin were often connected by a mass of lagging chromatin. We refer to this as chromatin and not chromosomes because the material was amorphous, and little or no evidence of condensed mitotic chromosome morphology could be observed.

The first two phenotypes can be explained if centromeres fail to disjoin at anaphase onset. Under these circumstances, centromeres of bioriented chromosomes would tend to accumulate near the spindle equator—later, near the midbody—and be stretched apart by the spindle forces. Monooriented kinetochores would move as pairs to one or the other spindle pole. If this occurred in cells that had attained metaphase, then the bulk of kinetochores would remain as pairs in the spindle midzone. However, as described above, abrogation of INCENP and/or Aurora B function prevents cells from reaching metaphase and would therefore be expected to lead to the observed phenotype, with most centromeres at poles and only a few remaining in the midzone. The presence of massive amounts of lagging chromatin is highly characteristic of anaphase/telophase after loss of INCENP and/or Aurora B function. This lagging chromatin is distinct from that seen when problems with sister chromatid disjunction are caused by loss of topoisomerase II function. Loss of topo II function in *S. pombe* resulted in anaphase cells with a small subset of the chromatin, including centromeres at the poles and the bulk of the tangled chromatin trapped at the spindle midzone (Funabiki et al., 1993). In vertebrates, inhibition of topo II function with drugs causes a similar phenotype (Gorbsky, 1994). In contrast, RNAi for INCENP or Aurora B did not appear to prevent the bulk of the chromatin from moving towards the spindle poles. This lagging chromatin might arise from difficulties in sister chromatid disjunction, but we believe it more likely that it represents a failure of the chromosomes to move as integral units under the physical stress of anaphase movement. If the dumpy chromosomes observed in prometaphase cells (Adams et al., 2001b) lack an organized scaffold then when centromeres begin to move polewards, the chromatin of the arms might simply unravel and be left behind as a smear of amorphous chromatin. This would be

consistent with the observation that depletion of Aurora B function in *Drosophila* cells interferes with the binding of the condensin subunit *barren* to mitotic chromosomes (Giet and Glover, 2001). Indeed, mutations both in barren and smc4, two subunits of condensin exhibit chromatin bridges during syncytial and somatic mitosis (Bhat et al., 1996; Steffensen et al., 2001). It is possible that action of INCENP/Aurora B on other chromosomal components in addition to condensin subunits contributes to a loss of chromosomal integrity during anaphase.

4. Materials and Methods

Scoring of RNAi Phenotypes

Cell cycle parameters in INCENP and Aurora B RNAi experiments were determined as described in Chapter II. When possible, only INCENP or Aurora B negative cells were scored for mitotic parameters in the RNAi experiments (as determined by immunofluorescence). To determine the mitotic index in the presence of microtubule poisons for INCENP and Aurora B RNAi, Dmel2 cells were incubated for 12 hours in the presence of 1μg/mL of Colcemid.

Blocking of Drosophila Aurora B Affinity Purified Antibodies

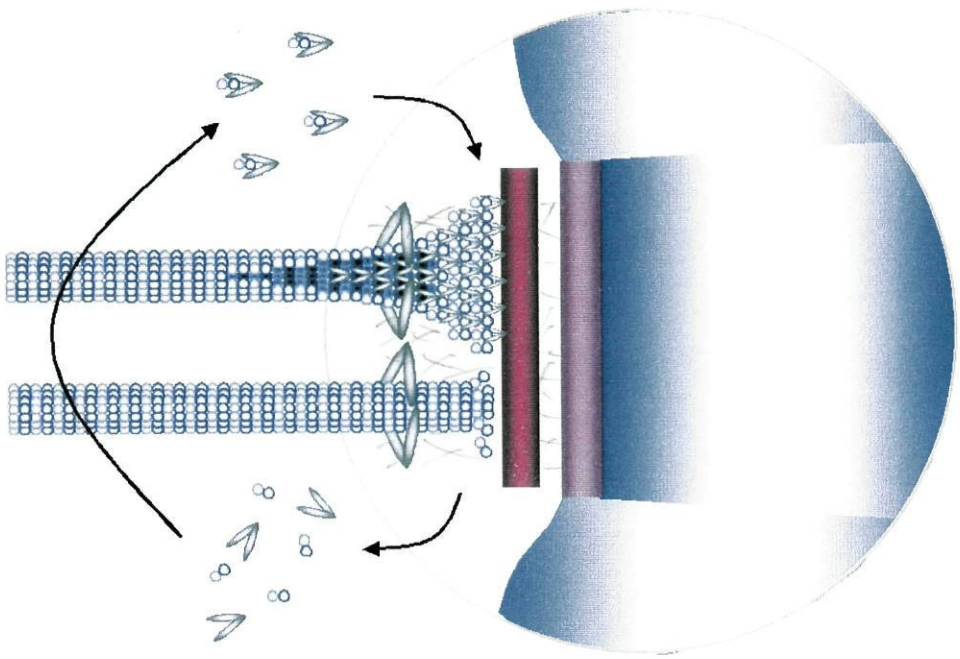
In order to ascertain the specificity of the serum used to detect Aurora B by immunofluorescence, affinity purified antibodies were diluted 1:50 in PBS + 0.1 % Triton X-100 (BioRad) with 10 % Fetal Bovine Serum, in the presence of GST-Aurora-B (0.3 mg/mL) and then incubated for 1 hour at RT before addition directly to Dmel2 cells and processed for immunofluorescence as usual. Secondary antibodies (Rabbit-FITC and Mouse Texas-Red) were purchased from Jackson Labs and used diluted 1:200.

