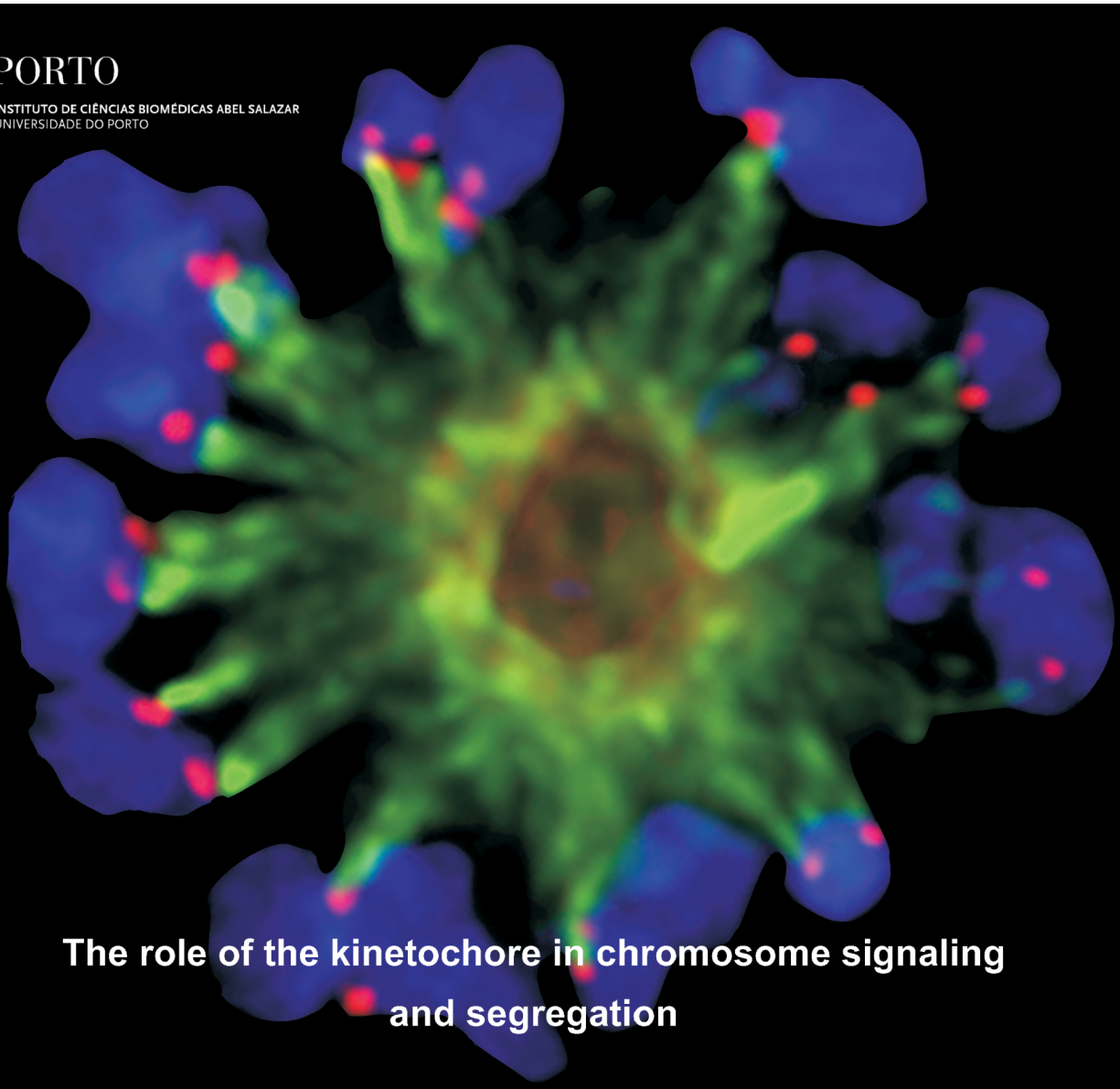
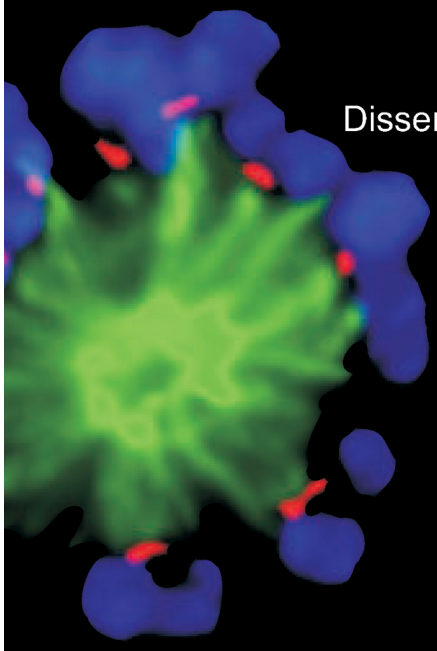
U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO



The role of the kinetochore in chromosome signaling and segregation

André Filipe Santos Maia

Dissertação de doutoramento em Ciências Biomédicas



2010

André Filipe Santos Maia

The role of the kinetochore in chromosome signaling and segregation

Dissertação de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador – Doutor Claudio E. Sunkel

Categoria – Professor Catedrático

Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

FCT
Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

Este trabalho foi financiado pela Fundação para a Ciência e Tecnologia (SFRH/BD/18038/2004)

De acordo com o disposto no nº 2 do Artº 8º do Decreto-Lei nº 388/70, nesta dissertação foram utilizados resultados das publicações abaixo indicadas. No cumprimento do disposto no referido Decreto-Lei, o autor desta dissertação declara que interveio na concepção e na execução do trabalho experimental, na interpretação dos resultados e na redacção dos manuscritos publicados ou em preparação, sob o nome de **Maia, A.** ou **Maia, A.F.**:

Martins, T., **Maia, A.F.**, Steffensen, S. and Sunkel C.E. (2009). Sgt1, a co-chaperone of Hsp90 stabilizes Polo and is required for centrosome organization. *EMBO Journal* **28**(3):234-47.

Vader, G., **Maia, A.F.** and Lens, S.M.A. (2008) The chromosomal passenger complex and the spindle assembly checkpoint: kinetochore-microtubule error correction and beyond. *Cell Division* 2008, **3**:10.

Maia, A.F., Lopes, C.S. and Sunkel, C.E. (2007). BubR1 and CENP-E have antagonistic effects upon the stability of microtubule-kinetochore attachments in *Drosophila* S2 cell mitosis. *Cell cycle*.**6**, 1367-1378.

Maia, A.F., Martins, T., Marinho, J. and Sunkel, C.E. (2007). Mitosis and spindle inhibitors. In *Molecular Biology in Cancer: Toward New Therapies* (ed. Jesús García-Foncillas), Prous Science, Barcelona and Philadelphia (in press).

Malmanche, N., **Maia, A.**, and Sunkel, C.E. (2006). The spindle assembly checkpoint: preventing chromosome mis-segregation during mitosis and meiosis. *FEBS Lett **580***, 2888-2895

Maia, A.F., Feijão, T., Vromans, M.J.M., Sunkel, C.E. and Lens, S.M.A. (2009). Aurora B kinase cooperates with CENP-E to promote timely anaphase onset. *(submitted)*

Acknowledgments / Agradecimentos

Deixei para escrever em último lugar as que são provavelmente as mais importantes palavras alusivas a esta longa jornada. Foram muitos os que comigo se cruzaram e todos tiveram um papel fundamental não só para a elaboração desta tese mas em especial no meu crescimento profissional e pessoal. A todos, sem excepção, eu agradeço! No entanto, não posso deixar de particularmente expressar a minha gratidão a alguns de vós:

Ao Professor Claudio Sunkel, por me ter aberto as portas do mundo da investigação científica. Em particular, pelas discussões construtivas, muitas vezes acompanhadas por esclarecedores esboços no quadro de giz, pelo espírito crítico, pela empenho em ensinar e por me incentivar a criatividade e gosto pela actividade científica.

Ao Hélder Maiato e a todos os elementos do seu grupo pelas discussões científicas e por serem uns óptimos "vizinhos" de corredor.

À Paula Coelho, pelos ensinamentos, pela ajuda, pelas "disputas" e acima de tudo por estar sempre presente.

À Carla Lopes, pelo espírito critico; à Paula Sampaio, pela ajuda com os microscópios; ao Bernardo Orr e à Joana Marinho pela boa disposição e "alto astral".

À Adelaide Santos, à Maria João Falcão e à Susana Aveiro pelo precioso apoio.

Ao Torcato, foi um prazer orientar-te nos teus primeiros meses na "mitose". Obrigado por teres levado a bom porto o meu "projecto secreto".

À Augusta Monteiro pela indispensável ajuda com as células e pela amizade.

À Filipa Sousa pelo companheirismo, pelas longas conversas e amizade.

À Tália Figueiredo, pela imprescindível ajuda na recta final desta tese.

À Susana Gouveia, pelo exemplo de força e determinação, pelo acolhimento e bons tempos passados na Holanda.

À Raquel Oliveira, a minha companheira de viagem nos congressos. Pelas frutíferas discussões científicas e amizade.

À Rita Reis, pelo companheirismo, pela amizade, pelos "ensinamentos", e acima de tudo por poder sempre contar contigo. Sempre que precisares cá estou para servir de agente de viagens!

I would like to thank to Susanne Lens for accepting me in her lab for the final year of my PhD training. There I had the opportunity to work in a very enthusiastic and sometimes even frenetic environment where I learnt a lot and had a great time. I also would like to thank to Martijn Vromans for helping me in the lab and all the members of the Lens, Kops and Medema groups from the UMC Utrecht for helpful discussions.

Ao meu primo Hélder Maia, pelo trabalho gráfico da tese e à minha família em geral pelo incondicional apoio.

Aos meus pais e à minha irmã, que mesmo não sabendo muito bem o que eu andava a fazer sempre acreditaram em mim e me "aturaram" nos momentos difíceis.

À Cláudia, pelo carinho, pela compreensão, pela ajuda a relativizar os maus momentos e por tornar os dias mais alegres. Sem ti teria sido muito difícil! Obrigado.

CONTENTS

Summary…… i

Resumo…….. iii

Résumé…….. v

PART I – GENERAL INTRODUCTION

1. The Cell Cycle…….. 3

1.1 The cell division cycle – a general description…… 3

1.2 Mitosis……. 5

1.3 The cell cycle control system…….. 7

2. The Kinetochore…….. 10

2.1 Structure…… 10

2.2 Protein constituents…….. 12

3. Microtubule-Kinetochore Attachment..... 15

3.1 Microtubule binding to kinetochores..... 15

3.2 Correction of defective microtubule attachments. Biorientation..... 16

3.3 Chromosome movements and congression..... 17

3.4 Coupling kinetochore-microtubule attachment to microtubule-
-polymerization dynamics and chromosome movements..... 19

3.5 Molecular machinery involved in kinetochore motility…… 20

4. The Kinetochore and the Spindle Assembly Checkpoint…….. 21

4.1 SAC and its molecular components…….. 22

4.2 SAC mechanism of action…….. 25

4.3 The kinetochore is a hub for kinase signaling…….. 26

5. A Weak Spindle Checkpoint Might Facilitate Tumorigenesis…… 28

6. Mitotic Progression as a Drug Target for Cancer Therapy……..... 30

7. Objectives…….. 31

PART II – EXPERIMENTAL WORK

Chapter 1 – Role of BubR1 and CENP-E in the stability of microtubule--kinetochore attachments

1. Introduction..... 37

2. Results..... 39

2.1 Depletion of BubR1 and CENP-meta from *Drosophila* cultured cells by dsRNA interference..... 39

2.2 Analysis of mitotic progression after depletion of BubR1 or CENP-meta.. 41

2.3 Analysis of prometaphase phenotypes after depletion of BubR1 or CENP-meta..... 45

2.4 Chromosome congression after simultaneous depletion of BubR1 and CENP-meta..... 47

2.5 Microtubule-kinetochore interaction after depletion of BubR1 or/and CENP-meta..... 49

2.6 Detailed analysis of microtubule-kinetochore interaction after depletion of CENP-meta..... 54

3. Discussion..... 57

4. Materials and Methods..... 61

4.1 Double-stranded RNA interference in *Drosophila* S2 cells..... 61

4.2 Immunofluorescence in S2 cells..... 62

4.3 Antibodies..... 62

4.4 Kinetochore-microtubule interaction (MG132-Taxol) assay..... 63

4.5 Time-lapse fluorescence imaging..... 63

Chapter 2 – Aurora B kinase cooperates with CENP-E to promote timely anaphase onset

1. Introduction..... 67

2. Results..... 68

2.1 CENP-E-depleted cells display two distinct mitotic phenotypes..... 68

2.2 Aurora B kinase is highly active on polar chromosomes..... 72

2.3 Aurora B delays mitotic progression upon CENP-E depletion..... 76

2.4 CENP-E-depleted cells do not slip out of mitosis but satisfy the SAC..... 78

3. Discussion..... 79

4. Materials and Methods..... 82

4.1 Cell culture and siRNA..... 82

4.2 Antibodies and reagents..... 82

4.3 Immunoblotting and immunofluorescence..... 83

4.4 Time-lapse microscopy..... 83

4.5 Quantification of active Aurora B..... 83

Chapter 3 – Preliminary studies on Sgt1, a newly described *Drosophila* kinetochore protein

1. Introduction..... 87

2. Results..... 88

2.1 Identification and sub-cellular localization of the *Drosophila* homologue of Sgt1..... 88

3. Discussion..... 90

4. Materials and Methods..... 92

4.1 Transfection of S2 cells..... 92

4.2 Immunostaining in S2 cells and antibodies..... 92

PART III – GENERAL DISCUSSION

General Discussion..... 95

PART IV – REFERENCES

References..... 101

PART V – APPENDIXES

Appendix 1 – Abbreviations..... 133

Appendix 2 – Recipes..... 141

Appendix 3 – Plasmids..... 145

Appendix 4 – Movies Legends..... 149

SUMMARY

Error-free chromosome segregation is crucial for the maintenance of genomic stability and prevention of tumorigenesis. The spindle assembly checkpoint (SAC) prevents exit from mitosis as long as not all chromosome pairs are properly attached to the mitotic spindle. Microtubules attach to chromosomes through binding at the kinetochore, a multi-protein structure that is organized over centromeric chromatin. The kinetochore plays an important role in the generation of the SAC signal that prevent mitotic exit while at the same time it is involved in chromosome movements during congression and segregation. The work reported in this thesis aims to further our understanding on how the combination of such functions ensures faithful chromosome segregation. The first part focuses on the phenotypic analysis of *Drosophila melanogaster* S2 cells depleted of the SAC protein BubR1 and the microtubule motor protein CENP-meta/CENP-E. The results show that while BubR1-depleted cells exit mitosis prematurely due to loss of SAC activity, CENP-meta-depleted cells are delayed in mitosis. Furthermore, a detailed analysis of microtubule-kinetochore interactions after depletion of BubR1 or CENP-meta clearly shows that without BubR1 cells contain a high frequency of monoriented and fully unattached chromosomes while most CENP-meta-depleted cells have chromosomes attached to spindle microtubules. Moreover, when both proteins are simultaneously depleted absence of CENP-meta is able to partly rescue microtubule-kinetochore attachments lost due to BubR1 depletion. The data shows that BubR1 is required to promote stable microtubule-kinetochore attachment. However, CENP-E appears to promote kinetochore-microtubule instability, suggesting a role of CENP-E in the mechanism that corrects inappropriate kinetochore attachment. Accordingly, the second part of the thesis concentrates in determining whether CENP-E and Aurora B kinase activities cooperate to promote efficient chromosome biorientation. The results, obtained in HeLa cells, do not support a regulation of Aurora B kinase activity by CENP-E. However, strongly suggest that Aurora B kinase destabilizes spindle pole proximal kinetochore-microtubule interactions keeping the SAC active to allow CENP-E-mediated congression of monoriented chromosomes. Taken together, the work presented in this thesis, through the study of two *bona fide* kinetochore proteins, provides insight in how chromosomes congress and how can they delay mitotic exit when errors occur thus avoiding aneuploidy.

RESUMO

A segregação correcta dos cromossomas é crucial para a manutenção da estabilidade genética e na prevenção da génese tumoral. O ponto de controlo do fuso mitótico (SAC) previne a saída de mitose enquanto todos os pares de cromossomas não tenham estabelecido uma ligação correcta com microtúbulos do fuso mitótico. Os microtúbulos ligam-se aos cromossomas através dos cinetocóros que são uma estrutura multi-proteica que se organiza na cromatina centromérica. O cinetocóro desempenha um papel muito importante na produção do sinal que mantém o SAC activo, prevenindo a saída de mitose, enquanto ao mesmo tempo está envolvido nos movimentos dos cromossomas durante a congressão para a placa metafásica e posterior segregação para pólos opostos da célula em divisão. O trabalho apresentado nesta tese pretende ampliar o conhecimento em como a combinação destas funções do cinetocóro assegura uma correcta segregação dos cromossomas. A primeira parte da tese descreve a análise fenotípica de células de cultura S2, de *Drosophila*, nas quais se depletou a proteína do ponto de controlo do fuso mitótico BubR1 e a proteína motora dos microtúbulos CENP-meta/CENP-E. Os resultados mostram que enquanto as células na ausência de BubR1 saem de mitose prematuramente devido à perda de actividade do SAC, as células depletadas de CENP-E permanecem em mitose por longos períodos. Para além disso, uma análise detalhada das ligações dos microtúbulos aos cinetocóros, após depleção de BubR1 ou CENP-meta, claramente demonstram que na ausência de BubR1 as células apresentam uma elevada frequência de cromossomas monorientados e até completamente desligados do fuso mitótico, enquanto a maioria das células depletadas de CENP-meta apresentam os cromossomas ligados a microtúbulos do fuso. Mais ainda, quando ambas as proteínas são simultaneamente depletadas, a ausência de CENP-meta resgata parcialmente a perda de ligações cinetocóro-microtúbulo geradas pela ausência de BubR1. Estes dados mostram que BubR1 é necessária para promover ligações estáveis entre os microtúbulos aos cinetocóros. No entanto, CENP-E parece promover instabilidade nesta ligação, sugerindo que esta proteína possa estar envolvida no mecanismo que corrige ligações inapropriadas dos microtúbulos aos cinetocóros. Consequentemente, a segunda parte da tese concentra-se em determinar se CENP-E e a actividade da cinase Aurora B cooperam para promover uma eficiente biorientação dos cromossomas. Os resultados, obtidos em células HeLa, não suportam uma regulação da actividade da cinase Aurora B por parte de CENP-E. No entanto, sugerem fortemente que a cinase Aurora B destabiliza as interacções cinetocóro-microtúbulo, que se estabelecem junto aos pólos da célula em divisão, mantendo a actividade do SAC e permitindo a congressão mediada por CENP-E dos cromossomas monorientados. Em suma, o

trabalho desenvolvido nesta tese, através do estudo de duas proteínas do cinetocóro, expande o conhecimento em como os cromossomas congregam e como conseguem atrasar a saída de mitose aquando da existência de erros evitando aneuploidias.

RÉSUMÉ

La ségrégation correcte des chromosomes est cruciale pour la maintenance de la stabilité génétique et dans la prévention de la origine de la tumeur. Le point de contrôle du fuseau mitotique (SAC) prévient la sortie de la mitose avant que toutes les paires de chromosomes n'aient pas établi une liaison correcte avec les microtubules du fuseau mitotique. Les microtubules se lient aux chromosomes à travers des kinétochore qui sont une structure multi-protéique qui s'organise dans la chromatine centromérique. Le kinétochore joue un rôle très important dans la production du signal qui maintient le SAC actif, en prévenant la sortie de la mitose, tandis qu'au même temps est enveloppé dans les mouvements de chromosomes pendant la congression pour la plaque métaphasique et ultérieure ségrégation vers les pôles opposés de la cellule en division. Le travail présenté dans cette thèse apport une meilleure compréhension pour le rôle de ces fonctions du kinétochore assure une correcte ségrégation (séparation) des chromosomes. La première partie de cette thèse décrit l'analyse phénotypique des cellules de culture S2, de *Drosophila*, a l'intérieur desquelles on a fait la déplétion de la protéine du point de contrôle du fuseau mitotique BubR1 et la protéine motrice des microtubules CENP-meta/CENP-E. Les résultats montrent que pendant que les cellules, dans l'absence de BubR1, sortent de la mitose prématurément due à la perte de l'activité du SAC, les cellules en déplétion de CENP-E demeurent en mitose par de longs moments. De plus, une analyse détaillée des liaisons des microtubules au kinétochore, après déplétion de BubR1 ou CENP-meta, démontre nettement qu'en absence de BubR1 les cellules présentent une fréquence élevée de chromosomes monorientés et mêmes complètement débranchés du fuseau mitotique, pendant que la plupart des cellules en déplétés de CENP-meta présentant les chromosomes liés à des microtubules du fuseau. En plus, quand les deux protéines sont simultanément déplétés, l'absence de CENP-meta délivre partiellement la perte de liaisons kinétochore-microtubules engendrées par l'absence de BubR1. Ces données montrent que BubR1 est nécessaire pour promouvoir les liaisons stables entre les microtubules et les kinétochores. Cependant, CENP-E semble promouvoir l'instabilité dans cette liaison en suggérant que cette protéine peut être entourée dans le mécanisme qui corrige des liaisons inappropriées des microtubules aux kinétochores. Par conséquent, la deuxième partie de cette thèse se centralise à déterminer si CENP-E et l'activité de la kinase Aurora B coopère pour promouvoir une efficace biorientation des chromosomes. Les résultats, obtenus dans les cellules HeLa, ne supportent pas un règlement de l'activité de la Kinase Aurora B de la part de CENP-E. Cependant, ils suggèrent fortement que la Kinase Aurora B déstabilise les interactions kinétochore-microtubule, qui se logent au niveau des pôles de la cellule en division, en

maintenance de l'activité du SAC et permettant la congression partagée par CENP-E des chromosomes monorientés. En un mot, le travaille développé dans cette thèse, par l'étude des deux protéines du kinétochore, répand la connaissance en expliquant comment les chromosomes se congrègue et comment arrivent à retarder la sortie de la mitose dès l'existence d'erreurs en évitant l'aneuploïdie.

PART I

GENERAL INTRODUCTION

1. THE CELL CYCLE

The invention of the microscope in the seventeenth century opened new doors to fascinating discoveries of generations of scientists. Robert Hooke, in 1665, made the first systematic account of the microscopic world in his book Micrographia (Hooke, 1665). Outstandingly, observing the honeycomb-like structure of cork he coined these structures as *cells*. Almost two centuries after, the cell was proposed to be the basic unit of life and that all living organisms are made of cells (Schleiden, 1838; Schwann, 1839). Soon after, Rudolf Virchow (1855) realized that every cell is derived from a pre-existing cell (*"omnis cellula e cellula"*). Later in 1880, Walther Flemming made for the first time a detailed description of cell division. He suggested that the nucleus of cells found in a resting state, can undergo metamorphosis into threads (later called chromosomes) that shorten and align in the equatorial plane of the cell, after which split into two halves and move to opposite sides of the cell. Then the threads become undistinguishable again and the resulting cells look like the resting mother cell. He termed this process of nuclear division as *mitosis* (from the Greek word for thread) (reedited in Flemming, 1965).

1.1 THE CELL DIVISION CYCLE – A GENERAL DESCRIPTION

The cell cycle is a fundamental area of investigation that can lead to a major understanding of the causes of tumorigenesis and eventually contribute to the development of treatments for cancer (Holland and Cleveland, 2009; Jackson et al., 2007). It is a highly ordered and tightly regulated process by which a single cell gives rise to two genetically identical daughter cells that can continue proliferating, grow and eventually differentiate. Therefore, the cell division cycle is an essential process for the development of multicellular organisms that start as a single founder cell and through innumerable rounds of divisions become more and more complex. In the adult, cell division provides renewal of old or damaged cells.

The eukaryotic cell cycle can be divided into two fundamental parts: the longest, interphase, where cells grow and DNA is replicated (S phase), and mitosis (or M phase) where segregation of the replicated genetic material occurs (Fig. 1). Upon nuclear division, individualization of the two genetically identical daughter cells takes place after division of the cytoplasm, known as cytokinesis. Concomitantly with DNA replication, cells also replicate their centrosomes (in animal cells centrosomes are the major microtubule

organizing centers – MTOC) but these remain together until the onset of mitosis. DNA replication and mitosis are separated in many cell types by two intervening phases called Gap 1, between the end of an M phase and start of the next S phase and Gap 2 between the end of S phase and the initiation of the next M phase. During these Gap phases cells continue to grow and serve as important regulatory periods known as cell cycle transitions.

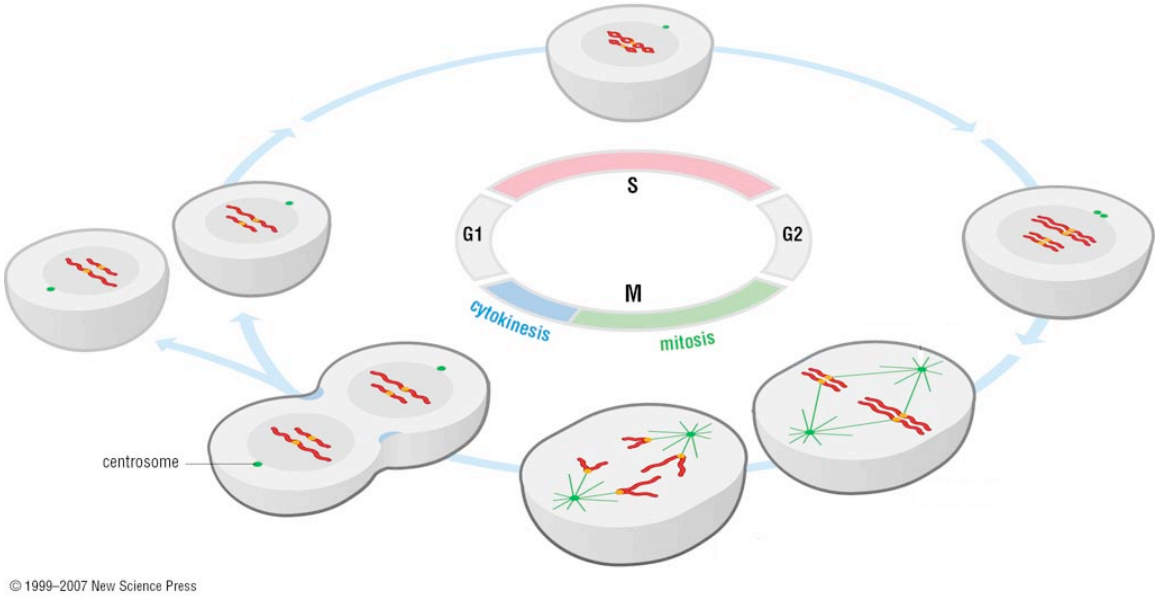


Figure 1. The eukaryotic cell cycle. The main events of cell reproduction are chromosome duplication, which occurs in the S phase, followed by chromosome segregation and nuclear division (mitosis) and cell division (cytokinesis), which are collectively called M phase. Centrosome replication also takes place during S phase, but they will not separate until the onset of mitosis. Following mitosis (M), cells enter G1 stage of interphase, initiating a new cycle. While in G1, cells can exit the cycle and enter a prolonged nondividing state G0 (G zero) and later return to G1. Or, continue through G1 and become committed to cell division. During G2 the cell ultimately prepares for mitosis. (Adapted from Morgan, 2007).

1.2 MITOSIS

Mitosis is a fascinating process that distributes equally the duplicated genome into the two daughter cells. In vertebrate cells, for purposes of description, mitosis is traditionally divided into five phases, based in the gross structural changes that take place: prophase, prometaphase, metaphase, anaphase and telophase (Fig. 2).

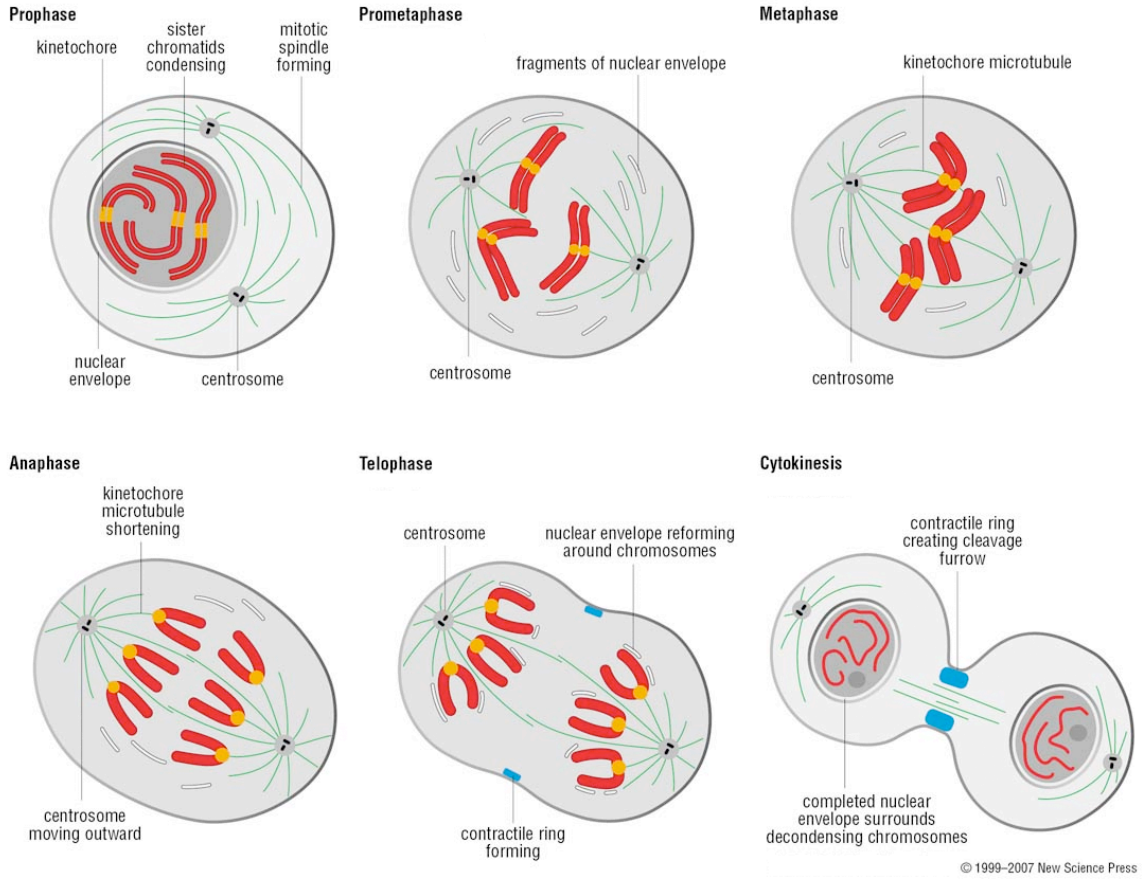


Figure 2. Stages of mitosis and cytokinesis in a vertebrate cell. In the very beginning of mitosis, each chromosome has a sister chromatid and the centrosome as also been replicated. As prophase initiates, the centrosomes begin moving to opposite poles of the cell. The chromatin, which is diffuse in interphase, slowly condenses into well-defined chromosomes. Disruption of the nuclear envelope sets the beginning of prometaphase. Chromosome condensation is now complete and each chromosome is composed of two chromatids held together at their centromeres. The spindle microtubules, which have been lying outside the nucleus, can now enter the nuclear region and interact with the chromosomes. Specialized protein complexes called kinetochores mature on each centromere and attach to some spindle microtubules, then called kinetochore microtubules. These interactions promote chromosome congression that ultimately leads to alignment at the equatorial plane of the cell. At this stage the cell is said to be in metaphase. At anaphase onset, the paired sister chromatids separate (now each called a chromosome) and move towards opposite poles of the cell. Mitosis is completed in telophase, when the chromosomes decondense and the nuclear envelope re-forms around each group of daughter chromosomes. Cytokinesis occurs concomitantly with the later mitotic stages giving rise to two genetically identical daughter cells. (Adapted from Morgan, 2007).

During prophase, the duplicated genetic material undergoes dramatic morphological changes. The DNA is progressively condensed into compact and flexible thread-like structures, known as chromosomes. By late prophase, each chromosome is visible as two identical filaments, the chromatids (or sister chromatids), held together at a region known as the centromere. Each sister chromatid contains a single kinetochore, a specialized protein complex that assembles on each centromere, where the microtubules of the spindle can establish stable interactions with the chromosomes. At the same time, the centrosomes separate and start migrating towards opposite sides of the cell. Concomitantly, extensive microtubule nucleation occurs and a bipolar microtubule array starts to form between them. In higher eukaryotes, prophase ends with breakdown of the nuclear envelope (NEBD) into membrane vesicles that persist around the spindle during mitosis. NEBD allows interaction of the microtubules with the chromosomes for the first time defining the beginning of prometaphase. Apart from the multiple roles that microtubules have during interphase, during mitosis microtubules play a fundamental role ensuring proper chromosome segregation. Microtubules are complex structures made up of α/β-tubulin dimers organized in head-to-tail orientation forming long protofilaments. *In vivo*, microtubules have 13 protofilaments that normally grow from centrosomes forming an open sheet structure that closes as the microtubule grows and thus contain well-defined longitudinal discontinuity. Microtubules grow by addition of α/β-tubulin dimers in the GTP-bound state at their plus-end away from the centrosome forming what is called a GTP-cap at the growing tip while the minus-end is anchored at the centrosome. As the microtubule grows and the sheet closes, at the microtubule lattice, GTP-bound to β-tubulin is rapidly hydrolyzed to GDP, which causes the protofilament to bend outwards favoring microtubule disassembly. Microtubules will tend to grow if the incorporation of GTP-bound subunits occurs faster than the hydrolysis of the GTP. If instead GTP is hydrolysed in all β-tubulin then the GTP-cap will be lost, the protofilaments will bend outwards and the whole sheet will collapse leading to rapid microtubule depolymerization. The balance between microtubule polymerization and depolymerization results in a behavior called microtubule instability where microtubules are constantly growing or shrinking and transitions from the two states are called either catastrophe, leading to microtubule depolymerization or rescue, leading to microtubule polymerization. During mitosis microtubules can be broadly divided into three classes: (1) kinetochore microtubules, those that establish attachment with the kinetochore, (2) polar microtubules, which overlap in the midzone of the spindle and are responsible for pushing the poles of the spindle apart and (3) astral microtubules, which radiate in all directions from the MTOCs towards de cell cortex where they help position the bipolar spindle and later contribute to determine the cleavage plane during cytokinesis. Eventually, stable interactions between

the spindle microtubules and the kinetochores are formed which will mediate chromosome movement. Prometaphase continues until all sister chromatids are attached to the spindle and have migrated to the equatorial plane of the cell, a process known as chromosome congression. When all chromosomes are aligned in the centre of the spindle, the cell is said to be in metaphase and is now ready to segregate the duplicated genome. Anaphase begins with disruption of the links that hold sister chromatids together so that they can now move to opposite poles of the cell due to shortening of kinetochore microtubules. Subsequently, the polar microtubules elongate and the two poles of the spindle move farther apart. Mitosis is completed in telophase, when nuclear envelope re-assembles around the decondensing chromosomes giving rise to two daughter nuclei. The spindle disassembles leaving a single centrosome associated with each nucleus. Cytokinesis or division of the cytoplasm, already starts during anaphase and continues in telophase through the formation of a contractile ring creating a cleavage furrow that ultimately leads to two separate daughter cells.

1.3 THE CELL CYCLE CONTROL SYSTEM

The eukaryotic cell cycle has to be tightly regulated in order to ensure faithful segregation of the genetic material. Accordingly, cells have controls that ensure the correct sequence of events. These control mechanisms are called checkpoints and certify that an event has been properly executed before proceeding to the next event (reviewed by Hartwell and Weinert, 1989). Three checkpoints have been amply documented: the DNA damage checkpoint, which arrests cells in G1, S, G2 or even in mitosis in case of lesions in the DNA (reviewed by Niida and Nakanishi, 2006); the DNA replication checkpoint, which ensures that mitosis is not initiated until DNA replication is complete and that no DNA is replicated twice during an S phase (reviewed by Takeda and Dutta, 2005), and the spindle assembly checkpoint (SAC), also called mitotic checkpoint or spindle checkpoint. The SAC delays metaphase-anaphase transition until all chromosomes are properly attached to the mitotic spindle (reviewed by Musacchio and Salmon, 2007). Failure of these checkpoints result in changes of ploidy and genetic damage that are likely to be critical determinants for cancer development and/or progression (reviewed by Kastan and Bartek, 2004).

The central components of the cell cycle control system are a family of enzymes called the cyclin-dependent kinases (Cdks) and their regulators. Oscillations in the activity of Cdks lead to changes in the phosphorylation state, and thus the state of activation of

proteins that are responsible for changes in the biochemical status of cellular machinery. The first steps towards the identification of these key regulators of the cell cycle were done with the identification, in *Xenopus* eggs, of the maturation-promoting factor, also called mitosis-promoting factor (MPF) (Masui and Markert, 1971; Smith and Ecker, 1971). Later on, activity of this factor that promotes entry of cells into mitosis has been found in mitotic cells of all species assayed. Almost two decades were required for the successful purification of MPF (Lohka et al., 1988), that turned out to be an heterodimeric complex composed of a Cdk (Arion et al., 1988; Dunphy et al., 1988; Gautier et al., 1988; Labbe et al., 1988; Simanis and Nurse, 1986) and a cyclin (Labbe et al., 1989). Concentrations of Cdk proteins are constant throughout the cell cycle and regulation of their activity depends primarily on corresponding oscillations in levels of the regulatory subunits known as cyclins, which bind tightly to Cdks and stimulate their catalytic activity. Cyclins were originally discovered in biochemical studies with sea urchin eggs and embryos due to their cyclical protein level oscillation every cell division (Evans et al., 1983). Different cyclin types are produced at different cell cycle stages, resulting in the formation and activation of different Cdk/cyclin complexes. There are cyclins associated with G1 (cyclin D), S phase (cyclins E and A), and mitosis (cyclins B and A) and each Cdk/cyclin complex promotes the activation of the next in the sequence, thus ensuring that the cell cycle progresses in an ordered fashion (reviewed by Murray, 2004). The activity of Cdk/cyclin complexes is further modulated by the addition or removal of inhibitory phosphorylation, and by changes in the levels of Cdk inhibitor proteins (CKIs) (reviewed in Pines, 1999).

Cell cycle progression is also controlled by proteolysis. Addition of ubiquitin-polymeric chains to specific cell cycle regulators, such as cyclins, is sufficient to target them for proteolytic degradation by abundant the 26S proteasome complex. Ubiquitination of a substrate requires an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an ubiquitin-ligase enzyme (E3). Recognition of targeting proteins is mainly governed by the E3 (reviewed in King et al., 1996). Two related E3 complexes are involved in degradation of cell cycle regulators: the SCF (Skp1/Cullin/F-box protein) and the Anaphase-Promoting Complex/Cyclosome (APC/C). The APC/C is mainly active in mitosis (upon activation by Cdc20/Fizzy) and in the G1 phase of the cell cycle (upon activation by Cdh1/Hct1/Fizzy-related), whose crucial functions are, respectively, initiation of sister chromatid separation at the metaphase-anaphase transition and resetting the cell-cycle clock by achieving and maintaining a low Cdk activity state. The SCF complexes appear to be more versatile, fulfilling a variety of functions at many stages of the cell cycle. Originally both complexes were thought to act in non-overlapping phases of the cell cycle, however, it is now known that there is a high level of crosstalk between APC/C and

different types of SCFs (reviewed by Vodermaier, 2004). Ubiquitin-mediated proteolysis can be additionally controlled by deubiquitinating enzymes (DUBs), that act by removing ubiquitin from ubiquitin-conjugated protein substrates (reviewed in Love et al., 2007).

Additionally, cell cycle control can be regulated by a balance of nuclear import and export of the components of the cell cycle machinery (reviewed by Pines, 1999), as well as by changes in the transcription of regulatory genes (reviewed by Breeden, 2003).

During G1 phase, S- and M-Cdks are kept inactive due to (1) suppression of the major cyclin genes, (2) to the presence of high concentrations of CKIs and (3) through the activation of the APC/C. G1/S cyclins are not targeted by the APC/C^{Cdh1} allowing their accumulation during G1, which leads to activation of G1/S-Cdks. This commits the cell to a new division cycle at the “restriction point” (“start point” in yeast). G1/S-Cdks primarily trigger the destruction of CKIs and inactivate the APC/C^{Cdh1}, leading to activation the S-Cdks. Meanwhile, G1/S-Cdks promote their own inactivation by stimulating destruction of G1/S cyclins. S-Cdk complexes initiate DNA replication and ensure that re-assembly of new pre-replication complexes is prevented, limiting DNA replication to once per cycle (reviewed by Diffley, 2004).

Mitotic Cdk complexes are formed during the S phase and G2, but kept inactive by phosphorylation until completion of DNA synthesis. These phosphorylations are catalyzed by the Myt1 and Wee1 kinases. At the G2/M transition, Myt1 and Wee1 are inactivated while the phosphatase Cdc25 is activated. Cdc25 dephosphorylates Cdk1, allowing for activation of the Cdk1/Cyclin B complex and entry into mitosis (reviewed by Perry and Kornbluth, 2007). Activated Cdk1/Cyclin B complexes phosphorylate several downstream targets inducing chromosome condensation, breakdown of the nuclear envelope, assembly of the mitotic spindle apparatus and alignment of condensed chromosomes at the metaphase plate (reviewed by Morgan, 1997). In addition to Cdk1, several other mitotic kinases (*e.g.* Polo, Aurora, BubR1, Mps1) are known to regulate cell division (for review see Nigg, 2001) and many more are still subject of extensive investigation (Bettencourt-Dias et al., 2004). As the cell reaches metaphase, M-Cdks activate the APC/C by phosphorylating core APC/C subunits, which enhances Cdc20 binding. APC/C^{Cdc20} stimulates the destruction of proteins that hold the sister chromatids together and M-Cdks, thereby inactivating M-Cdks. The resulting inactivation of M-Cdks leads to dephosphorylation of their mitotic substrates, which is required for spindle disassembly and the completion of M phase. Furthermore, as the activating binding of Cdc20 to the APC/C requires phosphorylation by M-Cdks, M-Cdk inactivation leads to decreased phosphorylation of the APC/C, dissociation of Cdc20 and the consequent inactivation of

APC/C^{Cdc20}. However, APC/C activity is maintained, through binding to Cdh1. Cdh1 is prevented from binding to the APC/C from S phase to metaphase due to phosphorylation by Cdks, and at the metaphase-anaphase transition, when M-Cdk is inactivated by the APC/C^{Cdc20}, Cdh1 is dephosphorylated and binds to the APC/C. APC/C^{Cdh1} activity remains high throughout G1, ensuring S- and M-Cdk inactivity until G1/S-Cdk activity rises again and the cell is committed to another cell cycle (Morgan, 2007).

