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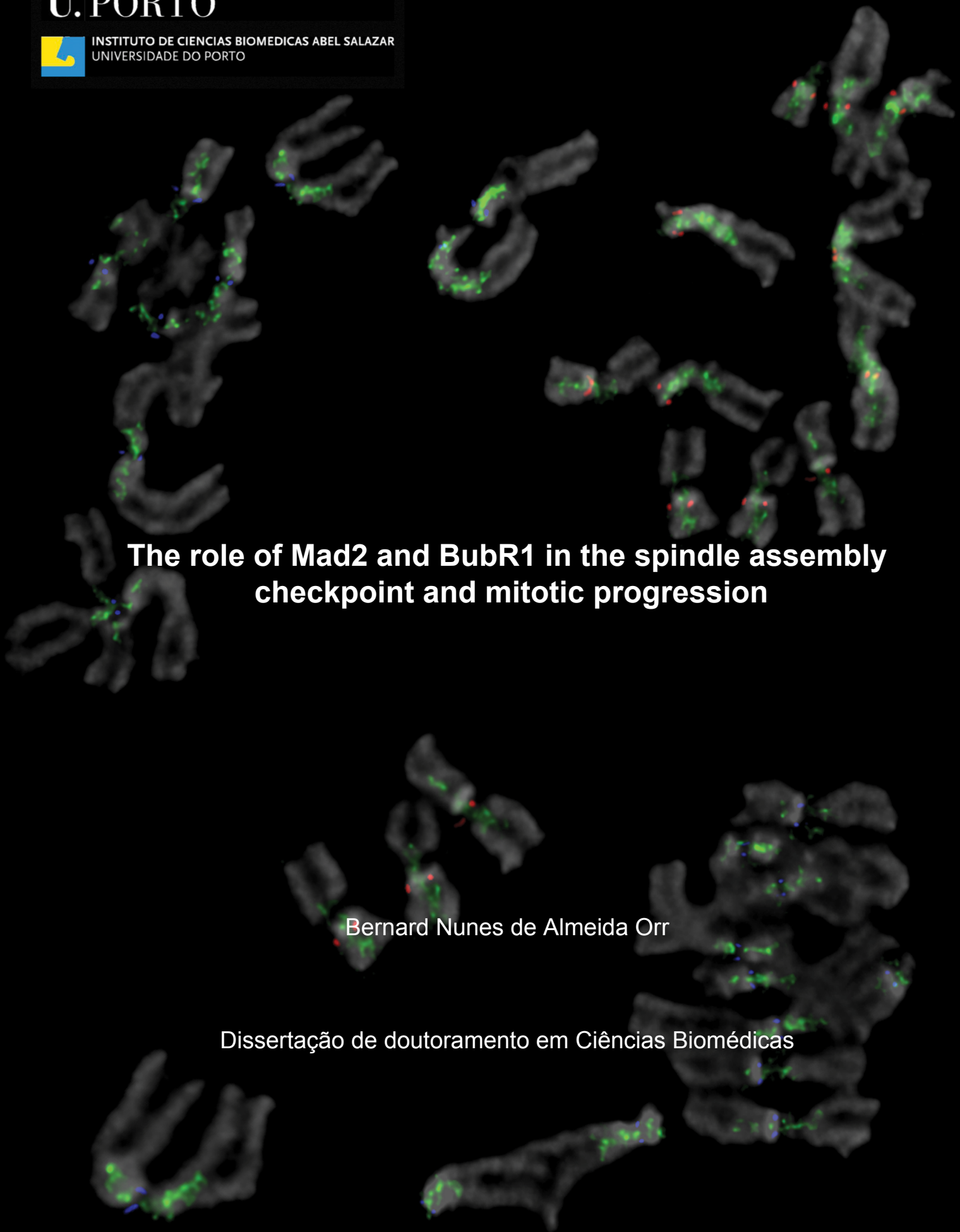

The role of Mad2 and BubR1 in the spindle assembly checkpoint and mitotic progression

Bernard Nunes de Almeida Orr

Dissertação de doutoramento em Ciências Biomédicas

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The role of Mad2 and BubR1 in the spindle assembly checkpoint and mitotic progression

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Orientador – Doutor Claudio E. Sunkel
Categoria – Professor Catedrático
Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

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Summary

Faithful chromosome segregation is essential for the maintenance of genomic stability during cell division. To ensure proper chromosome segregation, cells possess a surveillance mechanism that monitors the binding of chromosomes to the mitotic spindle. The Spindle Assembly Checkpoint (SAC) acts in mitosis to delay mitotic exit in the presence of chromosomes that either fail to attach or attach incorrectly to the spindle microtubules. The kinetochore, located at the centromere, is the chromosomal structure directly involved in the microtubule capture and is also required for generating the signals responsible for restraining anaphase onset. These signals are thought to involve several highly conserved proteins such as the Mad and Bub proteins that localize transiently to unattached kinetochores and promote the formation of inhibitory complexes that prevent the activation of the Anaphase Promoting Complex/Cyclosome (APC/C). The work presented in this thesis provides further understanding on the function of the SAC proteins suggesting that they participate in both kinetochore-dependent and -independent signalling. We show that loss of Mad2 causes an accelerated mitotic schedule accompanied by chromatin bridges during anaphase that result from premature chromosome decondensation. However, if provided with time, all Mad2-specific phenotypes are reverted and cells are now able to transit normally through mitosis and respond partially to microtubule poisons in a BubR1-dependent manner. We also show that Mad2 localization at kinetochores is dispensable for initial stages of SAC activity but is required for sustained SAC maintenance. These results indicate that SAC proteins appear to work in two separate stages of the SAC. First, during a kinetochore independent stage in which Mad2 is able to allow proper timing of prophase and early prometaphase by inhibiting the APC/C so that in a second, kinetochore-dependent phase, the inhibitory signals produced may directly block the APC/C. In the second part of the thesis we have analysed the role of the kinetochore protein CENP-C in the organization and function of kinetochores. Our results indicate that CENP-C plays a major role in *Drosophila* kinetochore assembly in a manner similar to CCAN proteins in higher eukaryotes. Furthermore, we show that CENP-C plays an important role in the stabilization of centromere determinants.

Resumo

A correcta segregação dos cromossomas é essencial para manter a estabilidade genética durante a divisão celular. Para assegurar a correcta segregação dos cromossomas, as células possuem um mecanismo de vigilância que monitoriza a ligação dos cromossomas aos microtúbulos do fuso mitótico. O ponto de controlo do fuso mitótico (SAC) actua na mitose de modo a atrasar a saída de mitose na presença de cromossomas que não estão ligados ou estão ligados de um modo incorrecto aos microtubulos do fuso. O cinetocóro, que se encontra localizado no centrómero, é a estrutura do cromossoma que está directamente envolvida em capturar os microtubulos, assim como em gerar sinais responsáveis por impedir o início da anafase. Pensa-se que esta sinalização envolve várias proteínas altamente conservadas, como as proteínas Mad e Bub, que se localizam temporariamente nos cinetocoros que estão livres e promovem a formação de complexos inibitórios que impedem a activação do complexo de promoção da anafase (APC/C). O trabalho apresentado nesta tese clarifica a função das proteínas do SAC, sugerindo que participam na sinalização dependente e independente do cinetocoro. Nós demonstramos que a perda da proteína Mad2 provoca uma aceleração na mitose acompanhada por pontes de cromatina durante a anafase, que resulta na descondensação precoce dos cromossomas. No entanto se, se der tempo, todos os fenótipos específicos de Mad2 são revertidos e as células podem deste modo, agora avançar normalmente na mitose e responder, parcialmente, a drogas de microtubulos de uma forma dependente de BubR1. Também demonstramos que, a localização de Mad2 nos cinetocoros é dispensável nas fases iniciais da actividade do SAC, mas é necessária para o manter. Estes resultados demonstram que as proteínas do SAC aparentam funcionar em duas fases separadas do SAC. Numa primeira fase, independente do cinetocoro, em que a Mad2 é capaz de permitir a correcta cronometragem da profase e da precoce prometafase, através da inibição do APC/C de modo a que numa segunda fase, dependente do cinetocoro, os sinais inibitórios produzidos possam directamente bloquear o APC/C. Na segunda parte da tese, analisamos a função da proteína presente nos cinetocoros, CENP-C, na organização e função destes. Os nossos resultados demonstram que a CENP-C desempenha uma função mais importante de montagem dos cinetocoros de *Drosophila,* de uma maneira semelhante às proteínas do CCAN nos eucariotas mais elevados. Além disso, demonstramos que a CENP-C desempenha uma função importante na estabilização de factores necessários para determinação do centrómero.

Part I

General Introduction

1. Cell Biology: a historical perspective

Biology is the study of the living world, and the concept of biology as an independent branch of scientific research arose in the 19th century. Since then, numerous important findings have marvelled the minds of world scientists. In the 17th century, Robert Hooke, one of the pioneers of the field of cell biology, first reported the discovery of cells and described them as small box-like structures (Hooke 1665), yet he was not able to grasp their biological significance. Ten years later, Anton van Leeuwenhoek was the first to observe a living cell and his work helped pave the way for cell biologists who first began to accept cells as the individual units of a larger organism. This statement was heavily influenced by work of Treviranus and Moldenhawer in plant cells (Treviranus 1811; Moldenhawer 1812), and a few years later, basing their observations on the previous reports, Schleiden and Schwann proposed that cells are the *quantum minimum* of life, thus classifying cells as individuals within a society (Schleiden 1839; Schwann 1839; Schwann and Schleiden 1847). Rudolph Virchow, a keen supporter of this hypothesis, corrected some of the fundamental flaws proposed by Schleiden and Schwann, and in 1858 formulated the hypothesis that all cells are derived from pre-existing cells, *Omins cellula a cellula* (Virchow 1858). Taken together, this work later served as a foundation for Henri Dutrochet's formulation of one of the most important theories of modern cell biology, "the cell is the fundamental element of organization" (Dutrochet 1824; reviewed in Tavassoli 1980).

1.1 The Cell Theory

The observations of Hooke, Leeuwenhoek, Schleiden, Schwann, Virchow and others, helped to develop the 'cell theory', which addresses the relationship between cells and living organisms and states that (a) cells are the building block of life, (b) all organisms are made of cells and (c) new cells are the product of the division of older cells. With the exception of viruses and mitochondria, the cell theory currently still holds true for all living organisms.

Around 40 years after the cell theory was proposed, Walther Flemming was the first to describe the stages of a process we currently know as the cell division cycle (Flemming 1879; Flemming 1965). Using newly synthesized aniline dyes, he discovered stainable, thread-like bodies in the cell nucleus, which Heinrich Waldeyer later named 'Chromosomes' **(Figure 1)**. Flemming described the process of the separation of the

threads along their lengths into two identical halves and named this process of the cell division cycle as *mitosis* (from the Greek word for 'separation of threads') and, inspired by Rudoph Virchow, coined the aphorism *omnis nucleus e nucleo* (Flemming 1879; Lukacs 1981; Paweletz 2001). However, Walther Flemming was unaware of the work on heredity developed by the geneticist Gregor Mendel, and it took almost 20 years for scientists to make the bridge between the nature of heritable traits and chromosomes. To this date, the field of cell biology has been extensively explored in several systems, and currently, the study of the several stages of the cell cycle is fascinating scientists every day.

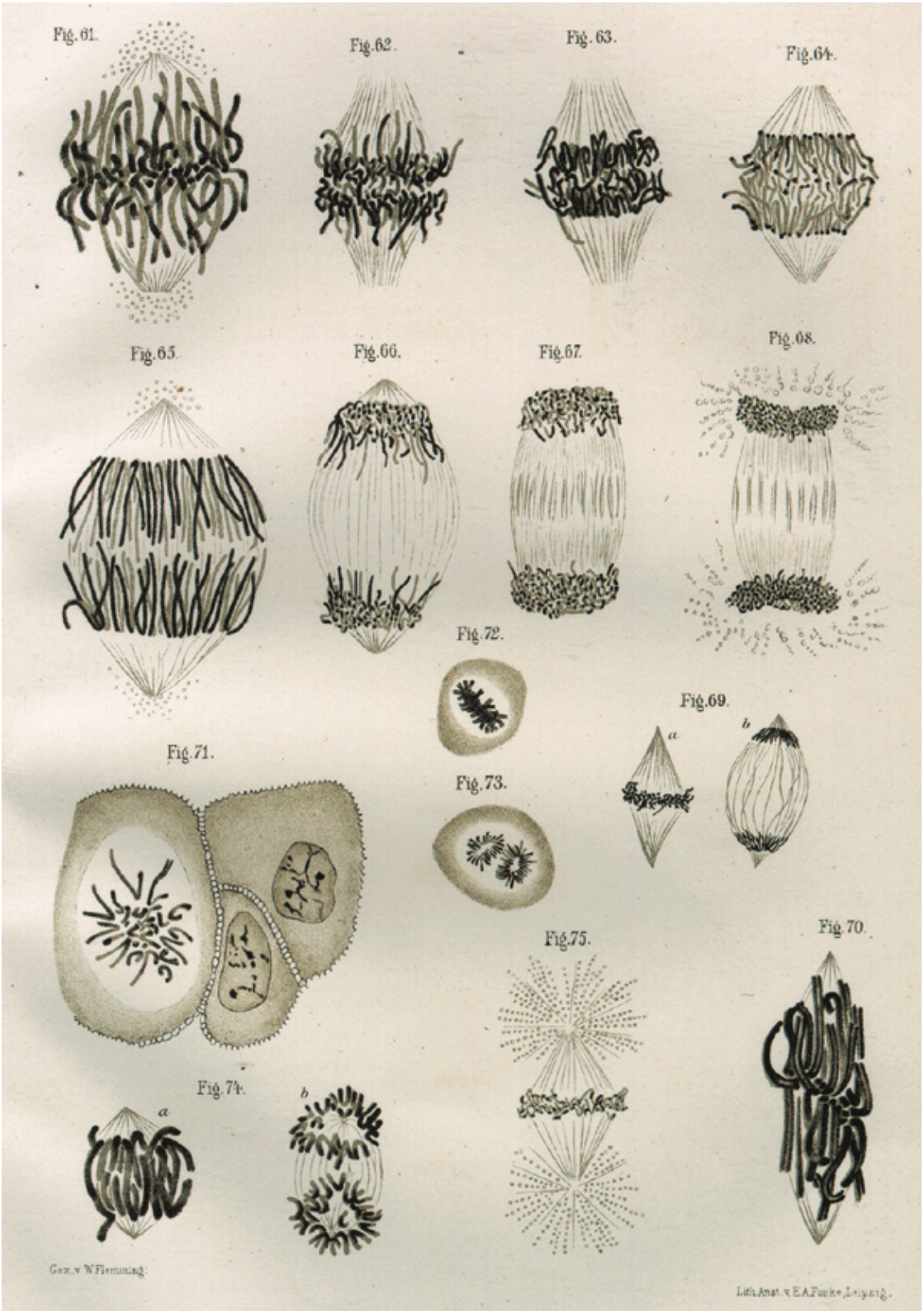


Figure 1 – Description of Mitosis by Walther Flemming. Using innovative microscopy techniques, German anatomist Walther Flemming discovered and explored the fibrous network within the nucleus, which he termed chromatin (stainable material). Flemming noted that during cell division, chromatin was organized into thread-like

bodies, and based on many observations of cells in various stages of division he correctly deduced the sequence of chromosome movements during mitosis. These movements were associated to different stages of mitosis and were confirmed decades later by microscopy of live cells. With high attention to detail, Flemming also noted that chromosomes split along their length during mitosis and correctly hypothesized that the split chromosomes were partitioned into different daughter cells at the end of mitosis (Adapted from Flemming 1879; Paweletz 2001).

2. The Cell Division Cycle

It was only in the 1950s that, based on the fundamental laws of the cell theory, the concept of a regulated 'Cell Cycle' gained strength amongst the scientific community. The cell cycle was proposed to be the process by which two identical daughter cells can be generated from a progenitor cell, thus enabling the faithful segregation of genetic material, one of the basic mechanisms by which multi-cellular organisms grow and survive.

The cell cycle is a highly regulated process that causes a non-reversible change in cell state and it can be sub-divided into several spatial and temporal events. The transitions between cell phases may be controlled by extra- or intra-cellular factors, and are essential for controlling the rates of cell proliferation and differentiation (reviewed in Morgan 2007). The cell cycle is composed of two phases, a short period known as M-phase that is responsible for ensuring the correct segregation of the genetic material and cytoplasm, and a longer period known as interphase that comprises all stages of cell division apart from M-phase **(Figure 2)**. Interphase is composed of three stages, an initial gap phase (G1), a DNA synthesis phase (S-phase) and a second gap phase following DNA replication (G2). During M-phase, two sequential events that correspond to: a) division of the nucleus (Mitosis) and b) division of the cytoplasm (Cytokinesis) take place, a process which results in the generation of two identical daughter cells.

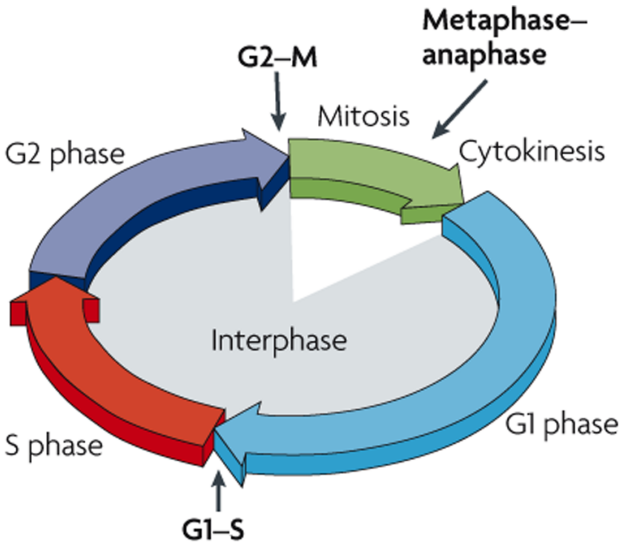


Figure 2 – The Cell Cycle. Cycling cells undergo three major transitions during their cell cycle: the switch of G1 to S-phase, G2 to M-phase and the metaphase-anaphase transition. The beginning of S-phase is marked by the onset of DNA replication. Upon the completion of G2, the start of M- phase is accompanied by nuclear envelope breakdown (NEBD) and chromosome condensation. The third transition involves the segregation of sister chromatids and marks the metaphase-to-anaphase transition. (Adapted from Hochegger *et al.* 2008).

2.1 Interphase

G1, known as gap or growth stage, is the first step of interphase and is essential for determination of cell fate. During G1, cells can choose to enter G0 (a reversible, quiescent state), become senescent (permanently excluded from the cell cycle; non-proliferative state), induce programmed cell death via apoptotic pathways or continue to progress into S-phase allowing the cell to enter the next round of the cell division cycle. For cells that are committed to proliferation, the late G1 events are responsible for preparing the cell for DNA replication (S-phase) and often include cell growth. If any DNA lesions are detected during G1, it is possible for cells to block DNA replication, or if DNA damage is detected during S-Phase, DNA replication can be halted via an essential mechanism termed the ‘DNA Damage Checkpoint’. G1 is followed by S-phase, in which cells faithfully duplicate their DNA to generate chromosomes composed of two identical sister chromatids. The second gap phase (G2) takes place after S-phase and precedes M-phase. Similarly to the G1 or S-phase, if DNA lesions are detected during G2, the DNA damage checkpoint is activated causing a cell cycle block

that prevents mitotic entry. During G2, cells continue to grow and protein synthesis rates increase, thus preparing cells for entry into M-phase (reviewed in Morgan 2007).

2.2 Mitosis

Mitosis is the first step of M-phase and during this period, cells undergo highly regulated and dramatic changes to ensure that chromosome segregation is achieved with high fidelity. The goal of mitosis is to faithfully partition the nucleus so that during cytokinesis, proper cytoplasmic division can then take place, giving rise to two identical daughter cells **(Figure 3)**. Mitosis is normally divided into five stages, Prophase, Prometaphase, Metaphase, Anaphase and Telophase, which occur sequentially in this order (reviewed in Morgan 2007).

Prophase (from the Greek word for 'phase before') is the first step of M-phase and is characterized by the initiation of chromatin compaction into structures that will eventually become the mitotic chromosome. Apart from DNA condensation, during prophase, centrosomes (sites for microtubule nucleation) become mature are separated to opposite sides of the intact nuclear envelope to direct spindle orientation at the following stage of mitosis.

Prometaphase is marked by nuclear envelope breakdown (NEBD) at the same time that full compaction of the chromatin is achieved resulting in the formation of the mitotic chromosomes. Mitotic chromosomes are composed of two identical sister chromatids (duplicated during the previous S-phase) which are held together by the 'Centromere', a DNA element that marks the primary constriction of mitotic chromosomes and is in many cases the binding site for specific proteins involved in kinetochore assembly. The 'Kinetochore' is a complex proteinaceous structure that provides the active interface required for binding microtubules that emanate from the mitotic spindle. NEBD exposes chromosomes and in particular kinetochores to growing microtubules and as microtubule nucleation takes place, the mitotic spindle (composed of highly dynamic microtubules) organizes around the condensed DNA. Chromosomes can then be captured by dynamic microtubules that bind kinetochores and when sister kinetochores are stably attached to microtubules anchored at opposite poles, chromosomes are eventually guided to the spindle equator by a microtubule-driven process.

The successful binding of kinetochores to microtubules nucleated from opposite poles, together with the contribution of microtubule-associated motors, is responsible for powering chromosome motion to the cell equator. Cells are at Metaphase when all chromosomes are stably attached to microtubules and positioned

at the equatorial region of the mitotic spindle. During prometaphase and metaphase, an essential quality control mechanism known as the Spindle Assembly Checkpoint (SAC), is responsible for delaying Anaphase onset until all chromosomes are stably attached to microtubules and located at the spindle equator. This checkpoint is active during mitosis and its silencing is essential for cells to undergo anaphase onset and exit mitosis. Thus, the SAC is a quality control mechanism required to prevent chromosome missegregation during mitosis.

Anaphase can be divided into two successive stages, A and B. Anaphase A is triggered by loss of cohesion between sister chromatids and is accompanied by microtubule shortening thus forcing sister chromatids to segregate to opposite spindle poles. The following step, Anaphase B, is characterized by the movement of centrosomes towards the cell cortex causing the entire spindle to elongate and thus contributing to further separation of the sets of sister chromatids.

The last stage of mitosis is known as Telophase and begins with DNA decondensation, followed by nuclear envelope re-assembly around daughter nuclei. The newly formed nuclear envelopes result from fusion of the remaining fragments of the mother cell nuclear envelope that was disassembled during prophase, and this step completes the process of nuclear division. Cytokinesis completes the final stages of cell division where a contractile actomyosin ring forms at the mid-spindle region between the newly segregated nuclei, and contracts until the cytoplasm is divided into two halves, eventually resulting in the process of abscission that separates the newly formed daughter cells. This step completes M-phase and the daughter cells are now ready to enter G1 for the following round of the cell cycle.

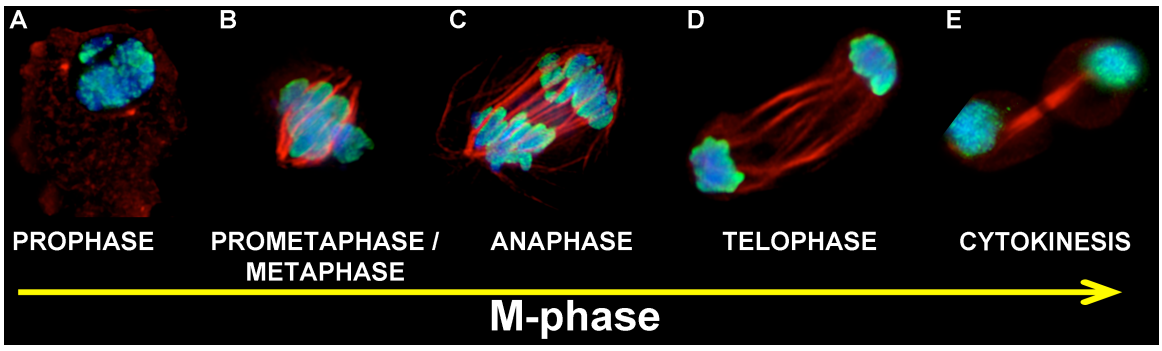


Figure 3 – Representation of M-phase. *Drosophila* S2 cells were fixed and stained to reveal DNA (blue), phospho-histone H3 (green) and mitotic spindle constituent α-tubulin (red). **(A)** Prophase is characterized by centrosome maturation, migration to opposite sides of the nuclear envelope and chromatin condensation. **(B)** Prometaphase begins with the disassembly of the nuclear envelope and the invasion

of dynamic microtubules into the nuclear compartment. At this stage chromosome condensation is completed and spindle microtubules can now interact with chromosomes by binding kinetochores. The interaction between kinetochores and microtubules is essential for inducing chromosome motion and promoting chromosome alignment at the equatorial plane of the cell. When all chromosomes are bi-oriented and aligned at the spindle equator, the cell is said to be in metaphase. **(C)** Anaphase onset is triggered by the removal of cohesion between paired chromatids and movement of sister chromatids to opposite sides of the cell. **(D)** During telophase, chromosomes decondense and the nuclear envelope forms around daughter nuclei. **(E)** Cytokinesis occurs concomitantly with the later mitotic stages and is characterized by the formation of an actomyosin ring that contracts to sever the cytoplasm and complete the process of cell division. The process by which cells achieve the division of both the nuclear material (mitosis) and the cytoplasm (cytokinesis) is generally referred to as M-phase.