DsRNA Interference in Drosophila Dmel2 Cells

INCENP and Aurora B RNAi in *Drosophila* Dmel2 cells was performed as described in Chapter II for RNAi in S2 cells, with the exception that we used 5 μg of dsRNA per ml of culture medium (Adams et al., 2001b). For immunofluorescence analysis cells were treated also as described in Chapter II. Treatment of Dmel2 cells with dsRNA synthesized from random human intronic sequence, to rule out unspecific effects of dsRNA, caused no visible defects and was indistinguishable from the untreated control. To determine the doubling time of the cells for each experiment, we calculated the best fit for the growth curves (semi-log scale) and the doubling time was calculated from the slope of the correspondent exponential equations (x=time/hrs and y=number of cells):

Experiment	Cell line	Exponential Equation	Doubling time/hrs
Control (no dsRNA)	Dmel2	$y=80.325e^{0.033x}$	21,0
Control (Hs intron dsRNA)	Dmel2	$y=85.318e^{0.0321x}$	21.6
INCENP RNAi	Dmel2	$y=89.934e^{0.0252x}$	27.5
Aurora B RNAi	Dmel2	$y=93.95e^{0.0192x}$	36.1

III. Conclusions



Conclusions

The work presented in this thesis provides further insights into the role of microtubule- associated proteins during mitosis.

Firstly, we have characterized the *Drosophila* MAST protein and found that it is absolutely essential for the formation and function of a bipolar spindle, as well as for chromosome congression. The results obtained by RNAi also suggest that MAST has a role at the kinetochores, either in allowing dynamic microtubules to remain associated to kinetochores or in regulating the dynamics of kinetochore microtubules.

Secondly, the analysis of CLASP1 function during mitosis indicated that this protein is likely to be the human homologue of MAST. CLASP1 defines a new compartment of the kinetochore that we have called the "outer corona". It is required for spindle stability and for normal behaviour of kinetochore-associated microtubules. Also, our results indicate that CLASP1 has a microtubule-bundling activity promoting microtubule stability. Most of the results could be incorporated into a model in which MAST/CLASP1 is required for the regulation of microtubule dynamics at the kinetochore. Therefore, MAST/CLASP1 represents the first MAP that is known to specifically affect the dynamic behaviour of this class of microtubules. In addition to CLASP1, CLASP2 may also have a role in these same processes since it showed the same pattern of localization during mitosis.

Thirdly, we have found that the chromosomal passenger proteins, including INCENP and Aurora B, have essential roles during mitosis, namely in chromosome congression and kinetochore disjunction.

Together, these observations suggest that MAPs may have unexpected essential functions during mitosis and strongly support a role in spindle formation and function, regulation of microtubule dynamics, microtubule-kinetochore attachment and chromosome movement during mitosis. Moreover, we have preliminary evidence indicating that MAPs may have a highly surprising function in mitotic-exit and may have a role in aneuploidy events such those implicated in human cancers. It would be important and highly significant to determine whether these proteins may be involved in such processes. Indeed, in this study, several distinct CLASP isoforms were identified from a human colon carcinoma cell line. Finally, this work have opened new lines of research namely in the role of microtubule-plus-end tracking proteins in kinetochore function, demanding further characterization of other related proteins in this process.