2. THE KINETOCHORE

2.1 STRUCTURE

Classical electron microscopy studies revealed the kinetochore as a plate-like trilaminar structure (Brinkley and Stubblefield, 1966; Jokelainen, 1967; McEwen et al., 1993; Rieder, 1982; Roos, 1973) (Fig. 3). In all eukaryotes, the kinetochore assembles on specialized nucleosomes (fundamental chromatin packing units), where histone H3 is replaced by the H3 variant CenH3 (also known as Cse4 in budding yeast, CID in *Drosophila* and CENP-A in humans). CENP-A is required for the recruitment of other inner kinetochore proteins, such as CENP-C, CENP-H and CENP-I/hMis6, forming the electron-dense inner-plate (for review see Chan et al., 2005). Separating the outer plate from the inner plate is a light-staining electron-translucent middle plate that is likely to be an artifact produced by conventional fixation and/or dehydration procedures (McEwen et al., 1998). The outer plate is composed of proteins whose primary function is to link the inner kinetochore to the microtubules. In the absence of microtubules, a meshwork of fibers, termed fibrous corona, can be seen extended from the surface of the outer plate (Ris and Witt, 1981). The fibrous corona and the outer plate contain the majority of the known kinetochore proteins that interact with microtubules (*e.g.* CENP-E, CLIP-170, Ndc80/Hec1) as well as spindle assembly checkpoint proteins (*e.g.* Bub1, Bub3, Mad1, Mad2, Mad3/BubR1, Mps1) (reviewed in Chan et al., 2005; Maiato et al., 2004a).

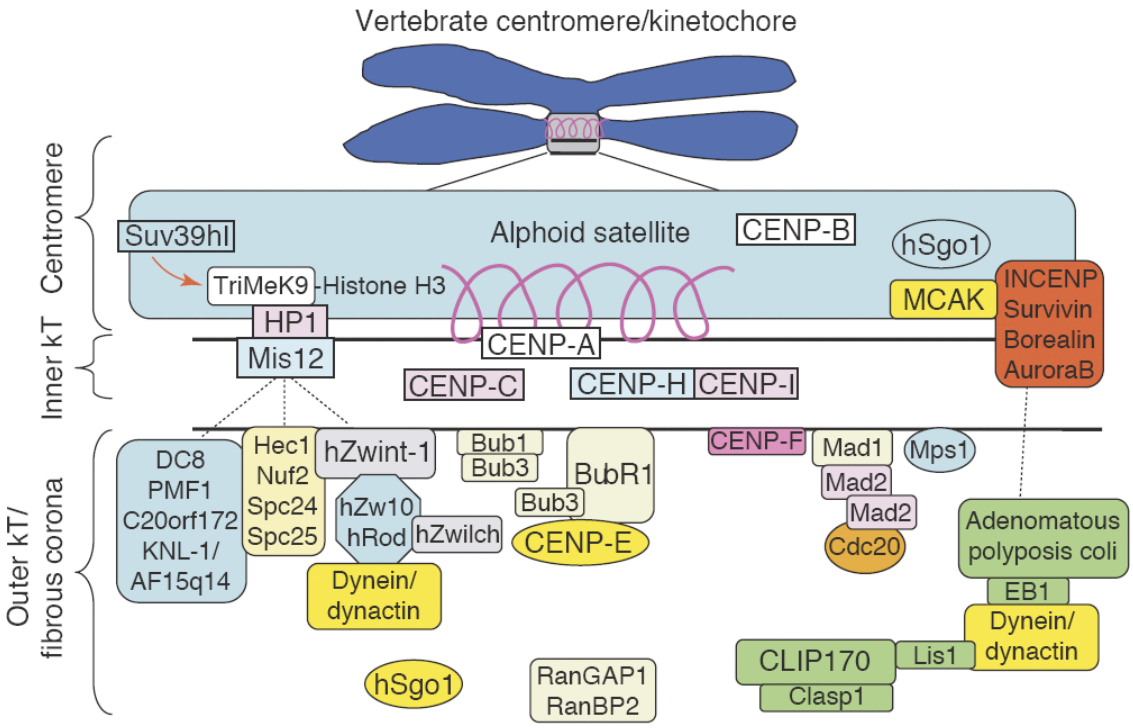


Figure 3. Organization of the vertebrate centromere/kinetochore. Schematic representation of the spatial distribution of centromeric and kinetochore proteins. The coil at the centromere depicts the proposed helical path organization of the chromatin fiber, where CENP-A-bearing nucleosomes are exposed as repeat subunits at the inner plate of the kinetochore. The majority of the kinetochore proteins are located at the outer kinetochore/fibrous corona and include microtubule-binding proteins (depicted in yellow), proteins that bind specifically to the growing ends of the microtubules (in green) and spindle assembly checkpoint proteins (adapted from Chan et al., 2005).

The size and complexity of kinetochores vary considerably among different species. The budding yeast, *Saccharomyces cerevisiae*, has very small kinetochores, which are not visible by standard light or electron microscopy and only bind a single microtubule. Nevertheless, despite their small size, these kinetochores are highly complex showing a hierarchical set of interdependencies among different kinetochore complexes (reviewed in McAinsh et al., 2003). *Drosophila melanogaster* S2 cells in culture have kinetochores that are similar to vertebrate kinetochores, although S2 mitotic kinetochores bind, on average, 11 microtubules compared with 17 microtubules that mitotic kinetochores of human cells in culture bind (Maiato et al., 2006; McEwen et al., 2001; Wendell et al., 1993). Some plants, *Caenorhabditis elegans,* and other animals have holocentric chromosomes, where the kinetochores cover the whole length of the chromosome, therefore the binding of microtubules is along the entire length of the

chromosome (reviewed by Dernburg, 2001). However, despite of the reported differences, many kinetochore proteins are conserved from yeast to humans including also species with holocentric chromosomes (Maiato et al., 2006; Oegema et al., 2001; Westermann et al., 2003).

2.2 PROTEIN CONSTITUENTS

Early efforts to identify the molecular composition of the kinetochores were hampered by the low abundance of constituent proteins and because these proteins are required for cell viability. The first steps towards their identification came with the use of anticentromere antibodies (ACA) in sera, derived from patients that developed the CREST Syndrome (a form of Systemic Sclerosis, showing Calcinosis, Raynaud's phenomenon, Esophageal dysmotility, Sclerodactyly, Telangiectasia). These sera recognized three major antigens, CENP-A, CENP-B and CENP-C (Earnshaw and Rothfield, 1985).

CENP-A is a histone-H3 variant (Palmer et al., 1991; Sullivan et al., 1994) and *in vivo* and *in vitro* experiments suggest that it is probably a centromeric nucleosome component (Vafa and Sullivan, 1997; Warburton et al., 1997). Subsequent studies, done in different model organisms, have shown that CENP-A is essential for kinetochore formation and function, as well as for centromere identity (Black et al., 2007; Blower and Karpen, 2001; Heun et al., 2006; Howman et al., 2000; Sullivan, 2001).

CENP-B binds to a 17 bp sequence (the CENP-B box) in alphoid DNA, a human centromeric satellite DNA, and forms a dimer (Earnshaw et al., 1987; Masumoto et al., 1989; Muro et al., 1992; Yoda et al., 1992). Studies done in knockout mice for *CENP-B* have revealed that CENP-B is not essential for mitosis or meiosis and no major defects were found regarding aspects of centromere assembly and function (Hudson et al., 1998; Kapoor et al., 1998; Perez-Castro et al., 1998). Therefore, it has been difficult to attribute a kinetochore function for CENP-B. However, studies with mammalian artificial chromosomes suggest that the CENP-B/CENP-B box interaction has some crucial roles for assembling other essential centromere/kinetochore components on alphoid DNA (Ikeno et al., 1998; Masumoto et al., 1998; Ohzeki et al., 2002; Suzuki et al., 2004).

CENP-C can bind the same alpha-satellite DNA as CENP-B but in distinct non-overlapping centromere domains (Politi et al., 2002). Antibody-injection experiments, analysis of conditional *CENP-C* knockouts in chicken cells and analysis of *CENP-C*-

knockout mice have shown that CENP-C is involved in kinetochore assembly, chromosome segregation and mitotic checkpoint function (Fukagawa and Brown, 1997; Kalitsis et al., 1998; Kwon et al., 2007; Tomkiel et al., 1994).

The identification of novel centromere-kinetochore associated proteins involved the use of monoclonal antibodies against human mitotic chromosome scaffolds (Compton et al., 1991; Earnshaw et al., 1984). One of the proteins identified by this method, was CENP-E (Yen et al., 1991; Yen et al., 1992). Immunoelectron microscopy studies combined with conventional fluorescence microscopy have revealed that CENP-E localizes to the fibrous corona, extending at least 50 nm away from the kinetochore outer plate, from prometaphase to anaphase (Cooke et al., 1997; Yao et al., 1997). In later stages of mitosis, CENP-E appears associated with the interpolar microtubules, at the midzone of the spindle, and finally with the cleavage furrow (Cooke et al., 1997; Yao et al., 1997; Yen et al., 1991). At the protein level, CENP-E has a cyclin-like accumulation and degradation, reaching its peak during G2 and early M phase followed by rapid degradation during the completion of mitosis (Brown et al., 1994). It is a kinesin motor protein (Yen et al., 1992) initially reported to contain minus end-directed motility (away from the kinetochores) (Thrower et al., 1995). However, later it was shown that the recombinant motor domain moves towards microtubule plus ends (growing ends of the microtubules) *in vitro* (Wood et al., 1997). Recently, CENP-E directionality was further investigated and demonstrated that full-length recombinant CENP-E is a genuine plus end-directed motor and that CENP-E tail, through direct interaction with the motor domain, is able to completely block CENP-E motility *in vitro* (Espeut et al., 2008; Kim et al., 2008). Furthermore, it was shown that CENP-E autoinhibition can be reversed by Mps1- or Cdk1-Cyclin B-mediated phosphorylation of the CENP-E tail (Espeut et al., 2008). Disruption of CENP-E function, by various methods, partially impairs metaphase chromosome alignment in *Xenopus* egg extracts (Wood et al., 1997), in *Drosophila melanogaster* (Goshima and Vale, 2003; Yucel et al., 2000) as well in vertebrate tissue cultured cells (Kapoor et al., 2006; Putkey et al., 2002; Schaar et al., 1997; Tanudji et al., 2004; Yao et al., 2000). However, other reports show that cells with reduced levels of CENP-E are able to align the chromosomes in a metaphase plate (Johnson et al., 2004; McEwen et al., 2001; Weaver et al., 2003), suggesting that there are redundant mechanisms for chromosome alignment (McEwen et al., 2001).

The next CENP- protein to be identified was CENP-F (also named Mitosin). It is a ≈400 kDa protein originally recognized by a human autoimmune serum and shown to have an expression and localization pattern similar to CENP-E (Liao et al., 1995; Rattner et al., 1993). Loss of function studies, by RNA interference, show that CENP-F-depleted

cells fail to accomplish metaphase chromosome alignment, anaphase chromosome segregation and cytokinesis. Moreover, repression of CENP-F causes a prolonged mitotic arrest that culminates in cell death before anaphase initiation through apoptosis (Bomont et al., 2005; Holt et al., 2005; Yang et al., 2005).

More recently, purification of CENP-A nucleosomes from human cells has identified a network of proteins that is constitutively present at centromeres and that includes CENP-C and 13 interacting proteins (CENP-H, CENP-I, CENP-K-U) (Foltz et al., 2006; Izuta et al., 2006; Obuse et al., 2004; Okada et al., 2006). This entire group of proteins, based on their colocalization and co-purification with CENP-A nucleosomes throughout the cell cycle in vertebrates, was proposed to be called the constitutive centromere-associated network (CCAN) (Cheeseman and Desai, 2008). Depletion of components of the CCAN often leads to cell-cycle arrest, kinetochore-assembly defects, severe chromosome missegregation and loss of kinetochore function (reviewed by Cheeseman and Desai, 2008).

In addition to centromere proteins (CENPs), several other proteins such as microtubule-associated proteins (MAPs) and SAC proteins associate with kinetochores during mitosis. Cytoplasmic dynein, a microtubule-associated motor protein that has microtubule minus end-directed motion (Lye et al., 1987), was also shown to localize to kinetochores (Pfarr et al., 1990; Steuer et al., 1990) but exclusively to the fibrous corona (Wordeman et al., 1991). Cytoplasmic dynein functions in close cooperation with the dynactin complex (dynamitin, Arp1 and $p150^{Glued}$) that also localizes to kinetochores (Faulkner et al., 1998). Another example is CLIP-170, that besides being a MAP is not a member of any motor family (Dujardin et al., 1998; Pierre et al., 1992). CLIP-170 has been shown to be important for proper chromosome congression (Dujardin et al., 1998; Tanenbaum et al., 2006). Likewise, the *bona fide* SAC proteins Bub1, Bub3, Mad1, Mad2, BubR1 and Mps1 localize to kinetochores (Abrieu et al., 2001; Basu et al., 1999; Basu et al., 1998; Chan et al., 1999; Chen et al., 1998; Chen et al., 1996; Jablonski et al., 1998; Li and Benezra, 1996; Martinez-Exposito et al., 1999; Taylor et al., 1998; Taylor and McKeon, 1997). The role of the SAC proteins during mitosis will be addressed later in this general introduction. Currently, around 80 kinetochore proteins have been identified in humans (for recent review see Cheeseman and Desai, 2008).

3. MICROTUBULE-KINETOCHORE ATTACHMENT

When the nuclear envelope breaks down, cytoplasmic microtubules gain access to the condensing chromatin, and although microtubules may eventually interact with the whole chromosome, the kinetochore is the major microtubule attachment site. The best understood mechanism of chromosome attachment is the one of animal cells, mainly dependent on the centrosomes. Centrosomes nucleate microtubules in all directions, which grow, elongate and then shrink back suddenly until become stabilized at the kinetochores. This process is known as “search-and-capture” and relies on the “dynamic instability” property of the microtubules (Heald and Nogales, 2002; Kirschner and Mitchison, 1986). However, the search-and-capture mechanism is not efficient enough to explain the typical observed duration of prometaphase (Wollman et al., 2005). Furthermore, this mechanism is unlikely to be effective in cells lacking centrosomes. A RanGTP gradient (Carazo-Salas et al., 2001; Kalab et al., 1999) may introduce a spatial bias into microtubule dynamics, thus improving the efficiency of search-and-capture (Wollman et al., 2005). More recently it has also been demonstrated that growth of microtubules from the kinetochores might play a role during spindle assembly and organization (Khodjakov et al., 2003; Maiato et al., 2004b; Tulu et al., 2006).

3.1 MICROTUBULE BINDING TO KINETOCHORES

Extensive work has been done in order to determine which are the components of the core kinetochore-microtubule attachment site. However, this as been a difficult task to accomplish since interference with most of the known kinetochore components results in chromosomes that are not attached to the mitotic spindle; not because these proteins are indeed an essential docking site for microtubule binding but as a secondary consequence of the Ipl1/Aurora kinase-mediated correction mechanism that destabilizes defective microtubule attachments (Cimini et al., 2006; Dewar et al., 2004; Lampson et al., 2004; Pinsky et al., 2006). The KNL1/Mis12 complex/Ndc80 complex (KMN) network, previously shown to be conserved in multiple organisms and essential for viability and kinetochore-microtubule interactions (reviewed in Kline-Smith et al., 2005), was suggested to be the core kinetochore-microtubule attachment site (Cheeseman et al., 2006). Two distinct microtubule-binding activities within the network were identified – one intrinsic to the Ndc80 and Nuf2 subunits of the Ndc80 complex, and a second intrinsic to KNL1

(Cheeseman et al., 2006). Furthermore, it was also shown that the N-terminus of Hec1/Ndc80 specifically regulates microtubule polymerization and depolymerization of the growing end, at kinetochores (DeLuca et al., 2006). Moreover, the crystal structure of the most conserved region of Hec1/Ndc80 was recently solved, and it was shown that an Ndc80p-Nuf2p heterodimer binds microtubules *in vitro*; and the less conserved, N-terminal segment of Ndc80p contributes to the interaction and may be a crucial regulatory element (Wei et al., 2007). These results suggest that Ndc80/Hec1 complex is the core kinetochore-microtubule binding site.

3.2 CORRECTION OF DEFECTIVE MICROTUBULE ATTACHMENTS. BIORIENTATION

Chromosomes can establish different types of interactions with spindle microtubules but only when sister kinetochores in a pair are attached to opposite spindle poles, resulting in biorientation or amphitelic attachment, the success of chromosome segregation is achieved. At the beginning of prometaphase, most attachments are monotelic, with one kinetochore bound to microtubules from one spindle pole and the other kinetochore unbound, these chromosomes are also called monoriented. Ideally, the unattached kinetochore then becomes attached to the opposite pole but that is not always the case and both sister kinetochores can attach to the same spindle pole, syntelic attachment, or one kinetochore can attach to both spindle poles, merotelic attachment. Incorrect attachments have then to be corrected but how the kinetochore detects these abnormalities is a matter of intense investigation. The generation of tension across sister kinetochores seems to play a major role. Tension, or some consequence of tension, stabilizes the proper spindle attachment (Nicklas and Ward, 1994) and increases the number of kinetochore microtubules, although within certain limits (King and Nicklas, 2000). Although biorientation results in the generation of tension at kinetochores, merotelic attachments are likely to also generate kinetochore tension, resulting in stable microtubule attachments, ultimately leading to aneuploidy (Cimini et al., 2001). Several lines of evidence suggest that the mitotic protein kinase Aurora B can correct attachments that do not generate tension (Hauf et al., 2003; Lampson et al., 2004; Pinsky et al., 2006; Tanaka, 2002) and even correct merotelic attachments (Cimini et al., 2006; Knowlton et al., 2006). Aurora B, together with its regulatory subunits, is located at the centromeres and phosphorylates numerous kinetochore components, including Ndc80/Hec1 complex (Cheeseman et al., 2002) leading to active destabilization of kinetochore microtubules (reviewed by Vader and Lens, 2008). In fact, *in vitro* phosphorylation of Ndc80 subunit of

the Ndc80 complex, by Ipl1/Aurora, reduces its microtubule-binding affinity (Cheeseman et al., 2006). Moreover, cells expressing a Hec1 N-terminal mutant that prevents Aurora B phosphorylation exhibit an increase in the frequency of erroneous microtubule-kinetochore attachments, inducing centromere hyper-stretch and decreasing the fidelity of chromosome segregation (DeLuca et al., 2006).

3.3 CHROMOSOME MOVEMENTS AND CONGRESSION

During mitosis, chromosomes undergo a series of orchestrated movements that ultimately lead to their alignment at the centre of the cell (Fig. 4), a process known as chromosome congression.

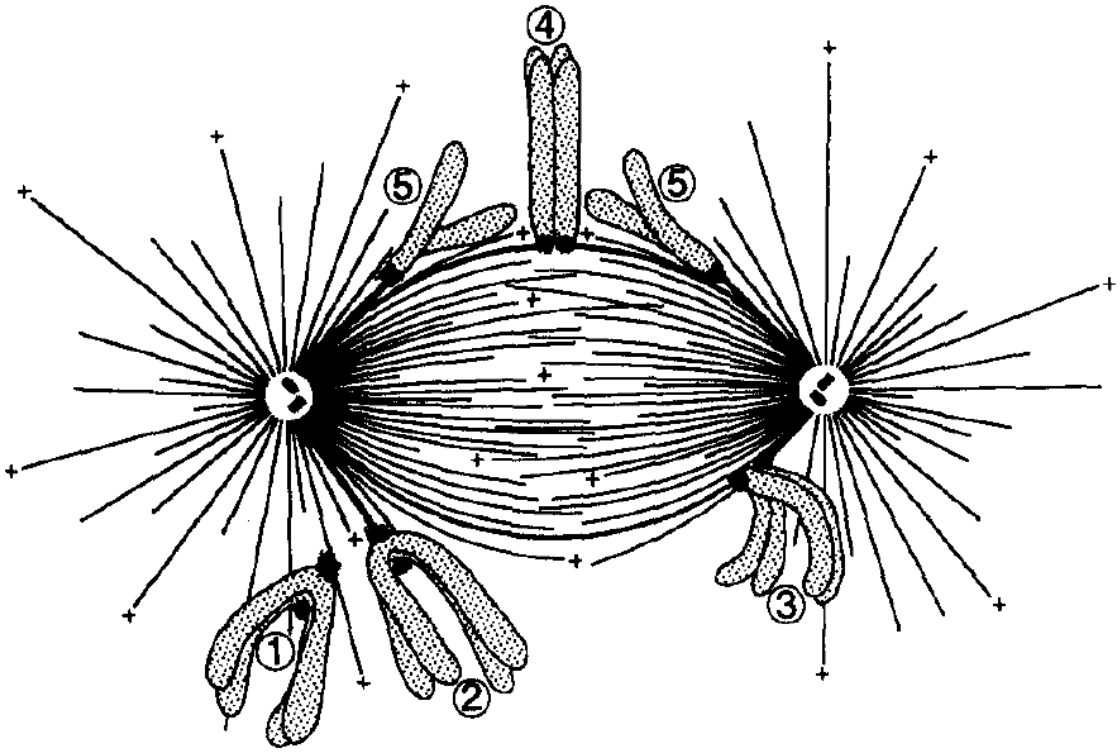


Figure 4. Chromosome movements during mitosis. (1) An attaching chromosome in which one kinetochore is exhibiting rapid poleward movement along the surface of an astral microtubule; (2) a monoriented chromosome oscillating between poleward and away from the pole phases of motion; (3) a bioriented chromosome, near one pole which is initiating congression; (4) a fully congressed metaphase chromosome that is oscillating around the spindle equator; and (5) chromatids in anaphase undergoing poleward motion (adapted from Rieder and Salmon, 1994).

Although, generally assumed that chromosome biorientation is a prerequisite for congression, it was recently shown that monoriented chromosomes could glide toward the spindle equator alongside kinetochore fibers attached to other already bioriented chromosomes (Kapoor et al., 2006), or even along any microtubule bundle without the requirement of the trailing kinetochore to be attached (Cai et al., 2009). Upon biorientation of all chromosomes sister chromatids move poleward during anaphase (Fig. 4) (Rieder et al., 1994).

Chromosome movements towards or away from the spindle poles are associated with depolymerization or polymerization of kinetochore microtubules, respectively. Switches between these two states are thought to be responsible for the alignment of the chromosomes in the spindle equator during prometaphase and known as kinetochore bi-stability. The trigger controlling this bi-stable system is thought to be tension at the kinetochore: low tension promotes the switch to kinetochore microtubules depolymerization and high tension promotes polymerization (reviewed in Maiato et al., 2004a). How these physical forces are translated into signaling at individual centromeres was recently addressed and found that centromere tension can be sensed by increased spatial separation of Aurora B from kinetochore substrates, which reduces their phosphorylation and stabilizes kinetochore microtubules (Liu et al., 2009).

There are three major forces acting on chromosomes to move them on the mitotic spindle: (1) Most of the poleward force is generated by the kinetochore due to microtubule depolymerization at the chromosome-bound end (also called “Pac-Man” mechanism) (Centonze and Borisy, 1991; McIntosh et al., 2002; Rieder and Salmon, 1998). (2) Microtubule flux, which depends on the active translocation of the entire microtubule toward the spindle pole coupled to minus end depolymerization and plus end polymerization (Cassimeris, 2004; Mitchison, 1989). (3) And the polar ejection force or polar wind. This force opposes the poleward force due to interaction of microtubules growing out from the spindle poles with the chromosome arms, pushing them away from the poles (Brouhard and Hunt, 2005; Rieder et al., 1986).

3.4 COUPLING KINETOCHORE-MICROTUBULE ATTACHMENT TO MICROTUBULE-POLYMERIZATION DYNAMICS AND CHROMOSOME MOVEMENTS

Kinetochores generate physical interactions with microtubules that have to be stable enough to resist the force exerted by the mitotic spindle but must also possess the ability to remain attached as the microtubules polymerize and depolymerize. Studies in budding yeast have identified a ten subunit microtubule-binding complex, the Dam1 complex (also called DASH) (Cheeseman et al., 2001; Jones et al., 2001) that oligomerizes to form rings that encircle the microtubules (Miranda et al., 2005; Miranda et al., 2007; Wang et al., 2007; Westermann et al., 2005). Follow-up studies have shown that the Dam1 complex slides along microtubule polymers (Asbury et al., 2006; Westermann et al., 2006) and couples movement of cargo to depolymerizing microtubules (Grishchuk et al., 2008). These particular characteristics create a dynamic kinetochore-microtubule interface that can explain how kinetochores remain attached to growing and shortening microtubules due to the existence of sleeves or collars proposed twenty-five years ago (Hill, 1985). Despite thorough searches, a Dam1 homologue in higher eukaryotes remained to be identified. However, very recently, four different groups identified a novel component of the Ska complex, Rama1/Ska3/C13orf3 previously shown to be required for the maintenance of the metaphase plate (Hanisch et al., 2006), and demonstrated that this complex is required to generate stable kinetochore-microtubule attachments (Gaitanos et al., 2009; Raaijmakers et al., 2009; Theis et al., 2009; Welburn et al., 2009). More interestingly, it was shown that the full Ska1 complex forms assemblies on microtubules that can facilitate the processive movement of microspheres along depolymerizing microtubules (Welburn et al., 2009). These characteristics are highly similar to the budding yeast Dam1 complex and so the Ska complex may have analogous functions in higher eukaryotes (Guimaraes and Deluca, 2009).The kinetochore localization of most of the proteins involved in the microtubule-kinetochore interface described so far is shown below (Figure 5).

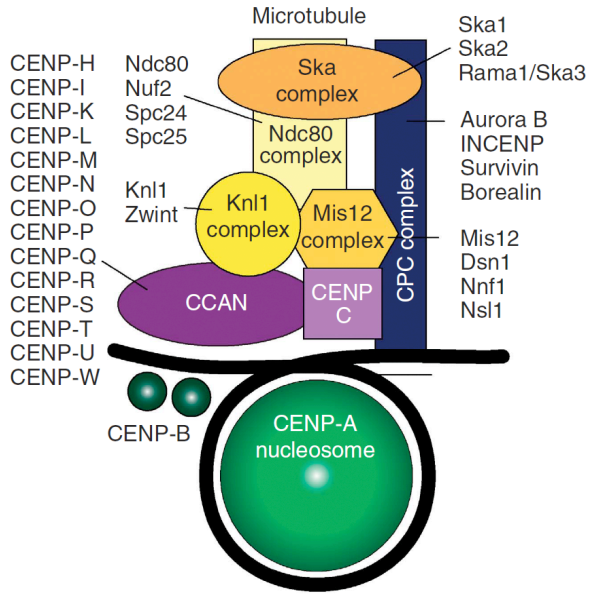


Figure 5. Schematic representation of the metazoans kinetochore-microtubule interface. Adapted from Santaguida and Musacchio, 2009 .

3.5 MOLECULAR MACHINERY INVOLVED IN KINETOCHORE MOTILITY

Several microtubule-associated proteins (MAPs) can associate transiently or constitutively with microtubules and are capable of modifying their dynamic properties thereby controlling kinetochore microtubule dynamics (reviewed in Maiato et al., 2004c). These include microtubule-depolymerizing proteins such as the kinesin-13 family (*e.g.* Kif2a and MCAK) (Desai et al., 1999; Homma et al., 2003). MCAK is the best-characterized member of the family. However, perturbation of its function, by several means, does not lead to severe defects in chromosome movement (Ganem et al., 2005; Kline-Smith et al., 2004; Maney et al., 1998). Instead, leads to increased frequency of lagging chromatids in anaphase; suggesting that MCAK utilizes microtubule depolymerase activity to perform other functions than chromosome motility, such as error-correction (Bakhoum et al., 2009; Knowlton et al., 2006; Lan et al., 2004; Wordeman et al., 2007). On the other hand, Kif2a (also called Klp10A in *Drosophila*) depolymerizes microtubules at their pole-associated minus ends contributing for poleward microtubule flux (Gaetz and Kapoor, 2004; Rogers et al., 2004). Also members of the kinesin-8 family can depolymerize microtubules but specifically at the kinetochore-associated plus end, contributing to chromosome congression (Gupta et al., 2006; Mayr et al., 2007; Stumpff et al., 2008). Conversely, perhaps counteracting the activity of the depolymerases, microtubule-plus-end-tracking proteins (+TIPs), such as APC, EB1, CLIP-170 and CLASPs, promote polymerization (reviewed by Schuyler and Pellman, 2001). The

adenomatous polyposis coli (APC) protein and its binding partner end-binding protein 1 (EB1) localize to the kinetochores in a microtubule-dependent manner (Fodde et al., 2001; Kaplan et al., 2001; Su et al., 1995; Tirnauer et al., 2002). Binding of EB1 to microtubule plus ends at the kinetochore interface is restricted to polymerizing microtubules (Tirnauer et al., 2002), promoting the stabilization of the microtubule lattice (Sandblad et al., 2006) and stimulating nucleation, sheet growth and closure (Vitre et al., 2008). CLIP-170 and its binding partners the CLASPs (CLIP-associated proteins) (Akhmanova et al., 2001) can localize to kinetochores even in the absence of microtubules (Dujardin et al., 1998; Lemos et al., 2000). CLIP-170 facilitates the formation of kinetochore-microtubule attachments (Tanenbaum et al., 2006) and it was recently found that its plus-end-tracking requires EB1 (Bieling et al., 2008; Dixit et al., 2009). CLASPs have been shown to promote the growth of kinetochore-bound microtubules (Maiato et al., 2003a; Maiato et al., 2005) and the available data are fully consistent with a model in which MAST/CLASP1 accumulates at the outer kinetochore corona and maintain attachment to the microtubules plus ends, while allowing the incorporation of new tubulin subunits (Maiato et al., 2004c).

4. THE KINETOCHORE AND THE SPINDLE ASSEMBLY CHECKPOINT

Evidence that progression through mitosis is carefully monitored was first obtained with the use of drugs that depolymerise microtubules and promote a prolonged mitotic arrest in vertebrate cells (Brues AM, 1936; Brues AM, 1937; Zieve et al., 1980). Afterwards, the existence of a quality-control checkpoint at metaphase was proposed (Nicklas and Arana, 1992). While it is now widely accepted that the SAC delays metaphase-anaphase transition until all chromosomes are properly bioriented in the mitotic spindle, the sensors, the nature of the signals, and the transduction pathways that promote the delay are still subject of intense investigation. The first evidence that the SAC monitors centromere function was obtained in budding yeast (Spencer and Hieter, 1992). It was shown that the presence of an abnormal centromere could induce a mitotic delay. Subsequently, live analysis of vertebrate cells and laser microsurgery unequivocally demonstrated that kinetochores that fail to form proper microtubule attachment send a “wait-anaphase” signal (Rieder et al., 1995; Rieder et al., 1994).

4.1 SAC AND ITS MOLECULAR COMPONENTS

Genetic screens designed to isolate mutations for which the mitotic arrest is overridden in the presence of microtubule depolymerising drugs allowed the identification of the Mad1, -2 and -3 (mitotic arrest deficient) and Bub1, -2 and -3 (budding uninhibited by benomyl) genes in budding yeast (Hoyt et al., 1991; Li and Murray, 1991). The Mad and Bub proteins are conserved throughout evolution and are essential to impose a mitotic arrest in response to microtubule damage in all organisms and cell lines tested so far (Basu et al., 1999; Bernard et al., 1998; Gorbsky et al., 1998; Li and Benezra, 1996; Taylor and McKeon, 1997). The only exception was the identification in higher eukaryotes of the Bub-Related-1 kinase (BubR1) which displays homology in its N-terminal part with the yeast Mad3 protein and in its C-terminal part with the kinase domain of Bub1 (Taylor et al., 1998). Immunolocalization studies of the Mad and Bub proteins in several model systems have shown that they localize to kinetochores that are unattached or under reduced microtubule tension (Chen et al., 1998; Logarinho et al., 2004; Taylor et al., 1998). The kinetochore localization of these proteins is thought to play a central role in broadcasting the “wait-anaphase” signal (Musacchio and Salmon, 2007). Biochemical studies have shown that these proteins can be purified from a variety of complexes including Mad1-Mad2, Bub1-Bub3, and Bub3-BubR1/Mad3 during mitosis (Basu et al., 1998; Campbell and Hardwick, 2003; Campbell et al., 2001; Chung and Chen, 2002; Hardwick et al., 2000; Roberts et al., 1994; Tang et al., 2001; Taylor et al., 1998). Formation of these complexes appears to be essential for their targeting to kinetochores during prometaphase and to trigger a SAC response that delays mitotic progression (Musacchio and Salmon, 2007). At the molecular level, several studies suggest that activation of the SAC involves the formation of inhibitory complexes between Mad2 and/or Mad3/BubR1 and Cdc20, preventing Cdc20 from activating the E3 ubiquitin-ligase APC/C (Hwang et al., 1998; Kim et al., 1998; Wu et al., 2000). These inhibitory complexes are thought to form at unattached kinetochores where Mad2, BubR1 and Cdc20 show a very fast turnover (Howell et al., 2004).

Biochemical experiments at the G2-M transition also identified a multisubunit complex, the Mitotic Checkpoint Complex (MCC). This complex contains the BubR1-Mad2-Bub3-Cdc20 proteins and strongly inhibits APC/C activity causing a delay in mitotic exit (Sudakin et al., 2001; Tang et al., 2001). Interestingly, the formation of the MCC does not require unattached kinetochores since the complex is present well before NEBD. Thus, a “two-step” model for the activation and maintenance of SAC activity has been

proposed (Chan et al., 2005). In this model, a first step involves the formation of the MCC during cell cycle progression from G2 to M to allow cyclin accumulation and mitotic entry. The second step takes place after NEBD when the SAC proteins can bind unattached kinetochores, producing additional MCC inhibitory complexes to sustain SAC activity until all kinetochore pairs are properly attached and congression is achieved (Figs. 6 and 7). Subsequent studies both in yeast (Fraschini et al., 2001) and *Drosophila* (Lopes et al., 2005) strongly support this model.

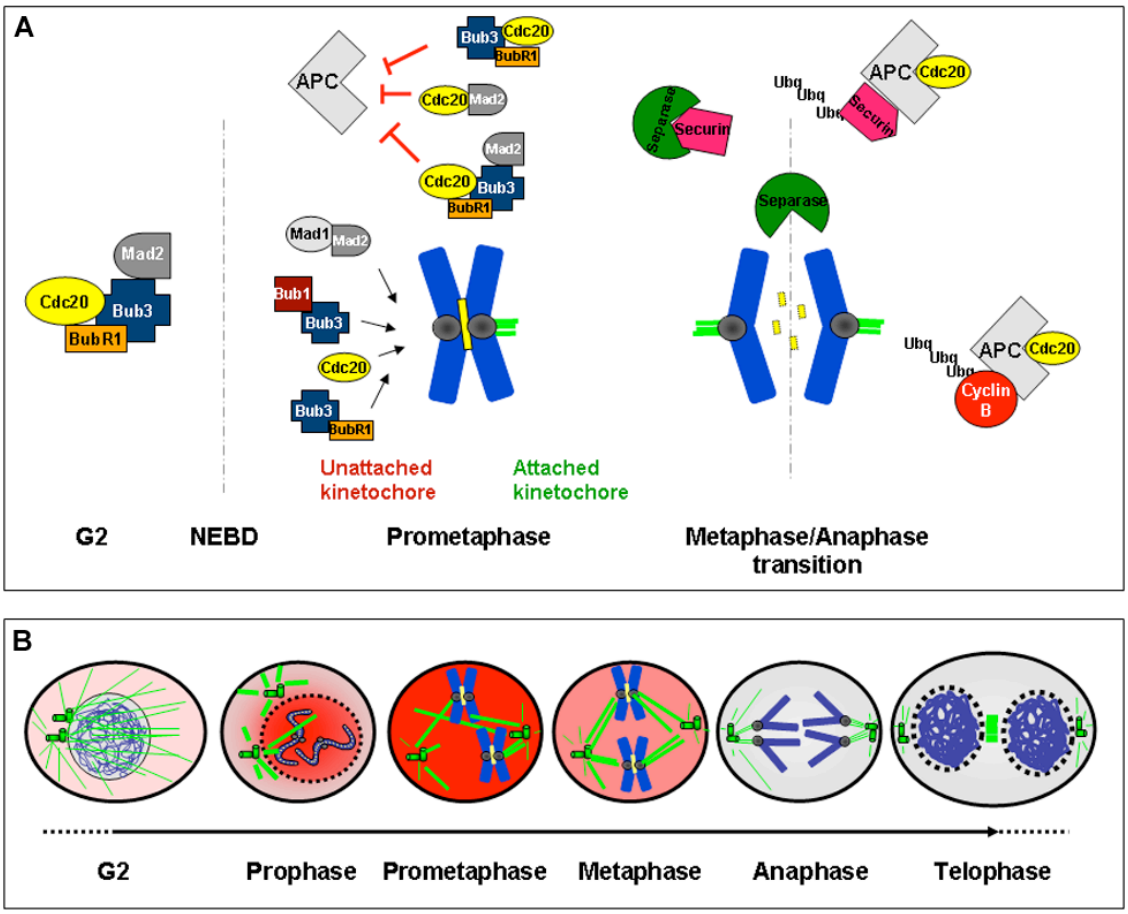


Figure 6. Diagram showing mitotic progression and the Spindle Assembly Checkpoint. (A) Before entering mitosis, the MCC (Mad2-BubR1-Bub3-Cdc20) is already formed. After NEBD, checkpoint protein complexes are recruited to unattached kinetochores originating more MCC that inhibits the APC/C. This ensures sufficient timing for chromosome congression. When achieved, APC/C is no longer inhibited and triggers the ubiquitination of securin and cyclin B targeting them for destruction by the 26S proteasome. Separase becomes active and cohesin is cleaved leading to anaphase onset. **(B)** Schematic representation of mitosis highlighting the accumulation and degradation of cyclin B, shown in background red.

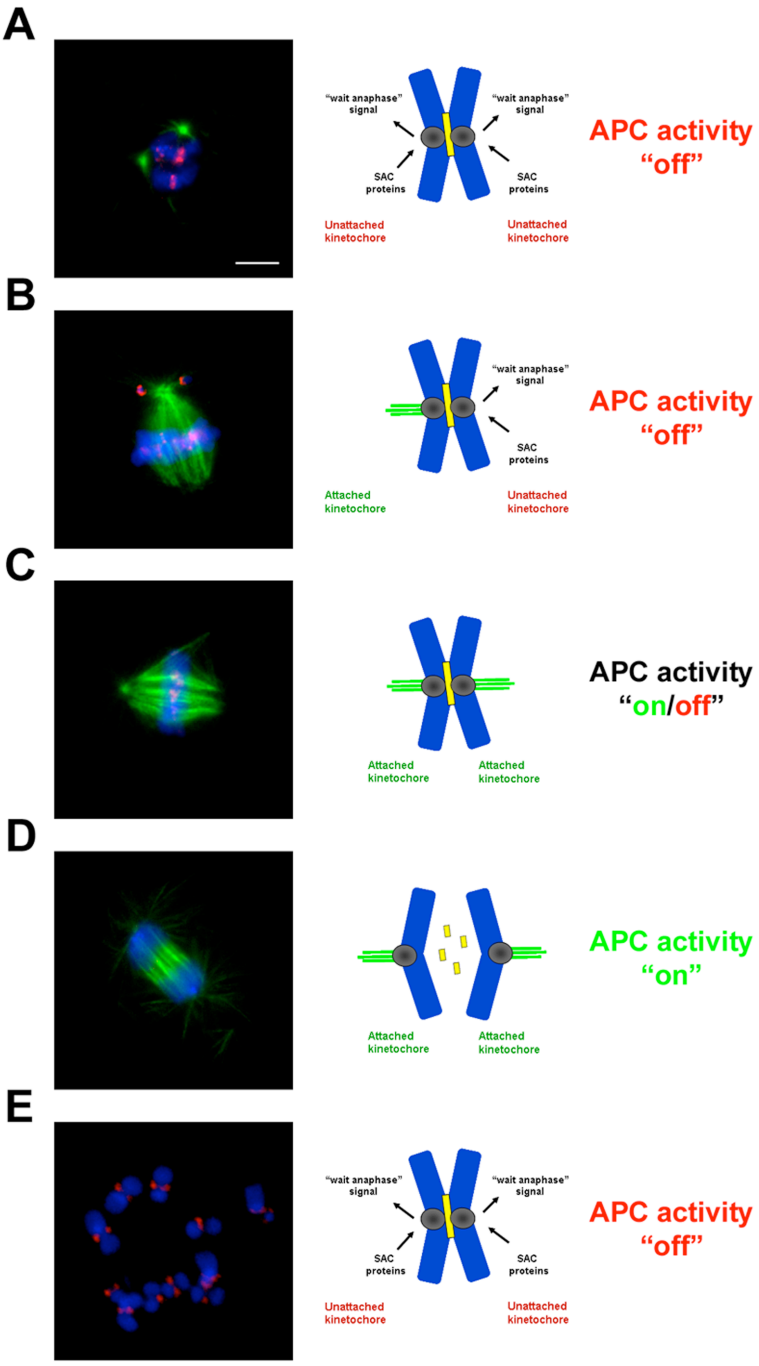


Figure 7. Example of a *bona fide* SAC protein (BubR1) kinetochore localization, at different mitotic stages, in *Drosophila* S2 cell line. (A) During prophase, since there is no attachment of microtubules to the kinetochores, BubR1 strongly localizes to the kinetochores of condensing chromosomes. Both kinetochores send a "wait-anaphase signal" inhibiting the APC/C. **(B)** Then, during prometaphase, the localization is diminished at the kinetochores of chromosomes that align at the centre of the cell. The lagging chromosomes show robust localization of BubR1 indicating that the SAC is giving extra time for chromosome alignment. **(C)** When all chromosomes align at the metaphase plate the signal is reduced. **(D)** At the metaphase-anaphase transition the inhibitory signal in no longer generated and APC/C becomes active ultimately promoting sister chromatid separation. **(E)** When cells are treated with microtubule depolymerising drugs the SAC proteins are highly recruited to the unattached kinetochores promoting an extensive mitotic arrest. DNA is in blue, α-Tubulin in green and *Dm*BubR1 in red. Bar is 5 μm.