2.3 The Mitotic Spindle

The observations documented by Flemming and others suggested that during anaphase, successful chromosome segregation was achieved with the aid of a transient fibrillar structure that begins to assemble around the DNA during prometaphase (Flemming 1965). This fibrous apparatus was shown to be highly dynamic and composed of continuous fibres that are either associated with chromosomes or form astral arrays (Inoue 1953; Inoue 1981). These fibres were later termed Microtubules and they are currently considered the basic constituents of the mitotic spindle.

The microtubule network is one of the structural components of the cell cytoskeleton, and plays important roles in several cell cycle processes that take place during M-phase and interphase. Microtubules are composed of α- and β-tubulin dimers, organized into imperfect helices that polymerize end-to-end to generate protofilaments. Protofilaments maintain a polar orientation since one end has the α-tubulin exposed (minus-end), whilst the other end exposes the β-tubulin subunit (plus-end). This α/β-tubulin arrangement ensures microtubule polarity which is an essential characteristic of dynamic microtubules. Since the microtubule minus-ends are anchored at centrosomes, microtubule growth takes place through the addition of α/β-tubulin dimers at microtubule plus-ends. Nevertheless, what determines whether microtubules grow or shrink is the rate-limiting reaction involving GTP hydrolysis into GDP which is required for the addition of α/β-tubulin dimers. This reaction takes place very fast, and microtubules will typically grow if the incorporation of the GTP-bound subunits occurs faster than GTP hydrolysis. The balance between microtubule polymerization and depolymerization rates causes high microtubule dynamicity, an

essential microtubule property required for several mitotic functions such as microtubule binding to chromosomes.

Microtubules can be classified into three functional groups: a) astral microtubules, b) interpolar microtubules or c) kinetochore-fibres (k-fibres). Astral microtubules are nucleated by centrosomes during early stages of mitosis (at NEBD) and contact the cell cortex to ensure the bipolar spindle is correctly anchored to the cell, thus reducing the probabilities of errors during cytokinesis. Interpolar microtubules extend from the spindle poles to the spindle mid-zone where they form a complex microtubule system that marks the connection between both spindle poles. These microtubules use anti-parallel sliding to maintain bipolar spindle morphology during mitosis. Kinetochore-fibres (k-fibres) are the third class of microtubules and these microtubules grow from kinetochores to make the connection between spindle poles and chromosomes. These microtubules interact with chromosomes at kinetochores, and form distinct microtubule bundles that are required for inducing chromosome motion during mitosis. Together, these three types of microtubules are the main constituents of the mitotic spindle. Although these classes of microtubule are stable, all microtubule types continuously undergo the addition of subunits at the plus end and loss at the minus end, a process that is essential for maintaining microtubule dynamicity.

2.4 Kinetochore-Microtubule attachment

One of the essential tasks performed before mitotic entry, is the disassembly of the interphase microtubule network and the consequent organization of microtubules into a dynamic mitotic spindle capable of powering chromosome movement during mitosis. At early stages of mitosis, microtubule turnover increases substantially and they become highly dynamic so that individual microtubules are constantly either growing or shrinking. This property is essential for spindle microtubules to bind chromosomes mainly through a mechanism known as ‘Search-and-Capture’ (Kirschner and Mitchison 1986) **(Figure 4)**. This mechanism takes into account the dynamic behaviour of microtubules and proposes that microtubules continuously grow and shrink until successful kinetochore binding is achieved. However, in systems lacking centrosomes, other mechanisms that promote microtubule-kinetochore interaction and spindle assembly are thought to play a major role. The model of ‘Spinde Self Organization’ was proposed to involve random microtubule nucleation near chromatin, that together with the activity of microtubule motor proteins, are then sorted into bipolar arrays focused at

spindle poles (Walczak *et al.* 1998). More recently, Maiato and co-workers re-assessed previous data indicating the existence of a chromosome-driven K-fibre assembly pathway that acts independently of centrosomes (Maiato *et al.* 2004b). In this "Combined" system, k-fibres nucleated from chromosomes interact with astral microtubules, resulting in the stabilization of the connection between chromosomes and spindle poles. A fourth pathway of spindle assembly has also been proposed. The 'Search-and-Transport' model, predicts that peripheral microtubules are transported to spindle poles via astral microtubules and are then incorporated into the mitotic spindle structure (Tulu *et al.* 2003). Despite numerous attempts to unravel the specific mechanisms by which cells achieve stable bi-polar attachment, it is most likely that cells are equipped with several redundant mechanisms of spindle assembly. Currently, it is thought that all these different mechanisms act in concert in most cell types to promote successful microtubule-kinetochore interaction and full spindle assembly.

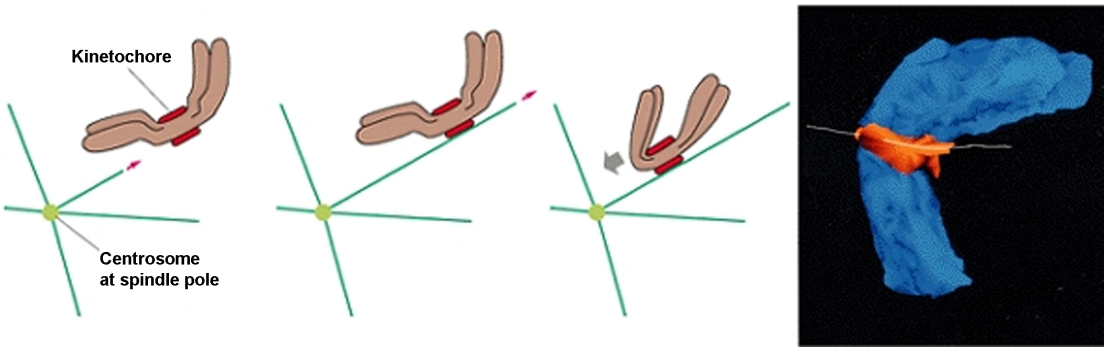


Figure 4 – Kinetochore capture of microtubules nucleated by centrosomes. When NEBD takes place, microtubules nucleated from centrosomes randomly probe the surrounding nuclear space until they interact with kinetochores. Initial capture appears to be by side interaction between kinetochores and microtubules allowing the chromosome to bind and then slide rapidly along it toward the spindle pole. The initial side-on interactions between kinetochores and microtubules are converted into end-on interactions when the chromosome reaches the centrosomes, a region where there is a high density of microtubules plus ends. (Adapted from Rieder and Alexander 1990).

2.5 Chromosome bi-orientation and congression

The interaction between chromosomes and microtubules is essential for positioning chromosomes at the spindle equator, a process commonly referred to as chromosome congression. When the spindle begins to assemble after NEBD, dynamic microtubules eventually bind to unattached kinetochores providing a connection that is

vital for ensuring chromosome congression to the spindle mid-region. However, the detailed mechanisms by which kinetochores and microtubules generate chromosome motion are not yet fully understood.

It is generally assumed that achieving bipolar attachment is a pre-requisite for allowing chromosome congression. However, this is a matter of much debate since chromosomes have also been reported to glide towards the spindle equator alongside kinetochore-fibres that are already attached to other bi-oriented chromosomes (Kapoor *et al.* 2006). This suggests that bi-orientation of a determined chromosome, may be required for generating the forces responsible for powering the movement of surrounding chromosomes.

3. Cell Cycle control

The duplication and division of cellular structures must be tightly controlled to ensure faithful cell division over numerous generations. The fidelity of cell reproduction is dependent on several mechanisms that ensure the accurate and timely transitions from one cell state to another. The cell cycle control system acts as a robust biochemical engine that is programmed to trigger specific cell cycle events in the correct order (reviewed in Murray 1993).

3.1 Cyclin-Dependent Kinases (Cdks)

The Cell Cycle is tightly regulated to certify that all processes are correctly completed before advancing to the following stages of cell cycle, an essential characteristic that ensures a unidirectional path of cell cycle progression. Current models propose that the cell cycle is mainly regulated by the activity of a family of enzymes named Cyclin-Dependent Kinases (Cdks). The role of Cdks in cell cycle control, is to activate/inactivate substrate proteins by catalyzing the attachment of phosphate groups to these proteins, thus causing changes in their enzymatic activities or affecting their interactions with other proteins. The catalytic properties of Cdks are essential for the regulation of cell cycle progression and these properties are governed by the binding of specific Cdk regulatory subunits. These subunits are known as ‘Cyclins’ and directly bind Cdks in order to stimulate their catalytic activity (Evans *et al.* 1983). The identification of these key regulatory units was initially obtained through the

discovery of the Maturation-Promoting Factor (MPF) (Masui and Markert 1971; Smith and Ecker 1971), a complex later described as a heterodimer composed of Cdk1 and Cyclin B (Labbe *et al.* 1988; Labbe *et al.* 1989). Since then, different types of cyclins have been identified and ascribed to different stages of the cell cycle **(Figure 5)**. For example, Cyclin A and B are essential for mitosis whilst Cyclin E and D are mostly required during interphase (reviewed in Murray 2004). The specific expression or activation of these proteins causes a biochemical switch that allows the cell machinery to correctly time cell-cycle processes, thus allowing cells to successfully progress to the following stage of the cell cycle.

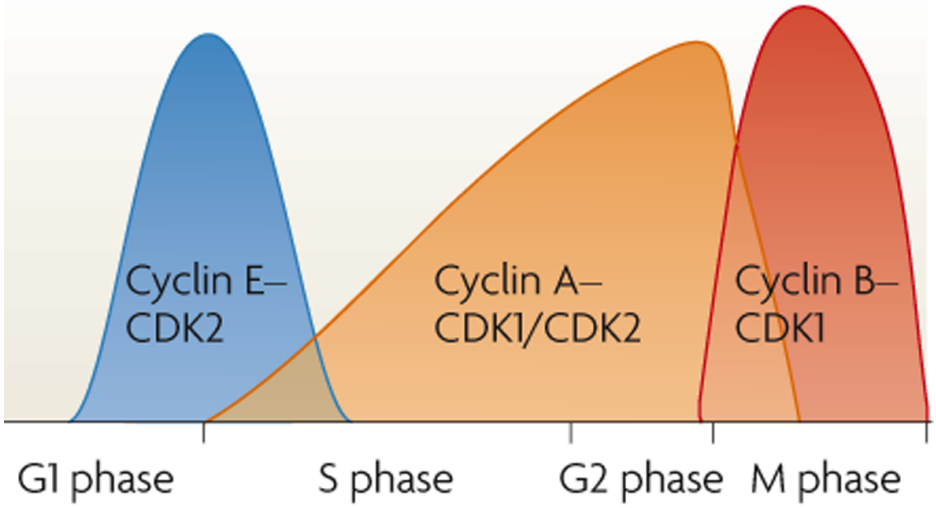


Figure 5 – Cell Cycle regulation by Cdk-Cyclin complexes. According to classical models of cell-cycle control, D-type cyclins and Cdk4 or Cdk6 regulate events in early G1-phase (not shown), Cyclin E–Cdk2 degradation triggers S-phase, Cyclin A–Cdk1/Cdk2 regulate the completion of S-phase, and the levels of Cdk1–Cyclin B are responsible for controlling mitotic exit (Adapted from Hochegger *et al.* 2008).

3.2 Cdk regulation: the role of Cyclins

Progression through G1 already requires activation of specific G1/S-Cdks and only then can the cell progress to reach the 'restriction point' that commits the cell to a new division cycle. Once the cell has progressed through the restriction point the cell is said to be committed to another complete round of proliferation since at this point it will only be able to block the cell cycle after completing mitosis and re-entering G1. From this stage onwards, S-Cdk complexes are activated thus triggering the initiation of DNA

replication. Interestingly, the initiation of this process also triggers S-Cdk inactivation, a mechanism that is required to guarantee that DNA replication is limited to once per cell cycle (reviewed by Diffley 2004).

Once S-phase is completed, mitotic cyclins are expressed to drive cells into mitosis. Mitotic entry is mostly governed by Cdk1, whose activation is dependent on binding to Cyclins A or B, and on the removal of two inhibitory phosphates at the Cdk1 ATP binding site (reviewed in Stark and Taylor 2006). When cells progress from G2 into mitosis, proteins responsible for inactivating M-Cdks are down-regulated, whilst the activity of other specific proteins is increased. One of the proteins up-regulated during mitotic entry is the Cdc25 phosphatase, whose activation causes Cdk1 dephosphorylation at specific inhibitory sites, considered to be an essential step in Cdk1/Cyclin B activation. The activation of the Cdk1/Cyclin B complex is responsible for triggering critical mitotic processes including chromosome condensation, NEBD, spindle assembly and chromosome alignment (reviewed by Morgan 1997). Although cell cycle control is mainly controlled by Cdk1 activity, other mitotic kinases such as Polo kinase, Aurora B, BubR1 and Mps1 are thought to play essential roles in the spatial-temporal order of cell cycle events (reviewed in Nigg 2001). These kinases act at later stages of nuclear division and are involved in the pathways responsible for triggering sister chromatid separation during metaphase. Once all chromosomes form stable, bi-polar attachments and are correctly positioned at the centre of the spindle equator, M-Cdks sensitize the Anaphase Promoting Complex/Cyclosome (APC/C) by phosphorylating core subunits that catalyze the formation of APC/C^{Cdc20} complexes containing Cdc20, a potent APC/C activator. The formation of APC/C^{Cdc20} complexes stimulates the destruction of specific proteins that ensure sister chromatid cohesion and inactivate M-Cdks, thus causing overall spindle disassembly and mitotic exit **(Figure 6)**. However, the formation of APC/C^{Cdc20} complexes has been shown to require APC/C phosphorylation through M-Cdks and therefore M-Cdk inactivation causes disassembly of APC/C^{Cdc20} complexes. Cdc20 dissociation is essential for the completion of M-phase and although APC/C activity is maintained high through Cdh1 binding, the cell is now allowed to progress to the following step of the cell cycle. APC/C^{Cdh1} activity remains high throughout G1 until the cell reaches S-phase, thus committing the cell to another round of division (reviewed in Irniger 2002; Morgan 2007).

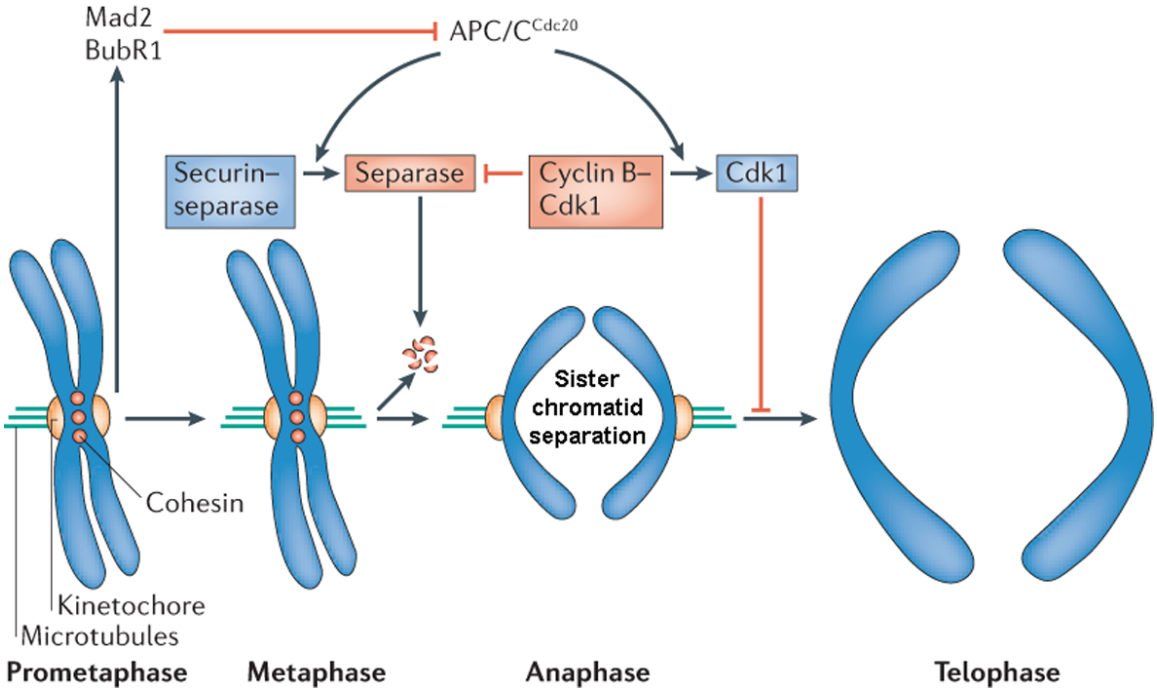


Figure 6 – Inhibition of mitotic exit through formation of APC/C^{Cdc20} complexes. During prometaphase, proteins involved in the spindle assembly checkpoint (such as Mad2 and BubR1) are recruited to kinetochores that are improperly attached to microtubules. Activated Mad2 and BubR1 can block mitotic exit by preventing the APC/C from targeting its substrates (Securin and Cyclin B) for destruction through two independent pathways. In metaphase, when all kinetochores are stably attached to microtubules, APC/C^{Cdc20} ubiquitylates Securin and Cyclin B thereby activating Separase and inactivating the Cyclin-dependent kinase 1 (Cdk1). Free Separase then cleaves cohesin complexes (shown as red circles) that are holding sister chromatids together thus triggering sister-chromatid separation. The second pathway involves the inactivation Cdk1 which leads to the dephosphorylation of Cdk1 substrates by protein phosphatases resulting in mitotic exit. Although these are two independent pathways of mitotic exit, in vertebrates, Cdk1 inactivation also contributes to the activation of Separase (Adapted from Peters 2008).

3.3 Anaphase onset requires APC/C activation through Cdc20 binding

Although cyclins are essential for conducting a timely cell cycle, proteolysis and transcription are two other important regulatory mechanisms that act in parallel with cyclins to control cell cycle progression. Proteolysis is essential for triggering specific, non-reversible events by targeting specific substrates for degradation by the 26S proteasome complex (reviewed in King *et al.* 1996). The proteolytic targeting of specific proteins is performed by ubiquitination, which requires an ubiquitin-activating enzyme (E1), an ubiquitin conjugating enzyme (E2) and an ubiquitin ligase enzyme (E3). This

mechanism relies mainly on the specificity of the E3 ubiquitin ligase enzymes that are directly involved in the destruction of essential cell cycle regulators (such as Securin and Cyclin B). Two essential E3 ubiquitin ligase complexes have been reported to perform essential functions in cell cycle progression: the SCF (Skp1/Cullin/F-box protein) complex and the Anaphase Promoting Complex/Cyclosome (APC/C). APC/C activation requires the binding of specific activator proteins, Cdc20/Fizzy in mitosis or Cdh1/Hct1/Fizzy-related in the G1 phase of the cell cycle, which are both required for correct substrate recognition. In mitosis, Cdc20-dependent APC/C activation is crucial for triggering sister chromatid separation at the metaphase-anaphase transition, while Cdh1/Hct1/Fizzy-related is responsible for maintaining low Cdk activity during G1. Although APC/C activity is essential for certain stages of cell cycle progression, the contribution of the SCF family of proteins appears to be more flexible since different SCF groups fulfill a number of functions at different stages of the cell cycle and inclusively, some groups may also act directly on APC/C (reviewed by Vodermaier 2004).

3.4 Cohesins: holding sister chromatids together

Chromosomes are composed of two identical sister chromatids which are the result of DNA replication. In order to guarantee the correct segregation of the two copies of the genome during cell division, these two identical sister chromatids must be tightly connected so that during mitosis, sister kinetochores are able to bind microtubules from opposite spindle poles. The cohesion between sister chromatids during metaphase is also essential to allow the pulling forces exerted by microtubules to generate tension between sister kinetochores. Only when tension is exerted can the system recognize proper bipolar attachment and allow the cell to exit mitosis. Cohesion is established during DNA replication through deposition of a multi-subunit protein complex that appears to encircle sister chromatids and acts as a molecular glue. This essential complex is termed the Cohesin complex and its localization between sister chromatids is essential for fidelity of chromosome segregation.

The identification of specific cohesin subunits was obtained through two independent screens in *S.cerevisiae,* and although their biological significance was not fully understood at the time, these studies suggested that cohesins play essential roles in proper chromosome segregation (Guacci *et al.* 1997; Michaelis *et al.* 1997). In mitosis, two Structural Maintenance of Chromosomes (SMC) proteins, SMC1 and SMC3 associate with two components of the cohesin complex, Scc1/Rad21 and

Scc3/SA at mitotic centromeres to provide cohesion between sister chromatids (reviewed in Nasmyth and Haering 2005). The timely removal of cohesins from centromeres at the metaphase-anaphase transition was later shown to be dependent on APC/C activity (Ciosk *et al.* 1998) and essential for anaphase onset. Subsequent studies demonstrated that cleavage of the Scc1/Rad21 cohesin subunit is a key event in sister chromatid separation, which is essential for triggering successful anaphase onset (Uhlmann *et al.* 1999; Uhlmann *et al.* 2000). Homologues for budding yeast cohesin subunits have been identified in all eukaryotes studied so far and most reports conclude that disruption of the cohesin complex results in precocious sister chromatid separation (PSCS), suggesting a highly conserved role for cohesins (Losada *et al.* 1998; Bhatt *et al.* 1999; Losada *et al.* 2000; Sumara *et al.* 2000; Sonoda *et al.* 2001; Mito *et al.* 2003; Vass *et al.* 2003). Consistently, the expression of non-cleavable forms of Scc1/Rad21 was shown to prevent or delay sister chromatid separation in different systems, even under active APC/C conditions (Uhlmann *et al.* 1999; Tomonaga *et al.* 2000; McGuinness *et al.* 2005).

The cohesin complex has been proposed to form a ring-like structure that entraps DNA within its coiled-coil arms, thus maintaining sister chromatids together during most periods of the cell cycle (Haering *et al.* 2002; Gruber *et al.* 2003). More recently, a study proposed a two-ring handcuff model for cohesin organization in which ring complexes are paired sideways in a manner analogous to handcuffs **(Figure 7)**, thus providing centromeres with the required flexibility for a controlled metaphase-anaphase transition (Zhang *et al.* 2008). Whether cohesin complex adopts single or double ring structures is still a controversial issue. Importantly, the proteolytic cleavage of Scc1/Rad21 at the metaphase-anaphase transition is a highly regulated APC/C-dependent process that induces the disassembly of the cohesin ring-like structure(s), ultimately resulting in sister chromatid separation. Once the SAC is satisfied in mitosis, the APC/C targets one of its substrates (Securin, the separase inhibitor) for proteasome-mediated destruction thus allowing activated separase to cleave Scc1/Rad21 thereby inducing sister chromatid separation at the metaphase-anaphase transition.

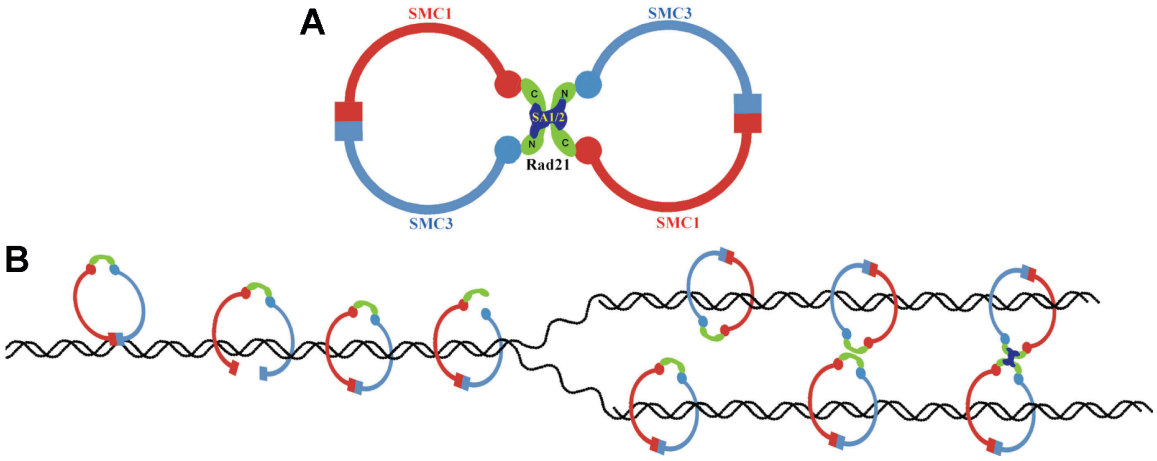


Figure 7 – Handcuff model of cohesin complex. **(A)** Handcuff model consists of two cohesin complex rings. **(B)** Establishment of sister chromatid cohesion: single-ring cohesin ring complexes are loaded onto the chromosomes at any stage of the cell cycle. During S phase, as the DNA is being replicated, each of the rings entraps one chromatid. Cohesion between sister chromatids is only established when the two Rad21 molecules are paired and tethered by SA1 or SA2, presenting a tight handcuff configuration capable of withstanding strong pulling forces (Adapted from Zhang *et al.* 2008).

4. Cell Cycle Checkpoints

The faithful segregation of genetic material is an essential step for successful cell division and therefore, cell cycle progression must be tightly regulated. To ensure the fidelity of all cell cycle processes, cells are equipped with quality control mechanisms, which prevent progression into subsequent stages of the cell cycle if the previous stage was not successfully completed. These mechanisms are referred to as 'checkpoints' and are essential for maintaining genomic stability (reviewed in Hartwell and Weinert 1989).