IV. Supplementary Information

1. Movies

2. Movie Legends

Movie 1 - Localisation of EGFP-CLASP1 to the microtubule plus-ends during prometaphase in living HeLa cells. Time-lapse sequences were collected by four-dimensional restoration microscopy every 30 s using a DeltaVision microscope. EGFP-CLASP1 dots seen near the metaphase plate most likely represent the kinetochores.

Movie 2 - Microtubule localisation of EGFP-CLASP1 at the metaphase-anaphase transition and during late mitotic events. Living HeLa cells expressing EGFP-CLASP1 were analysed by four-dimensional restoration microscopy and time-lapse series collected every 30 s. In metaphase, EGFP-CLASP1 was weakly associated with spindle microtubules overall. After anaphase onset, EGFP-CLASP1 remains associated with the shorting spindle and preferentially accumulates at the central spindle. During telophase and cytokinesis EGFP-CLASP1 concentrates with astral microtubules and ultimately accumulates in the midbody.

Movie 3 - Localisation of EGFP-CLASP1 to the microtubule plus-ends at late stages of cytokinesis in living HeLa cells. Time-lapse sequences were collected by four-dimensional restoration microscopy every 30 s using a DeltaVision microscope. EGFP-CLASP1 dots can be seen at the leading edges of the expanding cytoplasm.

Movie 4 - Behaviour of dominant-negative CLASP1 in the microtubules. Living HeLa cells expressing EGFP-CLASP1$_{250\text{-}943}$ were analysed by four-dimensional restoration microscopy and time-lapse series collected every 30 s. Note the bundling and the stabilization effect on the microtubules and the static behaviour of EGFP-CLASP1$_{250\text{-}943}$ along the entire length of the bundles and not in the usual comet-like fashion.

Movie 5 - Time-lapse video microscopy analysis by DIC of a human CF-PAC cell injected with pre-immune antibodies during late prophase. Chromosomes can be seen to fully congress to the metaphase plate within the first 15 min upon NEB. Metaphase lasts for ~25 min and the cell enters anaphase. During telophase/cytokinesis chromosomes in the two daughter cells start to decondense after ~1 hour from NEB. Time-lapse series collected every 30 s.

Movie 6 - Time-lapse video microscopy analysis by DIC of a human CF-PAC cell injected with anti-CLASP1 antibodies during late prophase. During prometaphase full congression of all chromosomes is not accomplished even after more than 2 h 30 m. Time-lapse series collected every 30 s.

Movie 7 - Time-lapse video microscopy analysis by DIC of another human CF-PAC cell injected with anti-CLASP1 antibodies during late prophase. Chromosome oscillations are abnormal and the chromosomes accumulate as a disorganized mass in the centre of the cell. The spindle can be seen to progressively collapse. Time-lapse series collected every 30 s.

Movie 8 - Four-dimensional analysis of GFP-Survivin (green) in living HeLa cells. Chromosomes are shown vitally stained with Hoechst (Blue). Time-lapse series collected every 30 s.

3. Tables

Table I. List of primary antibodies used and respective dilutions for immunofluorescence (IF) or western blot (WB).

Antigen	Serum	Dilution
HsCLASP1	Rabbit polyclonal	1:500 IF
HsCLASP2	Rabbit polyclonal	1:300 IF
DmMAST	Rabbit polyclonal	1:200 IF; 1:600 WB
α-tubulin	Mouse monoclonal (Sigma)	1:2000 IF; 1:10000 WB
ACA	Human CREST serum	1:1000 IF
DmINCENP	Rabbit polyclonal	1:500 IF
P-Histone H3	Rabbit polyclonal (NEB)	1:300 IF
P-Histone H3	Mouse monoclonal (NEB)	1:100 IF
γ-tubulin	Mouse monoclonal (Sigma)	1:500 IF
DmAurora B	Affinity purified rabbit polyclonal	1:50 IF
Cid	Rabbit polyclonal	1:2000 IF
DmBubR1	Rabbit polyclonal	1:1000 IF
CLIP-170	Rabbit polyclonal	1:300 IF
DmCyclin B	Rabbit polyclonal	1:500 IF
DmCP190	Rabbit polyclonal	1:500 IF
DmCP190	Mouse monoclonal	1:100 IF
HsCENP-C	Rabbit polyclonal	1:500 IF
HsCENP-E	Mouse monoclonal	1:50 IF
HsAPC	Rabbit polyclonal	1:300 IF
HsEB1	Mouse monoclonal (Transd. Labs)	1:100 IF
DmZW10	Rabbit polyclonal	1:500 IF
DmDHC	Mouse monoclonal	1:50 IF
D-CLIP-190	Affinity purified rabbit polyclonal	1:2000 IF
Hsp115	Rabbit polyclonal	1:300 IF

Table II. List of oligonucleotides used and respective sequences.