Additional molecular components that are required for SAC signaling in metazoans were further identified, including Mps1 (Hardwick et al., 1996; Weiss and Winey, 1996), the RZZ complex (reviewed in Lu et al., 2009), the deubiquitinating enzyme USP44 (Stegmeier et al., 2007), the human pre-messenger RNA processing 4 (PRP4) protein kinase (Montembault et al., 2007), the DNA-dependent adenosine triphosphatase (ATPase) Plk1-interacting checkpoint helicase (PICH) (Baumann et al., 2007) and the microtubule-affinity regulating kinase TAO1 (also known as MARKK) (Draviam et al., 2007). However, for the latter, PICH and TAO1, it was recently reported that the siRNA-based implication of these proteins in the SAC reflects an off-target effect on the SAC protein Mad2 (Hubner et al., 2009).

4.2 SAC MECHANISM OF ACTION

Studies on the Mad2 checkpoint protein put it as a central regulator of the mitotic checkpoint machinery (De Antoni et al., 2005; Luo et al., 2002; Luo et al., 2004; Xia et al., 2004; Yang et al., 2008). These results lead to the proposal of a molecular model for Mad2 activation and binding to Cdc20 thus explaining rapid APC/C inactivation (reviewed by Ciliberto and Shah, 2009). This model relies in the experimental evidence that Mad2 has two different native conformations, open Mad2 (O-Mad2, also referred to as $Mad2^{N1}$) and closed Mad2 (C-Mad2, also referred to as $Mad2^{N2}$). Before checkpoint activation, freely diffusible monomeric Mad2 is though to exist largely as O-Mad2, its inactive conformation. When bound to Mad1, Mad2 adopts the closed conformation (Luo et al., 2002; Sironi et al., 2002), the active form. Thus Mad2 activation requires a transient dimerization (Mapelli et al., 2007; Yang et al., 2008) that occurs at the unattached kinetochore. The closed Mad2 form can also induce a second Mad2 molecule, normally in the open conformation in the cytoplasm, to acquire the active conformation (De Antoni et al., 2005; Yang et al., 2008), thus contributing for the amplification of SAC signaling. Active Mad2 binds to Cdc20 resulting in a Mad2:Cdc20 complex that can become a full MCC complex either at the kinetochore (Howell et al., 2004; Shah et al., 2004) or in the cytoplasm (Essex et al., 2009; Kulukian et al., 2009) by binding the BubR1:Bub3 complex. Less well understood are the mechanisms for relieving APC/C inhibition and allowing the metaphase to anaphase transition. The current idea is that upon biorientation of the last unattached kinetochore the inhibitory signal of the APC/C is no longer generated and cells enter anaphase (Musacchio and Salmon, 2007). The RZZ complex (Rod-Zw10-Zwilch) is a key candidate to contribute for the silencing of the inhibitory signal generation. This

complex is recruited to the kinetochores where establishes a docking site for the molecular motor Dynein (Starr et al., 1998). In turn, Dynein, upon microtubule attachment, is able to transport Mad2 to the spindle poles (Howell et al., 2001; Sivaram et al., 2009) thus contributing to a reduction of Mad2 concentration at the kinetochores. Also, in mammalian cells, $p31^{comet}$ (formerly known as Cmt2) forms a transient complex with APC/C^{Cdc20}-bound Mad2 counteracting Mad2 function and thus contributes to checkpoint inactivation (Xia et al., 2004). Ubiquitination of Cdc20 in the context of the MCC:APC/C complex is also thought to play a role in this process. It was proposed that APC/C itself in complex with its E2 enzyme UbcH10 can ubiquitinate Cdc20 and possibly other components of the MCC:APC/C protein complex, leading to the release of Mad2 (Reddy et al., 2007). This process can be counteracted by the deubiquitinating enzyme USP44 that is able to remove ubiquitin from Cdc20 (Stegmeier et al., 2007). Furthermore, in yeast, evidence exists that APC/C-mediated proteosomal degradation of SAC components occurs leading to irreversible inactivation of the checkpoint in anaphase (Palframan et al., 2006).

4.3 THE KINETOCHORE IS A HUB FOR KINASE SIGNALING

Kinetochore localization of checkpoint proteins is hierarchical, meaning that the recruitment of some depends on the prior recruitment of others. Although not all studies are in full accordance, most probably due to the diversity of systems used in the different experiments (Chen, 2002; Johnson et al., 2004; Lampson and Kapoor, 2005; Orr et al., 2006), it is commonly accepted that Aurora B, Mps1, and Bub1 lie at the top of this hierarchy (Boyarchuk et al., 2007; Emanuele et al., 2008; Liu et al., 2006; Vigneron et al., 2004). These protein kinases and others, apart from constituting a platform over which a fully functional kinetochore is built, play critical roles in the spindle checkpoint signaling either participating in the generation and/or amplification of this signal. However, the mechanism (or mechanisms) by which they inhibit mitotic progression remains a matter of intense investigation (reviewed by Kang and Yu, 2009). The Aurora B kinase plays a major role in promoting biorientation, as described previously (section 3.2). However, in the process of doing so generates unattached kinetochores, a specific situation that is known to promote SAC activity (Pinsky et al., 2006). Furthermore, there are several lines of evidence indicating that the generation of unattached kinetochores is not the sole manner by which the chromosomal passenger complex (CPC), Aurora B and its regulatory subunits (INCENP, Survivin, Borealin), exerts control over the SAC. First, HeLa

cells blocked in mitosis by nocodazole (a situation where none of the kinetochores are attached and the microtubule destabilizing activity of Aurora B is not relevant) do eventually exit mitosis prematurely after chemical Aurora B inhibition (Hauf et al., 2003). Second, RNAi mediated-depletion of CPC components influences the robustness of the mitotic arrest of nocodazole-treated cells (Ditchfield et al., 2003; Lens et al., 2003; Vader et al., 2007). Third, Aurora B becomes critical for a nocodazole-induced mitotic checkpoint arrest in the absence of Bub1 (Morrow et al., 2005), suggesting it might collaborate with other kinases to enforce the mitotic arrest. Fourth, experiments in *Xenopus* tissue culture cells as well as studies in fission yeast have shown a requirement for Aurora B activity in spindle checkpoint function in response to unattached kinetochores (Kallio et al., 2002; Petersen and Hagan, 2003). And fifth, when Aurora B is in complex with an INCENP molecule that lacks its coiled-coil domain, Aurora B's microtubule destabilizing function and hence its capacity to create unattached kinetochores is still intact, yet is incapable of supporting checkpoint function (Vader et al., 2007). Together, these findings provide strong evidence that the CPC influences spindle checkpoint function also in a more direct function, independent of its well-established destabilizing effect on microtubule-kinetochore interactions (reviewed in Vader et al., 2008).

Mps1 was first described in *Saccharomyces cerevisiae* as an essential protein kinase for spindle pole body (SPB) duplication (Lauze et al., 1995; Winey et al., 1991). Subsequently, it was found to have a role in the mitotic checkpoint not only in budding yeast (Weiss and Winey, 1996) but also in higher eukaryotes (Abrieu et al., 2001; Liu et al., 2003; Stucke et al., 2002). Recently, a role in correction of improper chromosome attachments was also attributed. It was reported that Mps1 phosphorylates Borealin/DasraB on residues that are crucial for Aurora B activity and chromosome alignment (Jelluma et al., 2008). Interestingly, Mps1 is not the only kinase that apart from playing a role in the SAC also coordinates attachment error correction. The *bona fide* SAC proteins Bub1 and BubR1 are themselves required for proper kinetochore-microtubule interactions (reviewed by Logarinho and Bousbaa, 2008). Bub1 appears to regulate chromosome attachment through an Aurora B independent pathway (Meraldi and Sorger, 2005) whilst BubR1 seems to act by inhibiting Aurora B kinase (Lampson and Kapoor, 2005).

The number of kinases reported to be involved in the SAC machinery keeps expanding nowadays: The PRP4 protein kinase associates with kinetochores during mitosis and is required for the recruitment or maintenance of the checkpoint proteins Mps1, Mad1 and Mad2 at the kinetochores (Montembault et al., 2007).

Thus the molecular understanding of the SAC response while still far from clear has advanced significantly revealing a highly complex surveillance mechanism that at the centre appears to monitor microtubule-kinetochore interactions.

5. A WEAK SPINDLE CHECKPOINT MIGHT FACILITATE TUMORIGENESIS

It was Theodor Boveri who almost 100 years ago first proposed that abnormal distribution of chromosomes could be the cause for tumor development (Boveri, 1914; Harries, 2008). Indeed subsequent work has indicated that most solid tumors are aneuploid and many cell lines derived from cancer cells show chromosomal instability (CIN). Since the discovery of the SAC and some of its molecular components, a recurrent question has been whether aneuploidy is a cause or a consequence of malignant transformation (Holland and Cleveland, 2009).

Studies using Chinese hamster ovary cell lines exposed to microtubule poisons or Topoisomerase II drugs for prolonged periods of time were shown to undergo severe genomic instability associated with loss of the SAC response (Andreassen et al., 1996). Subsequently, oncogenic proteins such as the SV40 large T antigen (Chang et al., 1997) and the papillomavirus oncoproteins E6 and E7 (Thomas and Laimins, 1998) were shown to inactivate the SAC and promote aneuploidy. Also, it was shown that Mad1 is the target of the T-cell leukaemia virus oncoprotein Tax suggesting that down regulation of the SAC could be involved in the development of this tumor (Jin et al., 1998). In 1998, Cahill and colleagues reported mutations in Bub1 or BubR1 in colorectal cancer cell lines examined (Cahill et al., 1998). Later, several mutations or deletions were found in Bub1 and BubR1 genes in samples of leukemia and lymphoma cells (Ohshima et al., 2000; Ru et al., 2002) as well as in families with mosaic variegated aneuploidy (MVA) syndrome, in the case of the latter (Matsuura et al., 2006). Also, reduced levels of Bub1 were reported in some colon carcinoma (Shichiri et al., 2002) and acute myeloid leukaemia (Lin et al., 2002). Furthermore, studies have shown that altered Mad2 expression could be detected in nasopharyngeal carcinoma (Wang et al., 2000), breast cancer cell lines (Percy et al., 2000), ovarian cancer (Wang et al., 2002) and gastric cancers (Kim et al., 2005).

However, there are many studies that failed to detect mutations in any SAC protein when a large number of human samples were investigated (reviewed by (Lopes and Sunkel, 2003)), thus providing mixed results. In fact, there have been a small number of mutations (8/177) found on the Mad1 gene isolated from a panel of tumor derived cell

lines of primary cancer samples (Tsukasaki et al., 2001) and one mutation in a lung cancer (Nomoto et al., 1999).

Even though most cancer-derived samples do not contain mutations in SAC genes, and cancer cells exist that do have a robust SAC (Tighe et al., 2001), many tumor-derived cell lines do show partial or complete loss of SAC response for which the molecular nature of the defect underlying SAC inefficiency is not known. Why then, were mutations in SAC genes not found associated with the vast majority of human tumors? Conventional gene knockouts have been constructed for almost all known mitotic checkpoint genes, including Mad1, Mad2, Bub1, Bub3, BubR1 (Babu et al., 2003; Iwanaga et al., 2007; Kalitsis et al., 2000; Michel et al., 2001; Perera et al., 2007; Wang et al., 2004) and complete loss of function of these gene products leads to early embryonic lethality whereas heterozygous and hypomorphic mice are viable and fertile. It has been shown that deletions of either *mad2* (Dobles et al., 2000) or *bub3* (Kalitsis et al., 2000) cause severe chromosome mis-segregation during early mouse development leading to apoptosis and early developmental arrest. Similarly, mutation of either *bubR1* or *bub3* in *Drosophila* was shown to cause lethality in late stages of development (Basu et al., 1999; Lopes et al., 2005). Accordingly, complete inactivation of Mad2 (Michel et al., 2001) or BubR1 (Kops et al., 2004) in tumor derived cell lines causes massive chromosome missegregation and subsequence cell death within 2-6 divisions, leading to the suggestion that this might provide an interesting therapeutic approach (Kops et al., 2005). Nevertheless, *mad2*-null *Drosophila* is viable and fertile and their cells almost always divide correctly despite having no SAC (Buffin et al., 2007).

All together, these observations suggest that it is unlikely that loss of the SAC response could be the primary cause of tumor formation. Instead, the available data is much more consistent with the view that a weakened SAC response could facilitate tumor development in cells that are undergoing tumorigenesis. Actually, a recent study analyzing the consequences of gradual increases in chromosome segregation errors in the viability of tumor cells and normal human fibroblasts, found that partial inhibition of the human checkpoint proteins BubR1 and Mps1, both involved in checkpoint activation and chromosome alignment, sensitizes the tumor cell lines but not untransformed human fibroblasts to low doses of taxol due to increased severity of chromosome segregation errors (Janssen et al., 2009). Thus, suggesting that a weak spindle checkpoint and an increase in chromosome segregation errors might selectively kill tumor cells.

6. MITOTIC PROGRESSION AS A DRUG TARGET FOR CANCER THERAPY

Cancer cells have an increased dividing potential therefore significant effort has been spent on the discovery of new chemotherapeutic agents that specifically could kill cells in mitosis. Several drugs that interfere with microtubules often lead to failure in alignment of the daughter chromosomes and their bipolar attachment to the mitotic spindle. Targeted cells fail to pass through the checkpoints that ensure equal distribution of the genome to the daughter cells leading to mitotic arrest at the metaphase-anaphase transition, followed by apoptosis. Antimitotic drugs are a major group of antitumor agents that act either preventing microtubule assembly (tubulin polymerization inhibitors), such as Vinca alkaloids and Colchicine, or preventing the depolymerization of tubulin (microtubule-stabilizing agents), such as Taxanes. However, this classification is too simplistic because both microtubule-destabilizing and stabilizing agents, at low concentrations, potently suppress microtubule dynamics without affecting microtubule polymer mass, but they retain their ability to block cells in mitosis and induce apoptosis (Chen et al., 2003; Derry et al., 1995; Jordan et al., 1993; Jordan et al., 1996; Yvon et al., 1999). Microtubule depolymerising drugs when used at high concentrations inhibit incorporation of new tubulin subunits at the microtubule plus end resulting in a decrease in microtubule polymer mass. Yet, their anti-mitotic action seems to be more related to the suppression of microtubule dynamics than to a decrease in the microtubule polymer mass (Jordan et al., 1992). This suppression causes a metaphase-anaphase arrest often with chromosomes at the spindle poles rather than at the metaphase plate. After a prolonged arrest these cells eventually die by apoptosis (Jordan et al., 1996). Microtubule stabilizing agents unlike the Vinca alkaloids and Colchicine, promote tubulin polymerization, stabilizing microtubules (Schiff et al., 1979) and inducing microtubule assembly by lowering the critical concentration of tubulin required for polymerization (Schiff et al., 1979; Schiff and Horwitz, 1980). Despite of having a significant impact on the treatment of a wide variety of cancers, these agents suffer from significant problems. Since microtubules are crucial for a variety of important functions in cells, as axonal transport within neurons, these agents often cause severe neuropathies (Markman, 2003). Another concern is the development of resistance, including reduced intracellular drug accumulation due to increased drug efflux by multi-drug resistant (MDR) protein pumps, such as P-glycoprotein. These drug efflux transporters can efficiently pump anticancer agents out of cells lowering intracellular drug concentrations (Vinca alkaloids and Taxanes are both substrates for P-glycoprotein) (Gottesman, 1993; Greenberger et al., 1988; Liscovitch and Lavie, 2002). Another major mechanism underlying resistance is the direct alteration in tubulin, which includes

mutations that stabilize tubulin assembly (Hari et al., 2003), mutations in tubulin-binding sites (Giannakakou et al., 2000; Giannakakou et al., 1997; Gonzalez-Garay et al., 1999; Kavallaris et al., 2001), altered expression of tubulin isotypes (Kavallaris et al., 1997) and altered binding of microtubule-associated proteins (Kavallaris et al., 2001; Zhang et al., 1998). Resistance could thus be overcome with compounds that are not substrates for efflux pumps or with drugs that bind to novel sites on tubulin. Nevertheless, its is clear that due to the high mutation rate of cancer cells new mutations will arise resulting in synthesis of altered tubulins which will display drug resistance. Accordingly, a huge effort has been done in order to find target proteins that function exclusively during mitotic division. Among these proteins are Aurora kinases, Polo-like kinases and mitotic kinesins. The rational for targeting these proteins is that non-dividing differentiated cells should not be significantly affected and a therapeutic improvement is expected since cancer cells divide more frequently. These studies are at very preliminary stage yet they have revealed that targeting kinases that regulate spindle assembly and function or are involved with progression through mitosis in various ways could result in a major advance in cancer therapy (Gascoigne and Taylor, 2009). It is expected that in the very near future cancer chemotherapy will become increasingly more sophisticated and specific allowing a much more efficient control and management of tumor development and progression.

7. OBJECTIVES

The work reported in this thesis aims to understand the role of the kinetochore as a signal generator for the Spindle Assembly Checkpoint while at the same time functions as a vehicle through which the chromosome moves within the mitotic spindle during early stages of congression and how the combination of such functions ensures faithful chromosome segregation. In Chapter 1, we study the function of the *bona fide* checkpoint protein BubR1 and the kinesin-7 motor protein CENP-E in the SAC and in the stability of microtubule-kinetochore attachments. In Chapter 2, we continue addressing the role of CENP-E in chromosome congression and segregation. Finally, in Chapter 3, we identify an unknown *Drosophila* kinetochore protein and briefly describe its sub-cellular localization.

PART II

EXPERIMENTAL WORK

CHAPTER 1

Role of BubR1 and CENP-E in the stability of microtubule-kinetochore attachments

1. INTRODUCTION

Faithful inheritance of a complete genome requires proper chromosome segregation at each cell division. This process relies on the correct interaction between spindle microtubules and the kinetochore, a specialized structure localized on the surface of each sister chromatid (Brinkley and Stubblefield, 1966; Luykx, 1965). The kinetochores of each sister chromatid must establish stable functional interactions with microtubules from opposite spindle poles in order to allow chromosome congression and correct chromatid segregation at anaphase (Maiato and Sunkel, 2004; Rieder and Salmon, 1998). Kinetochores that fail to form a proper interaction with microtubules produce a "wait-anaphase" signal (Rieder et al., 1995) activating the spindle assembly checkpoint (SAC) that prevent exit from mitosis. Ultimately, the SAC is thought to provide time so that all chromosomes can attach correctly and congress to the metaphase plate. Several molecular mechanisms may contribute to these movements including action of kinetochore motors and the intrinsic dynamicity associated with stably-bound kinetochore microtubules (Rieder and Salmon, 1994). Unexpectedly, at the time this study was initiated, others have shown that SAC proteins also perform specific functions in chromosome congression. Human cultured cells depleted for BubR1 often show abnormal prometaphases with the chromosomes distributed along the length of the spindle rather than at the metaphase plate (Ditchfield et al., 2003; Lampson and Kapoor, 2005). Also, Bub1 depletion leads to permanent accumulation of misaligned chromosomes (Meraldi and Sorger, 2005; Morrow et al., 2005) in which both sister kinetochores are linked to microtubules abnormally (Meraldi and Sorger, 2005). Although these studies provided insights on the contribution of checkpoint proteins to chromosome congression, it is well known that the kinetochore-associated motor protein CENP-E, is also involved. CENP-E was first identified as localizing to the kinetochores of congressing chromosomes (Yen et al., 1991; Yen et al., 1992) and is required for the stabilization of kinetochore microtubules (Lombillo et al., 1995; Putkey et al., 2002). Disruption of CENP-E function was shown to impair metaphase chromosome alignment in *Xenopus* egg extracts (Wood et al., 1997), as well in *Drosophila melanogaster* (Goshima and Vale, 2003; Yucel et al., 2000) and vertebrate tissue cultured cells (Kapoor et al., 2006; Putkey et al., 2002; Schaar et al., 1997; Tanudji et al., 2004; Yao et al., 2000). Furthermore, early studies on the SAC components showed CENP-E to co-immunoprecipitate with BubR1 suggesting that both proteins may function as a complex at kinetochores (Chan et al., 1998; Yao et al., 2000). Interestingly, CENP-E was also shown to be required for the SAC in *Xenopus* egg extracts (Abrieu et al., 2000; Mao et al., 2003), but this was not the case in human tissue

cultured cells (McEwen et al., 2001; Schaar et al., 1997; Tanudji et al., 2004; Yao et al., 2000). Somewhat conflicting data was also obtained when it was shown that, in human cells, metaphase alignment could occur in the absence of CENP-E if cells are arrested in mitosis (Johnson et al., 2004; McEwen et al., 2001; Weaver et al., 2003). Together, all these results suggest that BubR1 and CENP-E play important roles in microtubule-kinetochore interaction, chromosome congression and SAC activity but given the diversity of systems used in the different studies it is difficult to determine their specific roles in these processes. To further study the role of BubR1 and CENP-E in microtubule-kinetochore attachments, both proteins were individually and simultaneously depleted from *Drosophila* S2 cells and a detailed phenotypic analysis during progression through mitosis was carried out. This analysis has revealed that BubR1 and CENP-meta have very different roles during mitotic progression with BubR1 acting essentially to promote microtubule-kinetochore attachment while CENP-meta appears to be required to destabilize microtubule-kinetochore attachments.

2. RESULTS

2.1 DEPLETION OF BUBR1 AND CENP-META FROM *DROSOPHILA* CULTURED CELLS BY DSRNA INTERFERENCE

In order to study the role of BubR1 and CENP-E during chromosome congression advantage was taken of the very efficient knockdown of protein levels in *Drosophila* cultured cells using double-stranded RNA interference (dsRNAi) (Clemens et al., 2000). *Drosophila* has two kinetochore CENP-E-like proteins, CENP-meta and CENP-ana (Yucel et al., 2000). However, CENP-meta seems to be the primary kinetochore kinesin used for congression since mutation of the CENP-ana gene leads to a dramatic increase in anaphase figures rather than an increased frequency of prometaphase/metaphase as seen in CENP-meta mutants (Yucel et al., 2000). Furthermore, simultaneous depletion of CENP-ana and CENP-meta by RNAi yielded an identical phenotype to CENP-meta single RNAi (Goshima and Vale, 2003). Therefore, in this study the analysis is concentrated on CENP-meta, which according to previous results and those obtained here, suggest that it is indeed the closest homologue to CENP-E. The *Drosophila* BubR1 homologue has been identified before (Logarinho et al., 2004). Analysis of BubR1 and CENP-meta protein levels after RNAi by western-blot showed that they were severely depleted (>90%) after 120h of treatment (Fig. 1.1A and 1.1B). Then immunofluorescence was done to confirm the level of depletion and to determine whether depletion of one affected the ability of the other to localize to kinetochores. To prevent exit from mitosis cells were arrested in a checkpoint independent manner by incubation with the proteasome inhibitor MG132 (Genschik et al., 1998; Oliveira et al., 2005) and microtubules depolymerized prior to fixation and immunostaining (Fig. 1.1D). Both BubR1 and CENP-meta fail to be detected by immunofluorescence confirming the very high level of depletion. Furthermore, under these conditions BubR1 and CENP-meta localized to kinetochores independently of each other (Fig. 1.1D). Simultaneous dsRNAi treatment against both proteins showed that BubR1 can be depleted to more than 90% while CENP-meta depletion reached 80% after 96h of treatment (Fig. 1.1C). Nevertheless, neither protein was detected by immunofluorescence (Fig. 1.1D).

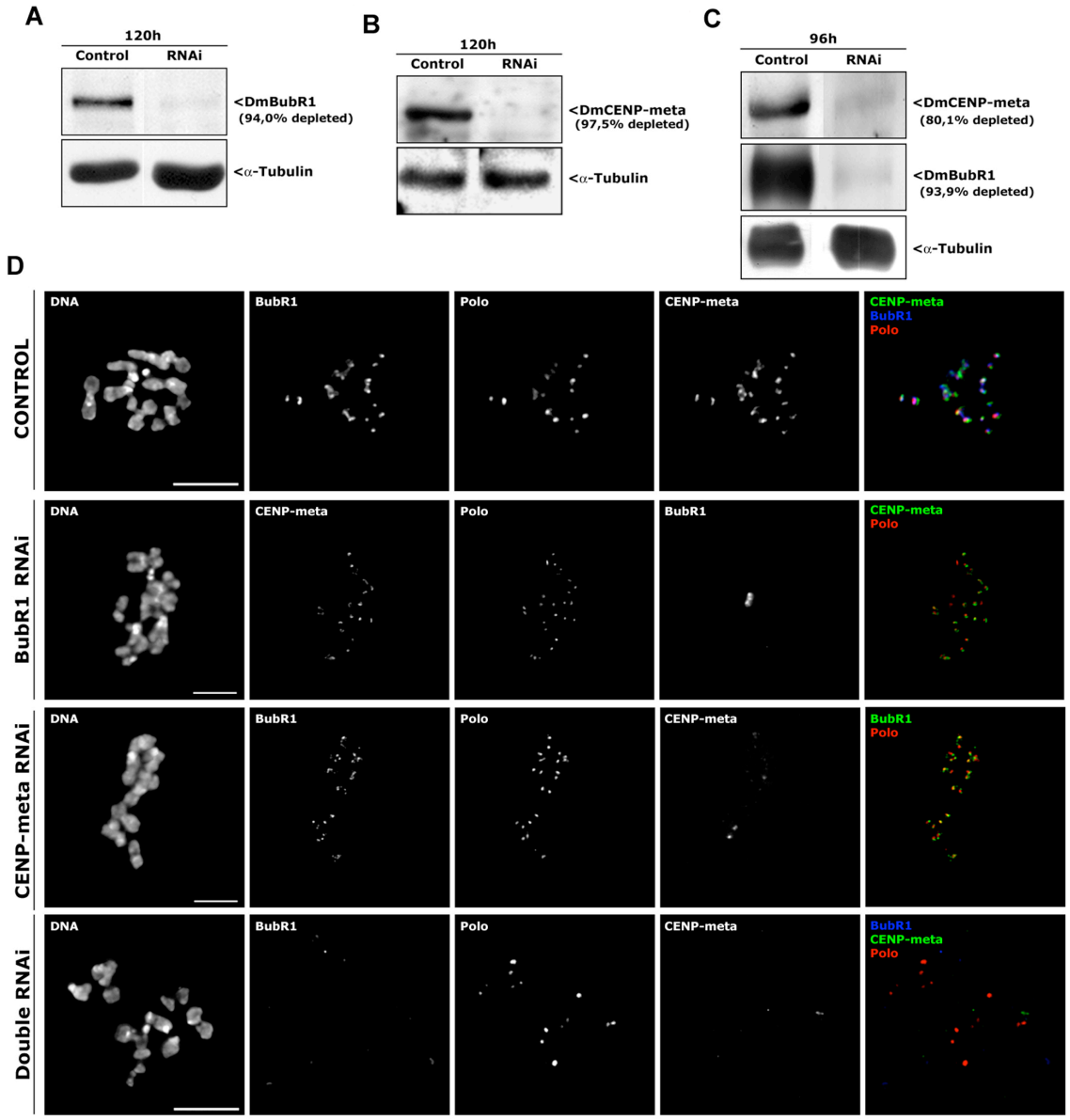


Figure 1.1 Depletion of BubR1 and CENP-meta from *Drosophila* S2 cells by dsRNA interference. Total protein extracts from 10^6 cells were separated by SDS-PAGE and the protein levels of **(A)** BubR1 and **(B)** CENP-meta or **(C)** both proteins was monitored by Western blot. α-tubulin was used as loading control and quantifications were performed using Image J software. **(D)** Cells depleted for either 120h (single RNAis) or 96h (double RNAi) were incubated in MG132 for 2h and then in colchicine for 3h to depolymerize microtubules prior to fixation and immunofluorescence. Depletion of BubR1 and CENP-meta was confirmed by immunofluorescence. Note that kinetochore localization of CENP-meta and BubR1 occurs independently of each other. Scale bar is 5 mm.

2.2 ANALYSIS OF MITOTIC PROGRESSION AFTER DEPLETION OF BUBR1 OR CENP-META

As observed for other cell types (Ditchfield et al., 2003; Lampson and Kapoor, 2005) depletion of BubR1 in *Drosophila* S2 cells causes abnormal chromosome congression. *In vivo* time-lapse microscopy in S2 cells stably expressing GFP-H2B showed that control cells align all the chromosomes in a metaphase plate before starting anaphase whilst BubR1-depleted cells undergo anaphase onset shortly after NEBD without aligning the chromosomes (Fig. 1.2 and Movies 1.1 and 1.2).

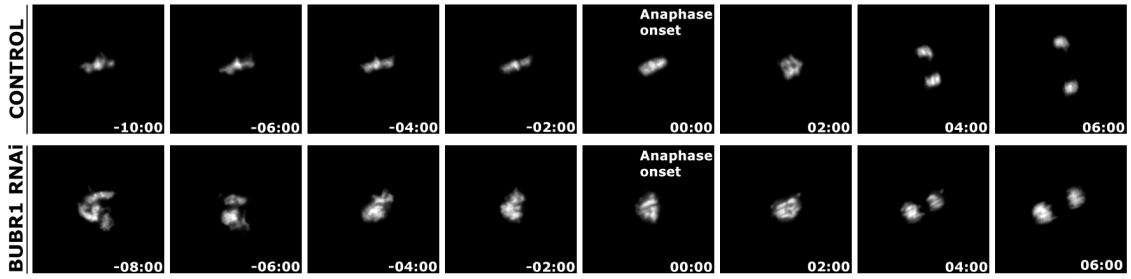


Figure 1.2 *In vivo* analysis of mitotic progression after depletion of BubR1. Selected images from time-lapse movies of control (upper panel) and BubR1-depleted cells (lower panel). Anaphase onset was set as time zero. Time mm:ss.

Given that BubR1 is required for the SAC, it is possible that in the absence of BubR1 cells progress and exit mitosis too rapidly to reach congression. Indeed, BubR1-depleted cells spend less time in prometaphase due to premature degradation of Cyclin B (Fig. 1.3A and 1.3B), sister chromatids separate prematurely (Fig. 1.3C) and exit mitosis faster than control cells (Fig. 1.3D-E and Movies 1.3 and 1.4). Therefore, in the absence of BubR1 cells might fail to reach metaphase just because there is not enough time to allow congression to take place.

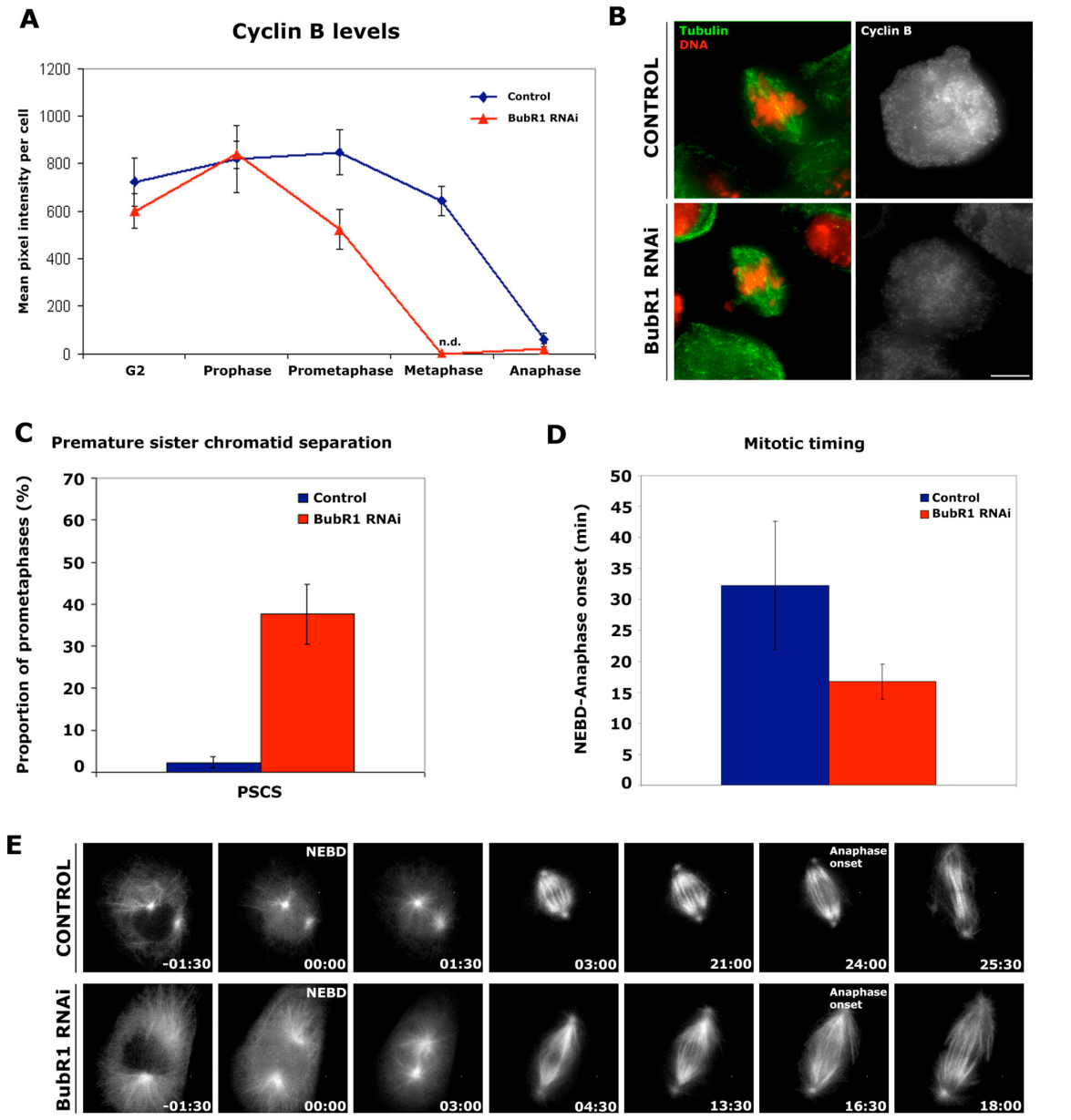


Fig. 1.3 Mitotic progression after depletion of BubR1. (A) Quantification of cyclin B levels during G2 and mitotic progression in control and BubR1-depleted cells. (*n.d.*, not determined because there are no cells in metaphase after depletion of BubR1). Error bars represent the SEM. **(B)** Prometaphase cells showing the levels of cyclin B in control and BubR1-depleted cells. Cells were stained for DNA (red) and immunostained to reveal tubulin (green) and cyclin B (right panel). Scale bar is 5 µm. **(C)** Percentage of prometaphases showing PSCS in control and RNAi-treated cells. **(D)** Quantification of the time either control or BubR1-depleted cells (120h), stably expressing GFP-tubulin, spent in prometaphase from nuclear NEBD to anaphase onset. **(E)** Control and dsRNA-treated cells were placed in an appropriate chamber and images taken every 90 sec using wide-field time-lapse microscopy. This figure shows selected images from movies of either control (see Movie 1.3) or BubR1 RNAi-treated cells (see Movie 1.4). The sequences of images have been aligned setting NEBD as time 0. In control cells, anaphase onset occurred 24 minutes after NEBD while in BubR1 RNAi-treated cells, anaphase started 16,5 minutes after NEBD, showing a significant decrease of the time spent in prometaphase. Time mm:ss. Quantification (D) shows that control cells (n=14) started anaphase 32±10 min after NEBD whereas BubR1-depleted cells (n=19) entered anaphase approximately 17±3 min after NEBD.

Indeed, previous results have suggested that if BubR1-depleted cells are arrested or delayed in mitosis then chromosome congression can be partially achieved (Ditchfield et al., 2003; Johnson et al., 2004). Thus, control or BubR1-depleted cells were incubated with the proteasome inhibitor MG132 in order to arrest the cells in a checkpoint-independent manner. As a control of the experiment simultaneous quantification was done without the use of the inhibitor. The results show that, unlike control cultures which contain about 40% cells in prometaphase and 12% cells in metaphase, after depletion of BubR1 the frequency of prometaphases is highly reduced (25%) and there were no cells in metaphase (Fig. 1.4A). After incubation with MG132, control cultures showed 55% of cells reaching the metaphase state (Fig. 1.4A-B) and only 23% remained in prometaphase, within the incubation period. On the other hand, only 10% of BubR1-depleted cells were able to reach the metaphase plate (Fig. 1.4A-B) and 45% remained in a prometaphase state (Fig. 1.4A).

These results clearly indicate that preventing BubR1-depleted cells from exiting mitosis prematurely only marginally restores the ability of chromosomes to congress. Given that in control cultures most cells reach metaphase, BubR1 is likely to have an essential role in chromosome congression.

In contrast, loss of CENP-meta appears to have very different consequences (Putkey et al., 2002; Schaar et al., 1997; Tanudji et al., 2004; Wood et al., 1997; Yao et al., 2000; Yucel et al., 2000). Depletion of CENP-meta delayed progression through prometaphase (60%) and only reduced by half (6%) the frequency of metaphases (Fig. 1.4A). Therefore, in the absence of CENP-meta chromosomes can congress, although at a reduced frequency. Furthermore, the fact that cells accumulate in prometaphase indicates that CENP-meta is not required for the SAC response. Finally, incubation of CENP-meta-depleted cells in MG132 caused a response in all respects similar to control cells with approximately 25% of prometaphases and almost 60% of cells in metaphase (Fig. 1.4A-B). Taken together, these observations strongly suggest that BubR1 and CENP-meta have very different roles in chromosome congression.

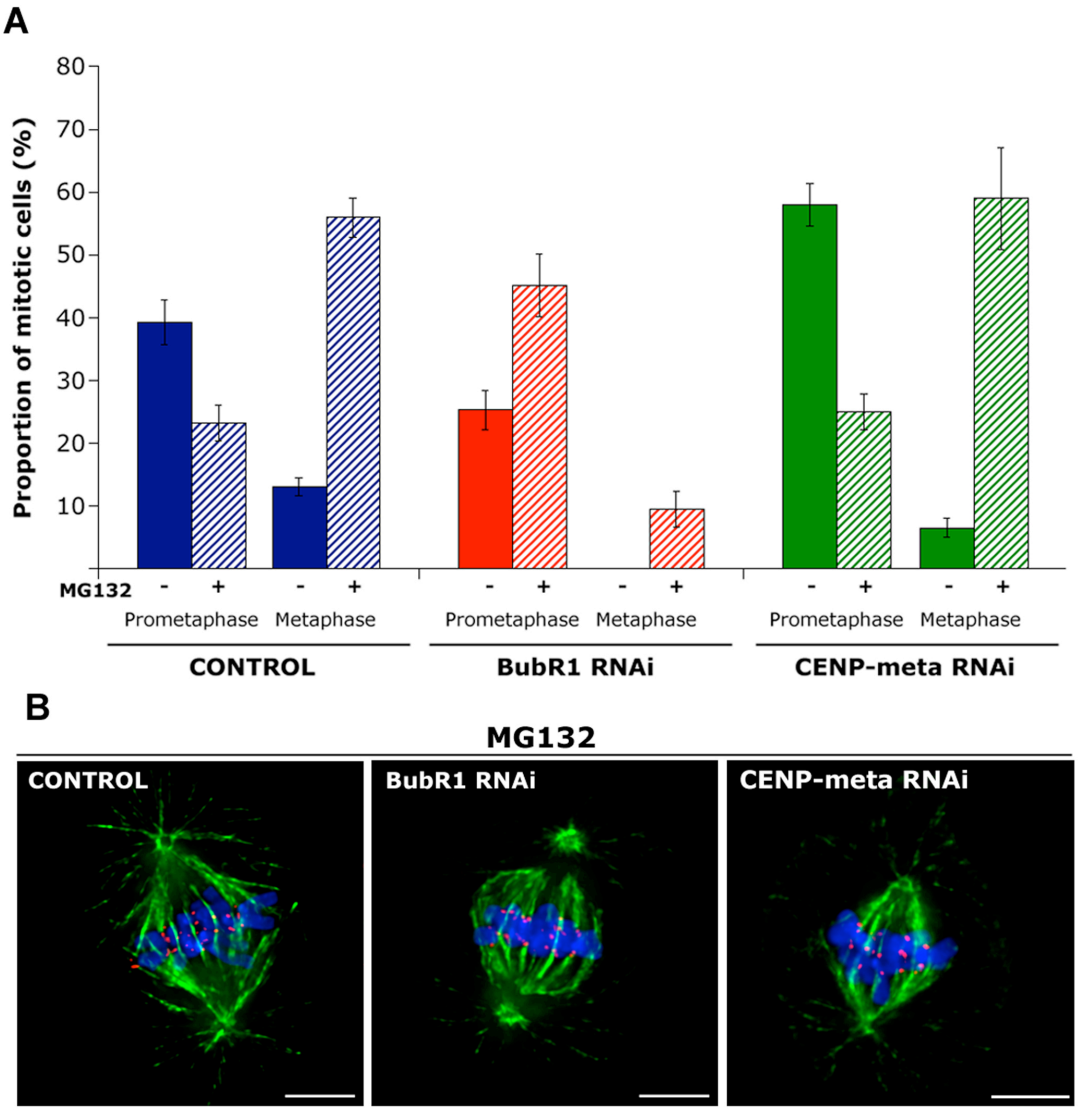


Figure 1.4 Mitotic progression after depletion of BubR1 or CENP-meta. (A) Quantification of the number of prometaphases and metaphases in control, BubR1 dsRNAi-treated cells and CENP-meta dsRNAi-treated cells incubated in the absence (-) or presence (+) of MG132 for 2h. The percentage of prometaphases and metaphases was calculated over the total number of mitotic cells. At least 300 mitotic cells were used from two independent experiments. Error bars represent the SEM. **(B)** Immunofluorescence images showing metaphase alignment observed in control, BubR1- or CENP-meta-dsRNAi-treated cells when mitotic exit is prevented by the use of the proteasome inhibitor. The images show DNA (blue), tubulin (green) and the centromeric marker CID (red). Scale bar is 5 mm.

2.3 ANALYSIS OF PROMETAPHASE PHENOTYPES AFTER DEPLETION OF BUBR1 OR CENP-META

During the analysis of mitotic progression after depletion of BubR1 or CENP-meta two very different types of prometaphase were observed. In control cells most (80%) prometaphases appeared with a "regular" organization in which the majority of the chromosomes were well aligned or localized within a clearly define rectangle at the cell equator (Fig. 1.5). However, a proportion (20%) of prometaphases in which the chromosomes were "scattered" along the length of the spindle and most of them did not fall within the clearly defined rectangle, was also found (Fig. 1.5). Interestingly, after depletion of either BubR1 or CENP-meta the frequency with which these two types of prometaphases are found is severely altered (Fig. 1.5B). The results show that after depletion of either BubR1 or CENP-meta the frequency of regular prometaphases is somewhat reduced (50-60%) while that of scattered prometaphases is significantly increased (40-50%) suggesting that they might affect chromosome congression through a common mechanism. In order to address this further, determination whether the frequency of the two types of prometaphases changes after a mitotic arrest caused by incubation in MG132, was carried out. Under these conditions, loss of BubR1 or CENP-meta causes very different effects. In control cultures incubation with the proteasome inhibitor leads to a small decrease in the frequency of regular prometaphases and a corresponding increase in the frequency of scattered prometaphases (Fig. 1.5B). After depletion of BubR1, similar behaviour with a small reduction in regular prometaphases and a small increase in scattered prometaphases is observed, although the absolute frequencies still remain very different from controls (Fig. 1.5B). However, depletion of CENP-meta causes an effect that is almost the opposite with an increase in regular prometaphases and a corresponding decrease in scattered prometaphases (Fig. 1.5B). These results suggest that arresting cells in mitosis with the proteasome inhibitor does have a minor effect upon prometaphase organization causing a small increase in the scattered conformation both in control and BubR1-depleted cells. However, given that depletion of CENP-meta causes the opposite effect allowing the formation of a more regular prometaphase conformation in which most chromosomes congress strongly suggests that loss of CENP-meta appears to be promoting the stability of microtubule-kinetochore interaction.