4.1 DNA damage and DNA replication Checkpoints

At different stages of the cell cycle, cells can prevent or delay entry into subsequent phases using molecular signal transduction pathways that are commonly referred to as checkpoints (reviewed in Hartwell and Weinert 1989). Three checkpoints have been thoroughly documented: a) the DNA damage checkpoint which delays cell cycle progression at G1, S or G2 if DNA lesions are detected (Nakanishi *et al.* 2006;

Niida and Nakanishi 2006), b) the DNA replication checkpoint which ensures that M-phase is not initiated before DNA replication is complete (reviewed in Takeda and Dutta 2005) and c) the Spindle Assembly Checkpoint, or SAC, which acts in mitosis to delay anaphase onset if chromosomes are not correctly attached to the mitotic spindle (reviewed in Musacchio and Salmon 2007).

The DNA damage checkpoint detects DNA lesions (single-strand DNA or double-strand breaks) and arrests cell cycle progression until the DNA is repaired. Double-strand breaks (DSBs) activate a checkpoint pathway mediated by the ATM kinase whereas single-stranded DNA (ssDNA) activates an ATR kinase-dependent checkpoint response. Activation of ATM or ATR may take place at several stages of the cell cycle. For example, if ATM/ATR pathways are activated in G1, the DNA damage checkpoint delays cell cycle progression by blocking Cdk2/Cyclin E which is required for entry into S-phase. If the DNA lesions are detected during G2, Cdk1/Cyclin B is inactivated and mitotic entry is inhibited until DNA repair takes place, thus ensuring that the previous step is completed before the following step begins.

The replication checkpoint is critical for ensuring the fidelity of DNA duplication and acts during S-phase to inhibit cell cycle progression. It is triggered by the malformation of the replication machinery or impaired progression of the replication forks (reviewed in Nyberg *et al.* 2002; Branzei and Foiani 2005). This checkpoint is also mediated by the ATR kinase that is actively recruited to sites where a DNA replication block has occurred and if DNA damage is detected, the ATR kinase may also activate the DNA damage checkpoint. When the formation of replication forks is halted, the DNA damage checkpoint is activated through the exposure of ssDNA. The crosstalk between the replication checkpoint and the DNA damage checkpoint makes it difficult to dissociate between the molecular players involved in both checkpoints and therefore these two signals may be integrated into a single converging pathway termed the S-phase checkpoint.

4.2 The Spindle Assembly Checkpoint (SAC)

The spindle assembly checkpoint, or SAC, is activated upon mitotic entry and is essential for triggering anaphase onset only when all chromosomes are stably attached to microtubules and correctly positioned at the spindle equator. This checkpoint has been amply documented in several systems, and is a pre-requisite for the faithful segregation of the genome (reviewed in Gorbsky 2001; Hoyt 2001; Musacchio and Hardwick 2002; Cleveland *et al.* 2003; Kops *et al.* 2005b; Musacchio and Salmon

2007). The SAC monitors the status of kinetochore-microtubule attachment, so that in the presence of unattached or improperly attached chromosomes, it is responsible for catalyzing the production of a 'wait-anaphase' signal capable of delaying mitotic exit. Since the trigger for anaphase onset is directly dependent on the formation of APC/C^{Cdc20} complexes, it is thought that the SAC operates by promoting the sequestration of the APC/C activator Cdc20, which in turn prevents APC/C activation.

The first evidence that supports the existence of a surveillance mechanism that acts to inhibit anaphase onset was obtained from early experiments with the use of microtubule deploymerizing agents, that causes vertebrate cells to arrest in mitosis for prolonged periods (Brues and Cohen 1936; Brues and Marble 1937). Several years later, the observed mitotic block caused by improperly attached chromosomes was proposed to be SAC-dependent (Nicklas and Arana 1992; Rieder *et al.* 1994; Rieder *et al.* 1995). Although it is now clear that the SAC delays anaphase onset until all chromosomes are properly bi-oriented and under tension, the identification and functional analyses of molecular players that regulate this activity is still under intense investigation.

4.3 Molecular players involved in SAC maintenance

The initial steps towards the identification of key regulators of the SAC were performed using genetic screens in budding yeast aimed at identifying mutations that cause cells to exit mitosis in the presence of spindle damage. Two groups of proteins were identified in these screens and were termed: a) 'mitotic-arrest deficient' (Mad) which include the proteins Mad1, Mad2 and Mad3 (BubR1 homologue in yeast) or b) 'budding uninhibited by benzimidazole' (Bub) which include the proteins Bub1, Bub2 and Bub3 (Hoyt *et al.* 1991; Li and Murray 1991). In a subsequent study, Mps1 (monopolar spindle) was also identified and classified as an active component of the checkpoint pathway (Weiss and Winey 1996). Accordingly, Mad and Bub proteins are highly conserved in both sequence and function, amongst several eukaryotic cell lineages (Li and Benezra 1996; Taylor and McKeon 1997; Bernard *et al.* 1998; Gorbsky *et al.* 1998; Basu *et al.* 1999). The only exception so far, is the identification of Bub-Related-1 kinase, also known as BubR1 in higher eukaryotes, whose N-terminal region displays significant homology with the yeast Mad3 protein and its C-terminus is very similar to the Bub1 kinase domain (Taylor *et al.* 1998). All SAC proteins identified so far have been shown to localize at the outer region of kinetochores that are improperly attached to microtubules (reviewed in Musacchio and Hardwick 2002). The kinetochore

localization of these proteins is thought to be essential for the generation of a diffusible, 'wait-anaphase' signal that is capable of inhibiting mitotic exit (reviewed in Musacchio and Salmon 2007) and accordingly, a number of studies have identified biochemical sub-complexes of SAC proteins that are important for APC/C inhibition during mitosis (Roberts *et al.* 1994; Basu *et al.* 1998; Taylor *et al.* 1998; Hardwick *et al.* 2000; Chung and Chen 2002; Campbell and Hardwick 2003). All identified SAC proteins have been shown to promote APC/C inhibition by preventing Cdc20 binding, however, at the molecular level several studies have suggested that this control is maintained by the fast turnover of Mad2 and/or Mad3/BubR1 at unattached kinetochores (Hwang *et al.* 1998; Kim *et al.* 1998; Wu *et al.* 2000; Howell *et al.* 2004; Shah *et al.* 2004). Nevertheless, it is widely accepted that the SAC promotes APC/C inhibition by regulating the ability of Cdc20 to bind and activate the APC/C, thus controlling the APC/C-mediated poly-ubiquitination and consequent destruction of substrates required for blocking cells in mitosis (reviewed in Musacchio and Salmon 2007).

4.4 The Mitotic Checkpoint Complex (MCC) and the "two-step" model

An elegant study by Rieder and colleagues using Ptk1 cells demonstrated that molecules that localized at or around kinetochores, were responsible for inhibiting mitotic exit in the presence of unattached kinetochores, thus providing evidence to suggest that the SAC signal is kinetochore-dependent during later stages of prometaphase (Rieder *et al.* 1995). However, biochemical studies also identified the Mitotic Checkpoint Complex (MCC), a protein complex composed of Mad2-BubR1-Bub3-Cdc20 and whose formation does not require unattached kinetochores since it is also present in interphase cells (Sudakin *et al.* 2001; Tang *et al.* 2001). The MCC was shown to be a highly potent APC/C inhibitor that acts at early stages of mitosis, before mitotic kinetochores assemble, and taken together with other reports, a "two-step" model of SAC activation and maintenance was proposed (Chan *et al.* 2005; Orr *et al.* 2007). This model proposes that in a first step, SAC maintenance is regulated by the MCC (kinetochore-independent step) so that in a second step, when cells undergo NEBD, the accumulation of SAC proteins at improperly attached kinetochores is sufficient to sustain SAC activity and prolong the mitotic arrest. Reports in yeast and *Drosophila* strongly support the "two-step" model, which takes into account the significance of kinetochores in SAC maintenance, yet argues in favor of an initial kinetochore-independent stage that provides APC/C inhibition when kinetochore assembly is taking place (Fraschini *et al.* 2001; Lopes *et al.* 2005; Orr *et al.* 2007).

Efficient APC/C regulation is achieved through a complex network of pathways, involving positive and negative control through the phosphorylation and binding of specific activator proteins, such as Cdc20 and Cdh1 (reviewed by Peters 2002). In support of the “two-step” model of SAC maintenance, two temporally distinct pathways have been demonstrated to control APC/C activity upon entry into M-phase: a) the binding of Emi1 to Cdc20 during prophase is proposed to be involved in the regulation of mitotic timing (after prophase it is degraded by SCF-mediated proteolysis) and b) the SAC which is responsible for delaying anaphase onset in the absence of stable bi-polar attachment, and is dependent on the binding of Mad and Bub proteins to Cdc20 (reviewed in Sczaniecka and Hardwick 2008).

4.5 Mad2-dependent mechanisms of SAC maintenance

The SAC is required to delay anaphase onset in the presence of improperly attached kinetochores and Mad2 is an essential component of this pathway that plays an active role in blocking mitotic exit (reviewed in Musacchio and Salmon 2007). The SAC is thought to regulate APC/C activity through Mad2- and BubR1-dependent Cdc20 sequestration **(Figure 8)** and although both proteins bind Cdc20 individually, Mad2 and BubR1 have also been shown to act synergistically to promote efficient APC/C inhibition (Fang 2002). Mad2 is recruited to unattached kinetochores during prometaphase and is shed from kinetochores upon microtubule attachment (Waters *et al.* 1998; Logarinho *et al.* 2004). Mad2 recruitment to unattached kinetochores is thought to be Mad1-dependent, and requires the formation of Mad1-Mad2 complex (Chen *et al.* 1998; Chung and Chen 2002). The Mad1-Mad2 complex is assembled in a tetrameric 2:2 configuration, and the formation of this complex has been shown to induce a conformational change in Mad2 that results in an increased affinity for Mad1 and Cdc20 (Sironi *et al.* 2001; Luo *et al.* 2002; Sironi *et al.* 2002; DeAntoni *et al.* 2005). Mad2 adopts the ‘Closed’ conformation (C-Mad2 or N2-Mad2) when bound to Mad1 or Cdc20, or the ‘Open’ conformation (O-Mad2 or N1-Mad2) when unbound to these ligands (Sironi *et al.* 2002; Luo *et al.* 2004; DeAntoni *et al.* 2005). Since Mad1 and Mad2 are respectively stable and cycle at unattached kinetochores (Howell *et al.* 2000; Howell *et al.* 2004; Shah *et al.* 2004), it is thought that the interaction between Mad1 and Mad2 is required for the formation of Mad2-Cdc20 complexes (Kallio *et al.* 2002).

Two models were proposed for the formation of Mad2-Cd20 complexes: a) the ‘exchange’ model, which states that Mad1 is required to chaperone Mad2 to unattached kinetochores, where both Mad2 molecules are released to bind Cdc20 (Ibrahim *et al.*

2008) and b) the 'template' model, which proposes that the Mad1-Mad2 tetrameric complex acts as a template for the formation of single Mad2-Cdc20 complexes. Although the exchange model provides an interesting mechanism for the rapid formation of Mad2-Cdc20 complexes, the 'template' model appears to be more consistent with the current data. In strong support of the 'template' model, an elegant studies using HeLa cells demonstrated that Mad1 bound to C-Mad2 acts as a receptor for O-Mad2, which after being recruited is converted into a C-Mad2-Cdc20 (De Antoni *et al.* 2005; DeAntoni *et al.* 2005). The interaction between Mad1-C-Mad2 and O-Mad2 appears to be essential for catalyzing the production of C-Mad2-Cdc20, and this molecular pathway offers a simple explanation as to how Mad2-dependent SAC signaling may be achieved away from kinetochores.

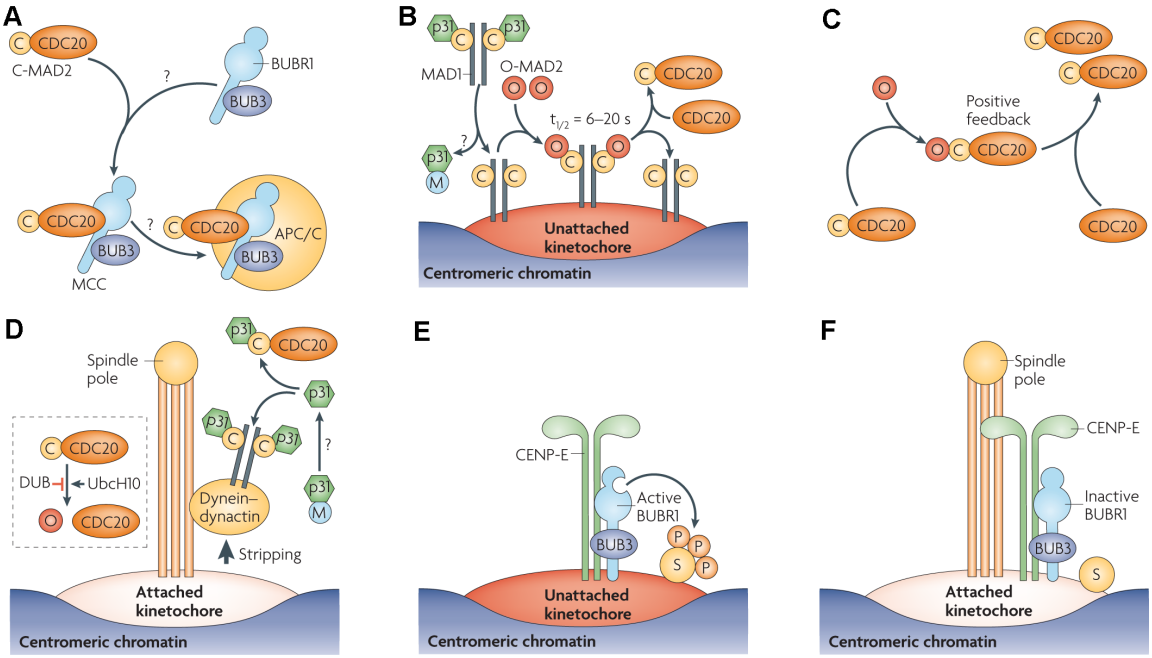


Figure 8 – Models illustrating the role of SAC proteins Mad2 and BubR1 at kinetochores. (A) The mitotic checkpoint complex (MCC) is composed of Mad2, BubR1–Bub3 sub-complexes and Cdc20. When bound to CDC20, Mad2 adopts a C-Mad2 (closed Mad2) conformation. Exactly how C-Mad2–Cdc20 and BubR1–Bub3 create the MCC is currently unclear, however it is thought that a KEN-box motif in BubR1 might regulate MCC binding to the APC/C. **(B)** The Mad2-template model proposes a mechanism for creating C-Mad2–Cdc20 complexes. Unattached kinetochores recruit C-Mad2-Mad1–p31^{comet}, and the consequent release of p31^{comet} allows the Mad1–C-Mad2 (template) to recruit O-Mad2 (open Mad2). O-Mad2 then binds Cdc20 and turns into C-Mad2, creating a structural copy of C-Mad2–Mad1. This model is highly consistent with the results obtained from fluorescence recovery after photobleaching (FRAP) of Mad2 at kinetochores. **(C)** C-Mad2–Cdc20 might be involved in a cytosolic auto-amplification reaction based on the same interaction which could

explain how a single unattached kinetochore is able to halt cell cycle progression. **(D)** At least three regulatory aspects might favor C-Mad2–Cdc20 dissociation along microtubules upon successful attachment. First, absence of unattached kinetochores might result in reactivation of the capacity of $p31^{comet}$ to inhibit the C-Mad2–O-Mad2 interaction thereby inhibiting the catalytic amplification of the SAC signal predicted by the template model. Second, non-degradative ubiquitylation of Cdc20 in a reaction that involves the E2 enzyme UbcH10 and the de-ubiquitylating protein (DUB) might accelerate the dissociation of C-Mad2–Cdc20. Third, the dynein–dynactin complex is responsible for powering the 'stripping' of C-Mad2-Mad1 complex and other proteins from kinetochores upon formation of kinetochore microtubules, thus inhibiting the formation of new C-Mad2–Cdc20 complexes. **(E)** During prometaphase, motor protein CENP-E is responsible for activating BubR1 kinase activity at unattached kinetochores. Although BubR1 substrates are currently unknown, candidate substrates are thought to be proteins involved in the regulation of kinetochore-microtubule attachment. **(F)** Upon stable, bi-polar attachment of microtubules to kinetochores, the kinase activity of BubR1 is switched off (Adapted from Musacchio and Salmon 2007).

4.6 Satisfying the SAC: Microtubule attachment or tension?

Although it is cleat that kinetochores play a pivotal role in SAC maintenance, whether the SAC senses microtubule occupancy or tension across sister kinetochores is still a matter of much debate (reviewed in Pinsky and Biggins 2005; Maresca and Salmon 2010). However, the observed variability between the dynamic localizations of different SAC proteins at kinetochores may provide clues for the understanding of this controversial issue. An elegant study in *Drosophila* cells demonstrated that Mad2 and Bub1 leave the kinetochore once microtubule attachment is achieved whilst Bub3 and BubR1 remain at attached kinetochores until sister kinetochores are under tension (Logarinho *et al.* 2004). However, since Mad1 and Mad2 have been shown to be required for SAC activation in response to lack of microtubule attachment (Shannon *et al.* 2002), the results suggest that the two sensing pathways may ultimately converge into a single mechanism. However, the importance of microtubule tension and attachment is confounded by the fact that they are interdependent. Although the precise kinetochore defects sensed by the SAC remain unclear, the emerging data suggest that several converging pathways are required for cells to exhibit a fully integrated SAC response.

Exactly how cells balance the rates of microtubule polymerization and depolymerization at kinetochores so that proper tension is exerted across sister chromatids is still under debate. However, it is known that low tension induces microtubule polymerization while high tension promotes microtubule polymerization (reviewed in Maiato *et al.* 2004c; Maiato and Sunkel 2004). One of the molecular

players involved in the regulation of this mechanism is Aurora B, a protein kinase that localizes between sister kinetochores during prometaphase (Adams *et al.* 2001). Upon chromosome bi-orientation, sister kinetochores move apart as a result of the high tension generated, thus causing Aurora B to be spatially separated from its kinetochore substrates which are important for stabilizing microtubule attachment (Liu *et al.* 2009; van der Waal and Lens 2010). Furthermore, it was recently proposed that Aurora B specifically phosphorylates distinct targets to differentially regulate kinetochore-microtubule interactions in a tension-dependent manner (Welburn *et al.* 2010). Importantly, the quality of the interaction between kinetochores and microtubules is essential for ensuring the faithful segregation of genetic material.

4.7 The Spindle Matrix

Due to several incompletely understood properties of microtubule dynamics, it has long been proposed that mitotic cells may require an additional structure forming a matrix that would be responsible for ensuring that microtubule associated proteins required for driving chromosome motion, are properly localized in the vicinity of the mitotic spindle (Pickett-Heaps *et al.* 1984). Although matrix-like structures have often been observed in fixed cell samples, there is no direct evidence supporting whether it plays a role in mitosis or even whether such a structure exists in living cells. Nevertheless if present in cells, a spindle matrix would be expected to (a) form a fusiform structure that coalesces with spindle microtubules, (b) persist even in the absence of microtubules, (c) be resilient in response to changes in spindle shape and/or length, and (d) affect spindle assembly and/or function if one or more of its components are perturbed.

In *Drosophila melanogaster*, a complex of at least four nuclear proteins, Skeletor, Megator (Mtor), Chromator, and EAST (Enhanced Adult Sensory Threshold), have been shown to form a putative spindle matrix that persists in the absence of microtubules in fixed preparations (Johansen and Johansen 2007). The interaction between these proteins is essential for the formation of a nuclear endoskeleton and importantly, these proteins are thought to be essential components of a putative spindle matrix during mitosis (Qi *et al.* 2005). However, the biological relevance of such structure has been questioned since is has never been observed in living cells suggesting that the observed matrix-like structure was the product of a fixation artifact.

From the identified four-protein complex, Mtor is the only protein that shows clear sequence conservation in other organisms, ranging from mammals to plants. Identified

Mtor homologues include the nuclear pore complex (NPC) protein translocated promoter region (Tpr) in mammals (Cordes *et al.* 1997; Zimowska *et al.* 1997), its respective counterparts Mlp1 and Mlp2 in yeast (Strambio-de-Castillia *et al.* 1999), and nuclear pore anchor in plants (Xu *et al.* 2007a). NPC proteins, including Mtor/Tpr orthologues in yeast, were shown to functionally interact with SAC components (Iouk *et al.* 2002; Scott *et al.* 2005). This functional interaction between Mtor/Tpr orthologues and SAC proteins suggests that the spindle matrix could provide an essential medium involved in the direct targeting of SAC proteins as well as microtubule-associated proteins during mitosis. However, the concept of a spindle matrix as a spatial determinant of key mitotic regulators that play essential roles in chromosome motion and SAC maintenance is not yet fully accepted amongst cell biologists.

5. Centromeres and Kinetochores

The centromere, initially described as the region on chromosomes where the primary constriction forms, is the site where kinetochore assembly takes place during early stages of mitosis. Apart from harbouring a large number of proteins essential for numerous mitotic tasks, the centromere also ensures sister chromatid cohesion until cells reach the metaphase-anaphase transition. Interestingly, while centromeric DNA is extremely diverse between species and to some extent even between chromosomes of the same species, kinetochore organization is mostly conserved amongst species.

5.1 Centromere-kinetochore interface

Eukaryotic centromeres are highly variable in size and sequence and centromeric DNA does not appear to be conserved either between different species or even between different chromosomes of the same species. Human and *Drosophila* chromosomes contain large regional centromeres as opposed to the point centromeres identified in budding yeast. The identification of the first centromere components came with the use of anti-centromere antibodies (ACA) in sera, which were derived from patients who developed CREST Syndrome (a type of Systematic Sclerosis, displaying 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).

All centromeres studied so far are characterized by the unique presence of CENP-A, a histone H3-variant that binds selectively to centromeric chromatin through a conserved CENP-A targeting domain (CATD) thought to serve as an epigenetic mark involved in the specification and maintenance of centromere identity. In all reported species, CENP-A (CID in *Drosophila*) has been shown to be essential for the recruitment of all other proteins required for kinetochore structure and function and currently, CENP-A is the only conserved centromere-specific protein identified in both *Drosophila* and humans (reviewed in Przewloka and Glover 2009). Above the centromere, at what has been described as the inner kinetochore, *Drosophila* and humans also share CENP-C (Earnshaw and Rothfield 1985; Saitoh *et al.* 1992), a large protein that in humans binds alpha-satellite DNA (Yang *et al.* 1996) by directly interacting with the non-conserved CENP-B protein (Suzuki *et al.* 2004). Both in *Drosophila* and humans, CENP-C has been proposed to play an essential role in kinetochore assembly (Liu *et al.* 2006; Przewloka *et al.* 2007; Erhardt *et al.* 2008) suggesting a conserved role between species.

5.2 Kinetochore structure

The main function of centromeres is to create an interface with centromeric chromatin and to provide a foundation layer for the assembly of mitotic kinetochores. Early studies using the electron microscope revealed the kinetochore as a multilayered structure composed of plate-like sections (Brinkley and Stubblefield 1966; Jokelainen 1967; Rieder 1982). The electron-dense inner plate is approximately 20-40 nm wide and is located on the surface of centromeric heterochromatin directly on CENP-A-bearing nucleosomes, which bind the inner kinetochore constitutively throughout the cell cycle **(Figure 9)**. The outer plate is approximately 35-40 nm and is separated from the inner plate by an electron-translucent middle zone. Most proteins involved in microtubule binding are thought to occupy this region of kinetochores. In the case of unattached kinetochores, the third layer, which is the most distal from centromeres, is composed of mesh of fibres that extend outwards from the surface of the outer kinetochore plate. This layer is termed the fibrous corona and most of the proteins involved in SAC signalling are located in this region (reviewed in Musacchio and Hardwick 2002; Chan *et al.* 2005; Musacchio and Salmon 2007). This mature tri-laminar structure is only acquired after NEBD when kinetochores become fully assembled, providing kinetochores with a conformational change that promotes the rapid monitoring of kinetochore-microtubule attachment and chromosome segregation.

Importantly, upon successful microtubule attachment, many proteins that reside at the fibrous corona dissociate from kinetochores (Maiato *et al.* 2004a) and this fibrous structure is no longer detectable by electron microscopy.