Oligo	Sequence	Length/bp
RT-622-F	TGCCTTTTCTATGCGCCTCAC	21
RT-622-R	TGTTAGCAAAGTCCTGGTGGG	21
RT-627-F	GCACAACAGTAAGCATAGAAC	21
RT-627-R	GATTTGTCATAGGCTTTCCCC	21
RC-622-1	CTCGGCTGGAATAAATCTG	19
RC-622-2	ATCCTCTTCATCAACAGCAC	20
RC-622-3	GGATGAACCAAGCCG	15
RC-627-1	TCATCTTCATCAACTGCTC	19
RC-627-2	CTGAATAGAAGGGACATC	18
RC-627-3	AGAAGCACCTCCAACCT	17
β-actin-F+1	GAGACCTTCAACACCCCA	18
β-actin-F2	GTTGCTATCCAGGCTGTG	18
β-actin-R1	AAGGAAGGCTGGAAGAGT	18
V40-i2-F	TAATACGACTCACTATAGGGCGAAGGACGAATAGACATT	39
V40-i3-R	TAATACGACTCACTATAGGGTCCTGTTTGACCTGGTCG	38
CD622XhoI-F	TGTACTCTCGAGAATGTTACTGGGGTTTCCACAGTC	36
CD+622SacII-R	TATGAACCGCGGCTCATATCTCCTCCTCACAGGC	34
FL-622-BspEI-F	TGTAGTTATCCGGACTGGATTTGAATTCCACTATGGAG	38
FL-622-AclI-R	ATGAACATAACGTTTCTCCGAGAACTTGGTGGAGCCTTGG ATGATGTAGAACTAGCAGAGGAAGGTCTGTT	71

Table III. List of CLASP1 constructs cloned into pEGFP-C1 vector and respective cloning sites

Construct	Cloning Sites
EGFP-CLASP1$_{1-1538}$	BspEI + SacII
EGFP-CLASP1$_{250-1538}$	XhoI/SalI + SacII
EGFP-CLASP1$_{1-1260}$	XhoI/SalI + SacII
EGFP-CLASP1$_{1-270}$	BspEI + AclI/AccI
EGFP-CLASP1$_{1256-1538}$	SmaI
EGFP-CLASP1$_{250-\Delta(406-1117)-1464}$	XhoI/SalI (Δ=NcoI)
EGFP-CLASP1$_{250-\Delta(406-1117)-1260}$	XhoI/SalI + SacII (Δ=NcoI)
EGFP-CLASP1$_{329-1538}$	SmaI/SspI
EGFP-CLASP1$_{1082-1538}$	EcoRI
EGFP-CLASP1$_{250-1081}$	XhoI/SalI + EcoRI
EGFP-CLASP1$_{250-943}$	XhoI/SalI + AflII
EGFP-CLASP1$_{509-817}$	XhoI + SacII

4. Abbreviations

Abs: Absorvance

ATP: Adenosine Triphosphate

CCD: Charge Coupled Device

DAPI: 4',6-diamidino-2'-phenylindole dihydrochloride

DIC: Differential Interference Contrast

dPBS: Dulbecco's Phophate Buffer Saline

ECL: Enhanced Chemi-Luminiscence

EGFP: Enhances Green-Fluorescent Protein

EM: Electron Microscopy

EST: Expressed Sequence Tag

FACS: Fluorescence Activated Cell Sorting

FBS: Fetal Bovine Serum

FCS2: Focht Chamber System 2

FITC: Fluorescein Isothiocyanate

GDP: Guanosine Diphosphate

GMPPCP: guanylyl 5'-(beta, gamma-methylenediphosphonate)

GST: Glutathione-S-Transferase

GTP: Guanosine Triphosphate

GTP-γS: guanosine 5'-O-(gamma[35S]thio)triphosphate

Df: Deficiency

DHC: Dynein Heavy Chain

NEB: Nuclear Envelope Breakdown

ORF: Open Reading Frame

PBS: Phosphate Buffer Saline

PCR: Polymerase Chain Reaction

P-H3: Phosphorylated Histone H3

Pi: Inorganic Phosphate

PI: Isoelectric Point

RACE: Rapid Amplification of cDNA Ends

RNAi: double-stranded RNA-mediated interference

rpm: rotations per minute

RT: Room Temperature

RT-PCR: Reverse Transcriptase Polymerase Chain Reaction

V. References

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Supplementary Information