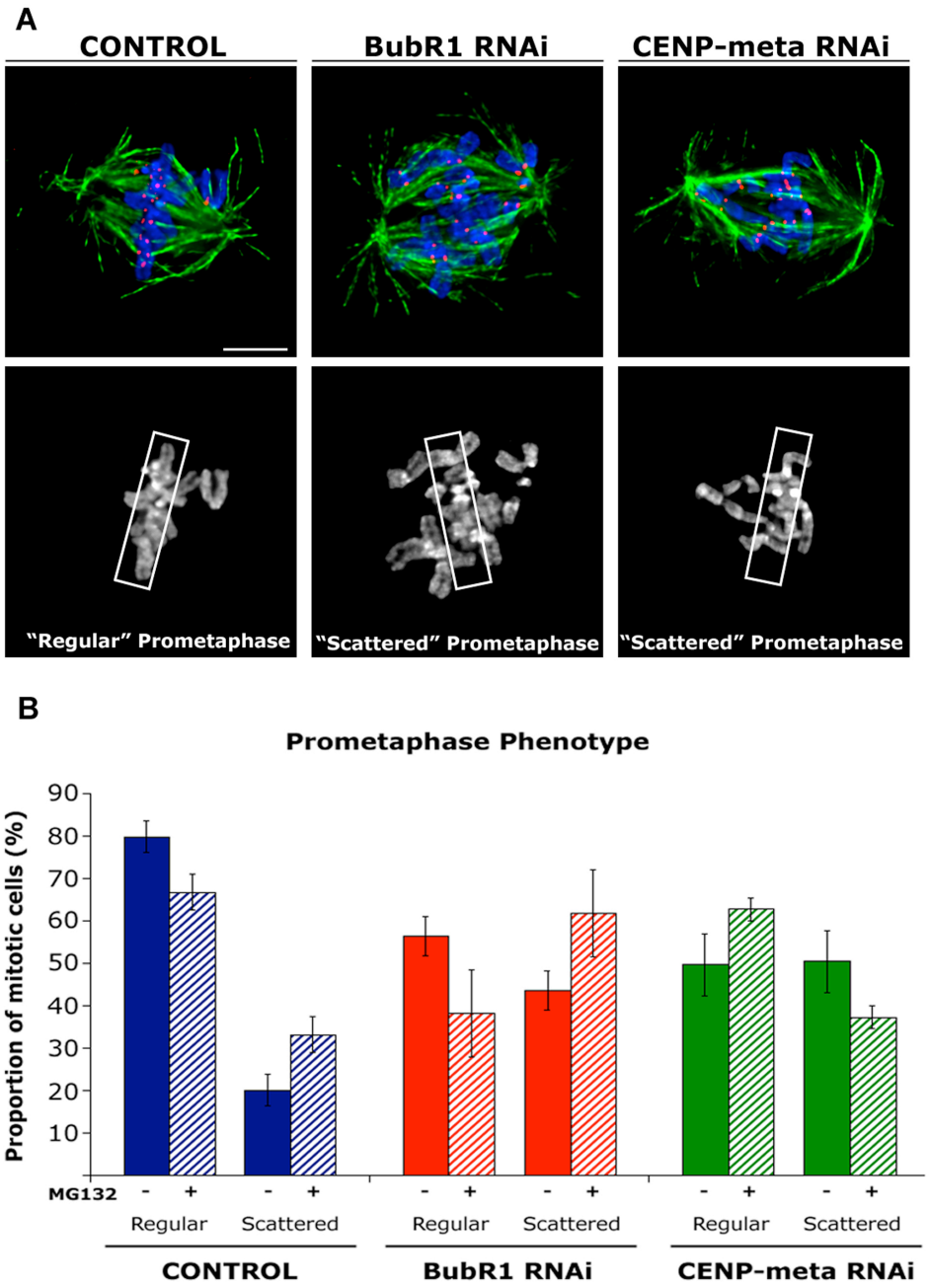


Figure 1.5 Prometaphase phenotypes after depletion of BubR1 or CENP-meta. (A) Immunofluorescence images showing prometaphase distribution of chromosomes after BubR1 or CENP-meta RNAi. The images show DNA (blue), tubulin (green) and the centromeric marker CID (red). Below, the images show only the corresponding DNA stain (white). In order to evaluate chromosome congression a rectangular box comprising most (85%) of aligned kinetochores in control metaphase cells was defined. The same box was placed perpendicularly to the spindle axis from BubR1 or CENP-meta-depleted prometaphases. Prometaphases showing chromosomes localized near the spindle poles were also considered as scattered prometaphases. Scale bar is 5 mm. **(B)** Quantification of prometaphase phenotypes in control, BubR1- and CENP-meta-depleted cells, before (-) or after (+) incubation in MG132 for 2 h. The percentage of each phenotype was calculated over the total number of prometaphases. At least 300 mitotic cells were quantified from two independent experiments. Error bars represent the SEM.

2.4 CHROMOSOME CONGRESSION AFTER SIMULTANEOUS DEPLETION OF BUBR1 AND CENP-META

So far, the results have shown that regarding mitotic progression and chromosome behavior, depletion of BubR1 has very different consequences when compared to depletion of CENP-meta. However, to determine if there is any specific epistatic relationship between the two proteins one must perform analysis of progression through mitosis in cells that were simultaneously depleted for both proteins. Significant level of depletion of both proteins was reached after 96 h of incubation with dsRNAs and neither protein was detected by immunofluorescence after MG132 incubation and microtubule depolymerization (Fig. 1.1C-D). Analysis of cells progressing through early stages of mitosis in the presence of MG132 showed that, unlike control cells, which accumulate in metaphase (45%), double-depleted cells accumulate in prometaphase (60%) and only very few (9%) reach metaphase (Fig. 1.6A-B). Analysis of prometaphase organization in the absence of both proteins showed a reduction in the regular prometaphase organization and a corresponding increase in the scattered phenotype (Fig. 1.6A and C). These observations indicate that with respect to both chromosome congression and prometaphase organization, cells lacking both BubR1 and CENP-meta behave much like cells lacking BubR1 alone.

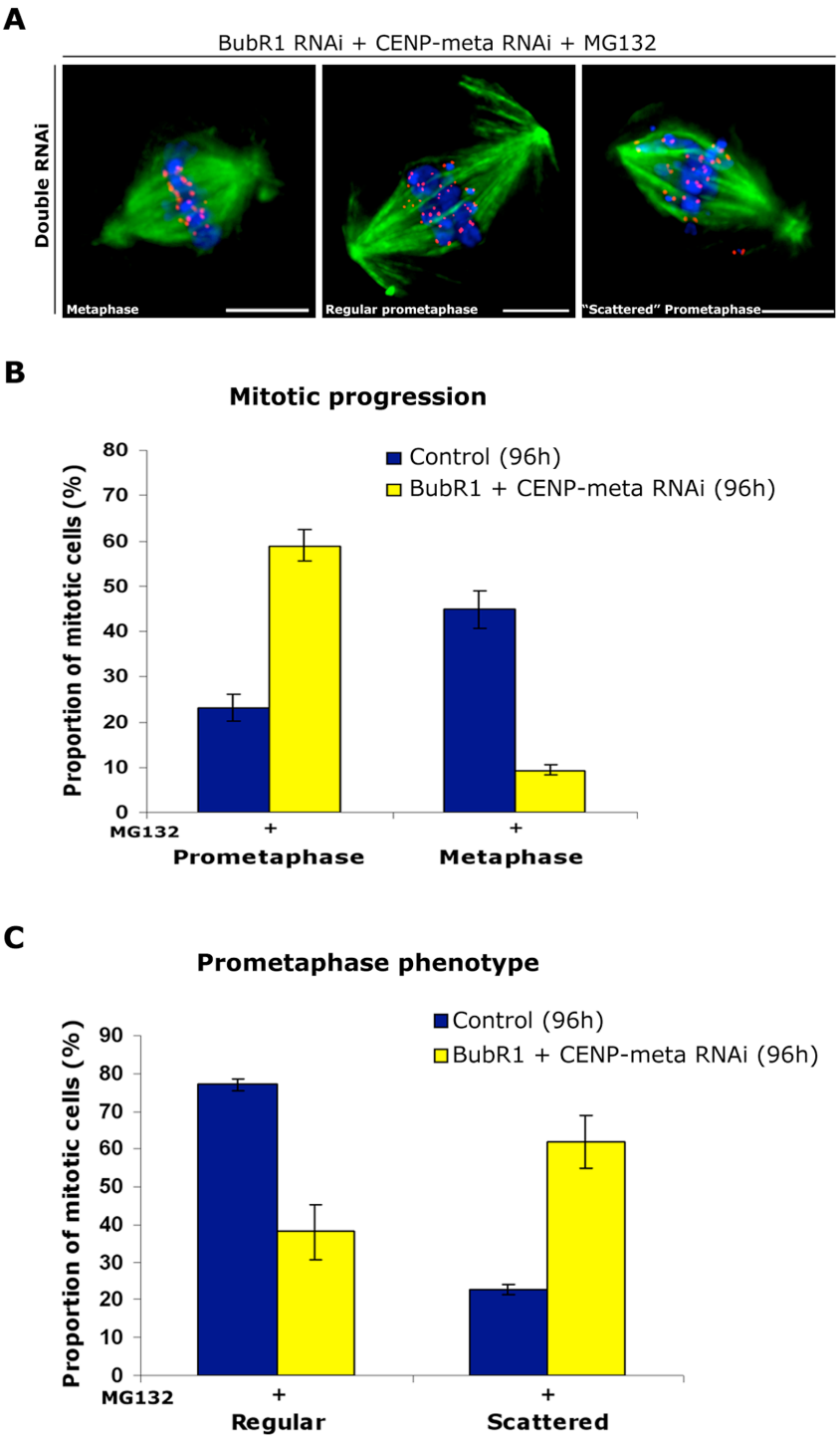


Figure 1.6 Chromosome congression after simultaneous depletion of BubR1 and CENP-meta. (A) Immunofluorescence images showing the distribution of chromosomes after depletion of BubR1 and CENP-meta. Cells were prevented from exiting mitosis by incubation in MG132 for 2h. Chromosome congression was evaluated as described before (see Fig. 1.5). The images show DNA (blue), tubulin (green) and CID (red). Scale bar is 5 µm. **(B)** Quantification of prometaphases and metaphases. **(C)** Quantification of prometaphase phenotypes. Error bars represent the SEM.

2.5 MICROTUBULE-KINETOCHORE INTERACTION AFTER DEPLETION OF BUBR1 OR/AND CENP-META

The analysis of double depletion suggested that BubR1 is epistatic over CENP-meta which is mostly consistent with the extensive publish data indicating that BubR1 and CENP-E interact and that CENP-E plays an important role in chromosome congression (Chan et al., 1998; Kapoor et al., 2006; Mao et al., 2003; Mao et al., 2005; Putkey et al., 2002; Schaar et al., 1997; Tanudji et al., 2004; Weaver et al., 2003; Wood et al., 1997; Yao et al., 2000; Yucel et al., 2000). However, the results presented in the previous sections on the analysis of individual depletions showed that the impaired chromosome alignment observed in the absence of BubR1 could not be explained simply by the loss of CENP-meta making any epistatic relationship unlikely. Therefore, in order to understand the basis of these contradictory results a characterization and quantification of the type of microtubule-kinetochore interaction in cells depleted for BubR1, CENP-meta or both was performed. Chromosomes can establish different types of interactions with spindle microtubules, including amphitelic (each sister kinetochore captured by microtubules emanating from opposite poles), syntelic (both kinetochores on sister chromatid pairs attached to kinetochore microtubule bundles from the same pole) or monotelic attachment (with only one kinetochore attached to microtubules and the other unattached). However, the complex nature of the prometaphase organization either in control or RNAi-depleted cells prevents from obtaining accurate quantification. Therefore, an assay that could discriminate with high accuracy at least some of the microtubule-kinetochore interactions, namely whether both kinetochores of a chromosome were attached (without discriminating syntelic from amphitelic attachment), monotelic or completely unattached was devised. Briefly, the assay consists in incubating cells with MG132 to prevent mitotic exit followed by treatment with taxol to promote the collapse of the mitotic spindle into a monopolar structure (see materials and methods). This treatment allows an easy read-out of the microtubule-kinetochore attachment orientation at the moment of spindle collapse by localizing all chromosomes at the periphery of the monoaster (Fig. 1.7A). Furthermore, it allows evaluating if the microtubule-kinetochore interaction is stable since a weak interaction would not resist the forces exerted by the collapse of the spindle into a monopolar structure resulting in an unattached kinetochore. *Drosophila* cells are an excellent tool to perform this kind of analysis due to the reduced number of chromosomes (11±3 kinetochore pairs per cell) allowing to determine the state of nearly all kinetochores present in the cell (Table 1.1). The results show that the majority of control cells (>95%) have either all chromosomes attached to the spindle or 1-2 monoriented chromosomes.

There were no cells found containing a fully unattached chromosome indicating that incubation with MG132 or with taxol is not able to destroy a stable microtubule-kinetochore attachment (Fig. 1.7A-B). However, BubR1-depleted cells show very different results in which only 50% of the cells contain either all chromosomes attached or 1-2 monoriented chromosomes. Nearly 40% of the cells contain 3-4 monoriented chromosomes and most of them (40%) also contain chromosomes that are completely unattached, a phenotype that had not been described before which suggest that microtubule-kinetochore interaction is very unstable (Fig. 1.7A-B). In order to discriminate if the unattached kinetochores were never attached to spindle microtubules or were in fact attached but more prone to destabilization, cells were treated with calcium before fixation. This procedure destabilizes less stable microtubules leaving mainly kinetochore-microtubules (Kapoor et al., 2000; Mitchison et al., 1986) (Fig. 1.8A). The data clearly shows that BubR1-depleted cells have microtubule fibers attached to the kinetochores (Fig. 1.8B). Importantly, in CENP-meta-depleted cells no unattached chromosomes were observed which is consistent with the ability of CENP-meta-depleted cells to organize normal metaphase plates if prevented from exiting mitosis. Moreover, most (75%) of the cells showed all chromosomes attached to the spindle and only some (25%) contained 1-2 monoriented chromosomes (Fig. 1.7A-B). These results clearly indicate that while loss of BubR1 leads to severe instability of microtubule-kinetochore interaction absence of CENP-meta causes a slight increase in the stability of microtubule attachment. Subsequently, cells depleted for both BubR1 and CENP-meta using the same assay were analyzed. Surprisingly, when cells are simultaneously depleted for BubR1 and CENP-meta the result obtained is not equal to BubR1, as would be expected given the proposed epistatic relationship between the two proteins, but it is an intermediate phenotype (Fig. 1.7A-B). More than 40% of double-depleted cells show all chromosomes attached, similar frequencies of 1-2 monoriented chromosomes and only 15% of cells with fully unattached chromosomes.

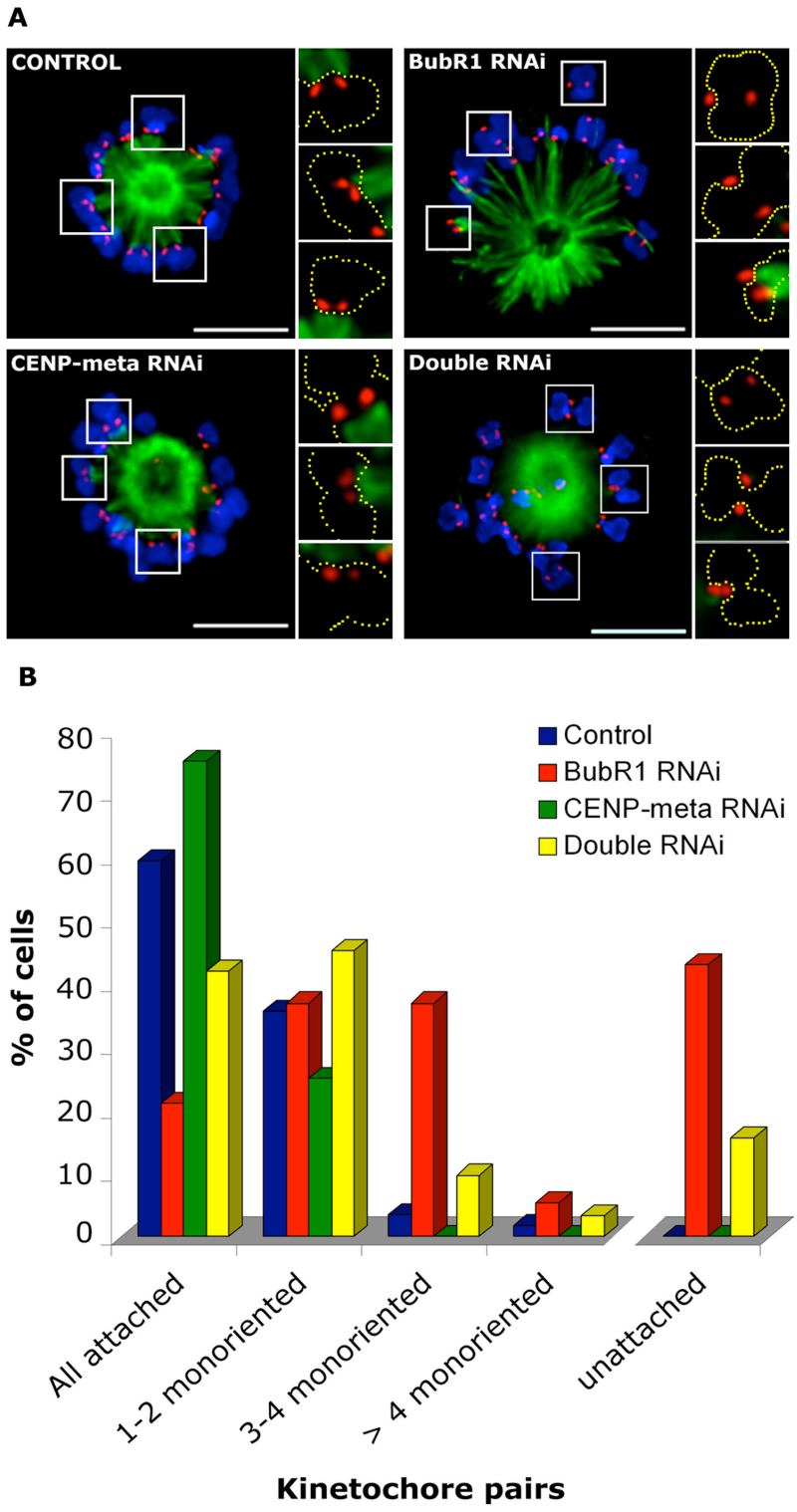


Figure 1.7 Analysis of microtubule-kinetochore interaction after depletion of BubR1 and/or CENP-meta. (A) Immunofluorescence images showing DNA (blue), tubulin (green) and CID (red) of either control or RNAi-treated cells after subjected to the MG132-Taxol assay. Note that the mitotic spindle collapses forming a large monopolar structure with all the chromosomes located on the outside allowing an unambiguous classification of their mode of interaction with spindle microtubules. Insets in the control figure show chromosomes with both kinetochores clearly attached. After depletion of BubR1 and double RNAi, insets show, from top to bottom, an unattached chromosome, a monoriented chromosome and a chromosome showing full attachment.

In CENP-meta-depleted cells, all insets show chromosomes completely attached. In all inserts yellow dots represent the limits of the chromosomes. Scale bar is 5 μm. **(B)** Quantification of microtubule-kinetochore attachment in control (n= 59), BubR1-depleted (n=21), CENP-meta RNAi-treated (n=32) and double RNAi-depleted cells (n=32) from two independent RNAi experiments. The graph shows the percentage of cells that present fully attached kinetochore pairs or with 1-2, 3-4 or more than 4 monoriented kinetochore pairs and the percentage of cells that show at least one unattached chromosome under the different experimental conditions.

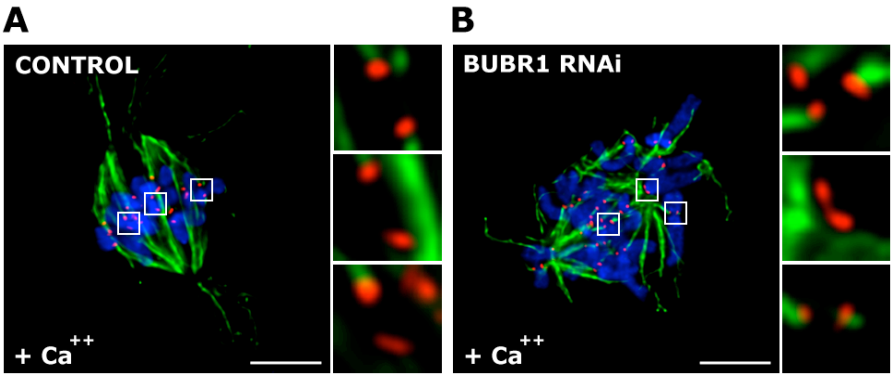


Figure 1.8 Analysis of microtubule-kinetochore interactions after depletion of BubR1 and calcium treatment. Immunofluorescence images showing DNA (blue), tubulin (green) and CID (red). **(A)** Control cell. Insets from top to bottom show amphitelic attachments. **(B)** BubR1-depleted cell. Insets show, from top to bottom, an amphitelic attachment, a synthelic attachment and a monoriented attachment. Scale bar is 5 mm.

Similar results were obtained if the quantitative analysis is done in terms of number of kinetochore pairs that are attached per cell (Table 1.1). BubR1-depleted cells have a significant ($P<0.01$) increase in the number of monoriented and unattached chromosomes whilst CENP-meta-depleted cells show no significant differences in microtubule-kinetochore attachment in relation to control cells. However, cells depleted for both proteins show a significant ($P<0.05$) increase in the number of fully attached chromosomes and a concomitant significant ($P<0.05$) decrease in the number of unattached chromosomes when compared to BubR1-depleted cells. These results strongly suggest that CENP-meta can partially rescue the unstable microtubule-kinetochore interaction phenotype observed when BubR1 is knocked down and therefore the two proteins are not under a simple epistatic interaction.

Table 1.1 Quantification of microtubule kinetochore attachment using the MG132-Taxol assay in control and RNAi-treated cells.

	Kinetochore pairs			
	Analysed per cell	**Fully attached (%)**	**Monoriented (%)**	**Unattached (%)**
Control (n=59)	12,0±2,8	94,0±10,1	6,0±10,1	0
BubR1 RNAi (n=21)	9,8±3,4	**75,1±20,4 *****	**16,9±13,6 ****	**8,0±11,9 ****
CENP-meta RNAi (n=32)	9,1±2,2	96,4±6,8	3,6±6,8	0
Double RNAi (n=32)	12,9±2,8	**88,4±15,6 *,•**	10,2±14,9	**1,4±3,7 *,•**

*** P<0,001; ** P<0,01; * P<0,05. Significance relative to control (*) or to BubR1 (•) RNAi.

2.6 DETAILED ANALYSIS OF MICROTUBULE-KINETOCHORE INTERACTION AFTER DEPLETION OF CENP-META

Previous results from studies in vertebrate cells have suggested that CENP-E is required to promote stable microtubule-kinetochore attachment since in its absence congression is severely affected (Goshima and Vale, 2003; Kapoor et al., 2006; Schaar et al., 1997; Wood et al., 1997; Yao et al., 2000; Yucel et al., 2000) and the number of microtubules per kinetochore reduced (McEwen et al., 2001; Page and Hawley, 2004; Putkey et al., 2002). Surprisingly, using the MG132-Taxol assay the opposite effect was found, since in the absence of CENP-meta microtubule-kinetochore attachment appears to be stabilized. In order to determine whether depletion of CENP-meta does lead to a more stable microtubule-kinetochore attachment this was further analyzed. It has been shown, in a variety of organisms that the SAC protein Mad2 accumulates strongly at kinetochores in the absence of microtubules (Chen et al., 1999; Chen et al., 1998; Logarinho et al., 2004) although a low level continues to enter the kinetochore and is removed mostly along spindle microtubules in a poleward direction (Buffin et al., 2005; Howell et al., 2004). Accordingly, control and CENP-meta-depleted cells were immunostained using anti-Mad2 antibodies (Fig. 1.9A-B). Consistent with the previous data, a significantly higher (19%) frequency of cells that show no Mad2 positive kinetochore labelling in CENP-meta-depleted cells were observed when compared to control cells, indicative of a more stable microtubule-kinetochore interaction. Then an accurate discrimination of the type of interaction that chromosomes depleted of CENP-meta establish with microtubules was done. For this, cells subjected to the MG132-Taxol assay were analyzed but instead of looking at cells in which the spindle had already collapsed completely the analyses was performed in cells just before the collapse of the spindle as the two asters are moving closer. In these cells, amphitelic chromosomes should localize in between the two asters and syntelic chromosomes should localize in the periphery of either aster together with monoriented chromosomes (Fig. 1.9C). Interestingly, this analysis allowed the observation that both in control and RNAi-treated cells some kinetochores are attached to a short microtubule bundle that is here called stub (Fig. 1.9C-D). Quantification of chromosome distribution in the collapsing spindle shows that CENP-meta RNAi-treated cells have significantly ($P<0.05$) more chromosomes located in the asters than control cells (Fig. 1.9E). This suggests in the absence of CENP-meta attachment is being established but then chromosomes fail to move to the centre of the spindle. Then quantification of the type of microtubule attachment was done and verified that CENP-meta-depleted kinetochores establish significantly ($P<0.05$) less

amphitelic interactions than control ones (Fig. 1.9F). Moreover, a significant ($P<0.05$) increase in the number of kinetochores that show a stub is observed (Fig. 1.9F). Interestingly, CENP-meta-depleted cells show a much higher frequency of chromosomes containing microtubule stubs compared to control cells (Fig. 1.9G). Finally, CENP-meta-depleted cells show less monoriented chromosomes when compared to control cells (Fig. 1.9H). Taken together these results suggest that rather promoting stable microtubule-kinetochore attachment directly, kinetochores of cells depleted of CENP-meta establish microtubule-kinetochore interactions that are less prone to be destabilized during progression through mitosis.

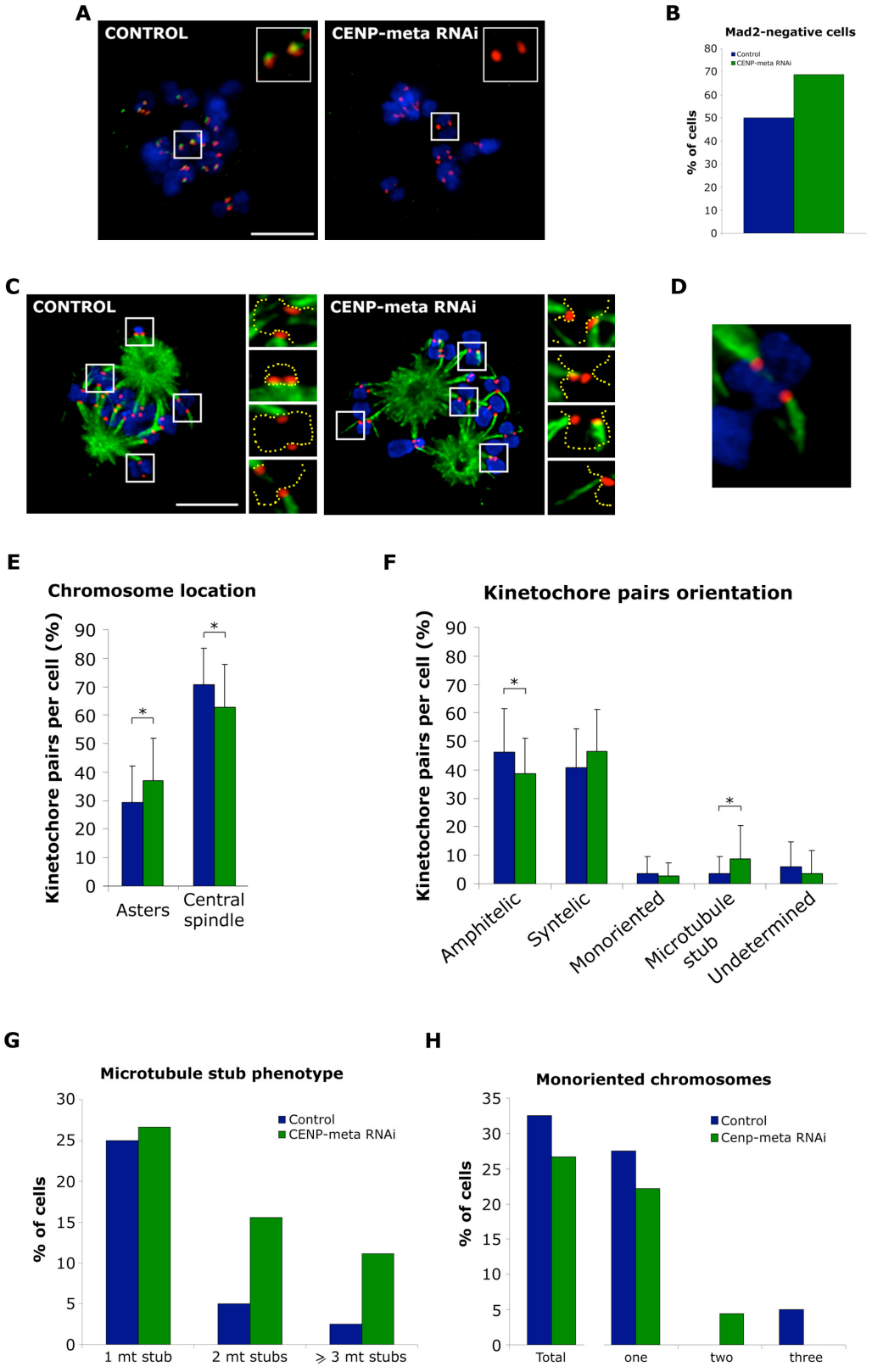


Figure 1.9 Detailed analysis of microtubule-kinetochore interaction after depletion of CENP-meta. (A) Immunofluorescence images showing DNA (blue), CID (red) and Mad2 (green) of either

control or CENP-meta-depleted cells. Insets show a higher magnification of a kinetochore pair. **(B)** Quantification of Mad2 in control (n=30) and CENP-meta-depleted cells (n=32). The graph shows the percentage of cells with no kinetochore staining of the Mad2 demonstrating that in the absence of CENP-meta more kinetochores appear bound to microtubules. **(C)** Immunofluorescence images showing DNA (blue), tubulin (green) and CID (red) of either control or CENP-meta-depleted cells subjected to the MG132-Taxol assay and analyzed just before the full collapse of the spindle as shown by the presence of the two asters. Insets show, from top to bottom, an amphitelic attachment, a syntelic attachment, a monoriented chromosome and a chromosome showing a microtubule stub at one kinetochore. In all inserts yellow outline represents the limits of the chromosomes. Scale bar is 5 μm. **(D)** Higher magnification of a chromosome showing a microtubule stub attached at one of the kinetochores. (E-H) Quantification of microtubule-kinetochore attachment in control (n=40) and CENP-meta-depleted cells (n=45) from three independent RNAi experiments. Error bars represent the SD. * P<0.05. **(E)** Quantification of chromosome distribution in the mitotic apparatus for control or CENP-meta-depleted cells. Chromosomes are considered to be in the central spindle if they lay between the two asters or in the asters if they appear attached on the periphery of the asters. **(F)** Quantification of kinetochore pairs orientation in control and after dsRNAi treatment for CENP-meta. Note that only less than 6% of the kinetochore pairs were impossible to determine the orientation. **(G)** Quantification of control and CENP-meta-depleted cells that show one, two, three or more than three microtubule stubs attached at one of the kinetochores. **(H)** Quantification of the number of cells that show monoriented chromosomes (Total) and cells that present one, two or three monoriented chromosomes in control and CENP-meta-depleted cells.

3. DISCUSSION

The present study provides evidence on the role of BubR1 and CENP-meta, the *Drosophila* homologue of CENP-E, on the process of attachment of chromosomes to the mitotic spindle. Individual or combined RNAi was performed in order to deplete the proteins allowing us to measure mitotic progression and to directly quantify the state and stability of microtubule-kinetochore attachment using a newly devised MG132-Taxol assay. The results show that while BubR1 appears to be essential for the stability of microtubule-kinetochore attachment, CENP-meta appears to be required to promote destabilization of microtubule-kinetochore attachments. Taken together the results obtained suggest that the stability of the microtubule-kinetochore interaction results, at least in part, from the balance of activities of BubR1 and CENP-E.

Previous studies have suggested that BubR1 and CENP-E interact physically (Chan et al., 1999; Chan et al., 1998; Mao et al., 2003; Mao et al., 2005; Yao et al., 2000). These reports lead to the suggestion that CENP-E was also involved in the SAC. Indeed, in *Xenopus* egg extracts CENP-E was shown to be essential to maintain SAC activity (Abrieu et al., 2000; Mao et al., 2003). However, MEFs-depleted of CENP-E are able to sustain a mitotic arrest although less robustly than wild-type cells (Putkey et al., 2002; Weaver et al., 2003). Moreover, depletion of CENP-E in human tissue cultured cells does not lead to inactivation of SAC activity (McEwen et al., 2001; Schaar et al., 1997; Tanudji

et al., 2004; Yao et al., 2000). Consistently with these latter results, here is reported that cells in which CENP-meta was depleted display an efficient SAC response and show no evidence of premature sister chromatid separation. Given the previously observed interaction between the two proteins, several studies have also attempted to determine whether there is any interdependency for their kinetochore localization (Johnson et al., 2004; Lampson and Kapoor, 2005; Weaver et al., 2003). Weaver et al., 2003 have shown that deletion of CENP-E reduces the amount of BubR1 at the kinetochores while Johnson et al., 2004 have shown that repression of CENP-E does not affect BubR1 but that BubR1 is required for kinetochore localization of CENP-E. More recently, data suggesting that the kinetochore localization of CENP-E is mostly independent from BubR1 has also been obtained (Lampson and Kapoor, 2005). However, given that depletion of BubR1 affects SAC activity and causes premature exit from mitosis most likely affects the results. Accordingly, all immunolocalization studies done here were with the cells arrested in a checkpoint-independent manner, through the use of the proteasome inhibitor MG132, followed by microtubule depolymerization to prevent microtubule attachment so that any interdependency could be unequivocally ascertained. The results clearly show that, in *Drosophila* S2 cells, the binding of BubR1 and CENP-meta to kinetochores is completely independent.

Analysis of chromosome congression in cells lacking BubR1 or CENP-meta indicate that these proteins play important roles during prometaphase. Previous data in human tissue cultured cells where BubR1 was depleted (Ditchfield et al., 2003; Johnson et al., 2004) showed that in its absence, most cells contained chromosomes that appeared scattered along the length of the spindle, few cells were found with a more regular prometaphase configuration and almost no cells were found in metaphase. Furthermore, analysis in *Xenopus* extracts, *Drosophila*, mouse fibroblasts and human cultured cells where CENP-E was knocked down or mutated (Goshima and Vale, 2003; Johnson et al., 2004; McEwen et al., 2001; Putkey et al., 2002; Schaar et al., 1997; Tanudji et al., 2004; Wood et al., 1997; Yucel et al., 2000) also showed an increase in the frequency of cells with scattered prometaphase chromosomes. To determine whether failure of congression in BubR1-depleted cells could be due to premature exit from mitosis, cells were treated with a proteasome inhibitor to block the metaphase-anaphase transition. Unlike control cells that accumulate to high frequency in metaphase, most BubR1-depleted cells were unable to fully align chromosomes, in accordance with previous studies in human cultured cells (Ditchfield et al., 2003; Johnson et al., 2004). Therefore, BubR1 appears to have a specific role in chromosome congression besides its function in the SAC response unlike CENP-meta which does not appear to affect

chromosome congression if blocked in M-phase as previously observed in human tissue cultured cells (Johnson et al., 2004). These observations fully support the view that metaphase alignment can occur via a CENP-E independent mechanism (McEwen et al., 2001; Weaver et al., 2003). However, while CENP-E is not essential for full chromosome alignment, the CENP-E independent mechanism is clearly not very efficient and requires an extended time in prometaphase for congression to occur.

Analysis of individual depletion of BubR1 or CENP-meta, clearly show different effects upon mitotic progression and chromosome congression suggesting that CENP-E does not contribute to the phenotype caused by depletion of BubR1. To determine if there is a possible functional interaction between the two proteins, BubR1 and CENP-meta were simultaneously depleted and mitotic progression and chromosome congression analyzed. Interestingly, the results show a congression phenotype that in all respects resembles the single BubR1 RNAi phenotype and again the microtubule-kinetochore interaction appeared abnormal suggesting that BubR1 might indeed act epistatically over CENP-meta. Nevertheless, without a more detailed analysis of the origin of the congression defect observed in cells depleted for BubR1 or CENP-meta it would be difficult to draw sound conclusions. Therefore, an assay based on the use of taxol in MG132-arrested cells, the MG132-Taxol assay, was devised which allowed carrying out a clear read-out of microtubule-kinetochore interaction in these cells. This assay allowed unveiling, for the first time, that cells lacking BubR1 apart from showing an increased number of monoriented chromosomes also have a high frequency of completely unattached chromosomes. There are two possible interpretations of these results, either the unattached kinetochores were never attached to spindle microtubules or were attached but are more prone to destabilization. We favour the second hypothesis, since calcium treatment, used to destabilize non-kinetochore microtubules, show that, in the absence of BubR1, kinetochore bundles do form and are relatively stable. Furthermore, it was recently suggested that human cultured cells depleted for BubR1 have an increased activity of the Aurora B kinase, which in turns, would promote destabilization of the microtubule-kinetochore interaction (Lampson and Kapoor, 2005). However, when cells depleted for CENP-meta were subjected to the MG132-Taxol assay, a very different response was observed: no cells were found containing completely unattached chromosomes and a very high frequency of cells in which all chromosomes are fully attached. These results differ from those previously described for murine fibroblasts lacking CENP-E, where completely unattached chromosomes were observed near the spindle poles despite the abundance of microtubules found in their vicinity (Putkey et al., 2002). We interpret this difference to be due to the nature of our assay in which cells are

subjected to an extended M-phase during which CENP-E-depleted kinetochores of both congressed and uncongressed chromosomes might slowly acquire kinetochore microtubules (McEwen et al., 2001). Thus, our results confirm that in the absence of CENP-E, an extended prometaphase is capable of revert the impaired chromosome congression phenotype observed and suggests that microtubule-kinetochore attachments are indeed more stable. These results were further confirmed by the analysis of cells depleted for both BubR1 and CENP-meta using the MG132-Taxol assay where was observed a significant increased stability of microtubule-kinetochore interaction with a significant reduction in the frequency of cells with completely unattached chromosomes.

But how is the absence of CENP-meta able to increase the stability of microtubule-kinetochore interaction? To address this question control and CENP-meta-depleted cells were immunostained with an anti-Mad2 antibody as a molecular marker for kinetochore attachment. Consistent with all the obtained data it was found that on average control cells contain almost 20 percent more Mad2-positive kinetochores than CENP-meta-depleted cells. Subsequently, it was applied the MG132-Taxol assay but analysing the cells just before the collapse of the spindle allowing to explore in detail the type of kinetochore attachment in control or CENP-meta-depleted cells. This showed that cells depleted of CENP-meta have an increased attachment rate fully consistent with the previous results. Interestingly, it was found a significant number of kinetochores showing a short microtubule bundle not bound to any spindle pole. Previous studies have indicated that vertebrate (Khodjakov et al., 2003; Tulu et al., 2006) and *Drosophila* S2 cells (Maiato et al., 2004b) can organize functional microtubule bundles from the kinetochore as an alternative pathway during the organization of the mitotic spindle. Therefore, in the absence of CENP-meta either the growth of microtubules from the kinetochore is favoured or alternatively the kind of interaction that is established with these kinetochores is so stable that during the collapse of the spindle the taxol-stabilized microtubules break giving rise to this stub. Whatever the nature of these short kinetochore bundles, they are consistent with more stable microtubule-kinetochore interaction in the absence of CENP-meta. Taken together this study does not support a simple model in which BubR1 is epistatic over CENP-E and support a different model where efficient chromosome congression may result from the concerted action of the opposing activities of BubR1 and CENP-E. On the one hand BubR1 appears to play an essential role in promoting stabilization of microtubule-kinetochore attachment possibly through inhibition of Aurora B (Lampson and Kapoor, 2005) and on the other CENP-E, independently of BubR1, appears to promote the destabilization of microtubule-kinetochore interactions. The mechanism by which CENP-E induces destabilization of kinetochore attachment is still

unknown, however, previous reports (Mao et al., 2003; Mao et al., 2005) have shown that unattached kinetochores stimulate association of BubR1 with CENP-E and that this association engages BubR1 kinase activity leading to autophosphorylation, to phosphorylation of CENP-E and maybe other yet unknown substrates. However, it is also possible that the increased kinetochore attachment we observe in the absence of CENP-meta is an indirect result from the inability to fully activate correction mechanisms. It is now clear that inappropriately attached kinetochores need to be released through the activity of Aurora B before the attachment can be corrected (Cahill et al., 1998; Cimini et al., 2006; Lampson et al., 2004; Pinsky et al., 2006). This is indeed fully consistent with the observation that in the absence of CENP-meta there are less amphitelic, more syntelic and less monooriented chromosomes than in control cells. Thus, CENP-E could have an important role in promoting the activation of the mechanisms that correct inappropriate kinetochore attachment.