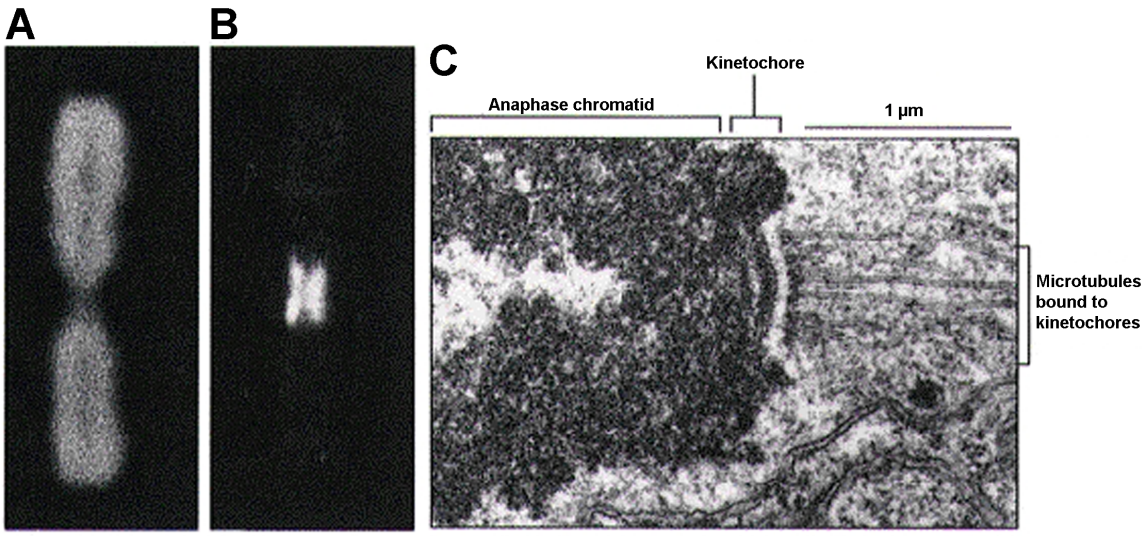


Figure 9 – Kinetochore ultra-structure. (A) A metaphase chromosome stained with a DNA-binding fluorescent dye. **(B)** A metaphase chromosome stained with human auto-antibodies that react with specific kinetochore proteins, displaying two kinetochores, each one associated with one chromatid. **(C)** Electron micrograph of an anaphase chromatid with microtubules attached to its kinetochore. While most kinetochores have been typically described as a trilaminar structure, the kinetochore depicted here (from a green alga) has an unusually complex structure with additional layers (Adapted from Brinkley and Stubblefield 1966; Alberts *et al.* 1994).

Although kinetochores share essential architectural characteristics and organization amongst species, the existence of a physical trilaminar structure is highly controversial. Since different fixation protocols yield variable results, the observation of a plate-like structure at mitotic chromosomes is thought to be an artefact caused by fixation and/or dehydration procedures (McEwen *et al.* 1998). Despite this, recent experiments have mapped the relative positions of proteins along the inter-kinetochore axis of *S.cerevisiae*, *Drosophila* and human kinetochores with nanometer accuracy (Schittenhelm *et al.* 2007; Joglekar *et al.* 2009; Schittenhelm *et al.* 2009; Wan *et al.* 2009; Ribeiro *et al.* 2010). Although the existence of physical kinetochore layers is arguable, these reports provide data arguing in favour of a shared eukaryotic kinetochore design, in which kinetochore organization is mainly governed by hierarchical rules.

5.3 The Constitutive Centromere-Associated Network (CCAN)

Recent molecular studies have identified a number of proteins that constitutively associate with the centromere-kinetochore interface and that are conserved in vertebrates but have not been identified in *Drosophila* (Chan *et al.* 2005; Foltz *et al.* 2006; Izuta *et al.* 2006; Okada *et al.* 2006) *or C.elegans* (Oegema *et al.* 2001; Cheeseman *et al.* 2004). Interestingly, in humans, while most of the constituents exhibit a temporal order of assembly at kinetochores, the emerging data on the Constitutive Centromere-Associated Network (CCAN), does not support a single linear assembly pathway. The multi-protein CCAN complex localizes at the inner kinetochore and is thought to perform essential functions in establishing centromeric organization and ensuring full kinetochore assembly (Hori *et al.* 2008).

CCAN constituents may be sub-divided into different sub-complexes based on their inactivation phenotypes and specific protein-protein interactions. CENP-N has been recently described as the first protein to selectively bind CENP-A nucleosomes through the CENP-A CATD domain (Carroll *et al.* 2009). CENP-N is implicated in the centromere assembly pathway of CENP-A and shown to direct the localization of the CENP-H complex via a direct interaction with CENP-L (Carroll *et al.* 2009). Furthermore, in HeLa cells CENP-N localization at centromeres is interdependent with CENP-T, a component of the CENP-T/W complex (Foltz *et al.* 2006) suggesting that CENP-N plays a central role in the early stages of centromere assembly. Accordingly, human CENP-T has also been shown to interact directly with CENP-A and CENP-B (Orthaus *et al.* 2008) and in chicken DT40 cells, disruption of the CENP-T/W complex causes chromosome missegregation and loss of kinetochore assembly (Hori *et al.* 2008) suggesting that the CENP-T/W complex acts as a platform connecting centromere specification to CCAN assembly. A recent study has identified two novel CCAN proteins that are present in human and DT40 cells, CENP-S and CENP-X (CENP-S complex) that are dependent on the CENP-T/W complex for localization to centromeres and whose depletions lead to several mitotic errors (Amano *et al.* 2009). One other CCAN sub-complex that also localizes to the centromere downstream of the CENP-T/W complex is the CENP-H complex, composed of CENP-H, -I and -K proteins. The centromere localization of the CENP-H complex has been shown to be dependent on CENP-A and CENP-N in HeLa cells (Carroll *et al.* 2009) and dependent on CENP-C and the CENP-T/W complex in DT40 cells (Okada *et al.* 2006). Furthermore, CENP-I inactivation in HeLa cells causes mislocalization of outer

kinetochore components Mad1, Mad2 and CENP-F and cells exhibit transient cell cycle delays in G2 and mitosis (Liu *et al.* 2003). The CENP-O complex is composed of CENP-O, -P, -Q, -R and –U (-50) and in human cells, CENP-O depletion has been shown to cause defects in spindle assembly and mitotic progression (Toso *et al.* 2009). Moreover, CENP-O has been proposed to play a role in generating correct microtubule attachment (McAinsh *et al.* 2006) although a recent study suggests a more direct role in microtubule interaction by demonstrating that CENP-Q, a member of the CENP-O complex, is able to bind microtubules *in vitro* (Amaro *et al.* 2010).

Currently, none of the human CCAN constituents have yet been identified in *Drosophila* (Przewloka and Glover 2009) or *C.elegans* (Oegema *et al.* 2001; Cheeseman *et al.* 2004). Instead, a *Drosophila* genome-wide screen has identified the proteins Cal1 and CENP-C as essential factors for assembly of CID-containing nucleosomes (Erhardt *et al.* 2008). CID, Cal1, and CENP-C co-immunoprecipitate and are mutually dependent for centromere localization and function arguing in favour of a much simpler centromere-kinetochore interface specific to *Drosophila* chromosomes. Since no Cal1 homologues have been identified in human cells, these results suggest that in *Drosophila*, CENP-C, CID and Cal1 may fulfil all essential CCAN functions. Alternatively, *Drosophila* kinetochores might posses other highly divergent proteins that may substitute for CCAN proteins. However, the interdependence between CENP-C and CID for their localization appears to be a feature exclusive to *Drosophila* chromosomes.

5.4 Kinetochore protein composition

Apart from the numerous CENP-proteins (including CCAN) identified in several species, many other proteins involved in microtubule attachment and SAC have been shown to localize to kinetochores of different species during specific stages of mitosis. It is known that the human kinetochore contains more than eighty different proteins that are hierarchically organized into functional classes suggesting that overall kinetochore organization is highly conserved amongst species (Musacchio and Salmon 2007; Cheeseman and Desai 2008; Welburn and Cheeseman 2008; Gascoigne and Cheeseman 2010). Importantly, the kinetochore is a highly complex structure and proteins involved in numerous mitotic tasks, transiently localize at mitotic kinetochores at specific stages of mitotic progression **(Figure 10)**. Current models propose that all proteins that localize to kinetochores can be divided into four functional classes. These proteins can be either involved in a) binding centromeric

chromatin, b) generating kinetochore-microtubule attachment, c) SAC control and maintenance, or d) correcting kinetochore-microtubule attachment (reviewed in Santaguida and Musacchio 2009).

Proteins such as CENP-A and a few CCAN components are responsible for binding centromeric DNA and providing a platform for kinetochore assembly where other proteins involved in several other mitotic tasks may bind. One set of proteins that is directly involved in generating a kinetochore-microtubule interface, is the KNL-1/Mis12/Ndc80 (KMN) network that localizes at the outer kinetochore region during mitosis (Cheeseman *et al.* 2004; Cheeseman *et al.* 2006). Besides directly binding microtubules, the KMN network is thought to form the basis for the loading of a number of other proteins that transiently localize at the fibrous corona during prometaphase and are involved in several other mitotic pathways (Przewloka *et al.* 2007; Schittenhelm *et al.* 2007).

Despite localizing at the fibrous corona, the plus-end directed kinesin-7 motor protein CENP-E is also involved in generating efficient kinetochore-microtubule attachment by ensuring the transport of a mono-oriented chromosome along the spindle fibres (reviewed in Mao *et al.* 2010). Similarly, CENP-F also localizes at the fibrous corona and was shown to interact directly with CENP-E (Chan *et al.* 1998) to promote its recruitment to kinetochores (Yang *et al.* 2005). Another set of proteins that associate with outer kinetochore regions during prometaphase, is a group of Microtubule-Associated Proteins, also known as MAPs. Dynein and CLIP-170 are two types of MAPs that localize at the fibrous corona and have been proposed to play essential roles in chromosome congression (Pfarr *et al.* 1990; Steuer *et al.* 1990; Wordeman *et al.* 1991; Pierre *et al.* 1992; Dujardin *et al.* 1998).

Moreover, the Rod-Zw10-Zwilch (RZZ) complex has also been demonstrated to localize at kinetochores only in the absence of microtubule attachment (Williams *et al.* 1992; Williams and Goldberg 1994) and has been implicated in SAC control, presumably by promoting Mad2 accumulation at unattached kinetochores (Basto *et al.* 2000; Buffin *et al.* 2005). Similarly, the *bona fide* SAC proteins Mad1, Mad2, Bub1, Bub3, BubR1 and Mps1 have all been shown to localize to the fibrous corona or outer kinetochore layers depending on the status of microtubule attachment (Basu *et al.* 1998; Chen *et al.* 1998; Jablonski *et al.* 1998; Taylor *et al.* 1998; Basu *et al.* 1999; Chan *et al.* 1999; Taylor *et al.* 2001; Logarinho *et al.* 2004). Nevertheless, despite a few minor organism-specific differences, kinetochore composition and organization appears to be highly conserved amongst several species (reviewed in Musacchio and Salmon 2007; Gascoigne and Cheeseman 2010).

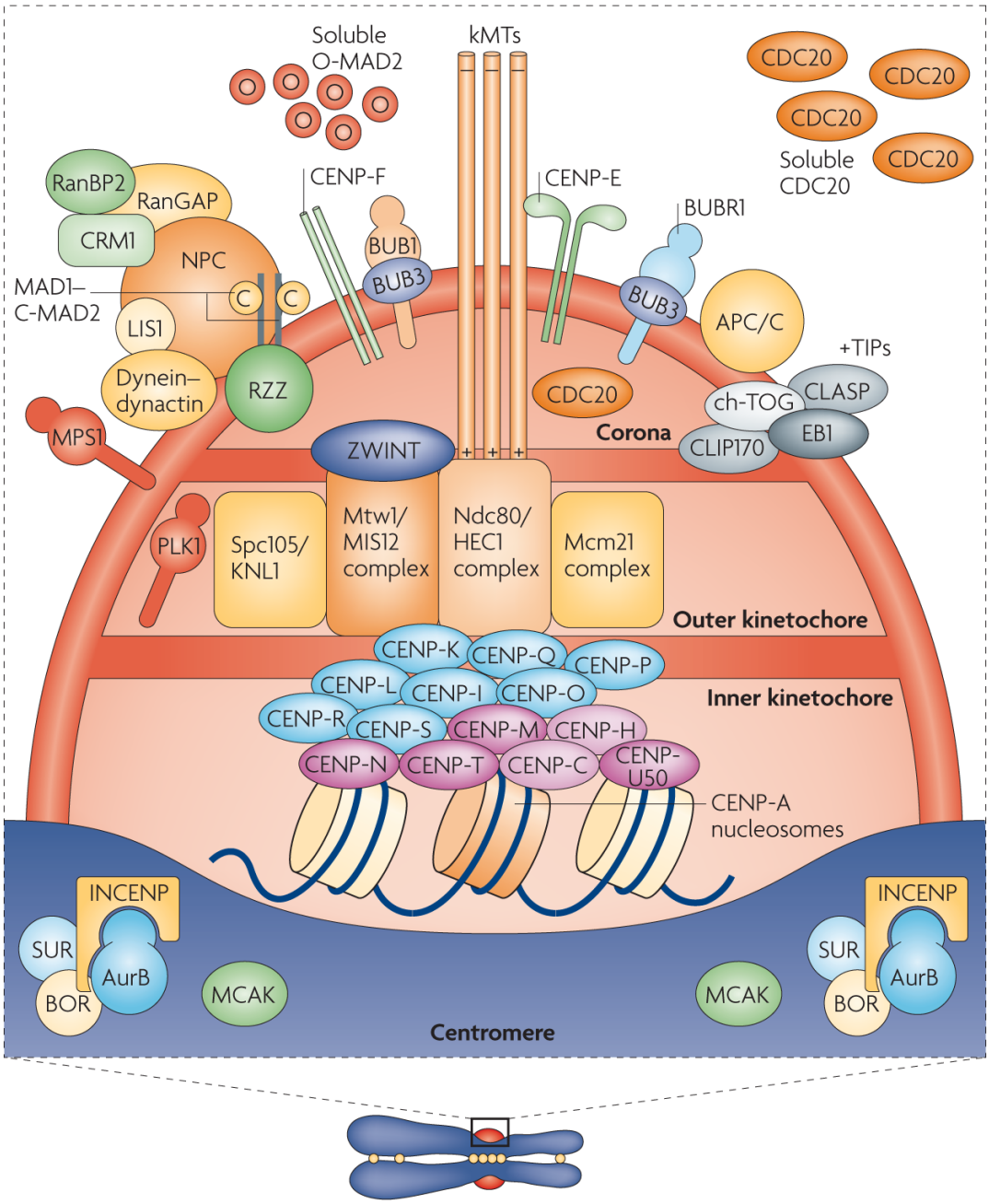


Figure 10 – Conserved centromere-kinetochore organization. Model depicting the organization of proteins recruited to kinetochores during mitosis. At the heart of the kinetochore is a specialized nucleosome that contains centromere protein CENP-A, a histone H3 homologue. Several inner-kinetochore components (cyan and purple ovals) associate with kinetochores constitutively throughout the cell cycle, whilst other proteins, including the Ndc80/Hec1, Mtw1/MIS12, minichromosome maintenance protein-21 (Mcm21) and spindle pole component Spc105/KNL1 proteins, are recruited to the outer kinetochore specifically in mitosis. These proteins not only provide a platform for the recruitment of SAC proteins but also seem to be directly involved in

microtubule binding. Moreover, other proteins such as Borealin, Survivin, Aurora B, inner centromere protein (INCENP) and mitotic centromere-associated kinesin (MCAK) preferentially populate the centromere region and are essential for regulating the stability of microtubule–kinetochore interactions through the correction of microtubule attachment errors. The APC/C is also recruited to mitotic kinetochores in a SAC-dependent manner and the Rod–Zw10–Zwilch (RZZ) complex is required for the kinetochore recruitment of Mad1–Mad2 complexes. However, large cytosolic pools of Mad2 and Cdc20 exist besides the populations that are recruited to the kinetochore and this may also be true for other SAC proteins such as BubR1 and Bub3, and for the APC/C itself. Most proteins represented in this figure are present at kinetochores in all metazoans: CLASP (CLIP-associating protein-1), CLIP170 (cytoplasmic linker protein-170) EB1 (end-binding protein-1), LIS1 (lissencephaly-1); Mps1 (multipolar spindle-1), PLK1 (polo-like kinase-1), RanBP2 (Ran-binding protein-2), RanGAP (Ran-GTPase-activating protein) and Zwint (Zw10 interactor) (Adapted from Musacchio and Salmon 2007).

5.5 Kinetochore-regulated microtubule binding

The interface responsible for the interaction between microtubules and chromosomes involves a conserved super-complex of proteins that localize at kinetochores, known as the KNL1/Mis12/Ndc80 (KMN) network, which is composed of the KNL1 protein (also named Spc105, Spc105R or Blinkin) and the Mis12 and Ndc80 sub-complexes.

Biochemical studies performed in human cells identified two distinct microtubule-binding activities within the KMN network: the first was shown to be associated with the Ndc80/Nuf2 subunits of the Ndc80 complex, and the second with KNL1 (Desai *et al.* 2003; Cheeseman *et al.* 2006). No co-sedimentation with microtubules was detected for the Mis12 complex alone, however, when in complex with KNL1, enhanced microtubule-binding activity was observed. The same behaviour was observed in the absence of the two other subunits of Ndc80 complex, Spc24 and Spc25 (Cheeseman *et al.* 2006). In support of this model, recent studies demonstrated that Aurora B kinase phosphorylates three spatially distinct targets within the KMN network, which are essential for generating different levels of microtubule-binding activity resulting in a tightly regulated mechanism (Welburn *et al.* 2010). Within the KMN network, the Ndc80 complex provides a direct interaction with microtubules (DeLuca *et al.* 2002). In HeLa cells Nuf2 and Ndc80 were shown to be necessary to form stable kinetochore-microtubule attachments (DeLuca *et al.* 2005). Moreover, the N-terminal regions of both proteins contain Calponin-homology (CH) domains that interact with microtubules (Cheeseman *et al.* 2006; Wei *et al.* 2007; Ciferri *et al.* 2008), and this specific microtubule binding appears to involve electrostatic interactions

mediated by the disordered N-terminal tail of Ndc80 (Guimaraes *et al.* 2008; Miller *et al.* 2008).

The Ndc80 complex in *Drosophila* is highly divergent in sequence when compared to other species. Despite this, loss of any Ndc80 constituent in *Drosophila* leads to the formation of elongated mitotic spindles with a scattered distribution of chromosomes and extensive missegregation (Przewloka *et al.* 2007). Due to the similar phenotypes observed after Ndc80 complex depletion in *Drosophila* and humans, it has been proposed that this complex plays a conserved role in kinetochore-microtubule binding in both species.

KNL1 also displays microtubule-binding ability. Interestingly, KNL1 depletion in human cells does not cause phenotypes as severe as those observed in *C.elegans* or *Drosophila* (Cheeseman *et al.* 2004; Przewloka *et al.* 2007), nevertheless, the stability of kinetochore-microtubule binding in KNL1 depleted cells was shown to be affected, as k-fibres in these cells were shown to be sensitive to low temperatures (Kiyomitsu *et al.* 2007). In *Drosophila,* depletion of Spc105R (the KNL1 homologue), causes a kinetochore-null phenotype with chromosomes scattered along the spindle, displaying impaired chromosome congression, alignment and segregation phenotypes (Przewloka *et al.* 2007) which altogether suggests that kinetochore-microtubule interactions are severely affected. One study performed in *Drosophila* embryos hypothesized that the repetitive middle region of Spc105R could contribute to regulated electrostatic interactions with spindle microtubules, similar to the N-terminal tails of Ndc80 (Schittenhelm *et al.* 2009). Although the human Mis12 complex (composed of Dsn1, Nnf1, Nsl1, and Mis12) does not interact with microtubules directly, it acts as a scaffold that bridges the interaction between the Ndc80 and KNL1 sub-complexes that have both been shown to have microtubule-binding ability (Cheeseman *et al.* 2006). In agreement, an elegant study in human cells has identified Nsl1 as a scaffold supporting interactions of the Mis12 complex with Ndc80 and KNL1 complexes (Petrovic *et al.* 2010). Although the Mis12 complex is not fully conserved between *Drosophila* and vertebrates (the former does not appear to contain the Dsn1 subunit), it has been shown that the depletion of different subunits leads to similar phenotypes including defects in chromosome alignment, orientation, and segregation (Goshima *et al.* 2003; Obuse *et al.* 2004; Kline *et al.* 2006). Moreover, human Nnf1 was found to be dispensable for chromosome attachment *per se*, but required for the metaphase alignment of chromosomes and for the correct generation of inter-kinetochore forces (McAinsh *et al.* 2006). Although the KMN network plays a fundamental role in kinetochore-microtubule binding, it is clear that the structure of this network is not fully conserved between humans and *Drosophila*. It is of crucial

importance to study the function of each individual component of KMN in *Drosophila* in order to understand the evolutionary adaptations that may have occurred in the KMN structure.

5.6 Forces involved in generating chromosome motion

Apart from the role of KMN in microtubule binding, there are other mechanisms capable of producing the forces that are necessary for chromosome motion upon microtubule attachment. Poleward force can be generated by microtubule depolymerisation at the chromosome-bound end, a phenomenon termed the 'Pac-Man' mechanism since kinetochores appear to 'chew up' kinetochore-microtubules (Centonze and Borisy 1991; Rieder and Salmon 1998; McIntosh *et al.* 2002). The movement of chromosomes towards the poles also appears to involve 'Microtubule Flux' which consists in a concerted flow of tubulin subunits from the spindle equator to the spindle poles caused by microtubule minus-end depolymerisation and plus-end polymerization while the chromosomes remain attached (Mitchison 1989; Cassimeris 2004; Matos *et al.* 2009). Kinetochore-bound molecular motors also appear to contribute to poleward chromosome motion. However, chromosomes not only move towards the spindle poles, they also congress at the spindle equator. However, polar ejection forces, which are generated by growing polar microtubules that push chromosomes away from spindle poles, have also been proposed to play a role in chromosome movement (Rieder *et al.* 1986; Brouhard and Hunt 2005).

5.7 Kinetochores and the SAC

Apart from regulating microtubule binding and chromosome motion, the kinetochore is also essential for maintaining SAC activity during prometaphase (Rieder *et al.* 1995). The composition of the kinetochore is dynamically regulated during the cell cycle and kinetochore are often considered to be a hub for the accumulation of a number of proteins involved in several cell cycle pathways. The transient localization of specific proteins during different stages of mitosis is thought to be essential for the regulation of mitotic progression **(Figure 11)**. Mad1, Mad2, BubR1, Bub1, Bub3 and Mps1 are the major SAC components involved in the assembly of inhibitory complexes at kinetochores that are not attached or under tension (reviewed in Musacchio and Hardwick 2002; Kops *et al.* 2005b; Musacchio and Salmon 2007; Santaguida and

Musacchio 2009). These inhibitory complexes prevent APC/C activation and block mitotic exit in the presence of unattached or improperly attached kinetochores.

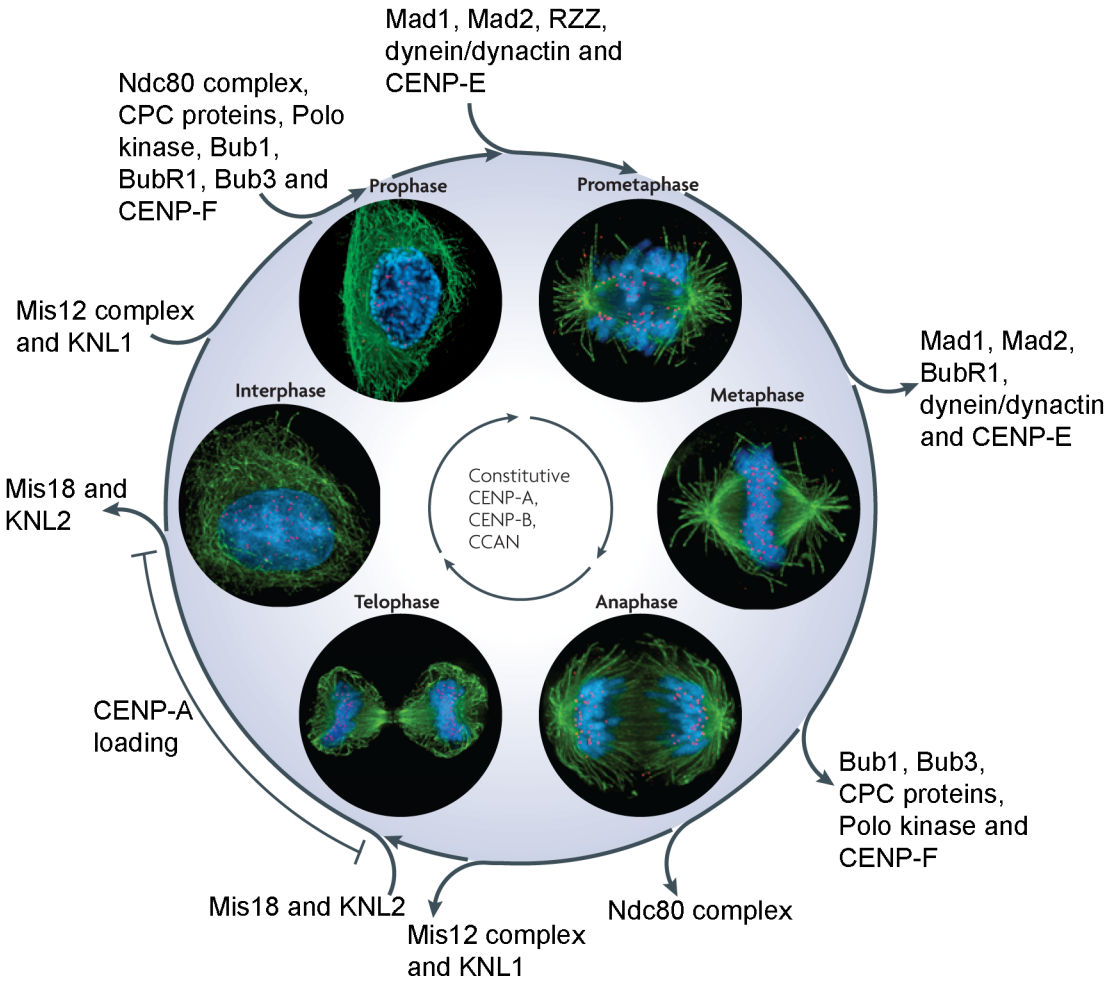


Figure 11 - Kinetochore composition is dynamically regulated during the cell cycle. Immunofluorescence images showing DNA (blue), microtubules (green) and kinetochores (red) throughout the cell cycle in human cells. Arrows on the periphery of the circle indicate at which cell cycle stage the indicated protein(s) are recruited or shed from the kinetochores. Arrows representing delocalization indicate the initial reduction of protein levels, but not necessarily the absolute loss of the components listed. The major themes in kinetochore localization that are highlighted in this figure are: constitutive localization (observed for CENP-A and CCAN); late interphase localization (Mis12 complex and KNL1); prophase accumulation (observed for a number of proteins, including the microtubule-binding Ndc80 complex); localization after nuclear envelope breakdown (observed for a number of proteins, including SAC proteins Mad1 and Mad2 and the motors dynein and CENP-E); delocalization following microtubule attachment (mostly checkpoint pathway components and motor complexes); delocalization at the metaphase–anaphase transition (observed for a number of proteins, including the chromosomal passenger complex (CPC) proteins); delocalization during late anaphase–telophase (observed for the stably bound Ndc80

complex, KNL1 and the Mis12 complex); and localization during late telophase–early G1 (observed for the CENP-A- loading factors Mis18 and KNL2 (also known as M18BP1)(Adapted from Cheeseman and Desai 2008).