4. MATERIALS AND METHODS

4.1 DOUBLE-STRANDED RNA INTERFERENCE IN *DROSOPHILA* S2 CELLS

In order to deplete BubR1 from *Drosophila* S2 tissue cultured cells, a fragment spanning the 5'UTR and including the ATG initiation codon of BubR1 cDNA (CG7838) was cloned into pSPT18 and pSPT19 expression vectors (Roche). RNA synthesis and RNAi experiments were performed as previously described by adding 15 µg of dsRNA to 10^6 cells (Coelho et al., 2003). To deplete CENP-meta, S2 cells were incubated with 15 µg of dsRNA, in the single RNAi experiment, and 20 g in the double RNAi experiment, corresponding to a fragment of CENP-meta (CG6392) defined by the primers (Forward) TGTTCCCGTCTTTCAACTGG and (Reverse) TCGCCTCTTTAGAGCCAACC. The PCR primers were kindly provided by Helder Maiato. RNA synthesis was performed as previously described (Maiato et al., 2003b). At 24 h intervals, cells were collected and processed for both immunofluorescence and immunoblotting. For immunoblotting, 10^6 cells were collected by centrifugation, resuspended in SDS-sample buffer and loaded on a 7.5% (for BubR1 RNAi) or 6.5% (for CENP-meta and double RNAi) SDS-PAGE. Treatment with drugs was performed as follows: 20 µM MG132 (Calbiochem) for 1-2 h; 30 µM colchicine (Sigma-Aldrich) for 2 hours and 100nM taxol (Sigma-Aldrich) for 3 h, before cells were collected for immunofluorescence analysis.

4.2 IMMUNOFLUORESCENCE IN S2 CELLS

For immunofluorescence, $2x10^5$ cells were centrifuged onto slides, fixed in 3.7% formaldehyde (Sigma-Aldrich), 0.5% Triton X-100 and 1 x PBS for 10 minutes followed by 3 washes in PBS-T (1x PBS, 0.05% Tween 20) for 5 minutes. Primary antibody incubation was performed in PBS-TF (1x PBS, 0.05% Tween 20, 10% FBS) for 1 hour at room temperature followed by 3 washes in PBS-T. Fluorescent-labeled secondary antibodies were used according to manufacturer instructions and then cells were washed 3 times with PBS-T for 5 minutes and mounted in Vectashield with 1 µg/ml of DAPI (Vector, UK). Images were collected in the Zeiss Axiovert 200M microscope (Carl Zeiss, Germany) using an Axiocam (Carl Zeiss, Germany). Data stacks were deconvolved, using the Huygens Essential version 3.0.2p1 (Scientific Volume Imaging B.V., The Netherlands) and projected using ImageJ 1.3v software (http://rsb.info.nih.gov/ij/).

4.3 ANTIBODIES

The primary antibodies used were anti-α-tubulin mouse B512 (Sigma-Aldrich) used at 1:4000 for immunofluorescence (IF), anti-α-tubulin mouse DM1A (Sigma-Aldrich) used at 1:8000 for immunoblotting (IB), anti-phosphorylated-Histone H3 rabbit (Upstate Biotechnology) used at 1:1000 (IF), anti-POLO mouse MA294 (Llamazares et al., 1991) used at 1:30 (IF), anti-BubR1 rabbit Rb666 (Logarinho et al., 2004) used at 1:3000 (IF) and 1:4000 (IB), anti-BubR1 rat used at 1:500 (IF), anti-Mad2 Rb1223 (Logarinho et al., 2004) rabbit used at 1:20 (IF), anti-CID chicken polyclonal (Blower and Karpen, 2001) used at 1:100 (IF), anti-CID rabbit (Henikoff et al., 2000) used at 1:1500 (IF), anti-Cyclin B rabbit (Jacobs et al., 1998) and anti-CENP-meta (a gift from Byron Williams and Michael Goldberg) used at 1:1000 (IF) and 1:2000 (IB). The secondary antibodies were from Molecular Probes and Jackson Immunoresearch and used accordingly to manufacturer instructions. For the generation of the rat anti-BubR1 antibody, a fragment encoding the aminoacids 103 to 697, from BubR1, was cloned in frame in the pQE30 expression vector. The recombinant protein was purified using the Ni-NTA Purification System (Qiagen), and used for immunisation in rats. This fragment of BubR1 is the same previously used for immunisation in rabbits (Logarinho et al., 2004). Both antibodies behave similarly in immunofluorescence and western blot.

4.4 KINETOCHORE-MICROTUBULE INTERACTION (MG132-TAXOL) ASSAY

Cells were incubated with 20 M of MG132 (Calbiochem) during 1 h and then with 100 nM of taxol (Sigma-Aldrich) for 3 h, which induces the collapse of the mitotic spindle into a monopolar structure with all the chromosomes localized around the aster. This organization allows an easy read-out of the microtubule-kinetochore attachments. Note that in this assay we cannot differentiate between amphitelic and syntelic attachment while monoriented and unattached chromosomes are easily scored. However, analysis of these cells just before the collapse of the spindle as the two asters are moving closer, allows determining whether the association with chromosomes is amphitelic, syntelic or monotelic, in between or in the periphery of the asters. Statistical independent two-sample (two-tailed) *t* test analysis of interaction with the spindle with each kinetochore pair was performed using the software SPSS v.14.0.

4.5 TIME-LAPSE FLUORESCENCE IMAGING

Time-lapse live imaging of mitosis was performed on S2 cells stably expressing GFP-α-Tubulin. Control or BubR1 RNAi-treated cells were grown for 72-120 hours and plated on glass coverslips previously coated with 100 µg/ml concanavalin A (Sigma-Aldrich). Time-lapse images were collected at 2 min intervals, starting immediately before NEBD using a Cell Observer System (Carl Zeiss, Germany). Image processing and movie assembly was processed using AxioVision 4.3 software (Carl Zeiss, Germany).

CHAPTER 2

Aurora B kinase cooperates with CENP-E to promote timely anaphase onset

1. INTRODUCTION

During mitosis, chromosomes need to biorient on the mitotic spindle to allow equal segregation of the genetic material. While attaching to the mitotic spindle, chromosomes make a series of orchestrated movements that ultimately result in alignment at the metaphase plate. Several molecular mechanisms contribute to these movements including the action of kinetochore- and microtubule-associated motor proteins, and the balance between microtubule polymerizing and depolymerizing factors (McIntosh et al., 2002). The plus-end-directed kinesin-7 motor protein CENP-E has a unique role in this process as it mediates the sliding of mono-oriented sister-chromatids along the kinetochore fibers of already bioriented sister-chromatid pairs (Kapoor et al., 2006) or even along any microtubule bundle without the requirement of the trailing kinetochore to be attached (Cai et al., 2009).

CENP-E associates with kinetochores in (pro)metaphase (Yen et al., 1992) and is involved in the capture and stability of kinetochore-microtubule (KT-MT) interactions (Maia et al., 2007; McEwen et al., 2001; Putkey et al., 2002). Disruption of CENP-E impairs metaphase chromosome alignment in *Xenopus* egg extracts (Wood et al., 1997), in *Drosophila melanogaster* (Goshima and Vale, 2003; Yucel et al., 2000) and in vertebrate tissue culture cells (McEwen et al., 2001; Putkey et al., 2002; Schaar et al., 1997; Tanudji et al., 2004; Weaver et al., 2003; Yao et al., 2000), resulting in a mitotic delay with a few unaligned chromosomes positioned very close to the spindle poles.

While CENP-E facilitates chromosome congression by mediating the lateral sliding of sister-chromatids along existing K-fibers, the mitotic kinase Aurora B promotes chromosome biorientation by correcting chromosome attachment errors. Through phosphorylation of several kinetochore substrates Aurora B selectively disassembles kinetochore-microtubule fibers that are not bioriented, allowing new microtubules to bind the kinetochores (Lampson et al., 2004; Tanaka et al., 2002). It is not known if CENP-E and Aurora B cooperate to promote efficient chromosome biorientation and timely anaphase onset and this works was set out to study this. The fate of the chromosomes that fail to align in the absence of CENP-E was explored by time-lapse and high-resolution fluorescence microscopy and found Aurora B to be highly active on these chromosomes. Aurora B kinase activity was required to destabilize pole-proximal KT-MT attachments and to delay mitotic progression. As such Aurora B allowed chromosome alignment by CENP-E independent pathways and it implies that by destabilizing pole-proximal syntelic

attachments Aurora B cooperates with CENP-E to mediate congression of mono-oriented polar chromosomes.

2. RESULTS

2.1 CENP-E-DEPLETED CELLS DISPLAY TWO DISTINCT MITOTIC PHENOTYPES

To time mitotic progression in cells depleted of CENP-E, HeLa cells stably expressing YFP-H2B were transfected with siRNA oligos to CENP-E and to Luciferase as a control. Knockdown of CENP-E was examined at the protein level in mitotic cells by western blotting and by immunofluorescence (IF) analysis. No CENP-E was detected in 50 μg of lysates of CENP-E siRNA-transfected cells (Fig. 2.1A) and in >90% of the cells CENP-E was undetectable by IF (Fig. 2.1B).

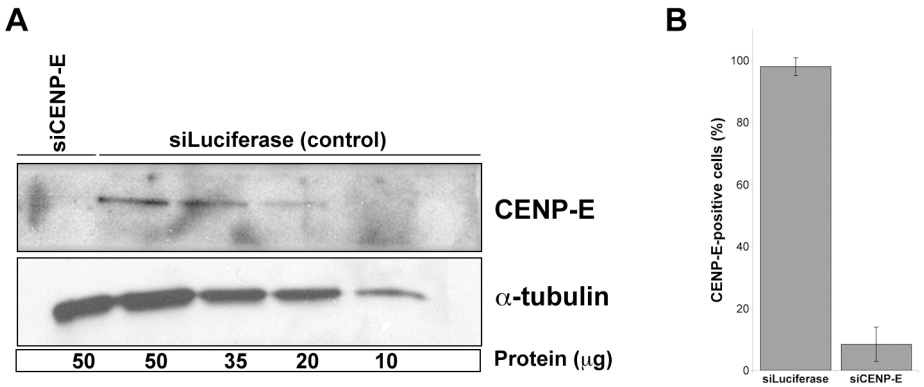


Figure 2.1 Depletion of CENP-E from HeLa cells by siRNA. (A): **(A)** Western blot for CENP-E. Different amounts of total protein was loaded of mock transfected cells to determine the detection limit of the CENP-E Ab. CENP-E siRNA transfected cells have at least 5 times less CENP-E protein than control cells. α-tubulin was used as loading control. **(B)** Quantification of the number of CENP-E-positive cells observed by IF in control (siLuciferase) and CENP-E-depleted cells. Error bars represent standard deviation (s.d.).

Time-lapse microscopy was used to measure the time cells spent in mitosis (TIM) (Fig. 2.2A), defined as the interval between nuclear envelope breakdown and anaphase onset/exit. To exclude any interference on the TIM by the siRNA oligo transfection, the TIM and mitotic progression profiles of non-transfected HeLa YFP-H2B cells were compared to those of cells treated with control siRNA (Fig. 2.2B and 2.5 and table 2.1).

No difference was found and all the subsequent studies were done either in cells treated with control siRNA or CENP-E siRNA.

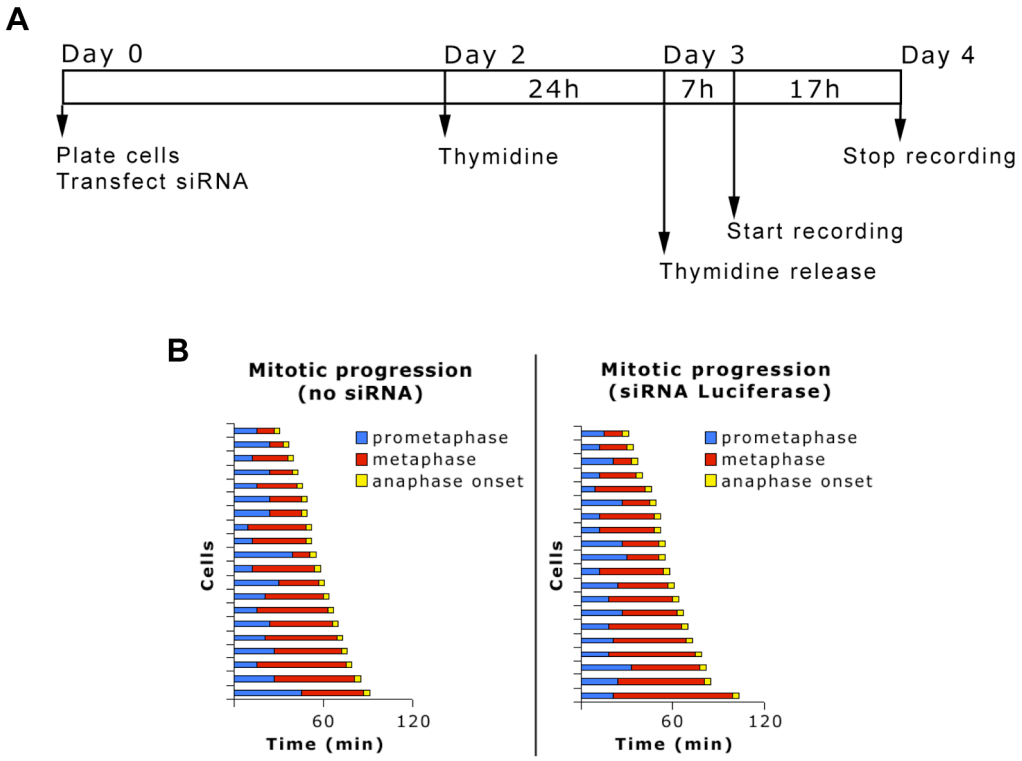


Figure 2.2 Mitotic progression comparisons between non-transfected and transfected HeLa YFP-H2B cells with control siRNA. (A) Experimental setup used to analyze mitotic progression by time-lapse microscopy in HeLa cells stably expressing YFP-H2B. Cells were continuously filmed 7h after the release of a thymidine block. Where indicated DMSO was added to account for the solvent of compounds that were used. **(B)** Time spent in different mitotic phases in 20 randomly selected HeLa YFP-H2B cells. Bars denote time in prometaphase (blue), time in metaphase (red) and time of anaphase onset (yellow).

Control transfected cells rapidly aligned all chromosomes in the metaphase plate (19.9±8.3 min, n=156) (Fig. 2.3 and Movies 2.1 and 2.2). However, in CENP-E-depleted cells two main phenotypes were consistently observed, referred to as phenotype a and b (Fig. 2.3).

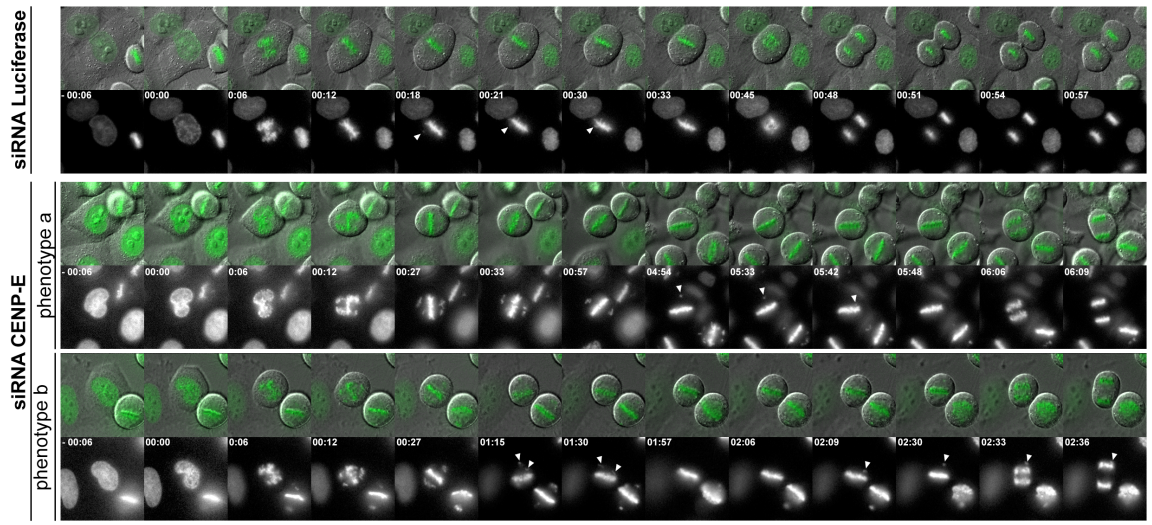


Figure 2.3 Images from live-cell movies of YFP-H2B cells either treated with control or CENP-E siRNA. Cells were transfected with Luciferase siRNA or CENP-E siRNA. Time from NEBD to chromosome alignment and anaphase onset/mitotic exit was measured. CENP-E-depleted cells show an extensive mitotic delay with a few chromosomes residing near the spindle poles (arrowheads). Eventually all chromosomes align in the metaphase plate and cells enter anaphase (phenotype a), or cells exit mitosis with uncongressed chromosomes (phenotype b). Upper panels, overlay of DIC (grey) and DNA (YFP-H2B, green). Lower panels, YPF-H2B. Time hr:min.

Chromosomes that persisted close to the spindle poles (polar chromosomes) were seen in all cells, but in 54% of the analyzed cells (n=182) these chromosomes eventually aligned into the metaphase plate after an extensive delay (Fig. 2.4-2.5, phenotype a: average TIM 238 ± 123 vs 50 ± 18 min for control cells, Table 2.1), and anaphase onset started after the last chromosome congressed into the plate (Fig. 2.3, phenotype a, Movies 2.3 and 2.4). In 37% of the cells mitosis was also significantly delayed (Fig. 2.4-2.5, phenotype b: average TIM 306 ± 121 min, Table 2.1), but anaphase onset started while a few chromosomes still resided near the spindle poles (Fig. 2.3, Movies 2.5 and 2.6). The remaining 9% of the cells were delayed in mitosis for more than 480 min of continuous filming and were therefore denominated as “stop” (Fig. 2.4).

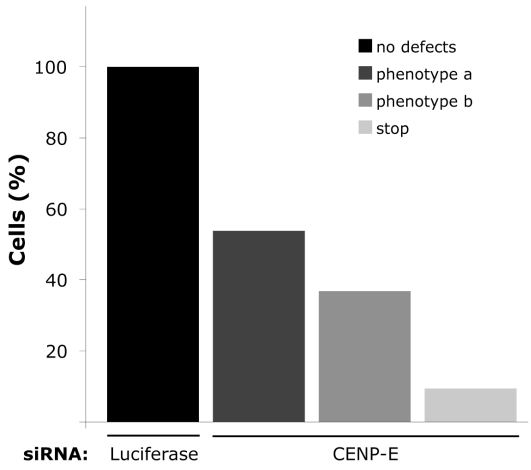


Figure 2.4 Mitotic phenotypes quantification in control and CENP-E-depleted cells. All control cells did anaphase with no alignment defects. CENP-E-depleted cells show mainly two distinct phenotypes (a and b). "stop" corresponds to cells that were still in mitosis after 8h of continuous recording.

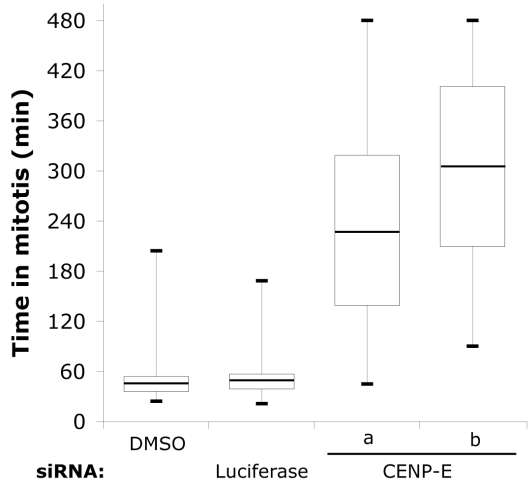


Figure 2.5 Box-and-Whisker plots quantifying time spent in mitosis (TIM) in the different experimental conditions. TIM is defined as the interval between NEBD and anaphase onset/mitotic exit, for HeLa YFP-H2B cells treated with DMSO (no siRNA), Luciferase siRNA and CENP-E siRNA. The TIM for CENP-E-depleted cells is sub-divided for the different phenotypes observed after CENP-E depletion: phenotype a and b. Median, interquartile range and full range are represented in the plot.

Thus, upon CENP-E knockdown, cells delay in mitosis for ± 4 hrs during which they manage to align the polar chromosomes, or they arrest for ≥ 5 hrs and then exit mitosis in the presence of misaligned chromosomes.

2.2 AURORA B KINASE IS HIGHLY ACTIVE ON POLAR CHROMOSOMES

The fact that for all CENP-E-depleted cells mitosis was severely delayed suggested that these cells have a functional mitotic checkpoint (Musacchio and Salmon, 2007). Indeed, earlier studies demonstrated that CENP-E-depleted cells arrest in mitosis when treated with the spindle poisons nocodazole, colcemid and taxol (Tanudji et al., 2004; Weaver et al., 2003). Immunofluorescence (IF) of the SAC protein Mad1 (Chen et al., 1998) and of CLIP-170, a microtubule "plus-end-tracking" protein that leaves the kinetochores upon microtubule attachment (Dujardin et al., 1998), confirmed that the polar chromosomes were unattached or poorly attached (Fig. 2.6A-B), and explains why mitotic progression is delayed in CENP-E-knockdown cells.

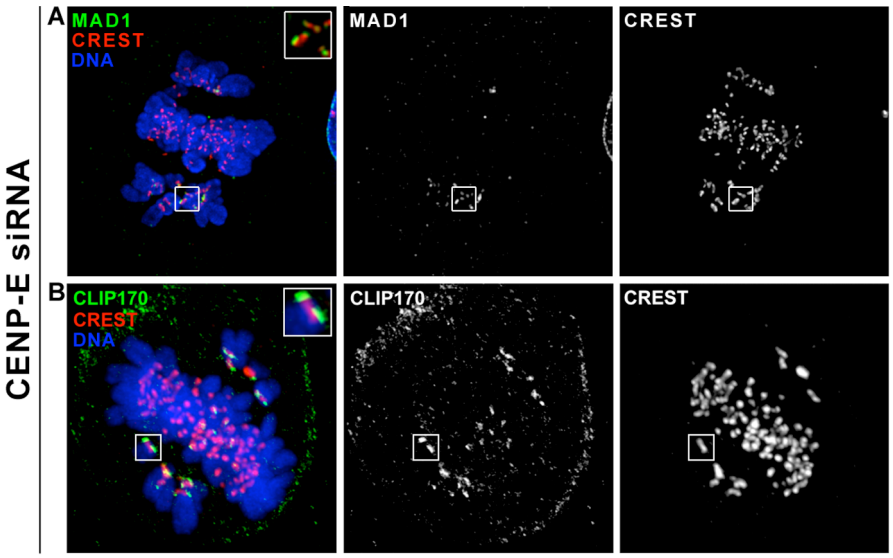


Figure 2.6 Immunolocalization of molecular markers that label unattached kinetochores in cells depleted of CENP-E. (A-B) Immunofluorescence images showing clear kinetochore localization of Mad1 **(A)** and CLIP-170 **(B)** in the unaligned chromosomes (inset, higher magnification) with almost complete absence on the aligned chromosomes.

It was then asked why kinetochores of the polar chromosomes remained unattached or poorly attached for such a long time. Polar chromosomes are located very close to the main microtubule organizing centers of the cell, the centrosomes, and therefore numerous KT-MT interactions are expected to occur. Indeed, the chromosomes that reside close to the spindle poles in CENP-E-knockdown cells are not static and movements can be observed suggesting that microtubules are able to interact with the chromosomes but these attachments are not properly stabilized (Movies 2.4 and 2.6). Aurora B prevents stabilization of KT-MT attachments that are not bioriented through phosphorylation of kinetochore substrates, such Hec1/Ndc80 and MCAK (Andrews et al., 2004; Cheeseman et al., 2006; DeLuca et al., 2006; Tanaka et al., 2002). To test if the failure to stabilize pole-proximal KT-MT attachments was due to local Aurora B activity, auto-phosphorylation of the kinase was monitored using phospho-specific antibodies for T232 (Yasui et al., 2004). Strikingly, the levels of T232 phosphorylation were significantly increased on the polar chromosomes of CENP-E-knockdown cells as compared to the aligned chromosomes, suggesting high levels of active Aurora B kinase at the polar chromosomes (Fig. 2.7A-B). This is most probably due to the increased protein levels of Aurora B (Fig. 2.8). The specificity of the phosphorylation signal was confirmed by inhibition of Aurora B kinase activity with the small-molecule inhibitor ZM447439 (Ditchfield et al., 2003) (Fig. 2.7A-B).

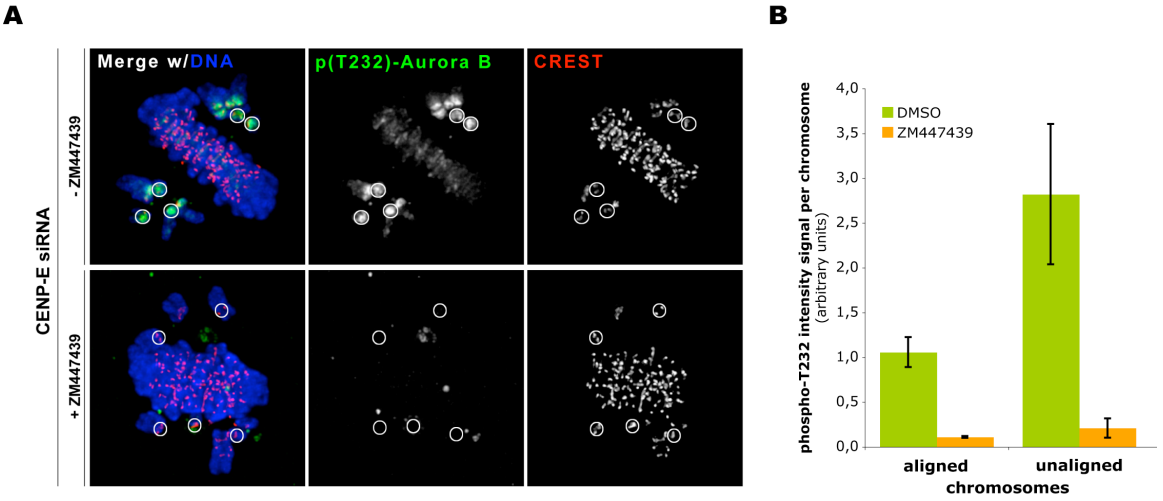


Figure 2.7 Aurora B kinase activity in CENP-E-depleted cells. (A) IF images for T232 auto-phosphorylation of Aurora B after CENP-E depletion (-ZM447439). Specificity of the phosphorylation signal is shown by decrease in the IF signal of pT232 upon inhibition of Aurora B kinase activity (+ZM447439). White circles indicate representative kinetochore pairs used for the quantification of the pT232 fluorescent signal **(B)**. Average of three independent experiments is shown.

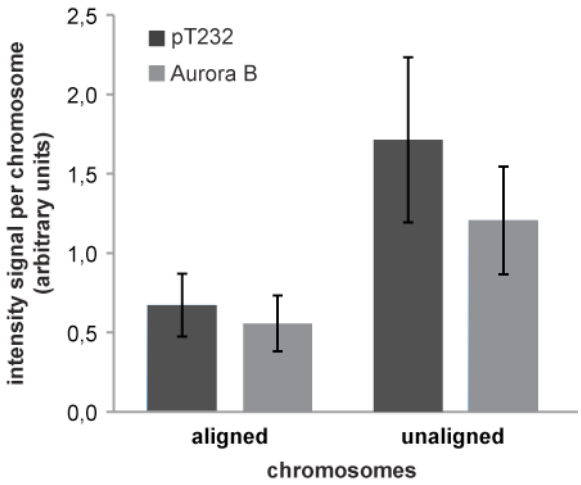


Figure 2.8 Aurora B kinase protein levels and activity in CENP-E-depleted cells. Quantification of pT232 and total Aurora B intensity levels in the aligned and unaligned chromosomes of CENP-E-depleted cells. See materials and methods for further details on the quantification. Error bars represent standard deviation.

To corroborate that the polar chromosomes in CENP-E-depleted cells fail to acquire stable attachments due to high local activity of Aurora B kinase, Aurora B was then inhibited with ZM447439 and the attachment status of the kinetochores was indirectly monitored by IF of Mad1 (Fig 2.9 A-B). In the majority of the polar chromosomes, kinetochores were either unattached (both kinetochores positive for Mad1) or mono-attached (one kinetochore positive for Mad1). Yet, after inhibition of Aurora B nearly all the polar chromosomes failed to accumulate Mad1 at their kinetochores, indicating that these kinetochores had acquired stable microtubule attachments (Fig. 2.9A-B).

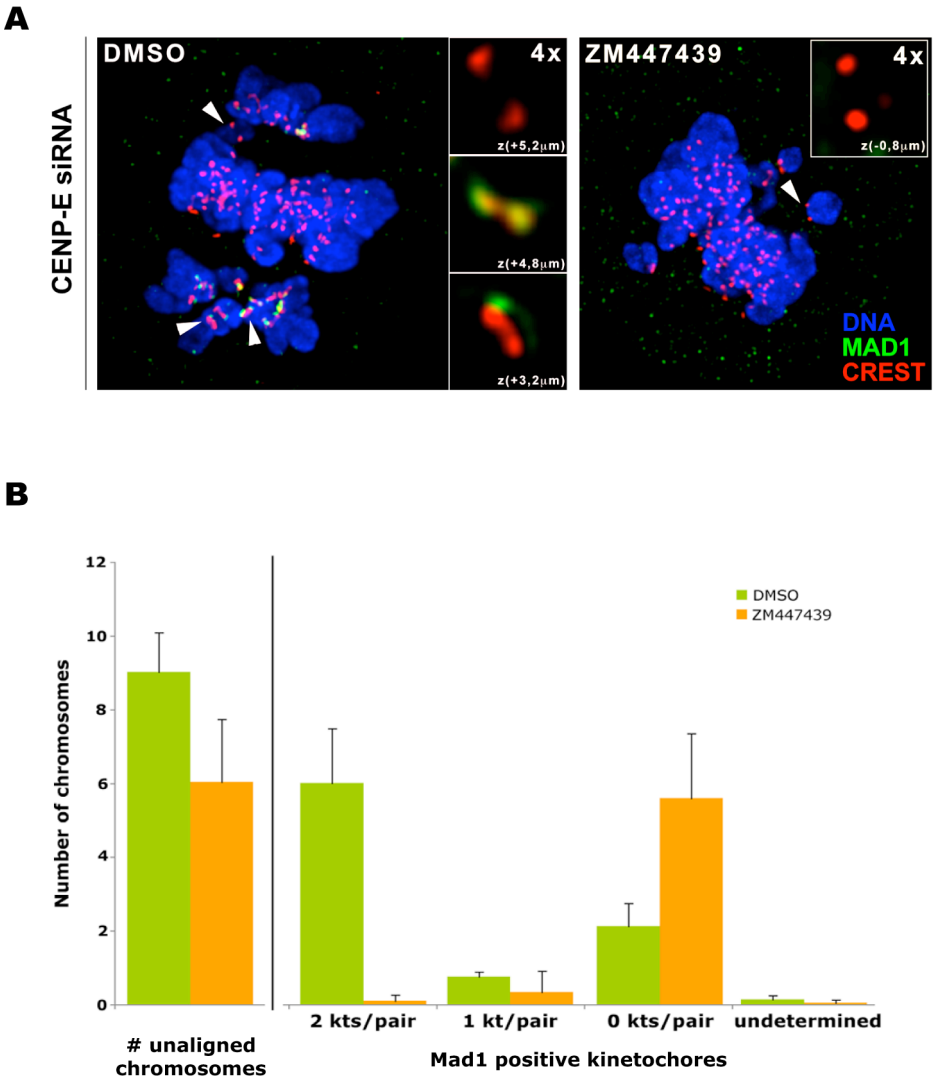


Figure 2.9 Microtubule-kinetochore attachments in cells depleted of CENP-E. (A) IF of Mad1 as an indirect marker for the attachment status of the kinetochore. Insets show higher magnifications of a single optical Z-stack of an unaligned chromosome with attached kinetochores (no Mad1, inset 1), with unattached kinetochores (Mad1 labels both kinetochores, inset 2) or with mono-attached kinetochores (Mad1 labels one kinetochore, inset 3). In cells treated with ZM447439 Mad1 is absent from all the unaligned chromosomes (inset shows higher magnification of a representative chromosome). **(B)** Quantification of the number of unaligned chromosomes in CENP-E-depleted cells with or without ZM447439 (left two bars). From these the number of chromosomes with unattached, mono-attached or attached kinetochores were scored. Average (± s.d.) of three independent experiments (n=27, DMSO; n=22, ZM447439).

Importantly, in the used experimental setup Mad1 levels were not reduced upon inhibition of Aurora B kinase by 35 minutes treatment with ZM447439 when microtubules were depolymerized by nocodazole (Fig. 2.10A-B). These results therefore demonstrate that Aurora B kinase activity destabilizes microtubule attachments of the kinetochores of polar chromosomes in CENP-E-depleted cells.

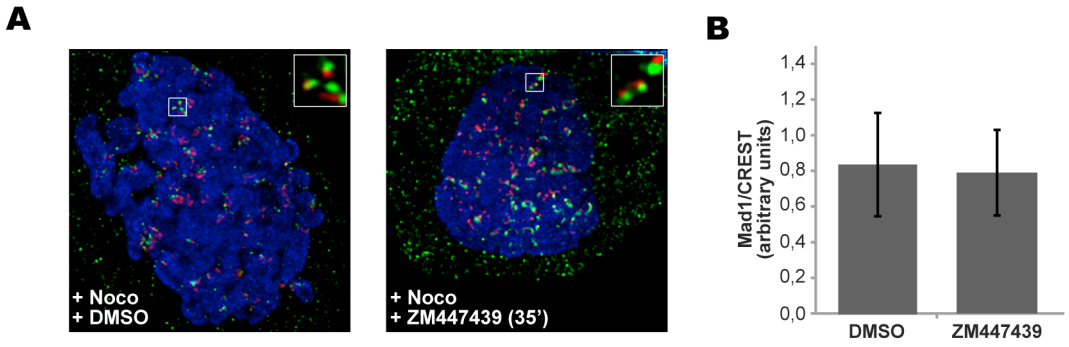


Figure 2.10 Mad1 localization before and after Aurora B kinase inhibition. (A) Immunofluorescence images of control siRNA-treated cells showing Mad1 localization (green) with or without 35 min. treatment with ZM447439. Cells were also treated with the microtubule depolymerizing drug nocodazole. DNA is shown in blue and CREST in red. **(B)** Bar graph plotting the fluorescence ratio of Mad1 to CREST signal under control (DMSO) or ZM447439 treatment. Error bars represent s.d.

2.3 AURORA B DELAYS MITOTIC PROGRESSION UPON CENP-E DEPLETION

Since the polar chromosomes in CENP-E-depleted cells remain unattached in an Aurora B dependent fashion it was hypothesized that Aurora B kinase activity was the molecular signal responsible for the mitotic delay. To accurately time mitotic exit after inhibition of Aurora B kinase activity CENP-E knockdown cells were first allowed to accumulate in mitosis with polar chromosomes and then ZM447439 was added (Fig. 2.11A). It was found that all the CENP-E-depleted cells entered anaphase with unaligned chromosomes 39.8±14.9 min. after addition of ZM447439 (Fig. 2.11B-C, Movies 2.7 and 2.8, Table 2.2). Furthermore, the TIM before addition of ZM447439 was also determined and this revealed that the duration of mitosis before Aurora B kinase inhibition did not influence the timing of anaphase onset after kinase inhibition (Fig. 2.11D). Interestingly, when CENP-E-depleted cells were treated with the microtubule depolymerizing drug nocodazole followed by addition of ZM447439 the SAC was not compromised and these cells remained arrested in mitosis (Fig. 2.11E).

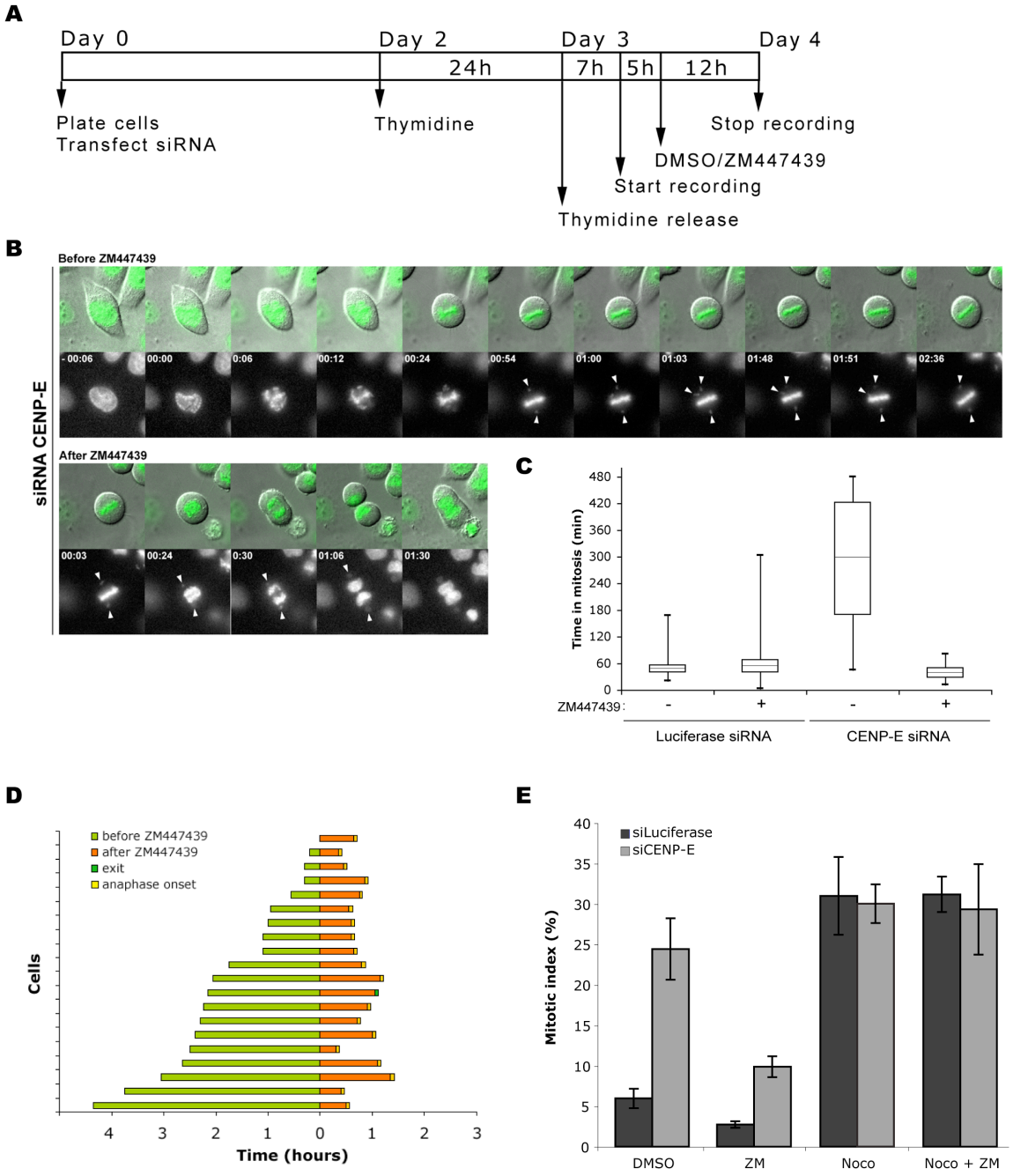


Figure 2.11 Aurora B kinase activity and CENP-E mitotic progression. (A) Experimental setup to determine the TIM of CENP-E-depleted cells after inhibition of Aurora B. Cells were released from a thymidine block and treated with DMSO/ZM447439 after they acquired a prometaphase configuration with polar chromosomes. **(B)** Live-cell video microscopy stills of HeLa YFP-H2B cells transfected with CENP-E siRNA "Before ZM447439" and "After ZM447439" addition. Arrowheads indicate the polar chromosomes. NEBD 00:00. Time hr:min. **(C)** Box-and-Whisker plots (median, interquartile range and full range) showing the TIM, here defined as the interval between addition of DMSO or ZM447439 and anaphase onset/mitotic exit. **(D)** TIM before and after addition of ZM447439 in CENP-E-knockdown cells. Yellow labels indicate cells undergoing anaphase and green labels indicate cells that decondensed the DNA and re-flattened. Graph shows 20 randomly chosen cells. **(E)** Quantification of the mitotic index of nocodazole-treated CENP-E-knockdown cells (± ZM447439). Error bars represent s.d.

Although, Aurora B can also influence the SAC in a more direct fashion (Vader et al., 2008) these data clearly show that in CENP-E-defective cells the SAC is active because high Aurora B kinase activity detaches microtubules from polar chromosomes.

2.4 CENP-E-DEPLETED CELLS DO NOT SLIP OUT OF MITOSIS BUT SATISFY THE SAC

Still, as shown in Fig. 2.4, after an approximately five-hour delay 37% of the cells undergo anaphase onset with a few misaligned chromosomes. This indicates that in the absence of CENP-E, the polar chromosomes can only prolong mitosis for some time. There are two possible ways by which cells exit mitosis after a long mitotic delay. Cells either exit because the SAC is silenced (Musacchio and Salmon, 2007) or the SAC is bypassed, a situation known as mitotic slippage (Andreassen and Margolis, 1994). In contrast to cells that silence the checkpoint, cells that bypass the SAC retain the checkpoint proteins Mad1, Mad2 and BubR1 at kinetochores in telophase and do not degrade the APC/C substrate Tpx2 (Brito and Rieder, 2006). To test if CENP-E-depleted cells silence or bypass the SAC cells were allowed to accumulate in mitosis for approximately 6 hours and then fixed and prepared for IF analysis. While Mad1 was localized on kinetochores of the polar chromosomes in mitosis (Fig. 2.12, inset 1; Fig. 2.6A), no kinetochore-associated signal was detected in the micronuclei that were formed by these misaligned chromosomes in telophase (Fig. 2.12, inset 2-3). Furthermore, high Tpx2 staining was found in mitotic cells but not in the micronuclei of cells that had undergone anaphase onset with unaligned chromosomes (Fig. 2.12C). The absence of both Mad1 and Tpx2 in the (micro)nuclei of CENP-E-depleted cells indicated that exit from mitosis was associated with SAC silencing and not due to mitotic slippage.

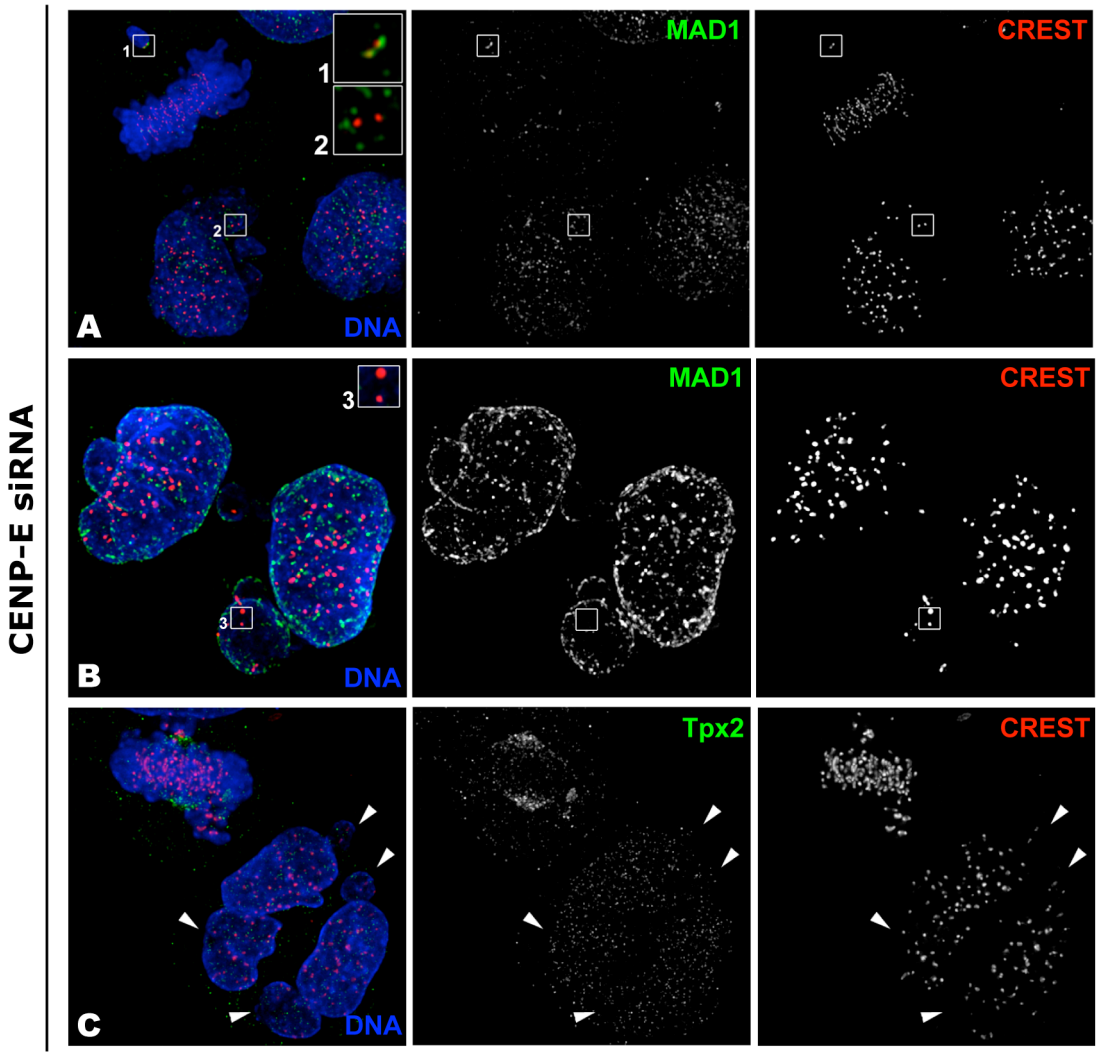


Figure 2.12 CENP-E-depleted cells silence the SAC. IF images for the SAC protein Mad1 **(A-B)** and the APC/C substrate Tpx2 **(C).** Note the presence of Mad1 on the kinetochores of the polar chromosomes (inset 1) and absence of Mad1 in telophase (inset 2-3). **(C)** Tpx2 localization in a CENP-E-depleted mitotic cell and absence of Tpx2 in micronuclei (arrowheads).

3. DISCUSSION

The data show that CENP-E facilitates efficient and timely chromosome congression but is not absolutely essential for full chromosome alignment. In the absence of CENP-E a few chromosomes experience congression difficulties and stay close to the spindle poles. Although, kinetochores of these polar chromosomes can interact with microtubules (a few polar chromosomes negative for Mad1 are always found, Fig. 2.9 A-

B), these microtubule attachments are not stabilized because of high local Aurora B kinase activity. Instead, these pole-proximal syntelic KT-MT attachments are destabilized, resulting in a SAC-dependent mitotic delay. In the majority of the CENP-E-knockdown cells this allows extra time and opportunity for chromosome congression by CENP-E independent mechanisms (McEwen et al., 2001), or by the remaining low levels of CENP-E, thus reducing chromosome segregation errors. Yet, in a subset of CENP-E-depleted cells the SAC was eventually silenced, and the question remains why.

Studies in *Xenopus* extracts showed a direct link between CENP-E and the mitotic checkpoint protein BubR1 suggesting that depletion of CENP-E could directly weaken the checkpoint (Mao et al., 2003; Yao et al., 2000). However, in human cells CENP-E-knockdown does not shorten the mitotic delay induced by the microtubule poisons taxol and nocodazole (Tanudji et al., 2004 ; Fig. 2.11 E), indicating that CENP-E is not required for a robust SAC response in these cells. In addition, it was found that inhibition of Aurora B only silenced the SAC in CENP-E-depleted cells when microtubules were present. In the presence of the microtubule depolymerizing drug nocodazole, co-inhibition of Aurora B and CENP-E did not further compromise the mitotic checkpoint. This indicates that in the CENP-E-depleted cells the mitotic delay is due to the microtubule destabilizing activity of Aurora B (Pinsky et al., 2006) and silencing of the SAC in a subset of the cells is most likely due to acquisition of microtubule attachments. So, why do the kinetochores of these polar chromosomes eventually become attached?

Since polar chromosomes still show high levels of active Aurora B kinase after a prolonged time in mitosis (data not shown), it is unlikely that these chromosomes acquired stable microtubule attachments because Aurora B kinase activity ceased over time. In light of recent data supporting a role for CENP-E in microtubule destabilization (Maffini et al., 2009; Maia et al., 2007), here it is favored the idea that because microtubules are more stable in the absence of CENP-E, the microtubule destabilization rate on the polar chromosomes will be slower increasing the likelihood that the final 1-2 polar chromosomes eventually become attached even when Aurora B kinase activity is high. This idea is supported by (Yang et al., 2009) suggesting that error correction can be attenuated over time while Aurora B is active if microtubules are stabilized by high taxol concentrations.

Finally, although a cooperative function for Aurora B in CENP-E dependent congression has been implied earlier (Kapoor et al., 2006), this work now in fact shows that high local Aurora B kinase activity liberates polar kinetochores from end-on attachments by destabilizing pole-proximal syntelic attachments. As such, Aurora B could

permit lateral attachment and CENP-E dependent sliding along microtubule bundles to promote efficient chromosome congression and timely anaphase onset.

Table 2.1 Quantification of time spent in mitosis (TIM) in the different experimental conditions. TIM is shown in minutes (min).

	TIM (min)							
Experiment	**Median**	**Average**	**STD[1]**	**SEM[2]**	**min[3]**	**max[3]**	**n_{cells}**	**nr. Exp**
DMSO	45	49,0	22,4	2,2	30	84	101	2
siLuciferase (5nM)	48	50,2	18,4	1,5	30	93	156	3
siCENP-E (5nM) Phenotype a	228	238,1	122,5	12,4	69	≥480	98	4
siCENP-E (5nM) Phenotype b	291	305,8	121,2	14,7	90	≥480	68	4

Table 2.2 Quantification of time spent in mitosis (TIM) in control and CENP-E-depleted cells after inhibition of Aurora B kinase activity. TIM is shown in minutes (min).

	TIM (min)							
Experiment	**Median**	**Average**	**STD[1]**	**SEM[2]**	**min[3]**	**max[3]**	**n_{cells}**	**nr. Exp**
siLuciferase (5nM) + ZM447439	48	60,5	38,2	3,5	27	156	120	3
siCENP-E (5nM) + ZM447439	39	39,8	14,9	1,5	18	66	95	4

[1] standard deviation

[2] standard error of the mean

[3] range after exclusion of the top and bottom 3% values

4. MATERIALS AND METHODS

4.1 CELL CULTURE AND SIRNA

HeLa cells were cultured in DMEM with 6% FCS and antibiotics, at 37ºC in a humidified atmosphere with 5% CO_2. For cell synchronization at the G1/S transition, cells were incubated with 2.5 mM thymidine for 24 hours. siRNA transfection was done with HiPerFect (Qiagen) according to the manufacturer's protocol. The CENP-E siRNA duplex (GAACUAAGAAGAAGCGUAU) and the Luciferase siRNA duplex (CGUACGCGGAAUACUUCGA) were from Dharmacon®. Drugs were used at the following concentrations: nocodazole, 0.25 μg/mL; MG132, 5 μM; ZM447439 (Tocris Bioscience, Bristol, UK), 2 μM. Incubation with ZM447439 was done for 35 minutes unless stated otherwise. To determine the mitotic index, RNAi-treated cells were released from a thymidine block to DMSO or nocodazole for 16 hours. Before fixation incubation with DMSO/ZM447439 was performed for 1 hour. Cells were fixed for 5 minutes in 8% formaldehyde, washed once with PBS, followed by 5 minutes incubation in ice-cold methanol. After washes with PBS, cells were counterstained with DAPI. Determination of the mitotic index was done in a Thermo Scientific Cellomics® ArrayScan® VTI, software version x.5. $>10^4$ cells were counted per condition.

4.2 ANTIBODIES AND REAGENTS

The following antibodies were used: Aurora B mAb (Transduction Laboratories, Lexington, KY), pT232-Aurora B pAb (Rockland, Philadelphia, PA), human CREST antiserum (Cortex Biochem, San Leandro, CA), Mad1 mAb (gift of A. Musacchio, European Institute of Oncology, Italy), Tpx2 mAb (BioLegend, San Diego, CA), CLIP-170 pAb (gift of N. Galjart, Erasmus Medical Center, The Netherlands), CENP-E (gift of D.Cleveland, Ludwig Institute for Cancer Research, CA), α-Tubulin (Sigma, St. Louis, MO). Secondary antibodies for immunofluorescence (Alexa-488, -568 and -647) were for Molecular Probes (Eugene, OR). Reagents were from Sigma unless stated otherwise.

4.3 IMMUNOBLOTTING AND IMMUNOFLUORESCENCE

Immunoblotting was performed as described (Smits et al., 2000). Prior to IF coverslips were washed with PEM buffer (100 mM Pipes, 10 mM EGTA, 1 mM $MgCl_2$), fixed for 10 minutes in 4% paraformaldehyde/0.2% sucrose, then washed once with PEM and permeabilized with 0.5% Triton X-100/PEM for 15 minutes. For IF, cells were washed with DPBS and incubated with the appropriate primary/secondary antibody combinations diluted in DPBS/3% BSA. Cells were counterstained with DAPI to visualize the DNA. Images were acquired using a Zeiss Axio Imager microscope (Carl Zeiss, Germany) using an Axiocam (Carl Zeiss, Germany). Data stacks were deconvolved using the Huygens Professional software (Scientific Volume Imaging BV, The Netherlands).

4.4 TIME-LAPSE MICROSCOPY

Cells were followed by time-lapse microscopy starting at 7 hours after release from the thymidine block. Lab-Tek TM II Chamber SlideTM System (NuncTM) was used to grow and film the cells on a Zeiss Axiovert 200M microcope (F-Fluar 40x/1.3 Oil) equipped with a motorized heated stage (37ºC), halogen lamp, Lambda DG-4 Ultra High Speed Wavelenght Switcher (Sutter Instruments) and CO2 controller. Phase contrast and fluorescent images were captured every 3 minutes using a Photometrics Coolsnap HQ camera (Scientific, Tucson, AZ). Images were processed using Metamorph software (Universal Imaging, Downington, PA).

4.5 QUANTIFICATION OF ACTIVE AURORA B

For quantification of Aurora B centromere activity, cells were stained with pT232-Aurora B and CREST antibodies. Images with multiple z-planes were captured and the integrated density of the T232 signal was quantified in individualized kinetochore pairs with ImageJ software (NIH) after deconvolution of the data stacks. Normalization for the background signal was performed. The average integrated density for pT232 was set in relation to average integrated density of the CREST signal. Five randomly chosen individual kinetochore pairs were analyzed per cell in five different cells, in 3 independent experiments.

CHAPTER 3

Preliminary studies on Sgt1, a newly described *Drosophila* kinetochore protein

1. INTRODUCTION

Accurate chromosome segregation requires organization and proper function of the mitotic apparatus including correct attachment of sister chromatids to spindle microtubules. Microtubule-kinetochore interaction is highly complex and involves many factors. It is now clear that kinetochores are highly conserved and contain a large number of proteins including internal structural components that ensure DNA binding, external components for microtubule binding and proteins involved in signalling processes like those required for the activation of the Spindle Assembly Checkpoint (SAC) (Musacchio and Salmon, 2007). Previously, it was suggested that in yeast and human cultured cells assembly or at least proper localization of some kinetochore components requires the activity of the Hsp90 cochaperone Sgt1 (Kitagawa et al., 1999; Steensgaard et al., 2004). Sgt1 was initially identified in a screen aimed to isolate suppressors of a temperature sensitive allele of skp1 and characterized as a subunit of both the core kinetochore and SCF (Skp1-Cul1-F-Box) ubiquitin ligase complexes (Kitagawa et al., 1999). Sgt1 contains protein interaction motifs like the tetracopeptide repeat domain (TPR Domain) and p23-like CHORD domain (also called CS domain), both found in proteins that interact with chaperones, as well as a Sgt1-specific domain (SGS Domain) required for its interaction with adenyl cyclase. As expected, Human Sgt1 was shown to bind chaperone Hsp90 by its CHORD domain that is very similar to the well-known Hsp90 co-chaperone p23 (Lee et al., 2004). The interaction between Sgt1 and Hsp90 was also described both in genetic and biochemical studies in yeast, where the complex was described as being important for assembly and turnover of the essential centromere binding factor 3 (CBF3) (Bansal et al., 2004; Catlett and Kaplan, 2006; Lingelbach and Kaplan, 2004; Rodrigo-Brenni et al., 2004).

In Human cells, a severe reduction of Sgt1 levels was shown to compromise kinetochore assembly and specifically localization of a number of SAC proteins including Mad1, Mad2 and BubR1 (Steensgaard et al., 2004). This resulted in a weaker SAC response when cells were challenged with spindle poisons that depolymerise microtubules. However, not only SAC proteins failed to accumulate but also other constituents of the human kinetochore, including Hec1, CENP-E, CENP-F and CENP-I were absent after depletion of Sgt1 (Steensgaard et al., 2004). Interestingly, studies in Human cells with an inhibitor of Hsp90 (17-AAG) suggested that this chaperone is important for kinetochore assembly since it causes delocalization of various centromeric proteins, mitotic arrest, failure of chromosome congression and aneuploidy (Niikura et al., 2006).

To address the role of Sgt1 further, first the Sgt1 homologue was identified and its sub-cellular localization during mitosis described. Surprisingly and opposite to what is reported for the human homologue *Drosophila* Sgt1 does have specific subcellular localizations at different stages of mitosis.

2. Results

2.1 Identification and sub-cellular localization of the *Drosophila* homologue of Sgt1

The *Drosophila* orthologue of Sgt1 was identified through blast searches using the human Sgt1 protein sequence. A single highly conserved putative protein encoded by the gene CG9617 was identified with 41% amino acid identity (Fig. 3.1A). Sequence analysis revealed that most members of the Sgt1 family are characterized by having three distinct domains: a TPR motif, a p23-like CHORD Domain (also called CS Domain) and the Sgt1 specific domain (SGS). While the *Drosophila* orthologue lacks the TPR domain, the function of Sgt1 as a co-chaperone (Bansal et al., 2004) is unlikely to be affected since the TPR and CS domain are both chaperone-interacting domains that may be redundant. Sgt1 has been reported in human cells to be a soluble protein without any particular localization during mitosis (Steensgaard et al., 2004). To determine if *Drosophila* Sgt1 localized in a similar manner, the coding sequence was tagged with EGFP, transfected into S2 *Drosophila* cells and expressed under the control of an inducible promoter (Fig. 3.1B-D). In asynchronous cultures, no specific accumulation of EGFP-Sgt1 was observed besides a clear localization at the mid body during very late mitosis (Fig. 3.1B). However, if cells were arrested in mitosis with the microtubule depolymerizing drug colchicine, EGFP-Sgt1 showed diffuse cytoplasmic staining but also strong accumulation at centrosomes and kinetochores (Fig. 3.1C and 3.1D). Expression of EGFP-Sgt1 in interphase or of EGFP alone in either interphase or mitosis gave no specific localization. These results indicate that unlike yeast and human cells, Sgt1 in *Drosophila* shows a specific subcellular localization at different stages of mitosis remarkably when spindle microtubules are depolymerised.

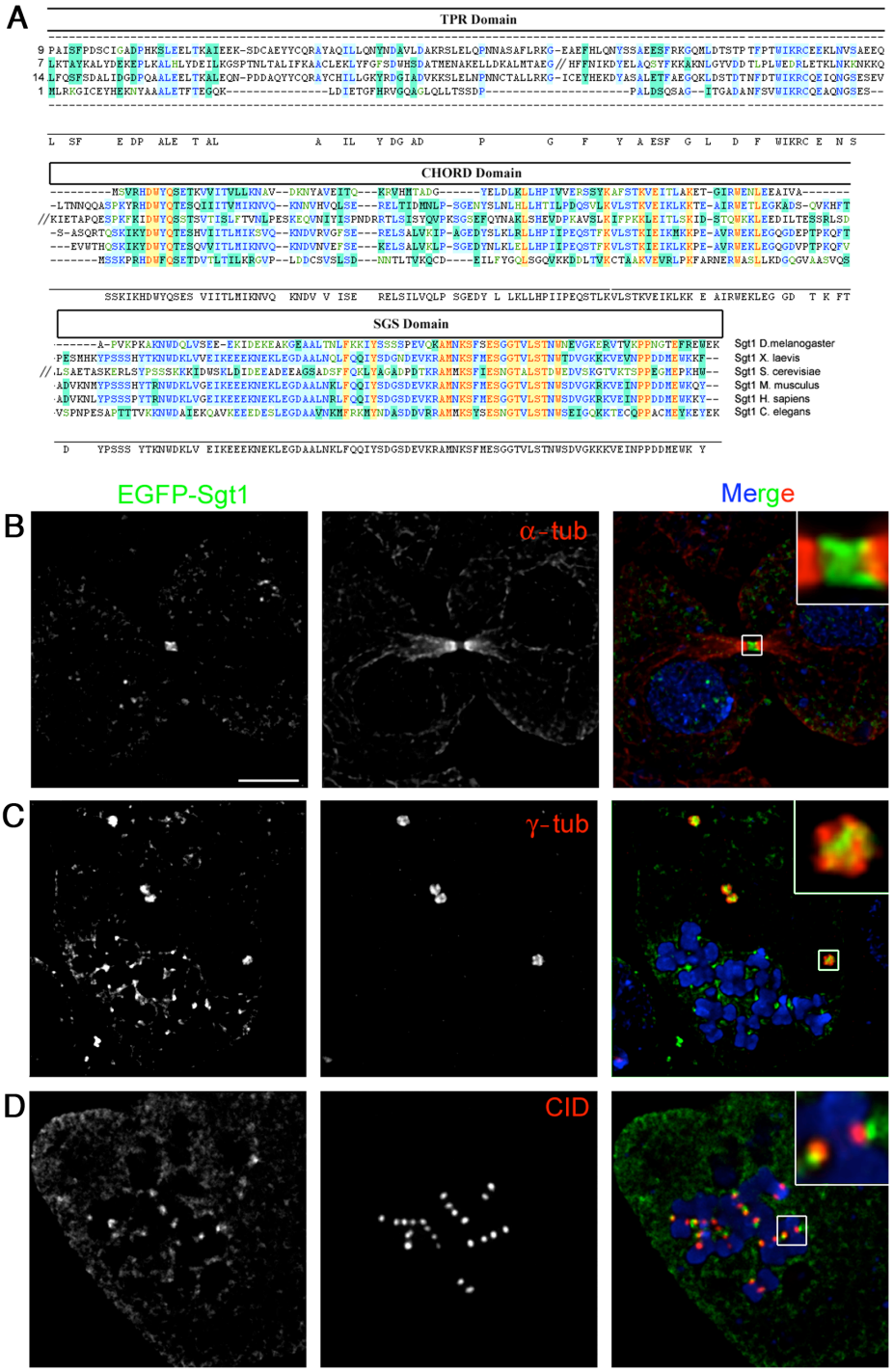


Figure 3.1 Identification of Sgt1 in *Drosophila* and cellular localization in S2 cells. (A) Sequence homology between Sgt1 proteins from different species. The conserved domains are indicated in boxes: Tetracopeptide domain (TPR Domain), p23-like CHORD Domain and Sgt1-specific domain (SGS Domain). **(B-D)** Localization of EGFP-Sgt1 in S2 cells. **(B)** In asynchronous cells EGFP-Sgt1 (green) can only be detected at the cleavage furrow during late mitosis as shown by the co-staining with α-tubulin (red). When cells are treated with colchicine, **(C)** accumulation of EGFP-Sgt1 (green) can be observe at the centrosomes identified by γ-tubulin (red) and **(D)** at the

outer region of the kinetochores as shown by co-staining with the centromeric marker CID (red). For all immunolocalizations DNA was stained with DAPI (blue). Bar is 5 µm.

3. DISCUSSION

The *Drosophila melanogaster* homologue of the human Sgt1 protein was identified and is encoded by the gene CG9617. This protein is partially conserved from yeast to human suggesting that it might play key roles in cellular processes. Members of the Sgt1 protein family contain three highly conserved domains in many species: TPR, p23-like CHORD and SGS domains. The TPR and p23-like CHORD domains are characteristic of proteins that interact with chaperones directing them to a specific group of proteins, named “client proteins”. Therefore, Sgt1 was classified as a co-chaperone since it interacts with the chaperone HSP90 through the p23-like domain. Moreover, Sgt1 is thought to form a complex that through the TPR domain activates Skp1, a protein involved in kinetochore assembly (Bansal et al., 2004; Kitagawa et al., 1999; Rodrigo-Brenni et al., 2004; Steensgaard et al., 2004). Although, *Drosophila* Sgt1 lacks the TPR domain, it is unlikely that its co-chaperone function is affected as in human cells the interaction with HSP90 is made through the p23-like CHORD domain (Lee et al., 2004), that is also conserved in the *Drosophila* protein. However, in *Drosophila*, Sgt1 protein is unlikely to interact with Skp1 and therefore might not be required for kinetochore assembly (Bansal et al., 2004; Rodrigo-Brenni et al., 2004).

Immunofluorescence studies done here show that Sgt1 localizes to the midbody in late mitosis and in the absence of microtubules to the outer kinetochore domain and also at centrosomes. This localization pattern suggests that Sgt1 might play a role in microtubule-kinetochore interaction and in centrosome function. While these results suggest that Sgt1 is required for somatic cell proliferation, meiosis might also require Sgt1 since ovaries and testis are highly abundant in Sgt1 mRNA (Chintapalli et al., 2007).

Although very preliminary, these results were a launching pad for a new project to be developed in Prof. Claudio Sunkel lab concerning the roles of Sgt1 during mitotic divisions in *Drosophila melanogaster*. At the moment of the writing of this thesis a paper with the follow-up studies, that were taken over by another PhD student in the lab, was published (Martins et al., 2009). Briefly, it was shown that mutations in *sgt1* do not affect overall kinetochore assembly or the spindle assembly checkpoint. *sgt1* mutant cells enter less frequently into mitosis and arrest in a prometaphase-like state. Mutations in *sgt1* severely compromise the organization and function of the mitotic apparatus. In these cells,

centrioles replicate but centrosomes fail to mature and pericentriolar material components do not localize normally resulting in highly abnormal spindles. Interestingly, a similar phenotype was previously described in Hsp90 mutant cells and correlated with a decrease in Polo protein levels. In *sgt1* mutant neuroblasts it was observed a decrease in overall levels of Polo. Overexpression of Polo resulted in a substantial rescue of the centrosome defects; most cells formed normal bipolar spindles and progressed through mitosis normally. Taken together, these findings suggest that Sgt1 is involved in stabilization of Polo allowing normal centrosome maturation, entry and progression through mitosis.

4. MATERIALS AND METHODS

4.1 TRANSFECTION OF S2 CELLS

Drosophila S2 cells were grown to a yield of $3x10^6$ cells in 3 mL of Schneider medium (Sigma) with 10% fetal bovine serum (FBS) at 25ºC and then transfected with pMT-EGFP-Sgt1 using calcium phosphate. Expression of the EGFP-Sgt1 construct was induced with $CuSO_4$ (1mM), and cells were collected after 16h for immunofluorescence analysis. Where indicated, cells were treated with 10 μM of colchicine for the desired period. The pMT-EGFP-Sgt1 vector was generated by cloning Sgt1 full-length cDNA into the *HincII*/*SacI* sites of the MCS of pMT-EGFP-C1 plasmid.

4.2 IMMUNOSTAINING IN S2 CELLS AND ANTIBODIES

Immunostaining was performed as previously described in the chapter 1 (section 4.2). The primary antibodies used were anti-α-tubulin mouse B512 (Sigma) used at 1:1000; anti-γ-tubulin mouse GTU88 (Sigma) used at 1:250; and anti-CID rat polyclonal (CE Sunkel, unpublished data) used at 1:1500. The secondary antibodies were obtained from Molecular Probes and Jackson Immunoresearch and used according manufacturer's instructions.

PART III

GENERAL DISCUSSION

Cell division cycle is vital for the development and function of all living organisms: from a simpler single-celled organism, where it grants the generation of an entire new organism, to a highly complex multicellular organism, where it provides the formation and subsequent maintenance of all sorts of specialized cells, tissues and organs.

One of the most visual phases of the cell cycle is the distribution of the chromosomes into two daughter cells. And although what can be observed, with state of the art technology and molecular biology techniques, is a gracious movement of all chromosomes to the equatorial plane of the cell and subsequent equal segregation generating two newly formed cells, this process is not error-free and therefore has to be highly regulated. Errors during cell division often lead to unequal segregation of the chromosomes (aneuploidy), known to be a major cause for the origin of birth defects (Ambartsumyan and Clark, 2008), and it has been argued that could underlay tumour development (Holland and Cleveland, 2009).

Appropriately, cells have evolved a mechanism, the Spindle Assembly Checkpoint, that significantly reduces chromosomal missegregation and thus contributes to the maintenance of genomic stability (Kops et al., 2005). The SAC is capable of delaying exit from mitosis until all kinetochores have acquired proper microtubule attachments. This requires that each sister kinetochore of a chromosome is attached to microtubules from opposite spindle poles, called biorientation or more specifically amphytelic attachment. Only when each sister chromatid from each chromosome is properly attached segregation of the genome will be correct.

However, as nuclear envelope breakdown takes place and microtubules emanated from centrosomes grow in all directions, they are able to bind indiscriminately to kinetochores and not always the chromosomes achieve biorientation during the first attachment attempt. Therefore, chromosomes can acquire abnormal attachments that need to be corrected. In order to correct defective kinetochore-microtubule interactions cells must be able to destabilize these erroneous attachments. Aurora B/Ilp1 is the mitotic protein kinase thought to be intimately involved in this correction mechanism. It has been shown that ultimately, this kinase leads to the destabilization of kinetochore microtubules thus creating unattached kinetochores that then trigger again the SAC (Pinsky et al., 2006) giving extra time and new opportunity for a new round towards biorientation.

After microtubules attach and chromosomes become bioriented they need to move towards the centre of the cell, a process known as congression. Chromosome congression is mostly understood in terms of polymerization and depolymerization of kinetochore microtubules. However, if a chromosome initially becomes monoriented with

only one sister kinetochore stably bound to one spindle pole, causing the chromosome to rapidly move towards that pole, how will it then become bioriented? Interestingly, chromosomes can also congress to the metaphase plate even if they are not bioriented, suggesting that other mechanisms are involved. It is known that the motor protein CENP-E plays a central role in this process as it mediates the gliding of mono-oriented kinetochores alongside adjacent k-fibers (Kapoor et al., 2006) or even alongside any microtubule bundle (Cai et al., 2009).

The work presented in this thesis aims to expand our knowledge on the molecular mechanisms that involve the kinetochore as a signal generator for the SAC while at the same time works as a vehicle through which the chromosome move within the mitotic spindle during early stages of congression. Also, we would like understanding how the combination of such functions ensures faithful chromosome segregation. More specifically, it focused on the study of the *bona fide* SAC protein BubR1 and the kinesin-7 motor protein CENP-E.

A part from being a core component of the mitotic checkpoint complex (Sudakin et al., 2001), BubR1 has also been implicated in chromosome congression independently of its function in the SAC (Ditchfield et al., 2003). Interestingly, BubR1 interacts with the microtubule motor CENP-E (Chan et al., 1998; Yao et al., 2000) suggesting that together they may play important roles at the kinetochore, namely in SAC activity and chromosome congression. In the first chapter of this thesis, a detailed comparative study of the knockdown of BubR1 and CENP-meta/CENP-E was carried out in *Drosophila* S2 cells. The results clearly show that the phenotypes after CENP-meta and BubR1 RNAi treatment differ. While depletion of BubR1 impaired the SAC, CENP-meta depletion had no effect in this mechanism. Moreover, the spindle defects observed after knockdown of each of these proteins was also very different. BubR1 was found to be involved in promoting the stability of the kinetochore-microtubule interactions, as previously observed in human cell lines (Ditchfield et al., 2003; Lampson and Kapoor, 2005), whilst unexpectedly CENP-meta/CENP-E promotes the destabilization of kinetochore attachment. Previously, CENP-E has been suggested to contribute for the stability of kinetochore microtubules (Putkey et al., 2002), therefore it was surprising that in the *Drosophila* S2 tissue culture system CENP-meta/CENP-E was somehow involved in creating a microtubule destabilizing environment at the kinetochore. However, correlative light electron microscopy studies have clearly shown that HeLa cells microinjected with anti-CENP-E antibodies, despite showing less microtubules bound to the kinetochores when the major peak of mitotic cells occurs, in cells that undergo a prolonged mitotic arrest a full complement of spindle microtubules were observed (McEwen et al., 2001).

These data suggests that in the absence of CENP-E, over time, kinetochore-microtubule attachment becomes more stable. Furthermore, it was recently determined, by measuring fluorescence loss after photoactivation of GFP-α-tubulin, that the calculated half-time turnover for kinetochore microtubules is significantly higher in cells depleted of CENP-E comparing with control RNAi cells (Maffini et al., 2009). This data, obtained in mammalian cells, nicely corroborates our results and suggest that CENP-meta/CENP-E normally contributes towards a partial instability of microtubule-kinetochore attachments. Neverheless, how CENP-E promotes destabilization of microtubule-kinetochore (MT-KT) attachments remains to be determined.

Our results show that depletion of BubR1, in *Drosophila* S2 cells, leads to destabilization of connections between chromosomes and spindle microtubules and that if BubR1 knockdown is combined with depletion of CENP-meta/CENP-E an increased stability of these connections is observed. These data together with the suggestion that BubR1 can inhibit Aurora B kinase activity (Lampson and Kapoor, 2005), allows several models for CENP-E function in the regulation of chromosome-spindle attachments:

One possibility is that CENP-E, directly or indirectly, could inhibit BubR1 thus leading to a situation where Aurora B kinase activity would be higher therefore decreased stability of the MT-KT interactions is observed. In fact, in *Xenopus* egg extracts a direct interaction between CENP-E and BubR1 was observed (Mao et al., 2003) and in HeLa cells CENP-E was shown to form a complex with BubR1 (Yao et al., 2000). However, the functional consequence of the association between these two proteins in human cells remains to be elucidated.

Another attractive hypothesis is that CENP-E might be involved in the correction of defective microtubule attachments through a direct or indirect regulation of Aurora B kinase. Since depletion of CENP-E leads to increased stability of kinetochore microtubules, this hypothesis predicts that Aurora B kinase should be diminished in the absence of CENP-E. However, the results obtained in chapter two of our study do not support this prediction. We have shown that in chromosomes that fail to reach the centre of the cell and become localized to the spindle poles, the polar chromosomes characteristic of CENP-E depletion, Aurora B kinase is highly active and as a consequence kinetochore attachment in these chromosomes is continuously being destabilized.

Finally, an alternative possibility might be that CENP-E itself or by recruiting and/or interacting with other key microtubule regulators at the kinetochores, could promote a microtubule-destabilizing environment. In fact, CENP-E is known to be required for the

recruitment of CLASP1 and CLASP2 (Maffini et al., 2009), two microtubule-associated proteins previously implicated in the regulation of kinetochore microtubule dynamics (Maiato et al., 2003a; Maiato et al., 2005; Pereira et al., 2006).

In chapter two of this thesis we continued to address the function of CENP-E at the kinetochore and we found that CENP-E and Aurora B cooperate closely. Our results allowed us to propose that Aurora B might assist CENP-E in its function of promoting the sliding of the kinetochores along microtubule bundles during chromosome congression (Cai et al., 2009; Kapoor et al., 2006). We have shown that Aurora B kinase activity destabilizes the attachments of polar chromosomes present in CENP-E-depleted cells, strongly suggesting that this destabilization, apart from triggering the SAC and promoting a mitotic delay, also frees the kinetochores from a synthelic attachment so they can slide towards the metaphase plate where they have an increased chance of becoming bioriented.

However, an intriguing question remains from all these studies (our results; Johnson et al., 2004; McEwen et al., 2001). How is that in approximately half of the cells depleted of CENP-E, after a long mitotic delay, all the chromosomes do move towards the metaphase plate, congress and exit mitosis? These results indicate very clearly that there are CENP-E independent mechanisms for chromosome congression, though these are surely much slower that when CENP-E is present. However, some of the studies that support this interpretation, including the one presented in this thesis, are based either on antibody injections or RNA mediated interference, where residual levels of the protein could still account for the observed chromosome congression. Nevertheless, genetic studies in *Drosophila* (Yucel et al., 2000) and in primary mouse fibroblasts obtained from a conditional *CENP-E* knockout (Putkey et al., 2002), showed different degrees of normal chromosome congression providing strong support for the existence of redundant mechanisms for such an important cellular function that is essential for the success of mitosis.

In conclusion, our studies have provided new insight on how chromosomes, through their kinetochores, make their way towards the metaphase plate and how can kinetochores delay mitotic exit when errors occur thus avoiding aneuploidy. Furthermore, as mitosis is an attractive target for anti-cancer strategies and Aurora B kinase and CENP-E inhibitors are currently under clinical development/trials, the functional data obtained here might help designing better anti-cancer therapies.

PART IV

REFERENCES

Abrieu, A., J.A. Kahana, K.W. Wood, and D.W. Cleveland. (2000). CENP-E as an essential component of the mitotic checkpoint in vitro. *Cell*. 102:817-26

Abrieu, A., L. Magnaghi-Jaulin, J.A. Kahana, M. Peter, A. Castro, S. Vigneron, T. Lorca, D.W. Cleveland, and J.C. Labbe. (2001). Mps1 is a kinetochore-associated kinase essential for the vertebrate mitotic checkpoint. *Cell*. 106:83-93

Akhmanova, A., C.C. Hoogenraad, K. Drabek, T. Stepanova, B. Dortland, T. Verkerk, W. Vermeulen, B.M. Burgering, C.I. De Zeeuw, F. Grosveld, and N. Galjart. (2001). Clasps are CLIP-115 and -170 associating proteins involved in the regional regulation of microtubule dynamics in motile fibroblasts. *Cell*. 104:923-35

Ambartsumyan, G., and A.T. Clark. (2008). Aneuploidy and early human embryo development. *Hum Mol Genet*. 17:R10-5

Andreassen, P.R., and R.L. Margolis. (1994). Microtubule dependency of p34cdc2 inactivation and mitotic exit in mammalian cells. *J Cell Biol*. 127:789-802

Andreassen, P.R., S.N. Martineau, and R.L. Margolis. (1996). Chemical induction of mitotic checkpoint override in mammalian cells results in aneuploidy following a transient tetraploid state. *Mutat Res*. 372:181-94

Andrews, P.D., Y. Ovechkina, N. Morrice, M. Wagenbach, K. Duncan, L. Wordeman, and J.R. Swedlow. (2004). Aurora B regulates MCAK at the mitotic centromere. *Dev Cell*. 6:253-68

Arion, D., L. Meijer, L. Brizuela, and D. Beach. (1988). cdc2 is a component of the M phase-specific histone H1 kinase: evidence for identity with MPF. *Cell*. 55:371-8

Asbury, C.L., D.R. Gestaut, A.F. Powers, A.D. Franck, and T.N. Davis. (2006). The Dam1 kinetochore complex harnesses microtubule dynamics to produce force and movement. *Proc Natl Acad Sci U S A*. 103:9873-8

Babu, J.R., K.B. Jeganathan, D.J. Baker, X. Wu, N. Kang-Decker, and J.M. van Deursen. (2003). Rae1 is an essential mitotic checkpoint regulator that cooperates with Bub3 to prevent chromosome missegregation. *J Cell Biol*. 160:341-53

Bakhoum, S.F., S.L. Thompson, A.L. Manning, and D.A. Compton. (2009). Genome stability is ensured by temporal control of kinetochore-microtubule dynamics. *Nat Cell Biol*. 11:27-35

Bansal, P.K., R. Abdulle, and K. Kitagawa. (2004). Sgt1 associates with Hsp90: an initial step of assembly of the core kinetochore complex. *Mol Cell Biol*. 24:8069-79

Basu, J., H. Bousbaa, E. Logarinho, Z. Li, B.C. Williams, C. Lopes, C.E. Sunkel, and M.L. Goldberg. (1999). Mutations in the essential spindle checkpoint gene bub1 cause chromosome missegregation and fail to block apoptosis in Drosophila. *J Cell Biol*. 146:13-28

Basu, J., E. Logarinho, S. Herrmann, H. Bousbaa, Z. Li, G.K. Chan, T.J. Yen, C.E. Sunkel, and M.L. Goldberg. (1998). Localization of the Drosophila checkpoint

control protein Bub3 to the kinetochore requires Bub1 but not Zw10 or Rod. *Chromosoma*. 107:376-85

Baumann, C., R. Korner, K. Hofmann, and E.A. Nigg. (2007). PICH, a centromere-associated SNF2 family ATPase, is regulated by Plk1 and required for the spindle checkpoint. *Cell*. 128:101-14

Bernard, P., K. Hardwick, and J.P. Javerzat. (1998). Fission yeast bub1 is a mitotic centromere protein essential for the spindle checkpoint and the preservation of correct ploidy through mitosis. *J Cell Biol*. 143:1775-87

Bettencourt-Dias, M., R. Giet, R. Sinka, A. Mazumdar, W.G. Lock, F. Balloux, P.J. Zafiropoulos, S. Yamaguchi, S. Winter, R.W. Carthew, M. Cooper, D. Jones, L. Frenz, and D.M. Glover. (2004). Genome-wide survey of protein kinases required for cell cycle progression. *Nature*. 432:980-7

Bieling, P., S. Kandels-Lewis, I.A. Telley, J. van Dijk, C. Janke, and T. Surrey. (2008). CLIP-170 tracks growing microtubule ends by dynamically recognizing composite EB1/tubulin-binding sites. *J Cell Biol*. 183:1223-33

Black, B.E., L.E. Jansen, P.S. Maddox, D.R. Foltz, A.B. Desai, J.V. Shah, and D.W. Cleveland. (2007). Centromere identity maintained by nucleosomes assembled with histone H3 containing the CENP-A targeting domain. *Mol Cell*. 25:309-22

Blower, M.D., and G.H. Karpen. (2001). The role of Drosophila CID in kinetochore formation, cell-cycle progression and heterochromatin interactions. *Nat Cell Biol*. 3:730-9

Bomont, P., P. Maddox, J.V. Shah, A.B. Desai, and D.W. Cleveland. (2005). Unstable microtubule capture at kinetochores depleted of the centromere-associated protein CENP-F. *Embo J*. 24:3927-39

Boveri, T. 1914. Zur Frage der Entstehung maligner tumoren. Jena:Gustav Fischer Verlag.