Recent data has suggested an emerging role for the human KMN network in SAC signalling. Initial reports demonstrated that depletion of the Ndc80 complex causes mis-localization of Mad1, Mad2 and Mps1 at kinetochores (Martin-Lluesma *et al.* 2002; Stucke *et al.* 2004). However, recent studies have proposed that the mislocalization of Mad1 and Mad2 is a consequence of a mis-regulated accumulation at kinetochores rather than loss of the Mad1 and Mad2 kinetochore-docking site (DeLuca *et al.* 2003). Interestingly, although in both studies Mad1 and Mad2 cannot be detected, cells still appear to arrest in mitosis in a prometaphase-like state, demonstrating that the SAC can still be activated under these conditions. Partial depletion of Hec1 of Nuf2 in HeLa cells may lead to a mitotic arrest while complete depletion appears to abolish SAC activity allowing cells to progress through mitosis with erroneous microtubule-kinetochore attachments (Meraldi *et al.* 2004). Accordingly, *Drosophila* Mitch (Spc25) mutant neuroblasts display a microtubule-dependent SAC response since they exit mitosis when incubated with colchicine, but delay in mitosis in asynchronous cell division (Williams *et al.* 2007). Moreover, in human cells Mps1 was shown to be required for Mad1 and Mad2 localization at kinetochores (Tighe *et al.* 2008) and also found to be required for SAC activity (Stucke *et al.* 2002). Although catalytically inactive Mps1 can restore kinetochore localization of Mad1, only the active kinase restores Mad2 localization suggesting that Mps1 kinase activity may regulate a transient Mad2 kinetochore localization. Thus, in human cells, Mps1 catalytic activity is required for the recruitment of Mad2 to kinetochores and consequent SAC function (Tighe *et al.* 2008). Nevertheless, the requirement of the Ndc80 complex for the kinetochore localization of Mad1, Mad2 and Mps1 in *Drosophila* still remains to be explored, despite current models proposing that the Mad1/Mad2 complex requires the RZZ complex to localize at kinetochores (Buffin *et al.* 2005).

The role of the Mis12 complex in SAC signalling has not yet been clearly addressed. Studies in human cells show that depletion of the four subunits of the Mis12 complex separately arrest cells in mitosis for long periods of time (Kline *et al.* 2006) although, this delay occurs despite a significant reduction in BubR1 levels at kinetochores, suggesting that the SAC may be partly compromised. However, no functional studies involving SAC behaviour and mitotic progression have thus far been reported for *Drosophila* Mis12 complex components.

The human KNL1 has been shown to contribute to the SAC through the interaction with the TPR motifs of Bub1 and BubR1. Bub1 requires both the amino and middle domains of KNL1 to be targeted to kinetochores while BubR1 binds mainly to the amino domain, which suggests that Bub1 may be the first to bind KNL1 and in a subsequent step BubR1 is recruited. The same study also showed that Zwint-1, a member of the RZZ complex (Wang *et al.* 2004a), requires the C-terminal domain of KNL1 to localize to kinetochores (Kiyomitsu *et al.* 2007). Accordingly, KNL1 depletion causes an accelerated mitosis with severe chromosome missegregation and micronuclei formation. The *Drosophila* homologue of KNL1, Spc105R, has been shown to interact with Bub1 in a yeast two-hybrid assay, however the interaction with BubR1 has not been confirmed (Schittenhelm *et al.* 2009). Considering that the *Drosophila* C-terminus of Spc105R interacts with Mis12 complex components and that Bub1 interacts with Nsl1 (a key component of the Mis12 complex) it is possible that the anchoring of BubR1 to the kinetochore may be dependent on the Mis12 complex (Schittenhelm *et al.* 2007; Schittenhelm *et al.* 2009). Taking the current data together, it is clear that the KMN network forms the base for the localization of SAC components and two distinct pathways involved in SAC signalling can be envisioned: one tension-sensitive pathway dependent on KNL1/Spc105R and possibly Mis12, and a second pathway involved in monitoring microtubule occupancy, presumably directed by the Ndc80 complex.

5.8 Human and *Drosophila* kinetochore organization

A comparison of the protein-protein interactions, dependencies **(Table 1)** and phenotypes resulting from the depletions of the human and *Drosophila* centromere-kinetochore interface proteins **(Table 2)**, reveals a number of subtle, yet important differences that support a model in which *Drosophila* chromosomes satisfy a minimal centromere-kinetochore interface. However, essential regulation of microtubule binding and SAC functions are equally conserved despite significant divergence between proteins **(Figure 12)**. Surprisingly, *Drosophila* centromeres do not appear to include the extensive CCAN protein complexes found in vertebrates, rather relaying on the structural role of CENP-C for stabilizing CID, the *Drosophila* CENP-A homologue proposed to serve as a foundation for kinetochore assembly. Although KMN function is conserved from humans to *Drosophila,* at least one subunit of each of the Mis12 and the Ndc80 complexes have not been found in flies whilst the *Drosophila* Spc105R has diverged significantly in comparison with its human counter part. As far as the human

kinetochore is concerned, two signalling pathways are clearly defined: one involving Bub1 and BubR1 through a direct interaction with KNL1 (and possibly Mis12) and a second involving Mad1, Mad2 and Mps1 through an Ndc80-dependent pathway, which together, are responsible for generating the kinetochore-based SAC signal. Due to the scarce data on the specific phenotypes resulting from depletion of individual *Drosophila* KMN components, it is unclear at this point whether the *Drosophila* KMN network shares significant homology with the human KMN, specifically in terms of function and outer kinetochore organization. Accordingly, the cumulative data argue that *Drosophila* chromosomes favour a simpler centromere-kinetochore interface that helps to identify a minimal chromosome segregation machine.

		Protein Name		Dependencies / Interactions	
		Human	*Drosophila*	*Human*	*Drosophila*
CENTROMERE		CENP -A	CID	Associated with CENP-T (Orthaus *et al.* 2008), CENP-B and CENP-N (Carroll *et al.* 2009). Dependent on CENP-N (Carroll *et al.* 2009).	Dependent on CENP-C and Cal1 (Erhardt *et al.* 2008). Independent of all outer KT proteins tested (Przewloka *et al.* 2007).
		CENP -B	n.d.	Interacts with CENP-C (Suzuki *et al.* 2004). Independent of CENP-A (Carroll *et al.* 2009) and CENP-I (Liu *et al.* 2006).	n.a.
		CENP -C	CENP-C	Dependent on CENP-A (Ando *et al.* 2002) and partially dependent on CENP-N (Carroll *et al.* 2009) and CENP-K (Ando *et al.* 2002). Interacts with CENP-B (Suzuki *et al.* 2004).	Dependent on CID and Cal1 (Erhardt *et al.* 2008).
		n.d.	Cal1	n.a	Dependent on CID and CENP-C (Erhardt *et al.* 2008).
CCAN		CENP-H Complex (H/I/K)	n.d.	Dependent on CENP-A (Liu *et al.* 2006) and CENP-N (Carroll *et al.* 2009). Complex components are interdependent (Liu *et al.* 2006). Required for the localization of CENP-F, Mad1 and Mad2 to KTs (Liu *et al.* 2003).	n.a.
		CENP-L	n.d.	Interacts with the C-terminus of CENP-N (Carroll *et al.* 2009).	n.a.
		CENP-N	n.d.	Required for the localization of CENP-A and required for CENP-H complex localization through CENP-L (Carroll *et al.* 2009). Interdependent with CENP-T (Foltz *et al.* 2006).	n.a.
		CENP-O Complex (O/P/Q/R and U(50))	n.d.	Binds MTs in vitro (Amaro *et al.* 2010). Localization of all CENP-O complex proteins is interdependent* (with the exception of CENP-R) (Hori *et al.* 2008).	n.a.
		CENP-S Complex (S/X)	n.d.	CENP-S Complex not required for the localization of any other CCAN proteins (Amano *et al.* 2009).	n.a.
		CENP-T/W Complex	n.d.	Dependent on CENP-A and interdependent with CENP-N (Carroll *et al.* 2009). CENP-T interacts with CENP-A and CENP-B (Orthaus *et al.* 2008).	n.a.
KMN NETWORK		Spc105/ Blinkin/ KNL-1	Spc105R	Required for the localization of Bub1, BubR1 and Zwint at KTs (Kiyomitsu *et al.* 2007). Associates to and targets PP1 to KTs (Liu *et al.* 2010). Required for CENP-F, Zwint and hDsn1 localization (Cheeseman and Desai, 2008)	Dependent on CID and CENP-C (Przewloka *et al.* 2007). Interacts with Nsl1 and Bub1
	Mis12 Complex	Nnf1	Nnf1R (Nnf1R-1/ Nnf1R-2)	Dependent on the other subunits of the Mis12 complex (Kline *et al.* 2006).	Dependent on Spc105R (Przewloka *et al.* 2007).
	Mis12 Complex	Nsl1/ Mis14	Nsl1R	Causes mislocalization of hSgo1 and AuroraB and interacts with hDsn1, Ndc80 and Blinkin (Liu *et al.* 2010; Petrovic *et al.* 2010).	Dependent on Spc105R and Mis12 subunits; independent of the Ndc80 complex; required for Nnf1R-1 localization but not Mis12 (Przewloka *et al.* 2007; Schittenhelm *et al.* 2007).
	Mis12 Complex	Mis12	Mis12	Dependent on CENP-A and CENP-C (Liu *et al.* 2006). Interacts with Zwint-1 and HP1 (Kiyomitsu *et al.* 2010).	Dependent on CID, CENP-C, Spc105R and Nnf1R paralogues; independent of Nsl1R and the Ndc80 complex (Przewloka *et al.* 2007; Schittenhelm *et al.* 2007).
	Mis12 Complex	Dsn1/ Mis13	n.d.	Required for BubR1 and CENP-E localization; Dsn1 depletion affects levels of CENP-A and CENP-H; dependent on other subunits of the Mis12 subunits (Kline *et al.* 2006).	n.a.
	Ndc80 Complex	Spc24	n.d.	Dependent on all Mis12 subunits (Kline *et al.* 2006).	n.a.
	Ndc80 Complex	Spc25	Spc25	Dependent on all Mis12 subunits (Kline *et al.* 2006).	Dependent on the Mis12 complex and Spc105R, but localizes independently of Nuf2 or Ndc80 (Przewloka *et al.* 2007; Schittenhelm *et al.* 2007).
	Ndc80 Complex	Nuf2	Nuf2	Dependent on CENP-I and Hec1 (Liu *et al.* 2010). Dependent on all Mis12 subunits (Kline *et al.* 2006).	Dependent on the Mis12 complex, Spc24, Spc25 and Spc105R (Przewloka *et al.* 2007; Schittenhelm *et al.* 2007).
	Ndc80 Complex	Hec1	Ndc80	Hec1 localization dependent on KNL-1 and CENP-K coordinately (Liu *et al.* 2010). Dependent on CENP-C, CENP-I (Liu *et al.* 2006) and Mis12 (Kline *et al.* 2006).	Dependent on the Mis12 complex, Spc24, Spc25 and Spc105R (Przewloka *et al.* 2007; Schittenhelm *et al.* 2007).

Table 1 – Dependencies/ Interactions. Comparative analysis of protein-protein interactions and localization dependancies between centromere-kinetochore proteins in humans and flies. Asterisks represent data obtained in chicken DT40 cells; n.d. – not determined; n.a. – not applicable; KT – kinetochore; MT – microtubule.

	Protein Name			Phenotypes	
	Human	*Drosophila*		Human	*Drosophila*
CENTROMERE	CENP -A		CID	Effects on KT assembly and chromosome congression/ segregation. Overexpression is not sufficient for KT assembly (Van Hooser *et al.* 2001).	Effects on KT assembly and chromosome congression/ segregation; SAC-dependent mitotic delay; No effect on cell ploidy (Erhardt *et al.* 2008). Overexpression is sufficient for full KT assembly (Heun *et al.* 2006).
	CENP -B		n.d.	Binds to a CENP-B box. Not required for KT assembly as it is absent from functional neocentromeres.	n.a.
	CENP -C		CENP-C	Effects on KT assembly and chromosome congression/ segregation; Strong mitotic delay (Yang *et al.* 1996; Tomkiel *et al.* 2004).	Effects on KT assembly and chromosome congression/ segregation; Strong mitotic delay and aneuploidy (Erhardt *et al.* 2008)
	n.d.		Cal1	n.a.	Strong mitotic delay; no effect on cell ploidy (Erhardt *et al.* 2008)
CCAN	CENP-H Complex (H/I/K)		n.d.	Delay in G2; Transient cytoplasmic Mad2-dependent mitotic delay (Liu *et al.* 2003).	n.a.
	CENP-L		n.d.	n.a.	n.a.
	CENP-N		n.d.	Loss of KT assembly and chromosome congression (Carroll *et al.* 2009).	n.a.
	CENP-O Complex (O/P/Q/R and U(50))		n.d.	Defects in spindle assembly and mitotic progression (Carroll *et al.* 2009). Required for recovery from spindle damage* (Hori *et al.* 2008).	n.a.
	CENP-S Complex (S/X)		n.d.	Effects on KT assembly and chromosome congression/ segregation (Amano *et al.* 2009).	n.a.
	CENP-T/W Complex		n.d.	Effects on KT assembly and chromosome segregation; Strong mitotic delay* (Hori *et al.* 2008).	n.a.
KMN NETWORK	Spc105/ Blinkin/ KNL-1		Spc105R	Effects on KT-MT attachment and chromosome congression/segregation; loss of SAC and accelerated mitotic exit (Kiyomitsu *et al.* 2007). MT-binding ability enhanced by the presence of the other KMN constituents (Cheeseman *et al.* 2006).	Effects on outer KT assembly, KT-MT attachment and chromosome congression/ segregation; Complete block in cell proliferation (Przewloka *et al.* 2007).
	Mis12 Complex	Nnf1	Nnf1R (Nnf1R-1/ Nnf1R-2)	Effects on KT-MT attachment and chromosome congression/ segregation (Goshima *et al.* 2003; Obuse *et al.* 2004). Strong SAC-dependent mitotic delay (Kline *et al.* 2006).	Double depletions affect chromosome congression/ segregation and spindle length regulation (Przewloka *et al.* 2007).
		Nsl1/ Mis14	Nsl1R		Effects on KT-MT attachment, chromosome congression/ segregation and spindle length regulation (Przewloka *et al.* 2007).
		Mis12	Mis12		Effects on KT-MT attachment, chromosome congression/ segregation and spindle length regulation (Przewloka *et al.* 2007).
		Dsn1/ Mis13	n.d.		n.a.
	Ndc80 Complex	Spc24	n.d.	n.d.	n.a.
		Spc25	Spc25	n.d.	Effects on KT-MT attachment and chromosome segregation (Przewloka *et al.* 2007); SAC-dependent mitotic delay (Tighe *et al.* 2008)
		Nuf2	Nuf2	Mad2-dependent mitotic arrest and cell death (DeLuca *et al.* 2003). Loss of KT-MT attachments and outer KT plate (DeLuca *et al.* 2005). Contains a CH domain required for MT-binding (Cheeseman *et al.* 2006; Wei *et al.* 2007; Miller *et al.* 2008).	Effects on KT-MT attachment and chromosome segregation (Przewloka *et al.* 2007).
		Hec1	Ndc80	Mad2-dependent mitotic arrest and cell death (DeLuca *et al.* 2003). N-terminal CH domain required for SAC function and MT-binding (Cheeseman *et al.* 2006; Wei *et al.* 2007; Ciferri *et al.* 2008). Complete depletion causes loss of SAC (Meraldi *et al.* 2004).	Effects on KT-MT attachment and chromosome segregation (Przewloka *et al.* 2007).

Table 2 – Phenotypes. Comparative analysis of phenotypes resulting from the specific disruptions of the centromere-kinetochore interface in humans and flies. Asterisks represent data obtained in chicken DT40 cells; n.d. – not determined; n.a. – not applicable; KT – kinetochore; MT – microtubule.

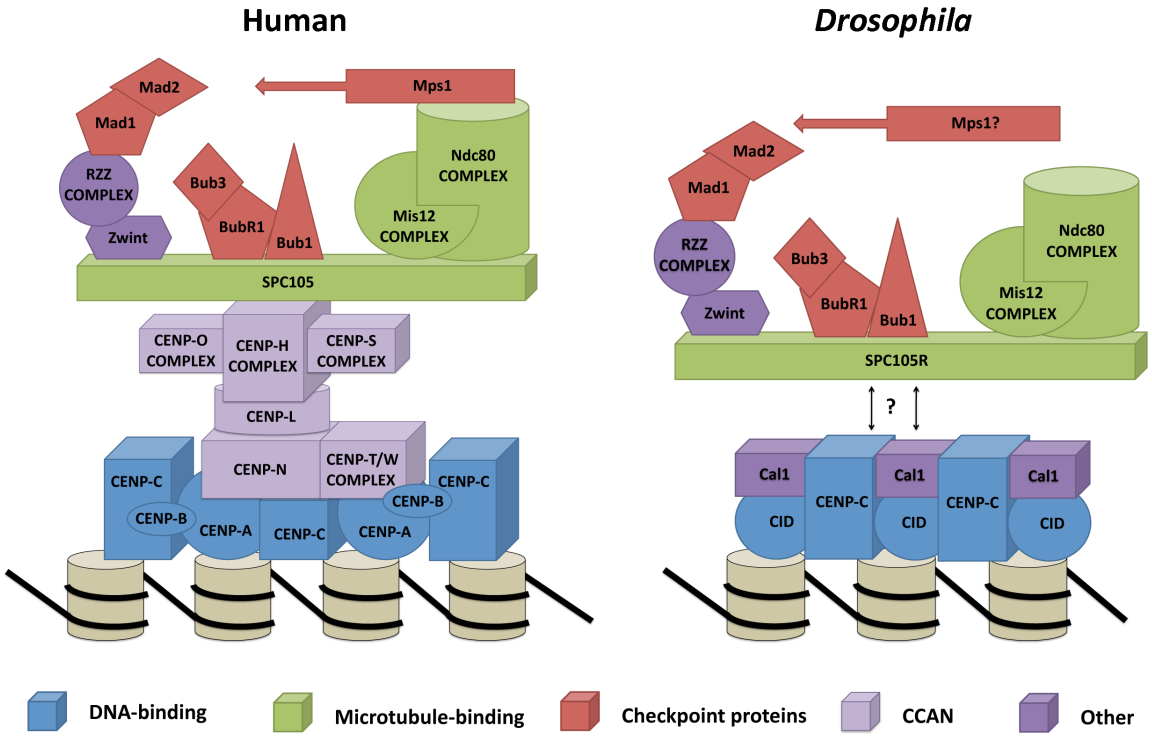


Figure 12 - Schematic model of the Centromere–Kinetochore interface in Human and *Drosophila* chromosomes. Essential regulation of microtubule binding and SAC functions are equally conserved between species, although the centromere–kinetochore structure appears distinct. The major differences are highlighted by the CCAN super-complex which has not yet been identified in *Drosophila*, raising the point as to whether there are other players between centromere proteins (CID, CENP-C and Cal1) and the KMN network or if these components are sufficient to fulfil the role of human CCAN. In both species the KMN network seems to act as a foundation for microtubule binding although in *Drosophila,* SAC functions have not yet been fully explored. While in human cells Mps1 is clearly dependent on Ndc80 for its kinetochore localization, this association has not been confirmed in *Drosophila*.

6. Targeting mitosis as an anti-cancer strategy

The observations of Theodor Boveri in the early 20th century, led to the proposal that chromosome missegregation could underlie the cause for tumour development (Boveri 1914). Since then, the fast-expanding field of cell biology has been largely devoted to the study of the relationship between Chromosomal Instability (CIN) and tumourigenesis. Although intimately related, whether aneuploidy is the cause or consequence of abnormal chromosome segregation is still a matter of intense investigation (reviewed in Holland and Cleveland 2009).

The relationship between the SAC and tumourigenesis has been amply studied in several systems. For example, the analysis of Chinese hamster ovary cell lines exposed to microtubule poisons or Topoisomerase II (Topo II) inhibitors was shown to cause genomic instability due to loss of SAC function (Andreassen *et al.* 1996). Furthermore, the study of oncogenic proteins such as the SV40 large T antigen and the papillomavirus oncoproteins E6 and E7, were shown to promote aneuploidy through SAC inactivation (Chang *et al.* 1997; Thomas and Laimins 1998). Unexpectedly, the *bona fide* SAC components reported so far have been shown to be either up- or down-regulated randomly in several tumour cell lines. Mad1 has been implicated in tumour progression, since it was shown to be a target of the T-cell leukaemia virus oncoprotein Tax suggesting that SAC down-regulation could be important for tumourigenesis (Jin *et al.* 1998). Interestingly, a few mutations have also been detected on the Mad1 gene in tumour-derived cell lines (Nomoto *et al.* 1999; Tsukasaki *et al.* 2001). Moreover, mutations in Bub1 or BubR1 have been reported in several colorectal cancer cell lines (Cahill *et al.* 1998) and also in samples of leukaemia and lymphoma cells (Oshima *et al.* 2000; Ru *et al.* 2002). Reduced levels of Bub1 have also been associated with colon carcinomas and acute myeloid leukaemia (Lin *et al.* 2002; Shichiri *et al.* 2002). Finally, Mad2 has also been associated with a variety of cancers, since altered Mad2 expression levels have been detected in breast cancer cell lines (Percy *et al.* 2000), nasopharyngeal carcinoma (Wang *et al.* 2000), ovarian cancer (Wang *et al.* 2002) and gastric cancers (Kim *et al.* 2005). Although there is a clear association between aneuploidy and the SAC, the analysis of large cancer populations does not allow for a direct correlation between these two events. Reports of cancer-derived cell lines have shown that most cell samples do not contain mutations in SAC proteins and surprisingly most cancer cells have a robust SAC (Tighe *et al.* 2001). This is an apparently contradictory result since we would expect that most tumours would be commonly characterized by specific mutations in SAC genes (Cahill *et al.* 1998).

Further studies have been focused on the analysis of several model organisms with conditional knockouts of essential SAC proteins. Specifically, Mad1, Mad2, Bub1, Bub3 and BubR1 were all shown to cause early embryonic lethality if complete loss of function was induced, but in the case of heterozygous mutations most species were found to be viable and fertile (Michel *et al.* 2001; Babu *et al.* 2003; Wang *et al.* 2004b; Iwanaga *et al.* 2007; Perera *et al.* 2007). In the case of mutations of *Mad2* (Dobles *et al.* 2000) or *Bub3* (Kalitsis *et al.* 2000) in mice, severe missegregation accompanied by apoptosis and early developmental arrest. Similarly, *Drosophila* mutations for BubR1 or Bub3 were demonstrated to cause lethality at late stages of larval development

(Basu *et al.* 1999; Lopes *et al.* 2005). In accordance, complete inactivation of BubR1 or Mad2 in tumour cell lines was shown to cause chromosome missegregation and consequently induce apoptosis within the following cell divisions (Michel *et al.* 2001; Kops *et al.* 2004; Michel *et al.* 2004). However, detailed analysis of *Mad2*-null *Drosophila* mutants suggests that Mad2 is not an essential gene since mutant flies are viable and fertile and cells present no apparent segregation defects (Buffin *et al.* 2007). Importantly, these studies propose that the correct targeting of genes involved in SAC maintenance could be exploited as a potential therapeutic approach designed at specifically killing cancer cells.

However, the cumulative data suggest that it is unlikely that loss of SAC function is the primary cause underlying the mechanisms involved in malignant transformation. Instead, it is more probable that tumourigenesis could be triggered by a weak checkpoint response, rather than by a complete inactivation of the SAC. Accordingly, an elegant study using human tumour cell lines demonstrated that merotelic kinetochore-microtubule attachment (one kinetochore is attached to microtubules from both spindle poles) causes chromosome missegregation in unstable cells, and that increasing merotely levels during successive cell cycles is responsible for generating CIN even in stable cells (Thompson and Compton 2008). Collectively, the cumulative data supports the hypothesis that a weak checkpoint that is unable to sense merotely is responsible for driving cells into aneuploidy and tumour development.