Boyarchuk, Y., A. Salic, M. Dasso, and A. Arnaoutov. (2007). Bub1 is essential for assembly of the functional inner centromere. *J Cell Biol*. 176:919-28

Breeden, L.L. (2003). Periodic transcription: a cycle within a cycle. *Curr Biol*. 13:R31-8

Brinkley, B.R., and E. Stubblefield. (1966). The fine structure of the kinetochore of a mammalian cell in vitro. *Chromosoma*. 19:28-43

Brito, D.A., and C.L. Rieder. (2006). Mitotic Checkpoint Slippage in Humans Occurs via Cyclin B Destruction in the Presence of an Active Checkpoint. *Curr Biol*. 16:1194-200

Brouhard, G.J., and A.J. Hunt. (2005). Microtubule movements on the arms of mitotic chromosomes: polar ejection forces quantified in vitro. *Proc Natl Acad Sci U S A*. 102:13903-8

Brown, K.D., R.M. Coulson, T.J. Yen, and D.W. Cleveland. (1994). Cyclin-like accumulation and loss of the putative kinetochore motor CENP-E results from

coupling continuous synthesis with specific degradation at the end of mitosis. *J Cell Biol*. 125:1303-12

Brues AM, C.A. (1936). Effects of colchicine and related substances on cell division. *Biochem J*. 30:1363

Brues AM, J.E. (1937). Nuclear abnormalities resulting from inhibition of mitosis by colchicine and other substances. *Am J Cancer*. 30:504-511

Buffin, E., D. Emre, and R.E. Karess. (2007). Flies without a spindle checkpoint. *Nat Cell Biol*. 9:565-72

Buffin, E., C. Lefebvre, J. Huang, M.E. Gagou, and R.E. Karess. (2005). Recruitment of Mad2 to the kinetochore requires the Rod/Zw10 complex. *Curr Biol*. 15:856-61

Cahill, D.P., C. Lengauer, J. Yu, G.J. Riggins, J.K. Willson, S.D. Markowitz, K.W. Kinzler, and B. Vogelstein. (1998). Mutations of mitotic checkpoint genes in human cancers. *Nature*. 392:300-3

Cai, S., C.B. O'Connell, A. Khodjakov, and C.E. Walczak. (2009). Chromosome congression in the absence of kinetochore fibres. *Nat Cell Biol*. 11:832-8

Campbell, L., and K.G. Hardwick. (2003). Analysis of Bub3 spindle checkpoint function in Xenopus egg extracts. *J Cell Sci*. 116:617-28

Campbell, M.S., G.K. Chan, and T.J. Yen. (2001). Mitotic checkpoint proteins HsMAD1 and HsMAD2 are associated with nuclear pore complexes in interphase. *J Cell Sci*. 114:953-63

Carazo-Salas, R.E., O.J. Gruss, I.W. Mattaj, and E. Karsenti. (2001). Ran-GTP coordinates regulation of microtubule nucleation and dynamics during mitotic-spindle assembly. *Nat Cell Biol*. 3:228-34

Cassimeris, L. (2004). Cell division: eg'ing on microtubule flux. *Curr Biol*. 14:R1000-2

Catlett, M.G., and K.B. Kaplan. (2006). Sgt1p is a unique co-chaperone that acts as a client adaptor to link Hsp90 to Skp1p. *J Biol Chem*. 281:33739-48

Centonze, V.E., and G.G. Borisy. (1991). Pole-to-chromosome movements induced at metaphase: sites of microtubule disassembly. *J Cell Sci*. 100 (Pt 1):205-11

Chan, G.K., S.A. Jablonski, V. Sudakin, J.C. Hittle, and T.J. Yen. (1999). Human BUBR1 is a mitotic checkpoint kinase that monitors CENP-E functions at kinetochores and binds the cyclosome/APC. *J Cell Biol*. 146:941-54

Chan, G.K., S.T. Liu, and T.J. Yen. (2005). Kinetochore structure and function. *Trends Cell Biol*. 15:589-98

Chan, G.K., B.T. Schaar, and T.J. Yen. (1998). Characterization of the kinetochore binding domain of CENP-E reveals interactions with the kinetochore proteins CENP-F and hBUBR1. *J Cell Biol*. 143:49-63

Chang, T.H., F.A. Ray, D.A. Thompson, and R. Schlegel. (1997). Disregulation of mitotic checkpoints and regulatory proteins following acute expression of SV40 large T antigen in diploid human cells. *Oncogene*. 14:2383-93

Cheeseman, I.M., S. Anderson, M. Jwa, E.M. Green, J. Kang, J.R. Yates, 3rd, C.S. Chan, D.G. Drubin, and G. Barnes. (2002). Phospho-regulation of kinetochore-microtubule attachments by the Aurora kinase Ipl1p. *Cell*. 111:163-72

Cheeseman, I.M., C. Brew, M. Wolyniak, A. Desai, S. Anderson, N. Muster, J.R. Yates, T.C. Huffaker, D.G. Drubin, and G. Barnes. (2001). Implication of a novel multiprotein Dam1p complex in outer kinetochore function. *J Cell Biol*. 155:1137-45

Cheeseman, I.M., J.S. Chappie, E.M. Wilson-Kubalek, and A. Desai. (2006). The conserved KMN network constitutes the core microtubule-binding site of the kinetochore. *Cell*. 127:983-97

Cheeseman, I.M., and A. Desai. (2008). Molecular architecture of the kinetochore-microtubule interface. *Nat Rev Mol Cell Biol*. 9:33-46

Chen, J.G., C.P. Yang, M. Cammer, and S.B. Horwitz. (2003). Gene expression and mitotic exit induced by microtubule-stabilizing drugs. *Cancer Res*. 63:7891-9

Chen, R.H. (2002). BubR1 is essential for kinetochore localization of other spindle checkpoint proteins and its phosphorylation requires Mad1. *J Cell Biol*. 158:487-96

Chen, R.H., D.M. Brady, D. Smith, A.W. Murray, and K.G. Hardwick. (1999). The spindle checkpoint of budding yeast depends on a tight complex between the Mad1 and Mad2 proteins. *Mol Biol Cell*. 10:2607-18

Chen, R.H., A. Shevchenko, M. Mann, and A.W. Murray. (1998). Spindle checkpoint protein Xmad1 recruits Xmad2 to unattached kinetochores. *J Cell Biol*. 143:283-95

Chen, R.H., J.C. Waters, E.D. Salmon, and A.W. Murray. (1996). Association of spindle assembly checkpoint component XMAD2 with unattached kinetochores. *Science*. 274:242-6

Chintapalli, V.R., J. Wang, and J.A. Dow. (2007). Using FlyAtlas to identify better Drosophila melanogaster models of human disease. *Nat Genet*. 39:715-20

Chung, E., and R.H. Chen. (2002). Spindle checkpoint requires Mad1-bound and Mad1-free Mad2. *Mol Biol Cell*. 13:1501-11

Ciliberto, A., and J.V. Shah. (2009). A quantitative systems view of the spindle assembly checkpoint. *EMBO J*. 28:2162-2173

Cimini, D., B. Howell, P. Maddox, A. Khodjakov, F. Degrassi, and E.D. Salmon. (2001). Merotelic kinetochore orientation is a major mechanism of aneuploidy in mitotic mammalian tissue cells. *J Cell Biol*. 153:517-27

Cimini, D., X. Wan, C.B. Hirel, and E.D. Salmon. (2006). Aurora kinase promotes turnover of kinetochore microtubules to reduce chromosome segregation errors. *Curr Biol*. 16:1711-8

Clemens, J.C., C.A. Worby, N. Simonson-Leff, M. Muda, T. Maehama, B.A. Hemmings, and J.E. Dixon. (2000). Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. *Proc Natl Acad Sci U S A*. 97:6499-503

Coelho, P.A., J. Queiroz-Machado, and C.E. Sunkel. (2003). Condensin-dependent localisation of topoisomerase II to an axial chromosomal structure is required for sister chromatid resolution during mitosis. *J Cell Sci*. 116:4763-76

Compton, D.A., T.J. Yen, and D.W. Cleveland. (1991). Identification of novel centromere/kinetochore-associated proteins using monoclonal antibodies generated against human mitotic chromosome scaffolds. *J Cell Biol*. 112:1083-97

Cooke, C.A., B. Schaar, T.J. Yen, and W.C. Earnshaw. (1997). Localization of CENP-E in the fibrous corona and outer plate of mammalian kinetochores from prometaphase through anaphase. *Chromosoma*. 106:446-55

De Antoni, A., C.G. Pearson, D. Cimini, J.C. Canman, V. Sala, L. Nezi, M. Mapelli, L. Sironi, M. Faretta, E.D. Salmon, and A. Musacchio. (2005). The Mad1/Mad2 complex as a template for Mad2 activation in the spindle assembly checkpoint. *Curr Biol*. 15:214-25

DeLuca, J.G., W.E. Gall, C. Ciferri, D. Cimini, A. Musacchio, and E.D. Salmon. (2006). Kinetochore microtubule dynamics and attachment stability are regulated by Hec1. *Cell*. 127:969-82

Dernburg, A.F. (2001). Here, there, and everywhere: kinetochore function on holocentric chromosomes. *J Cell Biol*. 153:F33-8

Derry, W.B., L. Wilson, and M.A. Jordan. (1995). Substoichiometric binding of taxol suppresses microtubule dynamics. *Biochemistry*. 34:2203-11

Desai, A., S. Verma, T.J. Mitchison, and C.E. Walczak. (1999). Kin I kinesins are microtubule-destabilizing enzymes. *Cell*. 96:69-78

Dewar, H., K. Tanaka, K. Nasmyth, and T.U. Tanaka. (2004). Tension between two kinetochores suffices for their bi-orientation on the mitotic spindle. *Nature*. 428:93-7

Diffley, J.F. (2004). Regulation of early events in chromosome replication. *Curr Biol*. 14:R778-86

Ditchfield, C., V.L. Johnson, A. Tighe, R. Ellston, C. Haworth, T. Johnson, A. Mortlock, N. Keen, and S.S. Taylor. (2003). Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores. *J Cell Biol*. 161:267-80

Dixit, R., B. Barnett, J.E. Lazarus, M. Tokito, Y.E. Goldman, and E.L. Holzbaur. (2009). Microtubule plus-end tracking by CLIP-170 requires EB1. *Proc Natl Acad Sci U S A*. 106:492-7

Dobles, M., V. Liberal, M.L. Scott, R. Benezra, and P.K. Sorger. (2000). Chromosome missegregation and apoptosis in mice lacking the mitotic checkpoint protein Mad2. *Cell*. 101:635-45

Draviam, V.M., F. Stegmeier, G. Nalepa, M.E. Sowa, J. Chen, A. Liang, G.J. Hannon, P.K. Sorger, J.W. Harper, and S.J. Elledge. (2007). A functional genomic screen identifies a role for TAO1 kinase in spindle-checkpoint signalling. *Nat Cell Biol*. 9:556-64

Dujardin, D., U.I. Wacker, A. Moreau, T.A. Schroer, J.E. Rickard, and J.R. De Mey. (1998). Evidence for a role of CLIP-170 in the establishment of metaphase chromosome alignment. *J Cell Biol*. 141:849-62

Dunphy, W.G., L. Brizuela, D. Beach, and J. Newport. (1988). The Xenopus cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis. *Cell*. 54:423-31

Earnshaw, W.C., N. Halligan, C. Cooke, and N. Rothfield. (1984). The kinetochore is part of the metaphase chromosome scaffold. *J Cell Biol*. 98:352-7

Earnshaw, W.C., and N. Rothfield. (1985). Identification of a family of human centromere proteins using autoimmune sera from patients with scleroderma. *Chromosoma*. 91:313-21

Earnshaw, W.C., K.F. Sullivan, P.S. Machlin, C.A. Cooke, D.A. Kaiser, T.D. Pollard, N.F. Rothfield, and D.W. Cleveland. (1987). Molecular cloning of cDNA for CENP-B, the major human centromere autoantigen. *J Cell Biol*. 104:817-29

Emanuele, M.J., W. Lan, M. Jwa, S.A. Miller, C.S. Chan, and P.T. Stukenberg. (2008). Aurora B kinase and protein phosphatase 1 have opposing roles in modulating kinetochore assembly. *J Cell Biol*. 181:241-54

Espeut, J., A. Gaussen, P. Bieling, V. Morin, S. Prieto, D. Fesquet, T. Surrey, and A. Abrieu. (2008). Phosphorylation relieves autoinhibition of the kinetochore motor Cenp-E. *Mol Cell*. 29:637-43

Essex, A., A. Dammermann, L. Lewellyn, K. Oegema, and A. Desai. (2009). Systematic analysis in Caenorhabditis elegans reveals that the spindle checkpoint is composed of two largely independent branches. *Mol Biol Cell*. 20:1252-67

Evans, T., E.T. Rosenthal, J. Youngblom, D. Distel, and T. Hunt. (1983). Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell*. 33:389-96

Faulkner, N.E., B. Vig, C.J. Echeverri, L. Wordeman, and R.B. Vallee. (1998). Localization of motor-related proteins and associated complexes to active, but not inactive, centromeres. *Hum Mol Genet*. 7:671-7

Flemming, W. (1965). Historical Paper. Contributions To The Knowledge Of The Cell And Its Vital Processes. *J Cell Biol*. 25:SUPPL:1-69

Fodde, R., J. Kuipers, C. Rosenberg, R. Smits, M. Kielman, C. Gaspar, J.H. van Es, C. Breukel, J. Wiegant, R.H. Giles, and H. Clevers. (2001). Mutations in the

APC tumour suppressor gene cause chromosomal instability. *Nat Cell Biol*. 3:433-8

Foltz, D.R., L.E. Jansen, B.E. Black, A.O. Bailey, J.R. Yates, 3rd, and D.W. Cleveland. (2006). The human CENP-A centromeric nucleosome-associated complex. *Nat Cell Biol*. 8:458-69

Fraschini, R., A. Beretta, L. Sironi, A. Musacchio, G. Lucchini, and S. Piatti. (2001). Bub3 interaction with Mad2, Mad3 and Cdc20 is mediated by WD40 repeats and does not require intact kinetochores. *Embo J*. 20:6648-59

Fukagawa, T., and W.R. Brown. (1997). Efficient conditional mutation of the vertebrate CENP-C gene. *Hum Mol Genet*. 6:2301-8

Gaetz, J., and T.M. Kapoor. (2004). Dynein/dynactin regulate metaphase spindle length by targeting depolymerizing activities to spindle poles. *J Cell Biol*. 166:465-71

Gaitanos, T.N., A. Santamaria, A.A. Jeyaprakash, B. Wang, E. Conti, and E.A. Nigg. (2009). Stable kinetochore-microtubule interactions depend on the Ska complex and its new component Ska3/C13Orf3. *EMBO J*. 28:1442-52

Ganem, N.J., K. Upton, and D.A. Compton. (2005). Efficient mitosis in human cells lacking poleward microtubule flux. *Curr Biol*. 15:1827-32

Gascoigne, K.E., and S.S. Taylor. (2009). How do anti-mitotic drugs kill cancer cells? *J Cell Sci*. 122:2579-85

Gautier, J., C. Norbury, M. Lohka, P. Nurse, and J. Maller. (1988). Purified maturation-promoting factor contains the product of a Xenopus homolog of the fission yeast cell cycle control gene cdc2+. *Cell*. 54:433-9

Genschik, P., M.C. Criqui, Y. Parmentier, A. Derevier, and J. Fleck. (1998). Cell cycle - dependent proteolysis in plants. Identification Of the destruction box pathway and metaphase arrest produced by the proteasome inhibitor mg132. *Plant Cell*. 10:2063-76

Giannakakou, P., R. Gussio, E. Nogales, K.H. Downing, D. Zaharevitz, B. Bollbuck, G. Poy, D. Sackett, K.C. Nicolaou, and T. Fojo. (2000). A common pharmacophore for epothilone and taxanes: molecular basis for drug resistance conferred by tubulin mutations in human cancer cells. *Proc Natl Acad Sci U S A*. 97:2904-9

Giannakakou, P., D.L. Sackett, Y.K. Kang, Z. Zhan, J.T. Buters, T. Fojo, and M.S. Poruchynsky. (1997). Paclitaxel-resistant human ovarian cancer cells have mutant beta-tubulins that exhibit impaired paclitaxel-driven polymerization. *J Biol Chem*. 272:17118-25

Gonzalez-Garay, M.L., L. Chang, K. Blade, D.R. Menick, and F. Cabral. (1999). A beta-tubulin leucine cluster involved in microtubule assembly and paclitaxel resistance. *J Biol Chem*. 274:23875-82

Gorbsky, G.J., R.H. Chen, and A.W. Murray. (1998). Microinjection of antibody to Mad2 protein into mammalian cells in mitosis induces premature anaphase. *J Cell Biol*. 141:1193-205

Goshima, G., and R.D. Vale. (2003). The roles of microtubule-based motor proteins in mitosis: comprehensive RNAi analysis in the Drosophila S2 cell line. *J Cell Biol*. 162:1003-16

Gottesman, M.M. (1993). How cancer cells evade chemotherapy: sixteenth Richard and Hinda Rosenthal Foundation Award Lecture. *Cancer Res*. 53:747-54

Greenberger, L.M., L. Lothstein, S.S. Williams, and S.B. Horwitz. (1988). Distinct P-glycoprotein precursors are overproduced in independently isolated drug-resistant cell lines. *Proc Natl Acad Sci U S A*. 85:3762-6

Grishchuk, E.L., A.K. Efremov, V.A. Volkov, I.S. Spiridonov, N. Gudimchuk, S. Westermann, D. Drubin, G. Barnes, J.R. McIntosh, and F.I. Ataullakhanov. (2008). The Dam1 ring binds microtubules strongly enough to be a processive as well as energy-efficient coupler for chromosome motion. *Proc Natl Acad Sci U S A*. 105:15423-8

Guimaraes, G.J., and J.G. Deluca. (2009). Connecting with Ska, a key complex at the kinetochore-microtubule interface. *EMBO J*. 28:1375-7

Gupta, M.L., Jr., P. Carvalho, D.M. Roof, and D. Pellman. (2006). Plus end-specific depolymerase activity of Kip3, a kinesin-8 protein, explains its role in positioning the yeast mitotic spindle. *Nat Cell Biol*. 8:913-23

Hanisch, A., H.H. Sillje, and E.A. Nigg. (2006). Timely anaphase onset requires a novel spindle and kinetochore complex comprising Ska1 and Ska2. *EMBO J*. 25:5504-15

Hardwick, K.G., R.C. Johnston, D.L. Smith, and A.W. Murray. (2000). MAD3 encodes a novel component of the spindle checkpoint which interacts with Bub3p, Cdc20p, and Mad2p. *J Cell Biol*. 148:871-82

Hardwick, K.G., E. Weiss, F.C. Luca, M. Winey, and A.W. Murray. (1996). Activation of the budding yeast spindle assembly checkpoint without mitotic spindle disruption. *Science*. 273:953-6

Hari, M., Y. Wang, S. Veeraraghavan, and F. Cabral. (2003). Mutations in alpha- and beta-tubulin that stabilize microtubules and confer resistance to colcemid and vinblastine. *Mol Cancer Ther*. 2:597-605

Harries, H. 2008. Concerning the Origin of Malignant Tumours.
By Theodor Boveri; Translated and annotated by Sir Henry Harris. *Co-published with The Company of Biologists*, New York.

Hartwell, L.H., and T.A. Weinert. (1989). Checkpoints: controls that ensure the order of cell cycle events. *Science*. 246:629-34

Hauf, S., R.W. Cole, S. LaTerra, C. Zimmer, G. Schnapp, R. Walter, A. Heckel, J. van Meel, C.L. Rieder, and J.M. Peters. (2003). The small molecule Hesperadin

reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. *J Cell Biol*. 161:281-94

Heald, R., and E. Nogales. (2002). Microtubule dynamics. *J Cell Sci*. 115:3-4

Henikoff, S., K. Ahmad, J.S. Platero, and B. van Steensel. (2000). Heterochromatic deposition of centromeric histone H3-like proteins. *Proc Natl Acad Sci U S A*. 97:716-21

Heun, P., S. Erhardt, M.D. Blower, S. Weiss, A.D. Skora, and G.H. Karpen. (2006). Mislocalization of the Drosophila centromere-specific histone CID promotes formation of functional ectopic kinetochores. *Dev Cell*. 10:303-15

Hill, T.L. (1985). Theoretical problems related to the attachment of microtubules to kinetochores. *Proc Natl Acad Sci U S A*. 82:4404-8

Holland, A.J., and D.W. Cleveland. (2009). Boveri revisited: chromosomal instability, aneuploidy and tumorigenesis. *Nat Rev Mol Cell Biol*. 10:478-87

Holt, S.V., M.A. Vergnolle, D. Hussein, M.J. Wozniak, V.J. Allan, and S.S. Taylor. (2005). Silencing Cenp-F weakens centromeric cohesion, prevents chromosome alignment and activates the spindle checkpoint. *J Cell Sci*. 118:4889-900

Homma, N., Y. Takei, Y. Tanaka, T. Nakata, S. Terada, M. Kikkawa, Y. Noda, and N. Hirokawa. (2003). Kinesin superfamily protein 2A (KIF2A) functions in suppression of collateral branch extension. *Cell*. 114:229-39

Hooke, R. 1665. Micrographia: or, some physiological descriptions of minute bodies made by magnifying glasses. *London: J. Martyn and J. Allestry.*

Howell, B.J., B.F. McEwen, J.C. Canman, D.B. Hoffman, E.M. Farrar, C.L. Rieder, and E.D. Salmon. (2001). Cytoplasmic dynein/dynactin drives kinetochore protein transport to the spindle poles and has a role in mitotic spindle checkpoint inactivation. *J Cell Biol*. 155:1159-72

Howell, B.J., B. Moree, E.M. Farrar, S. Stewart, G. Fang, and E.D. Salmon. (2004). Spindle checkpoint protein dynamics at kinetochores in living cells. *Curr Biol*. 14:953-64

Howman, E.V., K.J. Fowler, A.J. Newson, S. Redward, A.C. MacDonald, P. Kalitsis, and K.H. Choo. (2000). Early disruption of centromeric chromatin organization in centromere protein A (Cenpa) null mice. *Proc Natl Acad Sci U S A*. 97:1148-53

Hoyt, M.A., L. Totis, and B.T. Roberts. (1991). S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function. *Cell*. 66:507-17

Hubner, N.C., L.H. Wang, M. Kaulich, P. Descombes, I. Poser, and E.A. Nigg. (2009). Re-examination of siRNA specificity questions role of PICH and Tao1 in the spindle checkpoint and identifies Mad2 as a sensitive target for small RNAs. *Chromosoma*

Hudson, D.F., K.J. Fowler, E. Earle, R. Saffery, P. Kalitsis, H. Trowell, J. Hill, N.G. Wreford, D.M. de Kretser, M.R. Cancilla, E. Howman, L. Hii, S.M. Cutts, D.V.

Irvine, and K.H. Choo. (1998). Centromere protein B null mice are mitotically and meiotically normal but have lower body and testis weights. *J Cell Biol*. 141:309-19

Hwang, L.H., L.F. Lau, D.L. Smith, C.A. Mistrot, K.G. Hardwick, E.S. Hwang, A. Amon, and A.W. Murray. (1998). Budding yeast Cdc20: a target of the spindle checkpoint. *Science*. 279:1041-4

Ikeno, M., B. Grimes, T. Okazaki, M. Nakano, K. Saitoh, H. Hoshino, N.I. McGill, H. Cooke, and H. Masumoto. (1998). Construction of YAC-based mammalian artificial chromosomes. *Nat Biotechnol*. 16:431-9

Iwanaga, Y., Y.H. Chi, A. Miyazato, S. Sheleg, K. Haller, J.M. Peloponese, Jr., Y. Li, J.M. Ward, R. Benezra, and K.T. Jeang. (2007). Heterozygous deletion of mitotic arrest-deficient protein 1 (MAD1) increases the incidence of tumors in mice. *Cancer Res*. 67:160-6

Izuta, H., M. Ikeno, N. Suzuki, T. Tomonaga, N. Nozaki, C. Obuse, Y. Kisu, N. Goshima, F. Nomura, N. Nomura, and K. Yoda. (2006). Comprehensive analysis of the ICEN (Interphase Centromere Complex) components enriched in the CENP-A chromatin of human cells. *Genes Cells*. 11:673-84

Jablonski, S.A., G.K. Chan, C.A. Cooke, W.C. Earnshaw, and T.J. Yen. (1998). The hBUB1 and hBUBR1 kinases sequentially assemble onto kinetochores during prophase with hBUBR1 concentrating at the kinetochore plates in mitosis. *Chromosoma*. 107:386-96

Jackson, J.R., D.R. Patrick, M.M. Dar, and P.S. Huang. (2007). Targeted anti-mitotic therapies: can we improve on tubulin agents? *Nat Rev Cancer*. 7:107-17

Jacobs, H.W., J.A. Knoblich, and C.F. Lehner. (1998). Drosophila Cyclin B3 is required for female fertility and is dispensable for mitosis like Cyclin B. *Genes Dev*. 12:3741-51

Janssen, A., G.J. Kops, and R.H. Medema. (2009). Elevating the frequency of chromosome mis-segregation as a strategy to kill tumor cells. *Proc Natl Acad Sci U S A*. 106:19108-13

Jelluma, N., A.B. Brenkman, N.J. van den Broek, C.W. Cruijsen, M.H. van Osch, S.M. Lens, R.H. Medema, and G.J. Kops. (2008). Mps1 phosphorylates Borealin to control Aurora B activity and chromosome alignment. *Cell*. 132:233-46

Jin, D.Y., F. Spencer, and K.T. Jeang. (1998). Human T cell leukemia virus type 1 oncoprotein Tax targets the human mitotic checkpoint protein MAD1. *Cell*. 93:81-91

Johnson, V.L., M.I. Scott, S.V. Holt, D. Hussein, and S.S. Taylor. (2004). Bub1 is required for kinetochore localization of BubR1, Cenp-E, Cenp-F and Mad2, and chromosome congression. *J Cell Sci*. 117:1577-89

Jokelainen, P.T. (1967). The ultrastructure and spatial organization of the metaphase kinetochore in mitotic rat cells. *J Ultrastruct Res*. 19:19-44

Jones, M.H., X. He, T.H. Giddings, and M. Winey. (2001). Yeast Dam1p has a role at the kinetochore in assembly of the mitotic spindle. *Proc Natl Acad Sci U S A*. 98:13675-80

Jordan, M.A., D. Thrower, and L. Wilson. (1992). Effects of vinblastine, podophyllotoxin and nocodazole on mitotic spindles. Implications for the role of microtubule dynamics in mitosis. *J Cell Sci*. 102 (Pt 3):401-16

Jordan, M.A., R.J. Toso, D. Thrower, and L. Wilson. (1993). Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations. *Proc Natl Acad Sci U S A*. 90:9552-6

Jordan, M.A., K. Wendell, S. Gardiner, W.B. Derry, H. Copp, and L. Wilson. (1996). Mitotic block induced in HeLa cells by low concentrations of paclitaxel (Taxol) results in abnormal mitotic exit and apoptotic cell death. *Cancer Res*. 56:816-25

Kalab, P., R.T. Pu, and M. Dasso. (1999). The ran GTPase regulates mitotic spindle assembly. *Curr Biol*. 9:481-4

Kalitsis, P., E. Earle, K.J. Fowler, and K.H. Choo. (2000). Bub3 gene disruption in mice reveals essential mitotic spindle checkpoint function during early embryogenesis. *Genes Dev*. 14:2277-82

Kalitsis, P., K.J. Fowler, E. Earle, J. Hill, and K.H. Choo. (1998). Targeted disruption of mouse centromere protein C gene leads to mitotic disarray and early embryo death. *Proc Natl Acad Sci U S A*. 95:1136-41

Kallio, M.J., M.L. McCleland, P.T. Stukenberg, and G.J. Gorbsky. (2002). Inhibition of aurora B kinase blocks chromosome segregation, overrides the spindle checkpoint, and perturbs microtubule dynamics in mitosis. *Curr Biol*. 12:900-5

Kang, J., and H. Yu. (2009). Kinase signaling in the spindle checkpoint. *J Biol Chem*. 284:15359-63

Kaplan, K.B., A.A. Burds, J.R. Swedlow, S.S. Bekir, P.K. Sorger, and I.S. Nathke. (2001). A role for the Adenomatous Polyposis Coli protein in chromosome segregation. *Nat Cell Biol*. 3:429-32

Kapoor, M., R. Montes de Oca Luna, G. Liu, G. Lozano, C. Cummings, M. Mancini, I. Ouspenski, B.R. Brinkley, and G.S. May. (1998). The cenpB gene is not essential in mice. *Chromosoma*. 107:570-6

Kapoor, T.M., M.A. Lampson, P. Hergert, L. Cameron, D. Cimini, E.D. Salmon, B.F. McEwen, and A. Khodjakov. (2006). Chromosomes can congress to the metaphase plate before biorientation. *Science*. 311:388-91

Kapoor, T.M., T.U. Mayer, M.L. Coughlin, and T.J. Mitchison. (2000). Probing spindle assembly mechanisms with monastrol, a small molecule inhibitor of the mitotic kinesin, Eg5. *J Cell Biol*. 150:975-88

Kastan, M.B., and J. Bartek. (2004). Cell-cycle checkpoints and cancer. *Nature*. 432:316-23

Kavallaris, M., D.Y. Kuo, C.A. Burkhart, D.L. Regl, M.D. Norris, M. Haber, and S.B. Horwitz. (1997). Taxol-resistant epithelial ovarian tumors are associated with altered expression of specific beta-tubulin isotypes. *J Clin Invest*. 100:1282-93

Kavallaris, M., A.S. Tait, B.J. Walsh, L. He, S.B. Horwitz, M.D. Norris, and M. Haber. (2001). Multiple microtubule alterations are associated with Vinca alkaloid resistance in human leukemia cells. *Cancer Res*. 61:5803-9

Khodjakov, A., L. Copenagle, M.B. Gordon, D.A. Compton, and T.M. Kapoor. (2003). Minus-end capture of preformed kinetochore fibers contributes to spindle morphogenesis. *J Cell Biol*. 160:671-83

Kim, H.S., K.H. Park, S.A. Kim, J. Wen, S.W. Park, B. Park, C.W. Gham, W.J. Hyung, S.H. Noh, H.K. Kim, and S.Y. Song. (2005). Frequent mutations of human Mad2, but not Bub1, in gastric cancers cause defective mitotic spindle checkpoint. *Mutat Res*. 578:187-201

Kim, S.H., D.P. Lin, S. Matsumoto, A. Kitazono, and T. Matsumoto. (1998). Fission yeast Slp1: an effector of the Mad2-dependent spindle checkpoint. *Science*. 279:1045-7

Kim, Y., J.E. Heuser, C.M. Waterman, and D.W. Cleveland. (2008). CENP-E combines a slow, processive motor and a flexible coiled coil to produce an essential motile kinetochore tether. *J Cell Biol*. 181:411-9

King, J.M., and R.B. Nicklas. (2000). Tension on chromosomes increases the number of kinetochore microtubules but only within limits. *J Cell Sci*. 113 Pt 21:3815-23

King, R.W., R.J. Deshaies, J.M. Peters, and M.W. Kirschner. (1996). How proteolysis drives the cell cycle. *Science*. 274:1652-9

Kirschner, M., and T. Mitchison. (1986). Beyond self-assembly: from microtubules to morphogenesis. *Cell*. 45:329-42

Kitagawa, K., D. Skowyra, S.J. Elledge, J.W. Harper, and P. Hieter. (1999). SGT1 encodes an essential component of the yeast kinetochore assembly pathway and a novel subunit of the SCF ubiquitin ligase complex. *Mol Cell*. 4:21-33

Kline-Smith, S.L., A. Khodjakov, P. Hergert, and C.E. Walczak. (2004). Depletion of centromeric MCAK leads to chromosome congression and segregation defects due to improper kinetochore attachments. *Mol Biol Cell*. 15:1146-59

Kline-Smith, S.L., S. Sandall, and A. Desai. (2005). Kinetochore-spindle microtubule interactions during mitosis. *Curr Opin Cell Biol*. 17:35-46

Knowlton, A.L., W. Lan, and P.T. Stukenberg. (2006). Aurora B is enriched at merotelic attachment sites, where it regulates MCAK. *Curr Biol*. 16:1705-10

Kops, G.J., D.R. Foltz, and D.W. Cleveland. (2004). Lethality to human cancer cells through massive chromosome loss by inhibition of the mitotic checkpoint. *Proc Natl Acad Sci U S A*. 101:8699-704

Kops, G.J., B.A. Weaver, and D.W. Cleveland. (2005). On the road to cancer: aneuploidy and the mitotic checkpoint. *Nat Rev Cancer*. 5:773-85

Kulukian, A., J.S. Han, and D.W. Cleveland. (2009). Unattached kinetochores catalyze production of an anaphase inhibitor that requires a Mad2 template to prime Cdc20 for BubR1 binding. *Dev Cell*. 16:105-17

Kwon, M.S., T. Hori, M. Okada, and T. Fukagawa. (2007). CENP-C is involved in chromosome segregation, mitotic checkpoint function, and kinetochore assembly. *Mol Biol Cell*. 18:2155-68

Labbe, J.C., J.P. Capony, D. Caput, J.C. Cavadore, J. Derancourt, M. Kaghad, J.M. Lelias, A. Picard, and M. Doree. (1989). MPF from starfish oocytes at first meiotic metaphase is a heterodimer containing one molecule of cdc2 and one molecule of cyclin B. *EMBO J*. 8:3053-8

Labbe, J.C., M.G. Lee, P. Nurse, A. Picard, and M. Doree. (1988). Activation at M-phase of a protein kinase encoded by a starfish homologue of the cell cycle control gene cdc2+. *Nature*. 335:251-4

Lampson, M.A., and T.M. Kapoor. (2005). The human mitotic checkpoint protein BubR1 regulates chromosome-spindle attachments. *Nat Cell Biol*. 7:93-8

Lampson, M.A., K. Renduchitala, A. Khodjakov, and T.M. Kapoor. (2004). Correcting improper chromosome-spindle attachments during cell division. *Nat Cell Biol*. 6:232-7

Lan, W., X. Zhang, S.L. Kline-Smith, S.E. Rosasco, G.A. Barrett-Wilt, J. Shabanowitz, D.F. Hunt, C.E. Walczak, and P.T. Stukenberg. (2004). Aurora B phosphorylates centromeric MCAK and regulates its localization and microtubule depolymerization activity. *Curr Biol*. 14:273-86

Lauze, E., B. Stoelcker, F.C. Luca, E. Weiss, A.R. Schutz, and M. Winey. (1995). Yeast spindle pole body duplication gene MPS1 encodes an essential dual specificity protein kinase. *EMBO J*. 14:1655-63

Lee, Y.T., J. Jacob, W. Michowski, M. Nowotny, J. Kuznicki, and W.J. Chazin. (2004). Human Sgt1 binds HSP90 through the CHORD-Sgt1 domain and not the tetratricopeptide repeat domain. *J Biol Chem*. 279:16511-7

Lemos, C.L., P. Sampaio, H. Maiato, M. Costa, L.V. Omel'yanchuk, V. Liberal, and C.E. Sunkel. (2000). Mast, a conserved microtubule-associated protein required for bipolar mitotic spindle organization. *EMBO J*. 19:3668-82

Lens, S.M., R.M. Wolthuis, R. Klompmaker, J. Kauw, R. Agami, T. Brummelkamp, G. Kops, and R.H. Medema. (2003). Survivin is required for a sustained spindle checkpoint arrest in response to lack of tension. *EMBO J*. 22:2934-47

Li, R., and A.W. Murray. (1991). Feedback control of mitosis in budding yeast. *Cell*. 66:519-31

Li, Y., and R. Benezra. (1996). Identification of a human mitotic checkpoint gene: hsMAD2. *Science*. 274:246-8

Liao, H., R.J. Winkfein, G. Mack, J.B. Rattner, and T.J. Yen. (1995). CENP-F is a protein of the nuclear matrix that assembles onto kinetochores at late G2 and is rapidly degraded after mitosis. *J Cell Biol*. 130:507-18

Lin, S.F., P.M. Lin, M.C. Yang, T.C. Liu, J.G. Chang, Y.C. Sue, and T.P. Chen. (2002). Expression of hBUB1 in acute myeloid leukemia. *Leuk Lymphoma*. 43:385-91

Lingelbach, L.B., and K.B. Kaplan. (2004). The interaction between Sgt1p and Skp1p is regulated by HSP90 chaperones and is required for proper CBF3 assembly. *Mol Cell Biol*. 24:8938-50

Liscovitch, M., and Y. Lavie. (2002). Cancer multidrug resistance: a review of recent drug discovery research. *IDrugs*. 5:349-55

Liu, D., G. Vader, M.J. Vromans, M.A. Lampson, and S.M. Lens. (2009). Sensing Chromosome Bi-Orientation by Spatial Separation of Aurora B Kinase from Kinetochore Substrates. *Science*

Liu, S.T., G.K. Chan, J.C. Hittle, G. Fujii, E. Lees, and T.J. Yen. (2003). Human MPS1 kinase is required for mitotic arrest induced by the loss of CENP-E from kinetochores. *Mol Biol Cell*. 14:1638-51

Liu, S.T., J.B. Rattner, S.A. Jablonski, and T.J. Yen. (2006). Mapping the assembly pathways that specify formation of the trilaminar kinetochore plates in human cells. *J Cell Biol*. 175:41-53

Llamazares, S., A. Moreira, A. Tavares, C. Girdham, B.A. Spruce, C. Gonzalez, R.E. Karess, D.M. Glover, and C.E. Sunkel. (1991). polo encodes a protein kinase homolog required for mitosis in Drosophila. *Genes Dev*. 5:2153-65

Logarinho, E., and H. Bousbaa. (2008). Kinetochore-microtubule interactions "in check" by Bub1, Bub3 and BubR1: The dual task of attaching and signalling. *Cell Cycle*. 7:1763-8

Logarinho, E., H. Bousbaa, J.M. Dias, C. Lopes, I. Amorim, A. Antunes-Martins, and C.E. Sunkel. (2004). Different spindle checkpoint proteins monitor microtubule attachment and tension at kinetochores in Drosophila cells. *J Cell Sci*. 117:1757-71

Lohka, M.J., M.K. Hayes, and J.L. Maller. (1988). Purification of maturation-promoting factor, an intracellular regulator of early mitotic events. *Proc Natl Acad Sci U S A*. 85:3009-13

Lombillo, V.A., C. Nislow, T.J. Yen, V.I. Gelfand, and J.R. McIntosh. (1995). Antibodies to the kinesin motor domain and CENP-E inhibit microtubule depolymerization-dependent motion of chromosomes in vitro. *J Cell Biol*. 128:107-15

Lopes, C.S., P. Sampaio, B. Williams, M. Goldberg, and C.E. Sunkel. (2005). The Drosophila Bub3 protein is required for the mitotic checkpoint and for normal accumulation of cyclins during G2 and early stages of mitosis. *J Cell Sci*. 118:187-98

Lopes, C.S., and C.E. Sunkel. (2003). The spindle checkpoint: from normal cell division to tumorigenesis. *Arch Med Res*. 34:155-65

Love, K.R., A. Catic, C. Schlieker, and H.L. Ploegh. (2007). Mechanisms, biology and inhibitors of deubiquitinating enzymes. *Nat Chem Biol*. 3:697-705

Lu, Y., Z. Wang, L. Ge, N. Chen, and H. Liu. (2009). The RZZ complex and the spindle assembly checkpoint. *Cell Struct Funct*. 34:31-45

Luo, X., Z. Tang, J. Rizo, and H. Yu. (2002). The Mad2 spindle checkpoint protein undergoes similar major conformational changes upon binding to either Mad1 or Cdc20. *Mol Cell*. 9:59-71

Luo, X., Z. Tang, G. Xia, K. Wassmann, T. Matsumoto, J. Rizo, and H. Yu. (2004). The Mad2 spindle checkpoint protein has two distinct natively folded states. *Nat Struct Mol Biol*. 11:338-45

Luykx, P. (1965). The structure of the kinetochore in meiosis and mitosis in Urechis eggs. *Exp Cell Res*. 39:643-57

Lye, R.J., M.E. Porter, J.M. Scholey, and J.R. McIntosh. (1987). Identification of a microtubule-based cytoplasmic motor in the nematode C. elegans. *Cell*. 51:309-18

Maffini, S., A.R. Maia, A.L. Manning, Z. Maliga, A.L. Pereira, M. Junqueira, A. Shevchenko, A. Hyman, J.R. Yates, 3rd, N. Galjart, D.A. Compton, and H. Maiato. (2009). Motor-Independent Targeting of CLASPs to Kinetochores by CENP-E Promotes Microtubule Turnover and Poleward Flux. *Curr Biol*

Maia, A.F., C.S. Lopes, and C.E. Sunkel. (2007). BubR1 and CENP-E have antagonistic effects upon the stability of microtubule-kinetochore attachments in Drosophila S2 cell mitosis. *Cell Cycle*. 6:1367-78

Maiato, H., J. DeLuca, E.D. Salmon, and W.C. Earnshaw. (2004a). The dynamic kinetochore-microtubule interface. *J Cell Sci*. 117:5461-77

Maiato, H., E.A. Fairley, C.L. Rieder, J.R. Swedlow, C.E. Sunkel, and W.C. Earnshaw. (2003a). Human CLASP1 is an outer kinetochore component that regulates spindle microtubule dynamics. *Cell*. 113:891-904

Maiato, H., P.J. Hergert, S. Moutinho-Pereira, Y. Dong, K.J. Vandenbeldt, C.L. Rieder, and B.F. McEwen. (2006). The ultrastructure of the kinetochore and kinetochore fiber in Drosophila somatic cells. *Chromosoma*. 115:469-80

Maiato, H., A. Khodjakov, and C.L. Rieder. (2005). Drosophila CLASP is required for the incorporation of microtubule subunits into fluxing kinetochore fibres. *Nat Cell Biol*. 7:42-7

Maiato, H., C.L. Rieder, and A. Khodjakov. (2004b). Kinetochore-driven formation of kinetochore fibers contributes to spindle assembly during animal mitosis. *J Cell Biol*. 167:831-40

Maiato, H., P. Sampaio, and C.E. Sunkel. (2004c). Microtubule-associated proteins and their essential roles during mitosis. *Int Rev Cytol*. 241:53-153

Maiato, H., and C.E. Sunkel. (2004). Kinetochore-microtubule interactions during cell division. *Chromosome Res*. 12:585-97

Maiato, H., C.E. Sunkel, and W.C. Earnshaw. (2003b). Dissecting mitosis by RNAi in Drosophila tissue culture cells. *Biol Proced Online*. 5:153-161

Maney, T., A.W. Hunter, M. Wagenbach, and L. Wordeman. (1998). Mitotic centromere-associated kinesin is important for anaphase chromosome segregation. *J Cell Biol*. 142:787-801

Mao, Y., A. Abrieu, and D.W. Cleveland. (2003). Activating and silencing the mitotic checkpoint through CENP-E-dependent activation/inactivation of BubR1. *Cell*. 114:87-98

Mao, Y., A. Desai, and D.W. Cleveland. (2005). Microtubule capture by CENP-E silences BubR1-dependent mitotic checkpoint signaling. *J Cell Biol*. 170:873-80

Mapelli, M., L. Massimiliano, S. Santaguida, and A. Musacchio. (2007). The Mad2 conformational dimer: structure and implications for the spindle assembly checkpoint. *Cell*. 131:730-43

Markman, M. (2003). Managing taxane toxicities. *Support Care Cancer*. 11:144-7

Martinez-Exposito, M.J., K.B. Kaplan, J. Copeland, and P.K. Sorger. (1999). Retention of the BUB3 checkpoint protein on lagging chromosomes. *Proc Natl Acad Sci U S A*. 96:8493-8

Martins, T., A.F. Maia, S. Steffensen, and C.E. Sunkel. (2009). Sgt1, a co-chaperone of Hsp90 stabilizes Polo and is required for centrosome organization. *EMBO J*

Masui, Y., and C.L. Markert. (1971). Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J Exp Zool*. 177:129-45

Masumoto, H., M. Ikeno, M. Nakano, T. Okazaki, B. Grimes, H. Cooke, and N. Suzuki. (1998). Assay of centromere function using a human artificial chromosome. *Chromosoma*. 107:406-16

Masumoto, H., H. Masukata, Y. Muro, N. Nozaki, and T. Okazaki. (1989). A human centromere antigen (CENP-B) interacts with a short specific sequence in alphoid DNA, a human centromeric satellite. *J Cell Biol*. 109:1963-73

Matsuura, S., Y. Matsumoto, K. Morishima, H. Izumi, H. Matsumoto, E. Ito, K. Tsutsui, J. Kobayashi, H. Tauchi, Y. Kajiwara, S. Hama, K. Kurisu, H. Tahara, M. Oshimura, K. Komatsu, T. Ikeuchi, and T. Kajii. (2006). Monoallelic BUB1B mutations and defective mitotic-spindle checkpoint in seven families with premature chromatid separation (PCS) syndrome. *Am J Med Genet A*. 140:358-67

Mayr, M.I., S. Hummer, J. Bormann, T. Gruner, S. Adio, G. Woehlke, and T.U. Mayer. (2007). The human kinesin Kif18A is a motile microtubule depolymerase essential for chromosome congression. *Curr Biol*. 17:488-98

McAinsh, A.D., J.D. Tytell, and P.K. Sorger. (2003). Structure, function, and regulation of budding yeast kinetochores. *Annu Rev Cell Dev Biol*. 19:519-39

McEwen, B.F., J.T. Arena, J. Frank, and C.L. Rieder. (1993). Structure of the colcemid-treated PtK1 kinetochore outer plate as determined by high voltage electron microscopic tomography. *J Cell Biol*. 120:301-12

McEwen, B.F., G.K. Chan, B. Zubrowski, M.S. Savoian, M.T. Sauer, and T.J. Yen. (2001). CENP-E is essential for reliable bioriented spindle attachment, but chromosome alignment can be achieved via redundant mechanisms in mammalian cells. *Mol Biol Cell*. 12:2776-89

McEwen, B.F., C.E. Hsieh, A.L. Mattheyses, and C.L. Rieder. (1998). A new look at kinetochore structure in vertebrate somatic cells using high-pressure freezing and freeze substitution. *Chromosoma*. 107:366-75

McIntosh, J.R., E.L. Grishchuk, and R.R. West. (2002). Chromosome-microtubule interactions during mitosis. *Annu Rev Cell Dev Biol*. 18:193-219

Meraldi, P., and P.K. Sorger. (2005). A dual role for Bub1 in the spindle checkpoint and chromosome congression. *Embo J*. 24:1621-33

Michel, L.S., V. Liberal, A. Chatterjee, R. Kirchwegger, B. Pasche, W. Gerald, M. Dobles, P.K. Sorger, V.V. Murty, and R. Benezra. (2001). MAD2 haplo-insufficiency causes premature anaphase and chromosome instability in mammalian cells. *Nature*. 409:355-9

Miranda, J.J., P. De Wulf, P.K. Sorger, and S.C. Harrison. (2005). The yeast DASH complex forms closed rings on microtubules. *Nat Struct Mol Biol*. 12:138-43

Miranda, J.J., D.S. King, and S.C. Harrison. (2007). Protein arms in the kinetochore-microtubule interface of the yeast DASH complex. *Mol Biol Cell*. 18:2503-10

Mitchison, T., L. Evans, E. Schulze, and M. Kirschner. (1986). Sites of microtubule assembly and disassembly in the mitotic spindle. *Cell*. 45:515-27

Mitchison, T.J. (1989). Polewards microtubule flux in the mitotic spindle: evidence from photoactivation of fluorescence. *J Cell Biol*. 109:637-52

Montembault, E., S. Dutertre, C. Prigent, and R. Giet. (2007). PRP4 is a spindle assembly checkpoint protein required for MPS1, MAD1, and MAD2 localization to the kinetochores. *J Cell Biol*. 179:601-9

Morgan, D.O. (1997). Cyclin-dependent kinases: engines, clocks, and microprocessors. *Annu Rev Cell Dev Biol*. 13:261-91

Morgan, D.O. 2007. The Cell Cycle: Principles of Control. *New Science Press Ltd*.