6.1 Chromosomal Instability (CIN) and Aneuploidy

Solid tumours generally exhibit large karyotypes, but how these cells acquire extra chromosomes and faithfully segregate them during several cell divisions is currently unknown. While some tumour cells stably maintain a high chromosome number, other tumour cells display frequent events of loss or gain of whole chromosomes, a process known as CIN (Lengauer *et al.* 1997b; Lengauer *et al.* 1997a; Storchova and Pellman 2004). It is thought that elevated CIN rates are responsible for allowing tumour cells to acquire the characteristics required for proliferation and metastasis (Kuukasjarvi *et al.* 1997). In accordance, it has always been assumed that persistent chromosome missegregation is the major cause of aneuploidy with CIN in tumour cells (Lengauer *et al.* 1998). Despite being closely related, the differences between CIN and aneuploidy become apparent when studying Down's Syndrome (trisomy of chromosome 21), since it is a condition in which there is

widespread aneuploidy but no CIN. Importantly, it is thought that a combination of errors in centromere cohesion, SAC function, dynamics of kinetochore-microtubule attachment and cell cycle regulation are required for promoting CIN in otherwise normal cells (Thompson *et al.* 2010). Accordingly, a recent study demonstrated that chromosome missegregation in stable cells causes a p53-dependent cell cycle delay that prevents CIN. However, deletion of the p53 gene allows these cells to combine CIN with aneuploidy, features resembling those of human tumour cells (Thompson and Compton 2010). Since the direct link between CIN and aneuploidy can only be directly established in a small population of tumour cells, the alternative paths leading to aneuploidy still remain unclear and therefore the study of specific mitotic pathways that promote CIN, is thought to provide essential information that may improve cancer therapy in the future.

7. Objectives

The work presented in this thesis aims to provide further understanding on the mechanisms of SAC maintenance and kinetochore assembly in *Drosophila*. In the first chapter of thesis we address the roles of Mad2 in mitotic progression and provide a functional characterization of Mad2 depletion in *Drosophila* cells. In the second chapter, we dissect the kinetochore-dependent and -independent roles of Mad2 in SAC activation. In Chapter 3, we characterize mitosis in kinetochore-null cells and provide data to support an unsuspected role for CENP-C in determining centromere identity. In the final chapter we address the roles of the spindle matrix in SAC maintenance.

Part II

Experimental Work

Chapter 1

Mad2-independent Spindle Assembly Checkpoint activation and Controlled Metaphase-Anaphase transition in *Drosophila* S2 cells

1. Introduction

The spindle assembly checkpoint, or SAC, is a carefully orchestrated quality control mechanism required to ensure accurate chromosome segregation during cell division. The SAC is responsible for preventing anaphase onset in cells whose chromosomes have not yet reached a stable bipolar attachment. SAC activation/maintenance is thought to be mediated by a signal continuously generated at unattached or improperly attached kinetochores during prometaphase (Rieder *et al.* 1995). Studies in primary spermatocytes demonstrated that not only microtubule occupancy but also tension across kinetochore pairs is required in order to satisfy the SAC (Nicklas *et al.* 1995; Nicklas *et al.* 1998; Pinsky and Biggins 2005). The delayed metaphase-anaphase transition imposed by the SAC is ultimately controlled by the anaphase promoting complex/cyclosome (APC/C), a multi-subunit E3 ubiquitin ligase that targets several mitotic substrates (including mitotic cyclins and securin) for destruction by the 26S proteasome to allow sister chromatid separation and mitotic exit (reviewed by Peters 2002).

Genetic screens in budding yeast originally identified the main components of the SAC molecular machinery. These included, MAD1-3 (Li and Murray 1991) and BUB1-3 (Hoyt *et al.* 1991) and were shown to be required for a mitotic arrest in the presence of spindle damage. These genes have been found to be conserved from yeast to man with the exception of Mad3, which in higher eukaryotes is called Bub1-related kinase (BubR1) since it is highly similar to Bub1 but unlike Mad3 contains a protein kinase domain within the C-terminal half (Jablonski *et al.* 1998; Taylor *et al.* 1998).

Significant progress has been made in unraveling the molecular mechanism by which SAC proteins like Mad2 impose the mitotic arrest in response to inappropriately attached kinetochores. Mad2 was shown to be required for the establishment of a checkpoint-mediated arrest in response to spindle damage in Xenopus egg extracts (Chen *et al.* 1996) and in mammalian cells in culture (Gorbsky *et al.* 1998). Studies in Xenopus and human cells have also shown that Mad2 blocks mitotic exit by sequestering Cdc20, an APC/C activator (reviewed in Musacchio and Hardwick 2002; Bharadwaj and Yu 2004). Mad2 localizes to kinetochores early in mitosis after binding Mad1 (Chung and Chen 2003) where it undergoes rapid turnover (Howell *et al.* 2000; Howell *et al.* 2004; Shah *et al.* 2004). This rapid turn over at kinetochores is thought to underlay the formation of Mad2-Cdc20 inhibitory complexes, which signal abnormal microtubule-kinetochore attachment (Sironi *et al.* 2001; Sironi *et al.* 2002). Extensive

data obtained from fixed preparations in a variety of organisms has supported this model by showing that Mad2 accumulates strongly at kinetochores in the absence of microtubules (Chen *et al.* 1998; Chen *et al.* 1999). However, recent studies in *Drosophila* using a GFP-Mad2 transgene and time-lapse microscopy have suggested an alternative view. The data indicates that even after microtubule kinetochore attachment takes place, a low level of Mad2 continues to enter the kinetochore and is removed mostly along spindle microtubules in a poleward direction (Howell *et al.* 2001; Howell *et al.* 2004; Buffin *et al.* 2005). These results suggest that perhaps the inhibitory signal provided by unattached kinetochores results not from the absence of kinetochore microtubule attachment *per se* but from the inability of Mad2 (and maybe other checkpoint proteins) to exit the kinetochore through microtubules, causing the accumulation of Mad2 at the kinetochore and the consequent formation of complexes that can now freely diffuse throughout the cytoplasm and inhibit the APC/C (see also Buffin *et al.* 2005).

Although the role of kinetochores in the generation of a soluble inhibitory signal that delays metaphase-anaphase transition is consistent with most published data, recent experiments have suggested that cytoplasmic Mad2 is also required for the proper timing of early prometaphase independently of kinetochores (Meraldi *et al.* 2004). Studies in human tissue culture cells show that when Mad2 is depleted in cells with disrupted kinetochores, sister chromatid separation follows very shortly after NEBD. However, if kinetochore-deficient cells now contain cytosolic Mad2, prometaphase is extended significantly, even though these cells still show a defective SAC response (Meraldi *et al.* 2004). These results suggest that Mad2 has a kinetochore-associated function in maintaining SAC activity and a kinetochore-independent function in timing mitotic progression (for discussion see Kops *et al.* 2005b). Mad2 might therefore perform additional, SAC unrelated functions during progression through mitosis. Interestingly, recent studies have also shown that besides their role in maintaining SAC activity, other checkpoint proteins perform additional roles during mitosis progression. Bub3 has been shown to be required for the accumulation of cyclins during G2 and early mitosis (Lopes *et al.* 2005), whilst BubR1 (Lampson and Kapoor 2005) and Bub1 (Meraldi and Sorger 2005) were shown to be required for maintaining proper microtubule-kinetochore interactions and chromosome congression.

Therefore, to gain insight into the primary role of Mad2 during mitosis, *Drosophila* S2 tissue culture cells were treated with double-stranded RNA against Mad2 and mitotic progression analyzed in detail. Consistent with previous studies in other organisms, we find that depletion of Mad2 causes loss of the SAC response in *Drosophila* S2 cells. Moreover, Mad2-depleted cells fail to reach metaphase, exit

mitosis very soon after NEBD and show highly abnormal chromosome segregation that is characterized by the formation of extensive chromatin bridges and severe aneuploidy. However, our results indicate that Mad2 is unlikely to have any specific role in either chromosome condensation or in establishing kinetochore-microtubule interactions since a checkpoint-independent arrest in mitosis allows normal chromosome condensation and congression. Also, release from the mitotic arrest allows cells to exit mitosis without chromatin bridges and with chromatid segregation profiles that are indistinguishable from controls. More significantly, if Mad2-depleted cells are released from the mitotic arrest into media containing the microtubule deploymerizing agent colchicine, cells arrest in mitosis with intact sister chromatid cohesion and strong kinetochore accumulation of other SAC proteins suggesting an active SAC response. Taken together our results suggest that Mad2 is a mitotic timer that delays mitotic progression during early stages of prometaphase so that the SAC can be maintained and chromosome segregation can be properly conducted.

2. Results

2.1 Depletion of Mad2 by RNAi in S2 *Drosophila* cells

To study the role of the *Drosophila* Mad2 putative homologue, the protein was depleted from S2 cells by dsRNAi. Addition of dsRNA against Mad2 caused protein levels to drop by more than 92% by 72 hours and was virtually absent by 96 hours (Figure 1.1A). Immunolocalization studies with anti-Mad2 antibodies (Figure 1.1B), confirms these observations and quantification of levels on control and Mad2-depleted cells after colchicine incubation (see also Figure 1.8C) further demonstrates that the RNAi treatment effectively depletes the protein. Given that Mad2 is significantly depleted after 72 hours of treatment, all our studies were carried out using this period of dsRNA incubation. Previous studies have shown that S2 *Drosophila* tissue culture cells are able to show a functional SAC response (Logarinho *et al.* 2004). Therefore, in order to determine whether the SAC is functional in the absence of the putative Mad2 homologue, dsRNA-treated cells were incubated for 2 hours with colchicine and the mitotic index determined. Spindle damage causes control cells to arrest at a prometaphase-like state while Mad2-depleted cells fail to arrest in the presence of colchicine (Figure 1.1D). Consistent with these results, analysis of asynchronous prometaphase figures indicate that Mad2-depleted cells show a significant proportion of premature sister chromatid separation (PSCS) as determined by the loss of the cohesin subunit DRad21 (Figure 1.1E and F). These results indicate that in *Drosophila* S2 cells, Mad2 has a conserved role that is essential to maintain normal SAC activity.

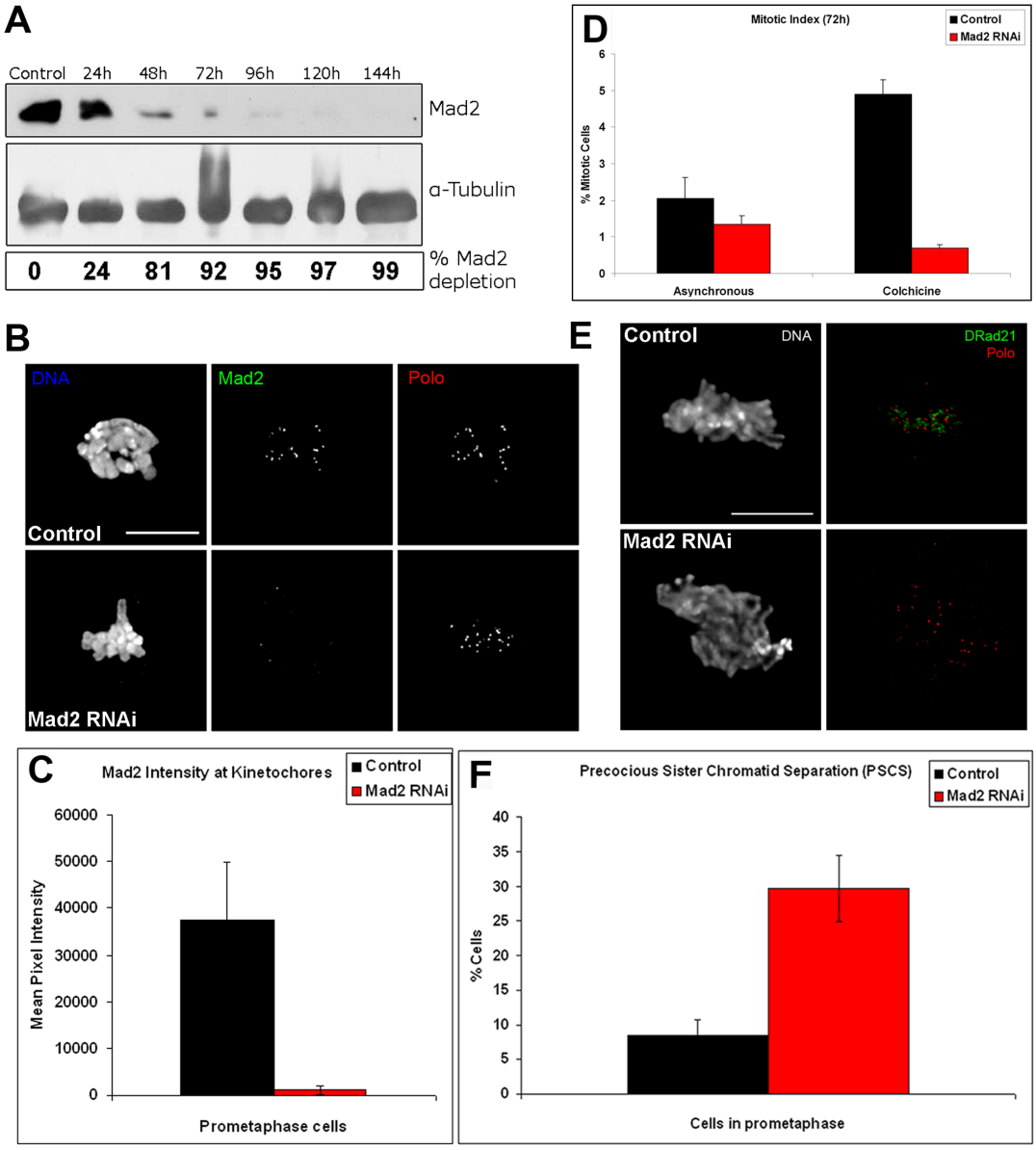


Figure 1.1 - Depletion of Mad2 by RNAi in S2 cells (A) Western blot analysis shows Mad2 depletion at different times after addition of Mad2 dsRNA. Below α-tubulin was used as a loading control. At 72h Mad2 depletion is 92%. **(B)** Immunolocalization of Mad2 in control and dsRNAi treated cells at 72h shows DNA (blue), Mad2 (green) and Polo (red). After 72h RNAi treatment cells do not contain Mad2 at their kinetochores while in control cells Mad2 staining is clearly visible. **(C)** Quantification of Mad2 levels in control and RNAi-treated prometaphase cells. At least 15 cells were analyzed in each case. **(D)** Control and RNAi-treated cells were incubated with 30μM Colchicine (2hr) and the mitotic index recorded. Cells lacking Mad2 fail to accumulate in mitosis. **(E)** These cells were also immunostained to reveal the cohesin subunit DRad21 to determine whether they exit mitosis prematurely. Note that there is a significant increase in the frequency of DRad21 negative cells after colchicine incubation (mitotic population only). **(F)** Immunolocalization of DRad21 in control and Mad2 RNAi-treated cells at 72 h followed by colchicine incubation revealed a threefold increase in PSCS. DNA (blue), DRad21 (green), and Polo (red) are shown. Note that after 72 h RNAi treatment most prometaphase cells do not contain centromere-associated DRad21, whereas in control cells the staining is clearly visible. In all images bar is 5 μm.

Previous studies have suggested that checkpoint proteins assemble at kinetochores in a well-defined order in which localization of Bub1 is essential for Mad2 binding (Johnson *et al.* 2004). However, this study analyzed cells that were actively progressing through mitosis without a functional SAC and was therefore confined to look only at early prometaphase. In order to determine whether Mad2 has indeed any role in the kinetochore localization of other checkpoint proteins, cells have to be prevented from exiting mitosis in a checkpoint-independent manner and microtubules depolymerized. Specifically, control and Mad2-depleted cells were prevented from exiting mitosis by incubation with the proteasome inhibitor MG132 (Genschik *et al.* 1998; Oliveira *et al.* 2005) and then treated with colchicine. Subsequently, control and Mad2-depleted cells were immunostained with antibodies against BubR1, Bub1 or Bub3 (Figure 1.2A-D). The results show that Mad2 is not required for the kinetochore localization of any of the checkpoint proteins tested.

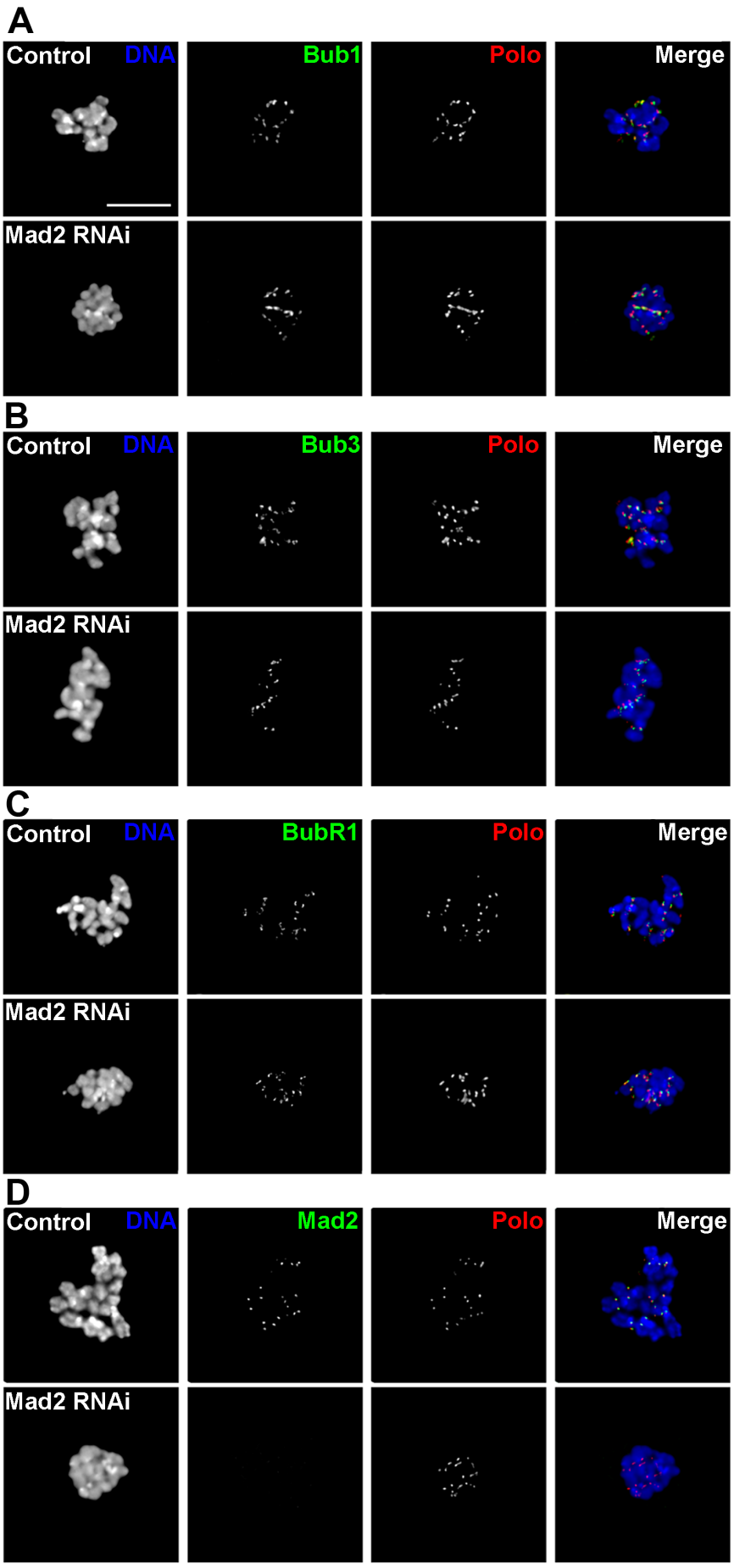
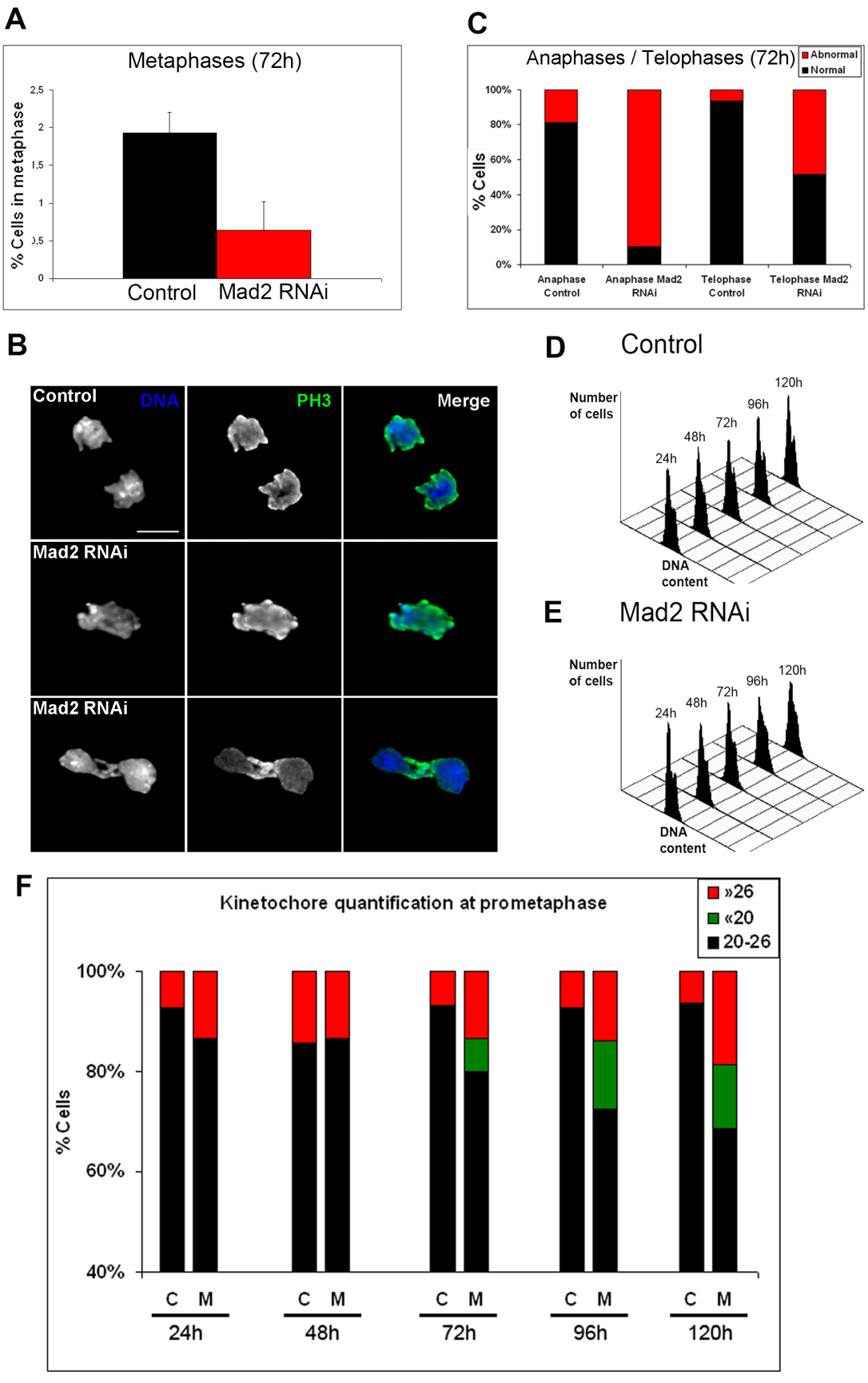


Figure 1.2 - Kinetochore accumulation of SAC proteins in the absence of Mad2.
In all experiments cells were treated with RNAi against Mad2 for 72 hours. Control and RNAi treated cells were then incubated with the proteasome inhibitor MG132 (20 μM) for 2 hours to prevent mitotic exit and then with Colchicine (30μM) during 45 min, fixed and stained to reveal DNA (blue) and **(A)** Bub1, **(B)** Bub3 **(C)** BubR1 or **(D)** Mad2 shown in green. Polo (used as a kinetochore marker) is shown in red. Bar is 5 μm. The results show that Mad2 is not required for the kinetochore localization of any of the checkpoint proteins tested.

2.2 Mitotic progression after depletion of Mad2

Although Mad2 has been extensively studied in a number of organisms, previous reports have failed to provide a comprehensive description of mitotic progression in the absence of Mad2. Accordingly, we depleted Mad2 from S2 cells and carried out a full phenotypic analysis. Mitotic cells were identified as being anti-phospho histone H3 (PH3) positive and immunolocalization of Polo was used to define successive mitotic stages (Llamazares *et al.* 1991). Overall quantification indicates that Mad2-depleted cells progress through early stages of mitosis normally and as expected there is a decrease in the number of prometaphases (data not shown). More significantly, we find that Mad2-depleted cells show a strong decrease in the frequency of metaphases (Figure 1.3A). Also, these cells exit mitosis with highly abnormal anaphases/telophases containing extensive PH3-positive chromatin bridges that remain until very late stages of cell division (Figure 1.3B). Quantitative analysis shows that most Mad2-depleted cells contain chromatin bridges during anaphase and approximately 50% of the cells are unable to resolve these bridges since they are still present during telophase (Figure 1.3C). Analysis of DNA content by FACS shows that depletion of Mad2 also results in severe aneuploidy (Figure 1.3D and E). DNA content profiles indicate that while control cultures show clearly defined 2N and 4N peaks throughout the experiment, from 72 hours onwards, cultures treated with dsRNA against Mad2 show a highly abnormal FACS profile and a clear separation between the 2N and 4N peaks is no longer observed, suggesting an increase in aneuploidy along time. To confirm this, we quantified the chromosome missegregation phenotype observed by FACS analysis, control and Mad2-depleted cells at different times were collected fixed and immunostained to reveal the number of kinetochores at prometaphase (Figure 1.3F). Our results show that at the start of the experiment most cells contain 20-26 kinetochores consistent with the expected average number of chromosomes in these cells (12 chromosomes). However, as the RNAi treatment progresses, we find a significant proportion of Mad2-depleted cells containing either more or less than 20-26 kinetochores.


A
Metaphases (72h)
% Cells in metaphase
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2
1,5
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60%
40%
20%
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Figure 1.3 - Mitotic progression after depletion of Mad2. (A-C) Cells were treated with dsRNAi for 72h before fixation and immunostaining to reveal DNA (blue), phospho-histone H3 (green) and Polo (red) and used for quantification. **(A)** Quantification of mitotic progression revealed that cells lacking Mad2 show a significant reduction in the frequency of metaphases amongst mitotic cells. **(B)** During anaphase and telophase cells show extensive PH3-positive chromatin bridges. Bar is 5 μm. **(C)** Quantification shows that after depletion of Mad2 most cells show chromatin bridges during anaphase or telophase. Analysis of DNA content in **(D)** control or **(E)** Mad2-depleted cells at different times. Note that after 72 hours Mad2-depleted cells fail to show a well defined 4N peak which becomes very broad indicating extensive aneuploidy. **(F)** Kinetochore quantification of cells at prometaphase. Numbers in the range of 20-26 kinetochores per cell was considered to be normal. C= control; M= Mad2 RNAi. Note the progressive shift in ploidy along time.

Taken together, these results suggest that cells lacking Mad2 that progress through mitosis are unable to organize proper metaphase plates, undergo PSCS and segregate their chromatids with extensive chromatin bridges that persist up to telophase resulting in severe aneuploidy. One possible hypothesis to explain these observations is that Mad2-depleted cells simply transit through prometaphase rapidly and exit mitosis prematurely. Previous results on the role of Mad2 in human tissue culture cells have shown that in its absence cells show a highly accelerated transit through mitosis characterized by a severe reduction in the time between NEBD and anaphase onset (Meraldi *et al.* 2004). Therefore, we determined whether loss of Mad2 alters the timing of mitotic progression in S2 cells (Figure 1.4 and Supplementary Movies 1 and 2). To address this directly we analyzed mitotic progression in Mad2-depleted cells by in vivo time-lapse microscopy using a S2 cell line stably expressing GFP-tubulin (Rodgers 2002) as previously described (Lopes *et al.* 2005). The results show very clearly that in the absence of Mad2 the time from NEBD to anaphase onset is significantly shortened (11 ± 2 min) when compared to control cells (33 ± 8 min). These results firstly indicate that in S2 cells, mitotic exit can only take place 11 min after NEBD independently of checkpoint activity, and secondly, that during normal progression through mitosis Mad2 allows cells to extend the length of prometaphase/metaphase up to 3-fold.

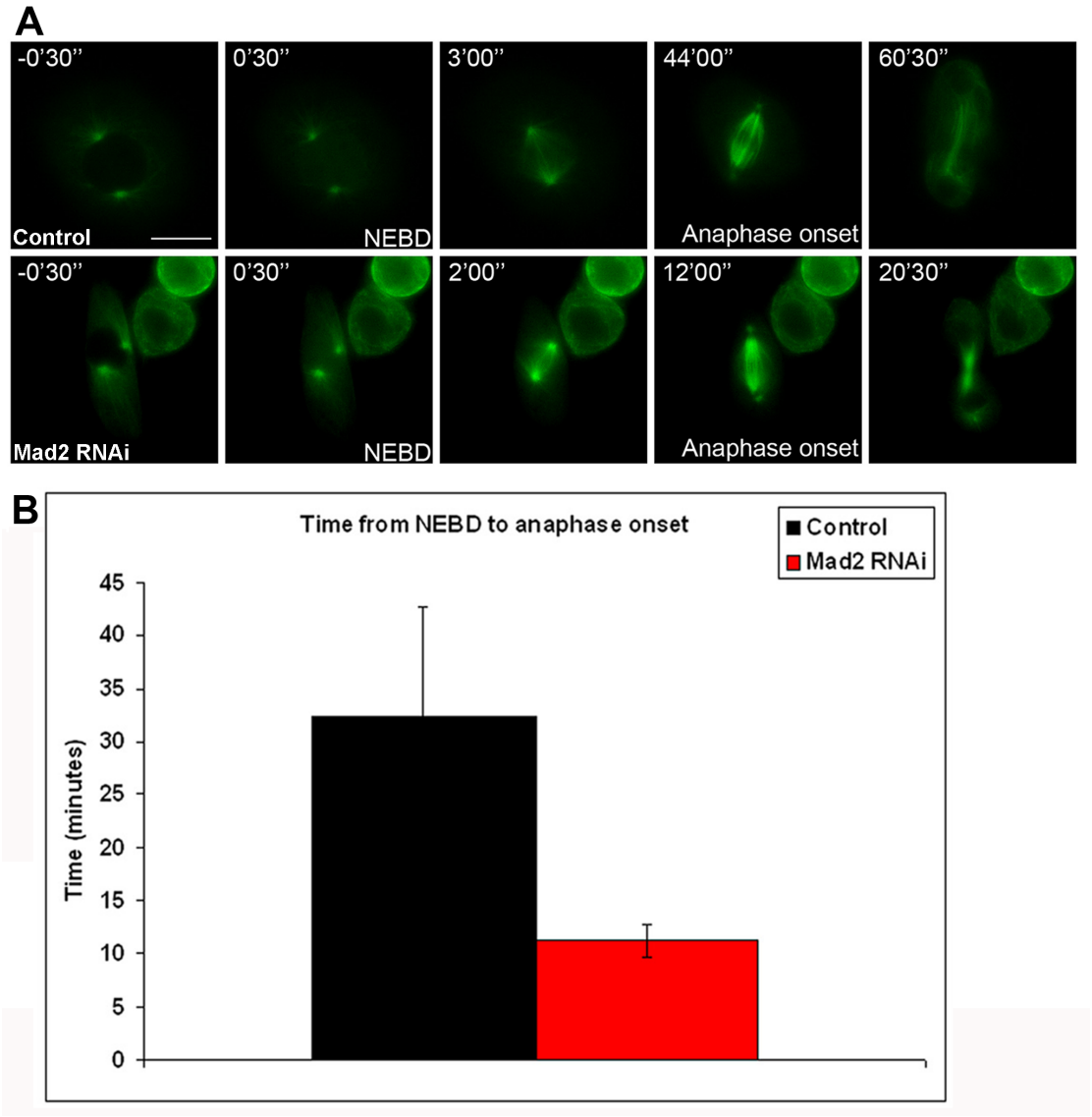


Figure 1.4 - Timing of mitotic progression in Mad2-depleted cells. The time between NEBD and anaphase onset in control and Mad2-depleted cells was determined by time-lapse fluorescence microscopy of S2 cells stably expressing GFP-Tubulin. **(A)** Selected frames of both control and RNAi-treated cells show that anaphase onset occurs earlier in cells lacking Mad2 than in the control cells. Note that NEBD can be easily determined because of the rapid entry of fluorescence tubulin to the nuclear space (see Supplementary Movies 1 and 2). Bar is 5 μm. **(B)** Quantitative analysis of mitotic timing from at least 10 cells. The results show that control cells take on average 33 ± 8 min from NEBD to anaphase onset displaying some variation in the timing of individual cells. However, Mad2-depleted cells complete NEBD to anaphase onset in only 11 ± 2 min displaying little or no significant variation between individual cells.

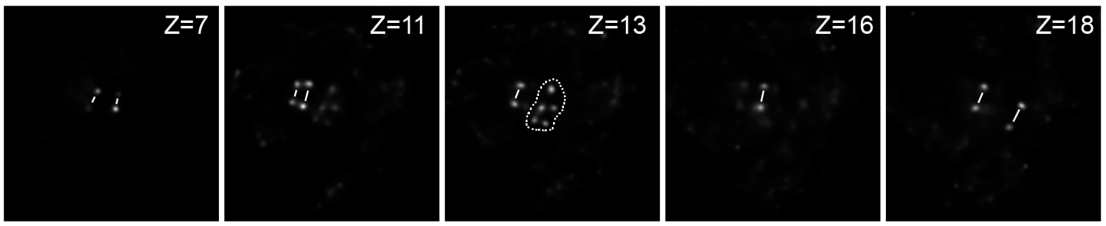


Figure 1.5 - Following kinetochore pairs through optical stacks. Kinetochores were followed through different optical stacks (Z) to determine how they are paired. Solid lines represent paired kinetochores and dotted line indicates a cluster of kinetochores in which the pairing between them is undetermined.

2.3 Chromosome congression in Mad2-depleted cells prevented from exiting mitosis

Quantitative analysis of mitotic progression of Mad2-depleted cells showed a strong reduction in the number of metaphases. To investigate whether failure of these cells to reach proper chromosome congression is only due to an accelerated anaphase onset, Mad2-depleted cells were prevented from exiting mitosis by incubation with MG132 as described above. Cells were then fixed and stained to detect chromosomes, kinetochores and spindle microtubules (Figure 1.6A and B). The results show that if Mad2-depleted cells are prevented from exiting mitosis prematurely, proper chromosome congression is achieved. Interestingly, abnormal chromosome congression has also been associated with improper microtubule-kinetochore attachment after depletion of other checkpoint proteins like Bub1 (Meraldi and Sorger 2005) or BubR1 (Lampson and Kapoor 2005). Therefore, to ascertain whether Mad2 has any specific role in this process, Mad2-depleted cells were prevented from exiting mitosis by incubation in MG132 and kinetochore-microtubule interaction analyzed. Fixed cells were stained for microtubules and kinetochores and imaged by deconvolution microscopy where each kinetochore pair was carefully followed through the stack of optical sections (see Materials and Methods and Figure 1.5 above). The results show that most chromosomes are able to establish correct amphitelic attachment (Figure 1.6C, D and F) suggesting that Mad2 does not have a specific role in establishing and/or maintaining microtubule kinetochore interactions. To determine whether microtubule kinetochore interactions are functional in the absence of Mad2, we quantified the inter-kinetochore distance in untreated late prometaphase cells and in metaphase cells treated with MG132 as an indication that tension was exerted (Figure

1.6E). As expected we find that the inter-kinetochore distance in prometaphase cells is on average half of that after chromosomes have fully congressed suggesting that proper tension is exerted upon kinetochore pairs even after Mad2 depletion. This is in full concordance with the amphitelic attachments observed in the absence of Mad2. Taken together, our results suggest that chromosome congression fails after Mad2-depletion simply because cells exit mitosis prematurely.

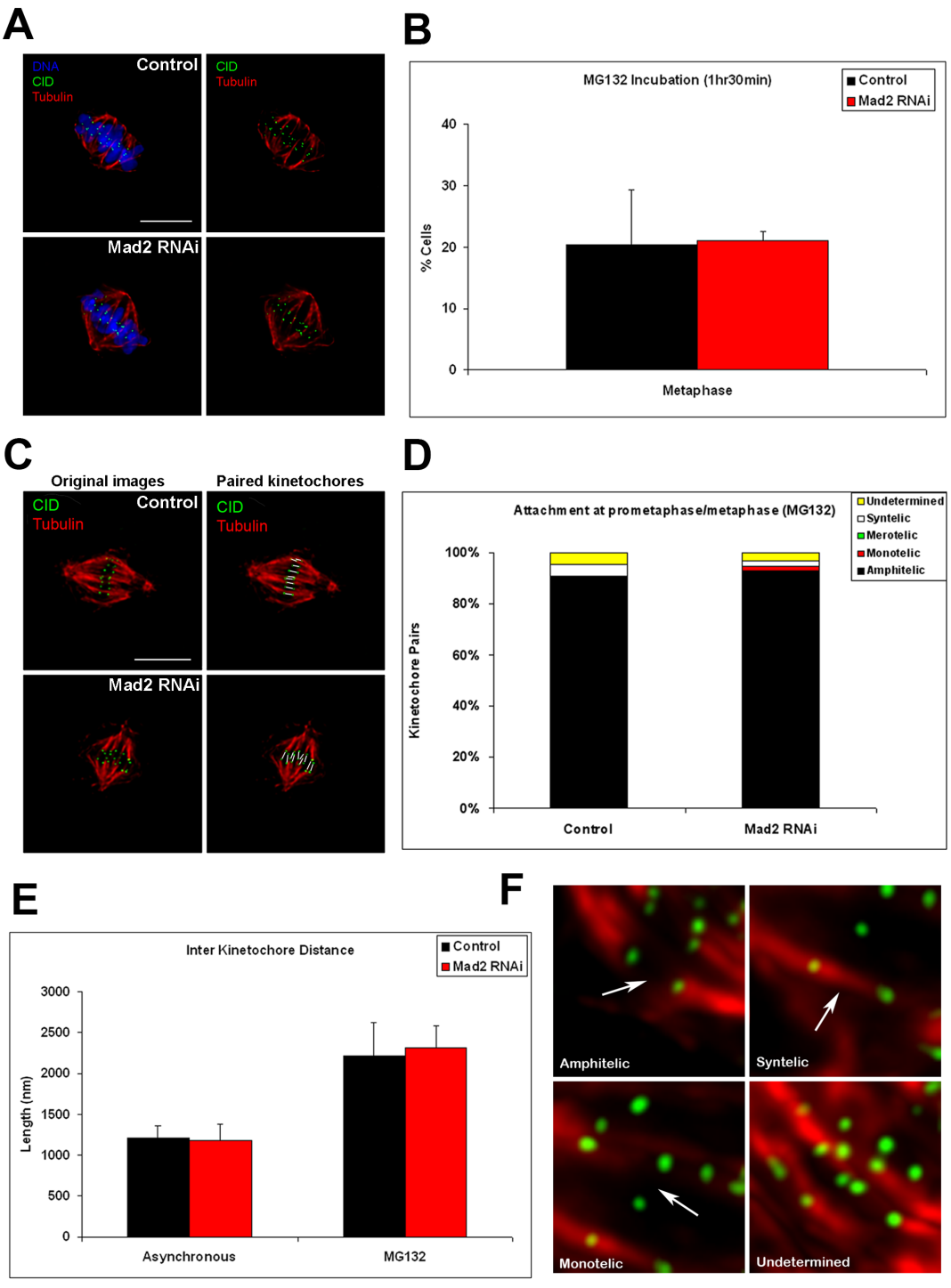


Figure 1.6 - Chromosome congression after checkpoint-independent mitotic arrest. (A, C) Cells were incubated with the proteasome inhibitor MG132 for 90 minutes before fixation and immunostained to reveal DNA (blue), kinetochores (green) and tubulin (red). **(B)** Quantification shows that when Mad2-depleted cells are incubated with MG132 the frequency of metaphases is similar to controls. **(C)** To determine the frequency of proper attachment of kinetochore pairs, control and Mad2-depleted cells were treated with Ca++ to reveal only kinetochore bundles, fixed and immunostained as before. Kinetochore pairing was determined by following individual chromosomes through different optical layers (see Figure 1.5). **(D)** Quantification of different kinetochore attachments in control and Mad2-depleted cells after incubation with MG132 to prevent exit from mitosis. Note that in the absence of Mad2 most kinetochore pairs appear to attach properly and only approximately 5% of kinetochore pairs could not be assigned to any particular type of attachment (n= >100 kinetochore pairs). **(E)** Inter-kinetochore distance measured in metaphase cells arrested with 20 μM MG132 and in asynchronous cells in culture. When treated with MG132, both control and Mad2-depleted cells are under tension and the inter-kinetochore distance is increased. **(F)** Panel showing types of attachment used for quantification shown in **(D)**; CID (green) and tubulin (red). Bar is 5 μm.

2.4 Analysis of chromosome condensation in Mad2-depleted cells

The analysis of mitotic progression after Mad2-depletion indicates that cells progress very rapidly during prometaphase and exit mitosis with PSCS leading to severe aneuploidy. Furthermore, during anaphase and telophase these cells show extensive chromatin bridges, which are highly reminiscent of phenotypes previously reported to be associated with abnormal chromosome condensation (Coelho *et al.* 2003; Oliveira *et al.* 2005). In order to determine whether Mad2 has any specific role in chromosome condensation and chromosome segregation or whether these phenotypes result exclusively from an accelerated transit through mitosis we devised a protocol that allowed a reversible checkpoint-independent transient mitotic arrest. Cells were incubated in a low dose of MG132 for up to 2 hours and then the drug was washed out by extensive dilution in fresh media. Samples were then collected every 30 minutes and mitotic progression analyzed by immunostaining (Figure 1.7A-C). Quantitative analysis shows that MG132 incubation causes a strong mitotic arrest and cells accumulate in metaphase while the frequency of anaphases and telophases is severely reduced. Accordingly, after the MG132 wash, cells rapidly exit mitosis and the number of anaphases and telophases increases while the frequency of metaphases is reduced (Figure 1.7A). The results clearly demonstrate that MG132 activity causes a transient accumulation of cells at metaphase, which can be reverted so that cells can then proceed through mitosis normally (Figure 1.7B). Additionally, our results show that the

additional time in mitosis provided by the reversible MG132 arrest allows Mad2-depleted cells to fully condense their chromosomes and exit mitosis without chromatin bridges at the same frequency as control cells (Figure 1.7C). Furthermore, if Mad2-depleted cells are arrested in mitosis and microtubules depolymerized to allow better visualization, chromosomes are able to condense and display a morphology that is indistinguishable from control cells (Figure 1.7D). In order to further confirm these observations, the localization of key components of the mitotic chromosome organization machinery was also analyzed in Mad2-depleted cells (Figure 1.7E and F). We find that in early prometaphase Mad2-depleted cells that were not arrested in mitosis, the condensin subunit Barren, essential for the structural integrity of chromosomes during mitosis (Bhat *et al.* 1996) and Topoisomerase II (Topo II), a protein responsible for modifying DNA topology (Swedlow *et al.* 1993), localize properly along a well organized chromosomal axis. These results demonstrate that Mad2 is unlikely to have any direct role in chromosome structure and suggest that the chromatin bridges observed in anaphase/telophase are exclusively due to a premature exit from mitosis.

Our phenotypic analysis of Mad2-depleted cells (see Figure 1.3D-F) also showed that over time, cells become severely aneuploid. Since all mitotic abnormalities caused by loss of Mad2 could be reverted either by a permanent or a transient mitotic arrest (Figure 1.7), we quantified kinetochore segregation in control and Mad2-depleted cells with and without a transient MG132-induced mitotic arrest (Figure 1.7G and H). In asynchronous cultures not treated with the proteasome inhibitor, the majority of control cells show a regular 1:1 kinetochore segregation, however, in cells depleted of Mad2, sister chromatids segregate unequally at a frequency almost 3-fold higher than in control cells. After a transient checkpoint independent mitotic arrest Mad2-depleted cells are able to segregate sister chromatids similarly to control cells (Figure 1.7H) suggesting that the unequal segregation seen in the absence of Mad2 is caused by the accelerated mitotic timing.

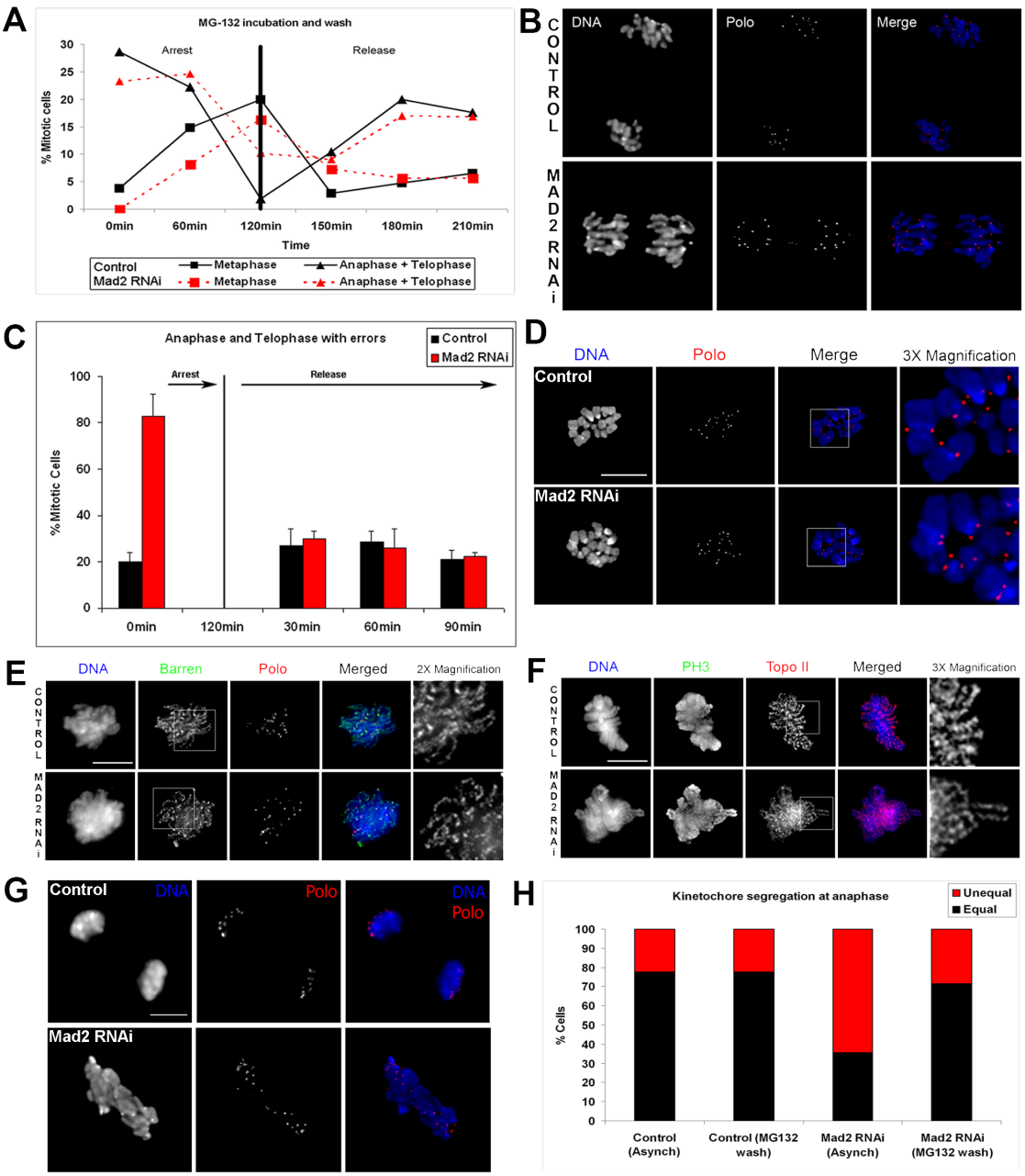


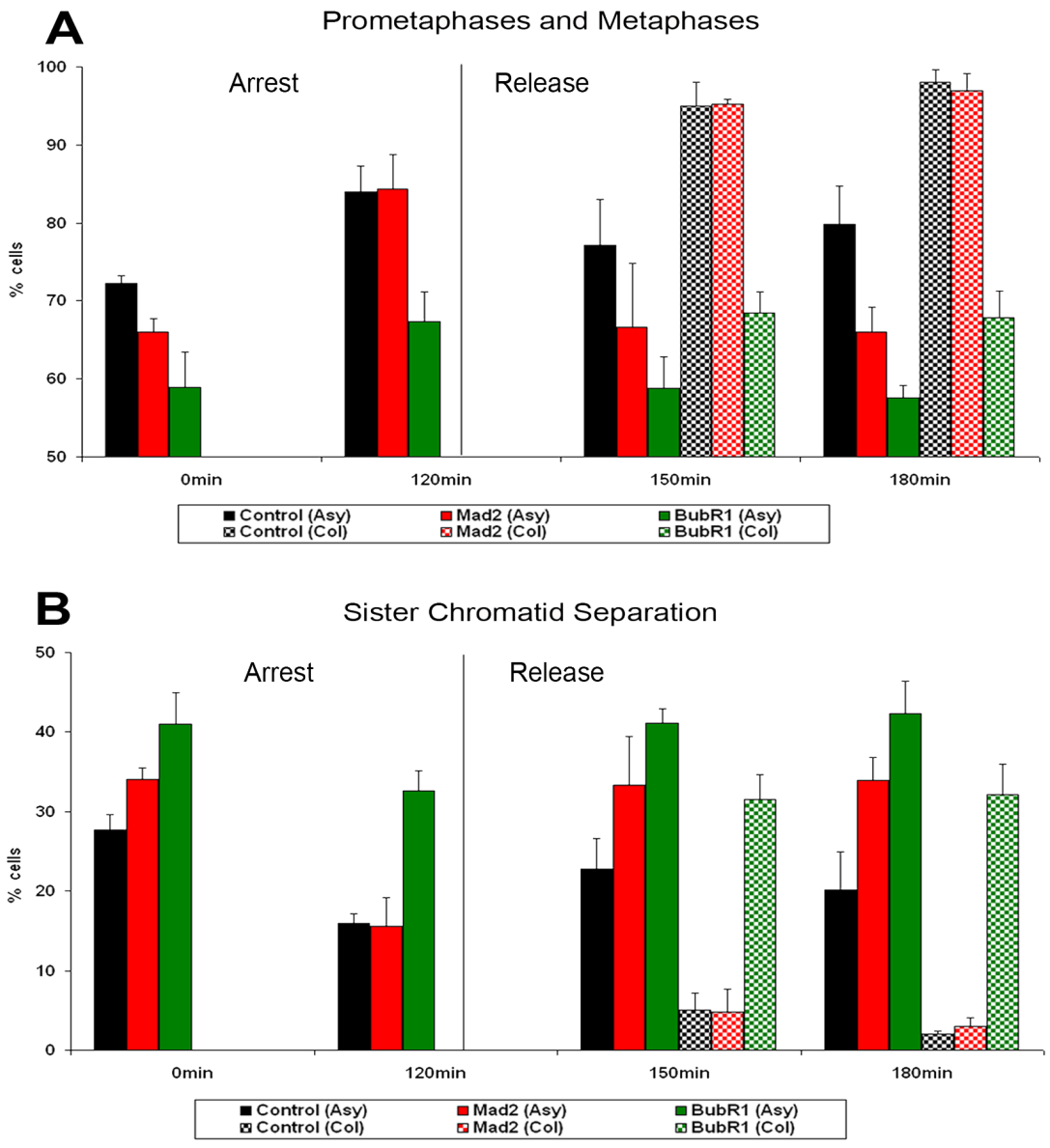
Figure 1.7 - Reversion of Mad2-asscoiated phenotypes. To determine whether the Mad2-associated phenotypes could be reverted by providing additional time in prometaphase/metaphase, S2 cells previously treated with dsRNA against Mad2 for 72h were incubated for 120 min with a low dose of MG132 (2μM) and then released from the block by performing a 3-fold dilution on the cell culture media with fresh media. Samples were then collected every 30 min for immunofluorescence analysis. **(A)** Quantitative analysis of mitotic progression in control and Mad2-depleted cells before and after reversion. From 0 to 120 min both control and Mad2-depleted cells show a strong decrease in anaphases and telophases and a marked increase in the number of metaphases. After washing MG132 (120min), the number of metaphases begins to decrease, and the number of anaphase and telophase figures increases. **(B)** Immunofluorescence shows that 180 min after washing the drug, most anaphases in Mad2-depleted cells do not show chromatin bridges. DNA is shown in blue and Polo in red. **(C)** Quantitative analysis of the anaphase and telophase figures before and after

the MG132 treatment shows a complete reversion of the phenotype. **(D)** Normally condensed chromosomes can be obtained in Mad2-depleted cells if cells are incubated with MG132 to prevent exit from mitosis. Before fixation cells were also treated with colchicine to depolymerize microtubules and induce a better chromosome spread. DNA is shown in blue and the kinetochore marker Polo in red. **(E, F)** Immunolocalization of essential chromosome structure and organization components. Barren and Topoisomerase II in control and Mad2-depleted prometaphases are properly localized to a well-defined sister chromatid axis, in asynchronous cell culture. **(G)** Analysis of kinetochore segregation in control and Mad2-depleted cells after 72 Hours of RNAi incubation was carried out on anaphase cells after immunostaining for DNA with anti-Polo antibody. **(H)** Quantification of chromosome segregation at anaphase shows that in control cells almost 80% of cells segregate kinetochores equally. After Mad2 depletion, nearly 70% of cells also show unequal kinetochore segregation. Providing extra time in prometaphase/metaphase to control cells by incubation in MG132 and then washing out the drug, does not alter the frequency of unequal kinetochore segregation. However, a similar treatment in Mad2-depleted cells reduces to almost control levels the frequency of unequal kinetochore segregation. In all panels bar is 5 μm.