Morrow, C.J., A. Tighe, V.L. Johnson, M.I. Scott, C. Ditchfield, and S.S. Taylor. (2005). Bub1 and aurora B cooperate to maintain BubR1-mediated inhibition of APC/CCdc20. *J Cell Sci*. 118:3639-52

Muro, Y., H. Masumoto, K. Yoda, N. Nozaki, M. Ohashi, and T. Okazaki. (1992). Centromere protein B assembles human centromeric alpha-satellite DNA at the 17-bp sequence, CENP-B box. *J Cell Biol*. 116:585-96

Murray, A.W. (2004). Recycling the cell cycle: cyclins revisited. *Cell*. 116:221-34

Musacchio, A., and E.D. Salmon. (2007). The spindle-assembly checkpoint in space and time. *Nat Rev Mol Cell Biol*. 8:379-93

Nicklas, R.B., and P. Arana. (1992). Evolution and the meaning of metaphase. *J Cell Sci*. 102 (Pt 4):681-90

Nicklas, R.B., and S.C. Ward. (1994). Elements of error correction in mitosis: microtubule capture, release, and tension. *J Cell Biol*. 126:1241-53

Nigg, E.A. (2001). Mitotic kinases as regulators of cell division and its checkpoints. *Nat Rev Mol Cell Biol*. 2:21-32

Niida, H., and M. Nakanishi. (2006). DNA damage checkpoints in mammals. *Mutagenesis*. 21:3-9

Niikura, Y., S. Ohta, K.J. Vandenbeldt, R. Abdulle, B.F. McEwen, and K. Kitagawa. (2006). 17-AAG, an Hsp90 inhibitor, causes kinetochore defects: a novel mechanism by which 17-AAG inhibits cell proliferation. *Oncogene*. 25:4133-46

Nomoto, S., N. Haruki, T. Takahashi, A. Masuda, T. Koshikawa, T. Takahashi, Y. Fujii, H. Osada, and T. Takahashi. (1999). Search for in vivo somatic mutations in the mitotic checkpoint gene, hMAD1, in human lung cancers. *Oncogene*. 18:7180-3

Obuse, C., H. Yang, N. Nozaki, S. Goto, T. Okazaki, and K. Yoda. (2004). Proteomics analysis of the centromere complex from HeLa interphase cells: UV-damaged DNA binding protein 1 (DDB-1) is a component of the CEN-complex, while BMI-1 is transiently co-localized with the centromeric region in interphase. *Genes Cells*. 9:105-20

Oegema, K., A. Desai, S. Rybina, M. Kirkham, and A.A. Hyman. (2001). Functional analysis of kinetochore assembly in Caenorhabditis elegans. *J Cell Biol*. 153:1209-26

Ohshima, K., S. Haraoka, S. Yoshioka, M. Hamasaki, T. Fujiki, J. Suzumiya, C. Kawasaki, M. Kanda, and M. Kikuchi. (2000). Mutation analysis of mitotic checkpoint genes (hBUB1 and hBUBR1) and microsatellite instability in adult T-cell leukemia/lymphoma. *Cancer Lett*. 158:141-50

Ohzeki, J., M. Nakano, T. Okada, and H. Masumoto. (2002). CENP-B box is required for de novo centromere chromatin assembly on human alphoid DNA. *J Cell Biol*. 159:765-75

Okada, M., I.M. Cheeseman, T. Hori, K. Okawa, I.X. McLeod, J.R. Yates, 3rd, A. Desai, and T. Fukagawa. (2006). The CENP-H-I complex is required for the efficient incorporation of newly synthesized CENP-A into centromeres. *Nat Cell Biol*. 8:446-57

Oliveira, R.A., P.A. Coelho, and C.E. Sunkel. (2005). The condensin I subunit Barren/CAP-H is essential for the structural integrity of centromeric heterochromatin during mitosis. *Mol Cell Biol*. 25:8971-84

Orr, B., H. Bousbaa, and C.E. Sunkel. (2006). Mad2-independent Spindle Assembly Checkpoint Activation and Controlled Metaphase-Anaphase Transition in Drosophila S2 Cells. *Mol Biol Cell*

Page, S.L., and R.S. Hawley. (2004). The genetics and molecular biology of the synaptonemal complex. *Annu Rev Cell Dev Biol*. 20:525-58

Palframan, W.J., J.B. Meehl, S.L. Jaspersen, M. Winey, and A.W. Murray. (2006). Anaphase inactivation of the spindle checkpoint. *Science*. 313:680-4

Palmer, D.K., K. O'Day, H.L. Trong, H. Charbonneau, and R.L. Margolis. (1991). Purification of the centromere-specific protein CENP-A and demonstration that it is a distinctive histone. *Proc Natl Acad Sci U S A*. 88:3734-8

Percy, M.J., K.A. Myrie, C.K. Neeley, J.N. Azim, S.P. Ethier, and E.M. Petty. (2000). Expression and mutational analyses of the human MAD2L1 gene in breast cancer cells. *Genes Chromosomes Cancer*. 29:356-62

Pereira, A.L., A.J. Pereira, A.R. Maia, K. Drabek, C.L. Sayas, P.J. Hergert, M. Lince-Faria, I. Matos, C. Duque, T. Stepanova, C.L. Rieder, W.C. Earnshaw, N. Galjart, and H. Maiato. (2006). Mammalian CLASP1 and CLASP2 cooperate to ensure mitotic fidelity by regulating spindle and kinetochore function. *Mol Biol Cell*. 17:4526-42

Perera, D., V. Tilston, J.A. Hopwood, M. Barchi, R.P. Boot-Handford, and S.S. Taylor. (2007). Bub1 maintains centromeric cohesion by activation of the spindle checkpoint. *Dev Cell*. 13:566-79

Perez-Castro, A.V., F.L. Shamanski, J.J. Meneses, T.L. Lovato, K.G. Vogel, R.K. Moyzis, and R. Pedersen. (1998). Centromeric protein B null mice are viable with no apparent abnormalities. *Dev Biol*. 201:135-43

Perry, J.A., and S. Kornbluth. (2007). Cdc25 and Wee1: analogous opposites? *Cell Div*. 2:12

Petersen, J., and I.M. Hagan. (2003). S. pombe aurora kinase/survivin is required for chromosome condensation and the spindle checkpoint attachment response. *Curr Biol*. 13:590-7

Pfarr, C.M., M. Coue, P.M. Grissom, T.S. Hays, M.E. Porter, and J.R. McIntosh. (1990). Cytoplasmic dynein is localized to kinetochores during mitosis. *Nature*. 345:263-5

Pierre, P., J. Scheel, J.E. Rickard, and T.E. Kreis. (1992). CLIP-170 links endocytic vesicles to microtubules. *Cell*. 70:887-900

Pines, J. (1999). Four-dimensional control of the cell cycle. *Nat Cell Biol*. 1:E73-9

Pinsky, B.A., C. Kung, K.M. Shokat, and S. Biggins. (2006). The Ipl1-Aurora protein kinase activates the spindle checkpoint by creating unattached kinetochores. *Nat Cell Biol*. 8:78-83

Politi, V., G. Perini, S. Trazzi, A. Pliss, I. Raska, W.C. Earnshaw, and G. Della Valle. (2002). CENP-C binds the alpha-satellite DNA in vivo at specific centromere domains. *J Cell Sci*. 115:2317-27

Putkey, F.R., T. Cramer, M.K. Morphew, A.D. Silk, R.S. Johnson, J.R. McIntosh, and D.W. Cleveland. (2002). Unstable kinetochore-microtubule capture and chromosomal instability following deletion of CENP-E. *Dev Cell*. 3:351-65

Raaijmakers, J., T. ME, A.F. Maia, and R.H. Medema. (2009). RAMA 1 ia a novel kinetochore protein involved in kinetochore-microtubule attachment. *J Cell Sci*. *in press*

Rattner, J.B., A. Rao, M.J. Fritzler, D.W. Valencia, and T.J. Yen. (1993). CENP-F is a .ca 400 kDa kinetochore protein that exhibits a cell-cycle dependent localization. *Cell Motil Cytoskeleton*. 26:214-26

Reddy, S.K., M. Rape, W.A. Margansky, and M.W. Kirschner. (2007). Ubiquitination by the anaphase-promoting complex drives spindle checkpoint inactivation. *Nature*. 446:921-5

Rieder, C.L. (1982). The formation, structure, and composition of the mammalian kinetochore and kinetochore fiber. *Int Rev Cytol*. 79:1-58

Rieder, C.L., R.W. Cole, A. Khodjakov, and G. Sluder. (1995). The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores. *J Cell Biol*. 130:941-8

Rieder, C.L., E.A. Davison, L.C. Jensen, L. Cassimeris, and E.D. Salmon. (1986). Oscillatory movements of monooriented chromosomes and their position relative to the spindle pole result from the ejection properties of the aster and half-spindle. *J Cell Biol*. 103:581-91

Rieder, C.L., and E.D. Salmon. (1994). Motile kinetochores and polar ejection forces dictate chromosome position on the vertebrate mitotic spindle. *J Cell Biol*. 124:223-33

Rieder, C.L., and E.D. Salmon. (1998). The vertebrate cell kinetochore and its roles during mitosis. *Trends Cell Biol*. 8:310-8

Rieder, C.L., A. Schultz, R. Cole, and G. Sluder. (1994). Anaphase onset in vertebrate somatic cells is controlled by a checkpoint that monitors sister kinetochore attachment to the spindle. *J Cell Biol*. 127:1301-10

Ris, H., and P.L. Witt. (1981). Structure of the mammalian kinetochore. *Chromosoma*. 82:153-70

Roberts, B.T., K.A. Farr, and M.A. Hoyt. (1994). The Saccharomyces cerevisiae checkpoint gene BUB1 encodes a novel protein kinase. *Mol Cell Biol*. 14:8282-91

Rodrigo-Brenni, M.C., S. Thomas, D.C. Bouck, and K.B. Kaplan. (2004). Sgt1p and Skp1p modulate the assembly and turnover of CBF3 complexes required for proper kinetochore function. *Mol Biol Cell*. 15:3366-78

Rogers, G.C., S.L. Rogers, T.A. Schwimmer, S.C. Ems-McClung, C.E. Walczak, R.D. Vale, J.M. Scholey, and D.J. Sharp. (2004). Two mitotic kinesins cooperate to drive sister chromatid separation during anaphase. *Nature*. 427:364-70

Roos, U.P. (1973). Light and electron microscopy of rat kangaroo cells in mitosis. II. Kinetochore structure and function. *Chromosoma*. 41:195-220

Ru, H.Y., R.L. Chen, W.C. Lu, and J.H. Chen. (2002). hBUB1 defects in leukemia and lymphoma cells. *Oncogene*. 21:4673-9

Sandblad, L., K.E. Busch, P. Tittmann, H. Gross, D. Brunner, and A. Hoenger. (2006). The Schizosaccharomyces pombe EB1 homolog Mal3p binds and stabilizes the microtubule lattice seam. *Cell*. 127:1415-24

Santaguida, S., and A. Musacchio. (2009). The life and miracles of kinetochores. *EMBO J*

Schaar, B.T., G.K. Chan, P. Maddox, E.D. Salmon, and T.J. Yen. (1997). CENP-E function at kinetochores is essential for chromosome alignment. *J Cell Biol*. 139:1373-82

Schiff, P.B., J. Fant, and S.B. Horwitz. (1979). Promotion of microtubule assembly in vitro by taxol. *Nature*. 277:665-7

Schiff, P.B., and S.B. Horwitz. (1980). Taxol stabilizes microtubules in mouse fibroblast cells. *Proc Natl Acad Sci U S A*. 77:1561-5

Schleiden, M.J. (1838). Beitraege sur phytogenesis (Contributions to Phytogenesis). *Mueller's Archiv.*

Schuyler, S.C., and D. Pellman. (2001). Microtubule "plus-end-tracking proteins": The end is just the beginning. *Cell*. 105:421-4

Schwann, T. (1839). Mikroscopische untersuchungen ueber die Uebereinstimmuting in der struktur und dem wachstum der thiere und planzen. (Microscope studies on the Uebereinstimmuting in the structure and the growth of animals and plants). *(Berlin)*

Shah, J.V., E. Botvinick, Z. Bonday, F. Furnari, M. Berns, and D.W. Cleveland. (2004). Dynamics of centromere and kinetochore proteins; implications for checkpoint signaling and silencing. *Curr Biol*. 14:942-52

Shichiri, M., K. Yoshinaga, H. Hisatomi, K. Sugihara, and Y. Hirata. (2002). Genetic and epigenetic inactivation of mitotic checkpoint genes hBUB1 and hBUBR1 and their relationship to survival. *Cancer Res*. 62:13-7

Simanis, V., and P. Nurse. (1986). The cell cycle control gene cdc2+ of fission yeast encodes a protein kinase potentially regulated by phosphorylation. *Cell*. 45:261-8

Sironi, L., M. Mapelli, S. Knapp, A. De Antoni, K.T. Jeang, and A. Musacchio. (2002). Crystal structure of the tetrameric Mad1-Mad2 core complex: implications of a 'safety belt' binding mechanism for the spindle checkpoint. *EMBO J*. 21:2496-506

Sivaram, M.V., T.L. Wadzinski, S.D. Redick, T. Manna, and S.J. Doxsey. (2009). Dynein light intermediate chain 1 is required for progress through the spindle assembly checkpoint. *EMBO J*. 28:902-14

Smith, L.D., and R.E. Ecker. (1971). The interaction of steroids with Rana pipiens Oocytes in the induction of maturation. *Dev Biol*. 25:232-47

Smits, V.A., R. Klompmaker, L. Arnaud, G. Rijksen, E.A. Nigg, and R.H. Medema. (2000). Polo-like kinase-1 is a target of the DNA damage checkpoint. *Nat Cell Biol*. 2:672-6

Spencer, F., and P. Hieter. (1992). Centromere DNA mutations induce a mitotic delay in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*. 89:8908-12

Starr, D.A., B.C. Williams, T.S. Hays, and M.L. Goldberg. (1998). ZW10 helps recruit dynactin and dynein to the kinetochore. *J Cell Biol*. 142:763-74

Steensgaard, P., M. Garre, I. Muradore, P. Transidico, E.A. Nigg, K. Kitagawa, W.C. Earnshaw, M. Faretta, and A. Musacchio. (2004). Sgt1 is required for human kinetochore assembly. *EMBO Rep*. 5:626-31

Stegmeier, F., M. Rape, V.M. Draviam, G. Nalepa, M.E. Sowa, X.L. Ang, E.R. McDonald, 3rd, M.Z. Li, G.J. Hannon, P.K. Sorger, M.W. Kirschner, J.W. Harper, and S.J. Elledge. (2007). Anaphase initiation is regulated by antagonistic ubiquitination and deubiquitination activities. *Nature*. 446:876-81

Steuer, E.R., L. Wordeman, T.A. Schroer, and M.P. Sheetz. (1990). Localization of cytoplasmic dynein to mitotic spindles and kinetochores. *Nature*. 345:266-8

Stucke, V.M., H.H. Sillje, L. Arnaud, and E.A. Nigg. (2002). Human Mps1 kinase is required for the spindle assembly checkpoint but not for centrosome duplication. *Embo J*. 21:1723-32

Stumpff, J., G. von Dassow, M. Wagenbach, C. Asbury, and L. Wordeman. (2008). The kinesin-8 motor Kif18A suppresses kinetochore movements to control mitotic chromosome alignment. *Dev Cell*. 14:252-62

Su, L.K., M. Burrell, D.E. Hill, J. Gyuris, R. Brent, R. Wiltshire, J. Trent, B. Vogelstein, and K.W. Kinzler. (1995). APC binds to the novel protein EB1. *Cancer Res*. 55:2972-7

Sudakin, V., G.K. Chan, and T.J. Yen. (2001). Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2. *J Cell Biol*. 154:925-36

Sullivan, K.F. (2001). A solid foundation: functional specialization of centromeric chromatin. *Curr Opin Genet Dev*. 11:182-8

Sullivan, K.F., M. Hechenberger, and K. Masri. (1994). Human CENP-A contains a histone H3 related histone fold domain that is required for targeting to the centromere. *J Cell Biol*. 127:581-92

Suzuki, N., M. Nakano, N. Nozaki, S. Egashira, T. Okazaki, and H. Masumoto. (2004). CENP-B interacts with CENP-C domains containing Mif2 regions responsible for centromere localization. *J Biol Chem*. 279:5934-46

Takeda, D.Y., and A. Dutta. (2005). DNA replication and progression through S phase. *Oncogene*. 24:2827-43

Tanaka, T.U. (2002). Bi-orienting chromosomes on the mitotic spindle. *Curr Opin Cell Biol*. 14:365-71

Tanaka, T.U., N. Rachidi, C. Janke, G. Pereira, M. Galova, E. Schiebel, M.J. Stark, and K. Nasmyth. (2002). Evidence that the Ipl1-Sli15 (Aurora kinase-INCENP) complex promotes chromosome bi-orientation by altering kinetochore-spindle pole connections. *Cell*. 108:317-29

Tanenbaum, M.E., N. Galjart, M.A. van Vugt, and R.H. Medema. (2006). CLIP-170 facilitates the formation of kinetochore-microtubule attachments. *EMBO J*. 25:45-57

Tang, Z., R. Bharadwaj, B. Li, and H. Yu. (2001). Mad2-Independent inhibition of APCCdc20 by the mitotic checkpoint protein BubR1. *Dev Cell*. 1:227-37

Tanudji, M., J. Shoemaker, L. L'Italien, L. Russell, G. Chin, and X.M. Schebye. (2004). Gene silencing of CENP-E by small interfering RNA in HeLa cells leads to missegregation of chromosomes after a mitotic delay. *Mol Biol Cell*. 15:3771-81

Taylor, S.S., E. Ha, and F. McKeon. (1998). The human homologue of Bub3 is required for kinetochore localization of Bub1 and a Mad3/Bub1-related protein kinase. *J Cell Biol*. 142:1-11

Taylor, S.S., and F. McKeon. (1997). Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage. *Cell*. 89:727-35

Theis, M., M. Slabicki, M. Junqueira, M. Paszkowski-Rogacz, J. Sontheimer, R. Kittler, A.K. Heninger, T. Glatter, K. Kruusmaa, I. Poser, A.A. Hyman, M.T. Pisabarro, M. Gstaiger, R. Aebersold, A. Shevchenko, and F. Buchholz. (2009). Comparative profiling identifies C13orf3 as a component of the Ska complex required for mammalian cell division. *EMBO J*. 28:1453-65

Thomas, J.T., and L.A. Laimins. (1998). Human papillomavirus oncoproteins E6 and E7 independently abrogate the mitotic spindle checkpoint. *J Virol*. 72:1131-7

Thrower, D.A., M.A. Jordan, B.T. Schaar, T.J. Yen, and L. Wilson. (1995). Mitotic HeLa cells contain a CENP-E-associated minus end-directed microtubule motor. *EMBO J*. 14:918-26

Tighe, A., V.L. Johnson, M. Albertella, and S.S. Taylor. (2001). Aneuploid colon cancer cells have a robust spindle checkpoint. *EMBO Rep*. 2:609-14

Tirnauer, J.S., J.C. Canman, E.D. Salmon, and T.J. Mitchison. (2002). EB1 targets to kinetochores with attached, polymerizing microtubules. *Mol Biol Cell*. 13:4308-16

Tomkiel, J., C.A. Cooke, H. Saitoh, R.L. Bernat, and W.C. Earnshaw. (1994). CENP-C is required for maintaining proper kinetochore size and for a timely transition to anaphase. *J Cell Biol*. 125:531-45

Tsukasaki, K., C.W. Miller, E. Greenspun, S. Eshaghian, H. Kawabata, T. Fujimoto, M. Tomonaga, C. Sawyers, J.W. Said, and H.P. Koeffler. (2001). Mutations in the mitotic check point gene, MAD1L1, in human cancers. *Oncogene*. 20:3301-5

Tulu, U.S., C. Fagerstrom, N.P. Ferenz, and P. Wadsworth. (2006). Molecular requirements for kinetochore-associated microtubule formation in mammalian cells. *Curr Biol*. 16:536-41

Vader, G., C.W. Cruijsen, T. van Harn, M.J. Vromans, R.H. Medema, and S.M. Lens. (2007). The chromosomal passenger complex controls spindle checkpoint function independent from its role in correcting microtubule kinetochore interactions. *Mol Biol Cell*. 18:4553-64

Vader, G., and S.M. Lens. (2008). The Aurora kinase family in cell division and cancer. *Biochim Biophys Acta*. 1786:60-72

Vader, G., A.F. Maia, and S.M. Lens. (2008). The chromosomal passenger complex and the spindle assembly checkpoint: kinetochore-microtubule error correction and beyond. *Cell Div*. 3:10

Vafa, O., and K.F. Sullivan. (1997). Chromatin containing CENP-A and alpha-satellite DNA is a major component of the inner kinetochore plate. *Curr Biol*. 7:897-900

Vigneron, S., S. Prieto, C. Bernis, J.C. Labbe, A. Castro, and T. Lorca. (2004). Kinetochore localization of spindle checkpoint proteins: who controls whom? *Mol Biol Cell*. 15:4584-96

Vitre, B., F.M. Coquelle, C. Heichette, C. Garnier, D. Chretien, and I. Arnal. (2008). EB1 regulates microtubule dynamics and tubulin sheet closure in vitro. *Nat Cell Biol*. 10:415-21

Vodermaier, H.C. (2004). APC/C and SCF: controlling each other and the cell cycle. *Curr Biol*. 14:R787-96

Wang, H.W., V.H. Ramey, S. Westermann, A.E. Leschziner, J.P. Welburn, Y. Nakajima, D.G. Drubin, G. Barnes, and E. Nogales. (2007). Architecture of the Dam1 kinetochore ring complex and implications for microtubule-driven assembly and force-coupling mechanisms. *Nat Struct Mol Biol*. 14:721-6

Wang, Q., T. Liu, Y. Fang, S. Xie, X. Huang, R. Mahmood, G. Ramaswamy, K.M. Sakamoto, Z. Darzynkiewicz, M. Xu, and W. Dai. (2004). BUBR1 deficiency results in abnormal megakaryopoiesis. *Blood*. 103:1278-85

Wang, X., D.Y. Jin, R.W. Ng, H. Feng, Y.C. Wong, A.L. Cheung, and S.W. Tsao. (2002). Significance of MAD2 expression to mitotic checkpoint control in ovarian cancer cells. *Cancer Res*. 62:1662-8

Wang, X., D.Y. Jin, Y.C. Wong, A.L. Cheung, A.C. Chun, A.K. Lo, Y. Liu, and S.W. Tsao. (2000). Correlation of defective mitotic checkpoint with aberrantly reduced expression of MAD2 protein in nasopharyngeal carcinoma cells. *Carcinogenesis*. 21:2293-7

Warburton, P.E., C.A. Cooke, S. Bourassa, O. Vafa, B.A. Sullivan, G. Stetten, G. Gimelli, D. Warburton, C. Tyler-Smith, K.F. Sullivan, G.G. Poirier, and W.C. Earnshaw. (1997). Immunolocalization of CENP-A suggests a distinct nucleosome structure at the inner kinetochore plate of active centromeres. *Curr Biol*. 7:901-4

Weaver, B.A., Z.Q. Bonday, F.R. Putkey, G.J. Kops, A.D. Silk, and D.W. Cleveland. (2003). Centromere-associated protein-E is essential for the mammalian mitotic checkpoint to prevent aneuploidy due to single chromosome loss. *J Cell Biol*. 162:551-63

Wei, R.R., J. Al-Bassam, and S.C. Harrison. (2007). The Ndc80/HEC1 complex is a contact point for kinetochore-microtubule attachment. *Nat Struct Mol Biol*. 14:54-9

Weiss, E., and M. Winey. (1996). The Saccharomyces cerevisiae spindle pole body duplication gene MPS1 is part of a mitotic checkpoint. *J Cell Biol*. 132:111-23

Welburn, J.P., E.L. Grishchuk, C.B. Backer, E.M. Wilson-Kubalek, J.R. Yates, 3rd, and I.M. Cheeseman. (2009). The human kinetochore Ska1 complex facilitates microtubule depolymerization-coupled motility. *Dev Cell*. 16:374-85

Wendell, K.L., L. Wilson, and M.A. Jordan. (1993). Mitotic block in HeLa cells by vinblastine: ultrastructural changes in kinetochore-microtubule attachment and in centrosomes. *J Cell Sci*. 104 (Pt 2):261-74

Westermann, S., A. Avila-Sakar, H.W. Wang, H. Niederstrasser, J. Wong, D.G. Drubin, E. Nogales, and G. Barnes. (2005). Formation of a dynamic kinetochore-microtubule interface through assembly of the Dam1 ring complex. *Mol Cell*. 17:277-90

Westermann, S., I.M. Cheeseman, S. Anderson, J.R. Yates, 3rd, D.G. Drubin, and G. Barnes. (2003). Architecture of the budding yeast kinetochore reveals a conserved molecular core. *J Cell Biol*. 163:215-22

Westermann, S., H.W. Wang, A. Avila-Sakar, D.G. Drubin, E. Nogales, and G. Barnes. (2006). The Dam1 kinetochore ring complex moves processively on depolymerizing microtubule ends. *Nature*. 440:565-9

Winey, M., L. Goetsch, P. Baum, and B. Byers. (1991). MPS1 and MPS2: novel yeast genes defining distinct steps of spindle pole body duplication. *J Cell Biol*. 114:745-54

Wollman, R., E.N. Cytrynbaum, J.T. Jones, T. Meyer, J.M. Scholey, and A. Mogilner. (2005). Efficient chromosome capture requires a bias in the 'search-and-capture' process during mitotic-spindle assembly. *Curr Biol*. 15:828-32

Wood, K.W., R. Sakowicz, L.S. Goldstein, and D.W. Cleveland. (1997). CENP-E is a plus end-directed kinetochore motor required for metaphase chromosome alignment. *Cell*. 91:357-66

Wordeman, L., E.R. Steuer, M.P. Sheetz, and T. Mitchison. (1991). Chemical subdomains within the kinetochore domain of isolated CHO mitotic chromosomes. *J Cell Biol*. 114:285-94

Wordeman, L., M. Wagenbach, and G. von Dassow. (2007). MCAK facilitates chromosome movement by promoting kinetochore microtubule turnover. *J Cell Biol*. 179:869-79

Wu, H., Z. Lan, W. Li, S. Wu, J. Weinstein, K.M. Sakamoto, and W. Dai. (2000). p55CDC/hCDC20 is associated with BUBR1 and may be a downstream target of the spindle checkpoint kinase. *Oncogene*. 19:4557-62

Xia, G., X. Luo, T. Habu, J. Rizo, T. Matsumoto, and H. Yu. (2004). Conformation-specific binding of p31(comet) antagonizes the function of Mad2 in the spindle checkpoint. *EMBO J*. 23:3133-43

Yang, M., B. Li, C.J. Liu, D.R. Tomchick, M. Machius, J. Rizo, H. Yu, and X. Luo. (2008). Insights into mad2 regulation in the spindle checkpoint revealed by the crystal structure of the symmetric mad2 dimer. *PLoS Biol*. 6:e50

Yang, Z., J. Guo, Q. Chen, C. Ding, J. Du, and X. Zhu. (2005). Silencing mitosin induces misaligned chromosomes, premature chromosome decondensation before anaphase onset, and mitotic cell death. *Mol Cell Biol*. 25:4062-74

Yang, Z., A.E. Kenny, D.A. Brito, and C.L. Rieder. (2009). Cells satisfy the mitotic checkpoint in Taxol, and do so faster in concentrations that stabilize syntelic attachments. *J Cell Biol*. 186:675-84

Yao, X., A. Abrieu, Y. Zheng, K.F. Sullivan, and D.W. Cleveland. (2000). CENP-E forms a link between attachment of spindle microtubules to kinetochores and the mitotic checkpoint. *Nat Cell Biol*. 2:484-91

Yao, X., K.L. Anderson, and D.W. Cleveland. (1997). The microtubule-dependent motor centromere-associated protein E (CENP-E) is an integral component of kinetochore corona fibers that link centromeres to spindle microtubules. *J Cell Biol*. 139:435-47

Yasui, Y., T. Urano, A. Kawajiri, K. Nagata, M. Tatsuka, H. Saya, K. Furukawa, T. Takahashi, I. Izawa, and M. Inagaki. (2004). Autophosphorylation of a newly identified site of Aurora-B is indispensable for cytokinesis. *J Biol Chem*. 279:12997-3003

Yen, T.J., D.A. Compton, D. Wise, R.P. Zinkowski, B.R. Brinkley, W.C. Earnshaw, and D.W. Cleveland. (1991). CENP-E, a novel human centromere-associated protein required for progression from metaphase to anaphase. *Embo J*. 10:1245-54

Yen, T.J., G. Li, B.T. Schaar, I. Szilak, and D.W. Cleveland. (1992). CENP-E is a putative kinetochore motor that accumulates just before mitosis. *Nature*. 359:536-9

Yoda, K., K. Kitagawa, H. Masumoto, Y. Muro, and T. Okazaki. (1992). A human centromere protein, CENP-B, has a DNA binding domain containing four potential alpha helices at the NH2 terminus, which is separable from dimerizing activity. *J Cell Biol*. 119:1413-27

Yucel, J.K., J.D. Marszalek, J.R. McIntosh, L.S. Goldstein, D.W. Cleveland, and A.V. Philp. (2000). CENP-meta, an essential kinetochore kinesin required for the maintenance of metaphase chromosome alignment in Drosophila. *J Cell Biol*. 150:1-11

Yvon, A.M., P. Wadsworth, and M.A. Jordan. (1999). Taxol suppresses dynamics of individual microtubules in living human tumor cells. *Mol Biol Cell*. 10:947-59

Zhang, C.C., J.M. Yang, E. White, M. Murphy, A. Levine, and W.N. Hait. (1998). The role of MAP4 expression in the sensitivity to paclitaxel and resistance to vinca alkaloids in p53 mutant cells. *Oncogene*. 16:1617-24

Zieve, G.W., D. Turnbull, J.M. Mullins, and J.R. McIntosh. (1980). Production of large numbers of mitotic mammalian cells by use of the reversible microtubule inhibitor nocodazole. Nocodazole accumulated mitotic cells. *Exp Cell Res*. 126:397-405

PART V

APPENDIXES

APPENDIX 1

Abbreviations

+TIPs: Microtubule Plus-end Tracking Proteins

Ab: Antibody

ACA: Anticentromere Antibodies

APC: Adenomatous Polyposis Coli

APC/C: Anaphase-Promoting Complex/Cyclosome

bp: base pairs

BSA: Bovine Serum Albumin

Bub: budding uninhibited by benzimidazole

***C. elegans*:** *Caenorhabditis elegans*

CBF3: Centromere Binding Factor 3

CCAN: Constitutive Centromere-Associated Network

cdc: cell division cycle

Cdks: Cyclin-dependent kinases

cDNA: complementary DNA

CENP: Centromere-associated Protein

CID: Centromere identifier

CIN: Chromosomal Instability

CKIs: Cyclin-dependent kinase inhibitor proteins

CLASP: CLIP-Associated Proteins

CLIP: Cytoplasmic Linker Protein

CPC: Chromosomal Passenger Complex

CREST: sera from a form of Systemic Sclerosis, showing Calcinosis, Raynaud's phenomenon, Esophageal dysmotility, Sclerodactyly, Telangiectasia. Recognizes CENP-A, CENP-B and CENP-C.

DAPI: 4',6'-diamino-2-phenylindole

DIC: Differential Interference Contrast microscopy

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DNA: deoxyribonucleic acid

DPBS: Dulbecco's Phosphate Buffered Saline

dsRNA: double stranded RNA

DTT: dithiothreitol

DUBs: Deubiquitinating enzymes

***e.g.*:** exempli gratia

EB1: End Binding protein 1

ECL: Enhanced ChemiLuminescence

EGFP: Enhanced Green Fluorescent Protein

EM: Electron Microscopy

Exp: Experiment(s)

FBS: Fetal Bovine Serum

FCS: Fetal Calf Serum

Fig.: Figure

g: gram

G1: Gap phase 1

G2: Gap phase 2

GFP: Green Fluorescent Protein

h(r): hour

HeLa: Human immortal cell line

Hsp: Heat-shock protein

IB: Immunoblotting

IF: Immunofluorescence

K-fiber: kinetochore fiber

kDa: kiloDalton(s)

Klp: Kinesin-like protein

KMN: KNL1/Mis12 complex/Ndc80 complex

KT: Kinetochore

L: Liter

LB: Luria-Bertani culture medium

M phase: Mitosis

M: Molar

Mad: Mitotic-arrest deficient

MAPs: Microtubule-Associated Proteins

MCAK: Mitotic Centromere Associated Kinesin

MCC: Mitotic Checkpoint Complex

MCS: Multiple Cloning Site

MDR: Multi-Drug Resistant

MEF: Mouse Embryonic Fibroblast

min: minutes

ml: milliliter

mM: miliMolar

mm: millimeter

mm:ss: minutes:seconds

MPF: Maturation/Mitosis-Promoting Factor

mRNA: messenger RNA

MT(s): Microtubule(s)

MTOC: Microtubule Organizing Center

MVA: Mosaic Variegated Aneuploidy

n: number of samples in the study

***n.d.*:** not determined

NEBD: Nuclear Envelope Breakdown

NIH: National Institutes of Health

nm: nanometer

nM: nanoMolar

ORF: Open Reading Frame

p(T232): antibody against phosphorylation in Threonine 232 on Aurora B

PBS: Phosphate-Buffered Saline

PCR: Polimerase Chain Reaction

PEM: PIPES-EGTA-Magnesium Chloride buffer

PSCS: Premature Sister Chromatid Separation

RNA: ribonucleic acid

RNAi: RNA interference

RT: Room Temperature

RZZ: Rod-ZW10-Zwilch

S phase: DNA synthesis phase

***S. cerevisiae*:** *Saccharomyces cerevisiae*

S. pombe: *Schizosaccharomyces pombe*

s.d.: standard deviation

S2: *Drosophila* Schneider 2 cell line

SAC: Spindle Assembly Checkpoint

SCF: Skp1/Cullin/F-box ubiquitin-protein ligase

SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrilamide Gel Electrophoresis

SDS: Sodium Dodecyl Sulphate

SEM: Standard Error of the Mean

siRNA: small interference RNA

SPB: Spindle Pole Body

STD: Standard Deviation

t-test: Student's *t* test

TIM: Time In Mitosis

TPR: Tetracopeptide domain

Tris: Tris(hidroximethyl)aminomethane

UTR: Untranslated Region

UV: ultraviolet

***X. laevis*:** *Xenopus laevis*

YFP: Yellow Fluorescent Protein

ZM447439: Aurora B kinase small-molecule inhibitor

μg: microgram

μl: microliter

μm: micrometer

μM: microMolar

APPENDIX 2

Recipes

LB Medium:

1% Tryptone
0.5% Yeast extract
1% NaCl

LB Agar:

1.5% (w/v) Agar in LB medium

Ampicillin plates:

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

Tetracycline plates:

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

PBS (Phosphate-Buffered Saline) pH 7.4:

137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4

PEM:

100 mM Pipes
10 mM EGTA
1 mM $MgCl_2$

2x SDS-PAGE sample buffer:

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

Protein Electrophoresis:

Stacking gel: 4% acrilamide; 125 mM Tris-HCl, pH 6.8; 0.1% SDS;
Separating gel: 7.5% acrilamide; 375 mM Tris-HCl, pH 8.8; 0.1% SDS;
Running buffer: 25 mM Tris, pH 8.3; 250 mM Glycine; 0.1% SDS

Ponceau S:

0.1% Ponceau
5% acetic acid

TBST:

50 mM Tris-HCl, pH7.5
150 mM NaCl
0.05% Tween 20

Transfer Buffer:

25 mM Glycine
192 mM Tris pH 8.3

Enhanced Chemiluminescent (ECL):

Solution A: 10 ml Tris 100 mM pH 8.5, 44 µl Cumaric acid (Sigma) 90 mM and 100 µl Luminol (FLUKA) 250 mM;
Solution B: 10 ml Tris 100 mM pH 8.5 and 6 µl H_2O_2 30% (Merck)
Solution A and B are mixed and incubated with the membrane at the time of ECL detection.

Schneider's Insect Medium:

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

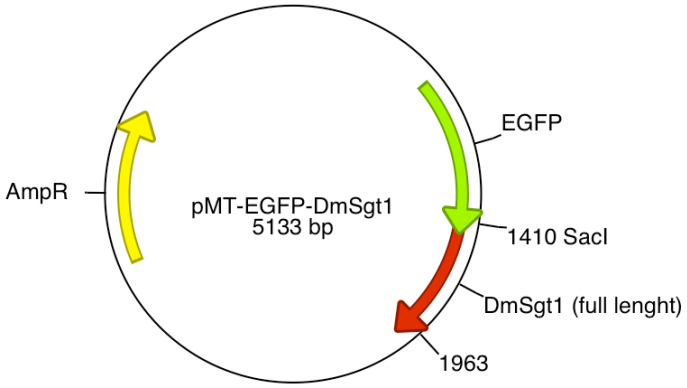
Calcium-Treatment Buffer:

100 mM PIPES, pH 6.8
1 mM $MgCl_2$
0.1 mM $CaCl_2$
0.1% Triton X-100

APPENDIX 3

Plasmids

pMT-EGFP-Sgt1



pMT-EGFP-Sgt1 was generated by cloning Sgt1 full-length cDNA into the *Hinc*II/*Sac*I sites of the MCS of pMT-EGFP-C1 plasmid.

APPENDIX 4

Movies Legends

Movie 1.1 Mitotic division of a control S2 cell stably expressing GFP-H2B recorded by time-lapse fluorescence microscopy. Times are relative to anaphase onset.

Movie 1.2 Mitotic division of a BubR1 depleted S2 cell stably expressing GFP-H2B recorded by time-lapse fluorescence microscopy. Times are relative to anaphase onset. Note that this cell undergoes anaphase onset without aligning the chromosomes in the metaphase plate.

Movie 1.3 Mitotic division of a control S2 cell stably expressing GFP-tubulin recorded by time-lapse fluorescence microscopy. Images were taken every 90 seconds. Times are relative to nuclear envelope breakdown.

Movie 1.4 Mitotic division of a BubR1 depleted S2 cell stably expressing GFP-tubulin recorded by time-lapse fluorescence microscopy. Images were taken every 90 seconds. Times are relative to nuclear envelope breakdown.

Movie 2.1 and Movie 2.2 Animated time-lapse images of a control (siLuciferase) HeLa cell stably expressing YFP-H2B. Images were taken every 3 minutes. Times are relative to nuclear envelope breakdown. Overlay of DIC Image (grey) and YFP-H2B (green), movie 2.1. YFP-H2B (grey), movie 2.2.

Movie 2.3 and Movie 2.4 Animated time-lapse images of a CENP-E-depleted HeLa cell stably expressing YFP-H2B. Phenotype a. Note that the cell only goes into anaphase after the last unaligned chromosome reaches the metaphase plate. Images were taken every 3 minutes. Times are relative to nuclear envelope breakdown. Overlay of DIC Image (grey) and YFP-H2B (green), movie 2.3. YFP-H2B (grey), movie 2.4.

Movie 2.5 and Movie 2.6 Animated time-lapse images of a CENP-E-depleted HeLa cell stably expressing YFP-H2B. Phenotype b. Note that the cell goes into anaphase with an unaligned chromosome. Images were taken every 3 minutes. Times are relative to nuclear envelope breakdown. Overlay of DIC Image (grey) and YFP-H2B (green), movie 2.5. YFP-H2B (grey), movie 2.6.

Movie 2.7 and Movie 2.8 Animated time-lapse images of a CENP-E-depleted HeLa cell stably expressing YFP-H2B. A black frame indicates that ZM447439 was added to the medium. Note that anaphase starts with several unaligned chromosomes. Images were taken every 3 minutes. Times are relative to nuclear envelope breakdown. Overlay of DIC Image (grey) and YFP-H2B (green), movie 2.7. YFP-H2B (grey), movie 2.8.