2.5 Analysis of the SAC in cells depleted of Mad2 or BubR1 after a transient mitotic arrest

In the previous section we showed that S2 cells can be arrested transiently using MG132 and that after washing the drug, cells exit mitosis normally even in the absence of Mad2. Surprisingly, we find that Mad2-depleted cells that are also arrested transiently in mitosis by the MG132 reversible treatment can also undergo a mostly normal progression through mitosis. This suggests that Mad2 has an essential role in providing time during early stages of prometaphase so that cells can complete chromosome condensation, microtubule-kinetochore attachment and anaphase onset. Therefore, given that these cells now show normal patterns of segregation it is important to determine if Mad2-depleted cells that are transiently arrested in prometaphase are able to respond to spindle damage. To study this further we performed a similar experimental procedure as described in the previous section with the exception that Mad2-depleted cells were released from the MG132 block into a media containing the microtubule depolymerizing drug colchicine (Figure 1.8). The results were highly surprising and show that after this treatment, Mad2-depleted cells behave just like wild type controls displaying a normal SAC response. Incubation in MG132 causes both control and Mad2-depleted cells to arrest and when they are released into a normal media the frequency of prometaphase and metaphases is reduced while that of sister chromatid separation increases (Figure 1.8A and B). However, if either control or Mad2-depleted cells are released from the block into media

containing colchicine to depolymerize spindle microtubules, they arrest in prometaphase at a high frequency (>95% of the mitotic population of cells) and the frequency of sister chromatid separation (determined by DRad21 immunostaining) is reduced significantly (Figure 1.8A and B). To determine whether the recovery of SAC activity after the transient mitotic arrest is specific to Mad2-depleted cells or a general behavior of cells depleted of any SAC protein, we performed the same experiment in cells depleted of BubR1 (Maia *et al.* 2007). Western blotting shows that most BubR1 (over 85%) can be effectively depleted by 120h (Figure 1.9) and could not be detected by immunofluorescence analysis (Maia *et al.* 2007). If BubR1-depleted cells are arrested with MG132, we observe an increase in accumulation at prometaphase and metaphase and a small reduction in the frequency of sister chromatid separation (Figure 1.8A and B). When released from the MG132, a reduction in the frequency of prometaphase and metaphases and a corresponding increase in the frequency of sister chromatid separation, was observed. Therefore in this experiment, whilst BubR1-depleted cells appear to respond less efficiently to the MG132 treatment, they do behave similarly to control and Mad2-depleted cells. However, if BubR1-depleted cells are released from MG132 into colchicine the behavior is radically different from either control or Mad2-depleted cells since the frequency of prometaphases and metaphase is significantly reduced and the frequency of sister chromatid separation significantly increased (Figures 1.8A and B). Thus, while a transient mitotic arrest causes Mad2-depleted cells to have a normal SAC response, BubR1-depleted cells fail to arrest after spindle damage suggesting that the recovery of SAC activity after these treatment is Mad2 specific. However, it is still possible that the behavior of Mad2-depleted cells could be the result of low levels of Mad2 present after RNAi treatment, which during the MG132 incubation and colchicine treatment have time to accumulate at kinetochores and provide SAC function. To explore this possibility, we took samples of cells treated with colchicine, MG132 or after the MG132 reversible protocol (180 min), fixed the cells and immunostained for Mad2. Subsequently the levels of immunofluorescence were quantified and the results indicate that unlike control cells, Mad2 is undetectable at the kinetochores of Mad2-depleted cells treated with colchicine and barely detectable after incubation in MG132 or when release into media containing colchicine (Figure 1.8C). Accordingly, given that Mad2 is virtually undetectable after Mad2-depletion, it is unlikely that the SAC activity we observed after the transient mitotic arrest is due to low Mad2 levels still present in RNAi treated cells.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Prometaphases and Metaphases
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Mad2 (Asy)
BubR1 (Asy)
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Mad2 (Col)
BubR1 (Col)
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Arrest
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10
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Mad2 (Col)
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Mad2 intensity at kinetochores
Control
Mad2 RNAi
Mean Pixel Intensity
80.000
60.000
40.000
20.000
0
Colchicine
MG132
MG132 wash
(Colchcine)

Figure 1.8 - Reactivation of the spindle assembly checkpoint in Mad2-depleted cells but not in cells lacking BubR1. To determine the status of the SAC in Mad2-depleted cells after a transient mitotic arrest, cells were incubated in MG132 for 2 hours and then released either in normal media or media containing colchicine and the frequency of **(A)** prometaphase and metaphases or **(B)** sister chromatid separation determined by DRad21 immunostaining and kinetochore pairing. Note that Mad2-depleted cells if released into normal media rapidly exit mitosis, however, if released into media containing colchicine accumulate in a prometaphase-like state similarly to control cells. In contrast, BubR1-depleted cells fail to accumulate at prometaphase/metaphase when released into colchicine after the wash suggesting an inactive SAC. **(C)** Quantification of Mad2 kinetochore signal by mean pixel intensity, in cells treated with colchicine, MG132 and in cells released from the MG132 wash into colchicine (180 min). Whilst almost no Mad2 was detected in any RNAi sample, in control cells there is a strong Mad2 accumulation in both cells treated with colchicine and those in which the low level of MG132 had been released into colchicine.

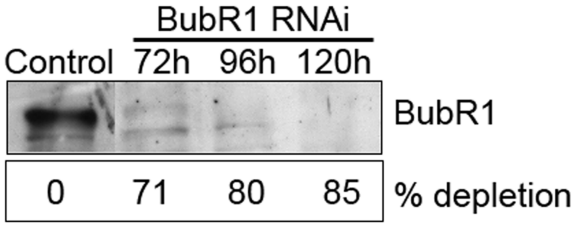


Figure 1.9 - Depletion of BubR1 by western blot analysis. BubR1 can be depleted by over 70% by 72h, reaching a maximum depletion of 85% at 120h after the addition of the dsRNA specific for BubR1.

2.6 Sister chromatid cohesion after a transient mitotic arrest in the absence of Mad2 or BubR1

To further explore whether Mad2-depleted cells are able to fully activate the SAC if transiently arrested in mitosis, we set out to determine whether the APC/C was still being inhibited properly in these cells. For this we chose to carry out immunolocalization of cohesin at the centromeric region between sister kinetochores after the different experimental protocols. Accordingly, control, Mad2 or BubR1-depleted cells in prometaphase from asynchronous cultures, after colchicine treatment or after the MG132 reversible treatment, were fixed and immunostained against Polo to label kinetochores and DRad21, one of the subunits of the cohesin complex (Figure 1.10). We find that in asynchronous control cultures all prometaphase cells display kinetochore pairs that are positive for DRad21 while most prometaphase cells depleted for either Mad2 or BubR1 do not show any DRad21 staining (Figure 1.10A). Loss of SAC activity was confirmed by immunostaining for DRad21 in cells incubated in

colchicine where we observed normal localization of cohesin in control cells while either Mad or BubR1-depleted cells show not cohesin localization in prometaphase (Figure 1.10B). However, analysis of cells subjected to the MG132 reversible protocol followed by release in colchicine show that both control and Mad2-depleted cells in prometaphase contain chromosomes with proper localization of DRad21 while the cohesin subunit is undetectable in BubR1-depleted cells (Figure 1.10C). These results fully support the hypothesis that if Mad2-depleted cells are transiently arrested in mitosis the SAC can be specifically re-activated to prevent premature activation of the APC/C.

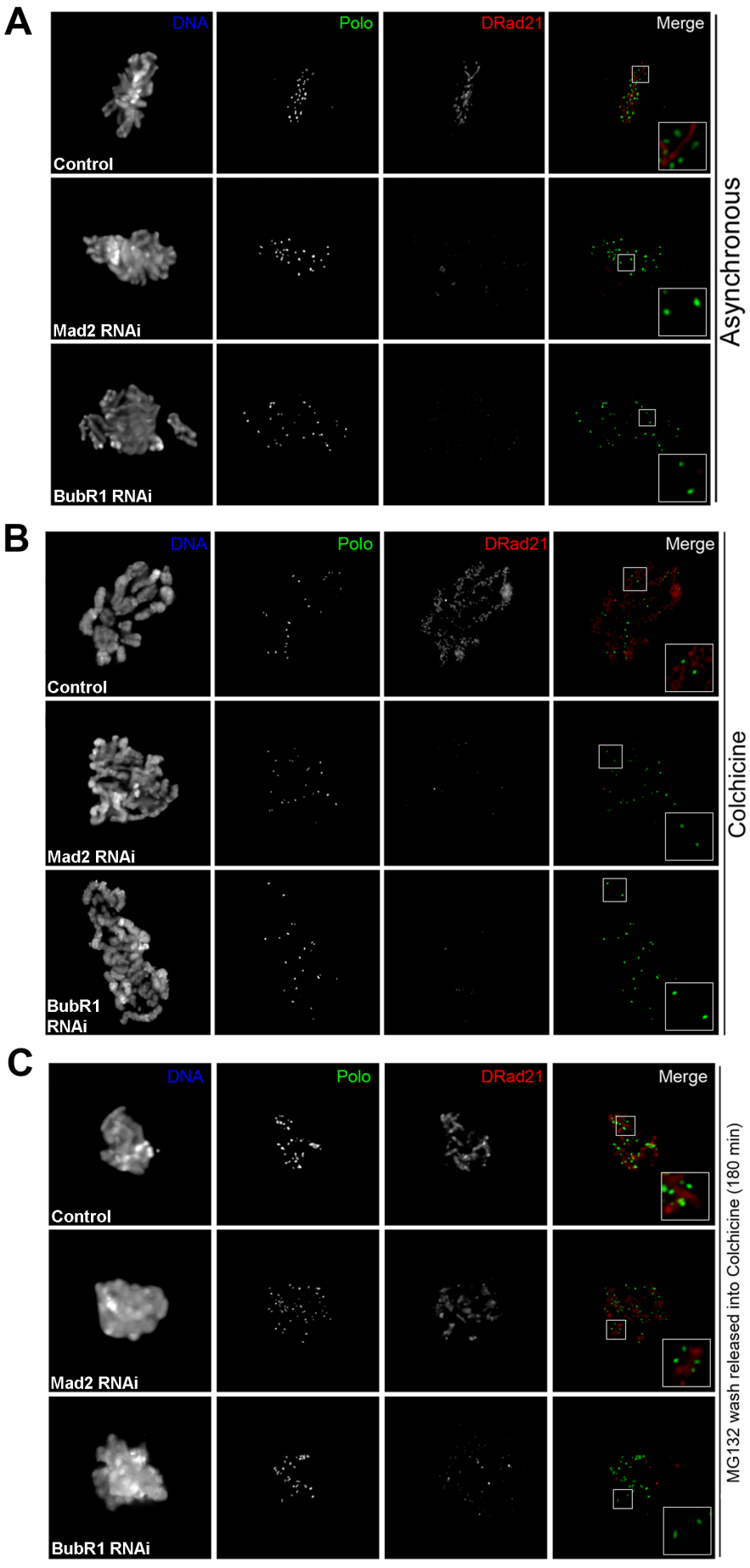

A
DNA
Polo
DRad21
Merge
Control
Mad2 RNAi
BubR1 RNAi
Asynchronous
B
DNA
Polo
DRad21
Merge
Control
Mad2 RNAi
BubR1 RNAi
Colchicine
C
DNA
Polo
DRad21
Merge
Control
Mad2 RNAi
BubR1 RNAi
MG132 wash released into Colchicine (180 min)

Figure 1.10 - Preventing DRad21 degradation in cells lacking Mad2. Cells were collected, fixed and stained to reveal DNA (blue), Polo (green) and the cohesin subunit DRad21 (red). Cells from control, Mad2 and BubR1 RNAi's were analysed in **(A)** asynchronous cells in culture, **(B)** colchicine treatment and **(C)** in cells subjected to the MG132 wash experiment and released into media containing colchicine (180 min). Note that Mad2-depleted cells released into this media are unable to degrade cohesion whilst BubR1-depleted cells fail to arrest in the presence of colchicine suggesting an inactive SAC response (see higher magnification inserts). Bar is 5 μm.

2.7 Analysis of BubR1 kinetochore accumulation in Mad2-depleted cells after a transient mitotic arrest

In the previous sections we have shown that in contrast to widely held views, cells that have been depleted of Mad2 can still activate the SAC and progress through a normal mitosis if they are prevented from premature mitotic exit by a transient arrest with the proteasome inhibitor MG132. Furthermore, we showed that this effect is unlikely to result from residual Mad2 levels after RNAi treatment and that it causes proper APC/C inhibition. To investigate whether the SAC re-activation presents other characteristics such as the high accumulation of other SAC proteins in the presence of unattached kinetochores, we set out to analyze the levels of BubR1 in Mad2-depleted cells before and after the transient arrest (Figure 1.11). We find that control or Mad2-depleted cells arrested in mitosis by MG132 incubation accumulate relatively low levels of BubR1 at kinetochores (Figure 1.11A and C). This is fully consistent with previous observations describing that after normal microtubule kinetochore attachment and tension BubR1 levels at kinetochores while still present is relatively low (Logarinho *et al.* 2004). However, if Mad2-depleted cells are then released into media containing colchicine where microtubule kinetochore is disrupted, kinetochore accumulation of BubR1 is significantly increased to levels that are comparable to that of control cells after spindle damage (Figure 1.11B and C). These results are in full accordance with our observations and further suggest that the transient arrest of Mad2-depleted cells allows a mostly normal SAC response after spindle damage.

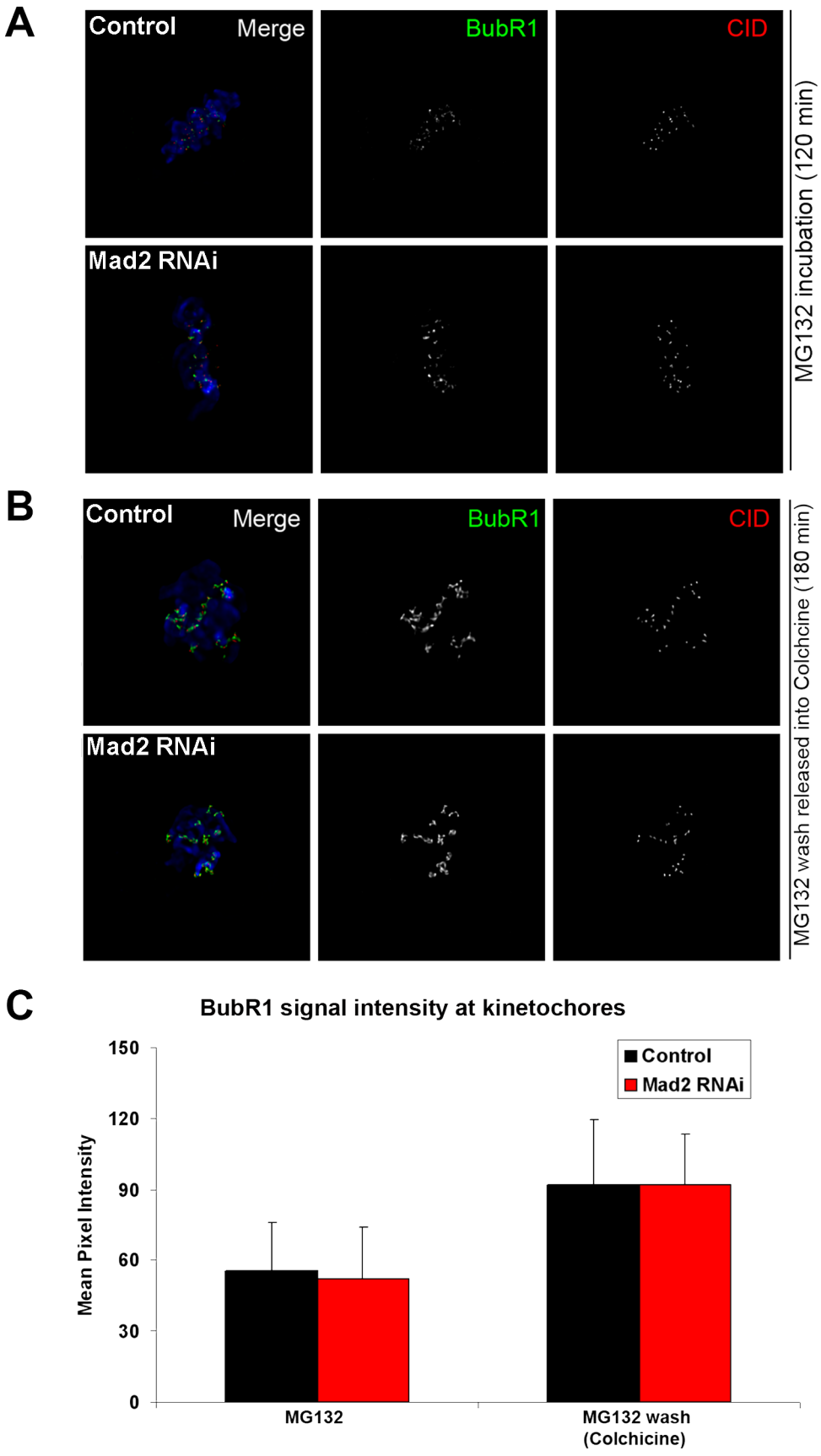


Figure 1.11 - Assaying SAC response in cells lacking Mad2. Cells from the MG132 wash were collected for analysis at **(A)** 2h after MG132 incubation (120 min) and **(B)** after the drug was washed with media containing colchicine (180 min). These cells were fixed and stained to reveal DNA (blue), BubR1 (green) and CID (red). **(C)** Quantification of the BubR1 kinetochore signal in control and Mad2-depleted cells shows an identical 2-fold increase in cells released into the colchicine. Note that cells lacking Mad2, similarly to control cells, accumulate BubR1 strongly when released into colchicine suggesting an active SAC. Bar is 5 µm.

3. Discussion

Recent studies on SAC components have provided significant understanding on the molecular mechanisms in which Mad2 is involved. However, it is surprising that little or no data has been obtained characterizing mitotic progression of cells that lack this essential SAC component. Here we provide the first detailed analysis of Mad2-depleted cells as they progress and eventually exit mitosis. Surprisingly, we find that all Mad2-associated phenotypes can be reverted and the checkpoint effectively re-activated by a transient mitotic arrest. Thus, contrary to current models which view Mad2 at the centre of the inhibition of the APC/C by the SAC, we hypothesize that Mad2 is only required for proper timing of mitotic progression early during prometaphase, allowing cells to fully engage the SAC through kinetochore accumulation of other checkpoint proteins so that complete chromosome condensation and congression can be achieved before a controlled metaphase-to-anaphase transition takes place.

3.1 Mad2 has a conserved role in *Drosophila*

Checkpoint proteins have been shown to be essential for the fidelity of mitosis as they are responsible for sensing errors in microtubule kinetochore interaction (Gorbsky *et al.* 1998; Canman *et al.* 2002; Mikhailov *et al.* 2002). Here we show that loss of the Mad2 homologue causes inactivation of the SAC in *Drosophila* S2 cells. To find out whether Mad2 is required for the kinetochore localization of other checkpoint components we performed immunolocalization studies against other checkpoint proteins. We find that all SAC proteins tested (Bub1, Bub3 and BubR1) show strong accumulation at kinetochores, demonstrating that they do not require Mad2 for their localization and also that proper kinetochore localization of these checkpoint components does not *per se* prevent premature mitotic exit. Previous studies in Xenopus and HeLa cells were performed in the presence of microtubule poisons and therefore it was unclear whether the absence of protein localization reflected a hierarchical relationship or the inability to analyze a large number of mitotic cells due to fast mitotic exit (Chen 2002; Johnson *et al.* 2004). Since individual depletion of Bub3 in *Drosophila* (Lopes *et al.* 2005), and analysis of the hypomorphic allele of BubR1 (Basu *et al.* 1999) resulted in a non-functional SAC response, it is very likely that these proteins work through parallel signaling pathways that are mutually required at some stage to sustain checkpoint activity. Consistent with previous work, it seems probable

that removing Mad2 may abrogate the spindle checkpoint not only because a sensor is being removed, but because the MCC (a far more potent APC/C inhibitor) (Sudakin *et al.* 2001) cannot form in its absence.

3.2 Mad2-depleted cells show abnormal progression through mitosis and aneuploidy

Previous results have shown that inactivation of Mad2 by antibody microinjection during prophase or prometaphase induced abnormal sister chromatid segregation in PtK1 cells (Gorbsky *et al.* 1998). Our phenotypic analysis revealed that *Drosophila* S2 cells lacking Mad2 also display severe abnormalities during mitotic progression. Mad2-depleted cells fail to reach metaphase and exit mitosis with extensive chromatin bridges. Moreover, the extensive anaphase bridges observed in Mad2-depleted cells appear to be exclusively due to a premature exit from mitosis since extending the time spent in mitosis is enough to revert this phenotype. This is fully consistent with recent data suggesting that proper chromosome condensation and sister chromatid resolution is only fully completed during early prometaphase (Maeshima and Laemmli 2003). Furthermore, our results suggest that in S2 cells, full chromosome condensation is only achieved late in prometaphase, after the minimal time cells spend in prometaphase when the SAC is inactivated. This is in full agreement with our previous analysis of mitotic progression in the absence of Bub3 (Lopes *et al.* 2005) where it was shown that in the absence of Bub3 the SAC is inactivated but cells do not exit mitosis with inappropriately condensed chromosome because of an extended period in prophase.

Several studies have shown that loss of SAC proteins causes PSCS and significant aneuploidy (Kops *et al.* 2005b). We find that loss of Mad2 causes premature degradation of cohesins during prometaphase resulting in high levels of PSCS. In addition, quantification of kinetochore segregation at anaphase shows that loss of Mad2 results in a high frequency of cells showing unequal kinetochore segregation. Furthermore, FACS analysis shows that the DNA content of the mitotic population changes significantly over time in the absence of Mad2. These results suggest that unlike in yeast where Mad2 is not essential for chromosome segregation (Cohen-Fix and Koshland 1997), *Drosophila* Mad2 is required to maintain the long viability of cells.

3.3 Mad2 role in timing of prometaphase is essential for SAC activation

Early studies on the role of Mad2 in the SAC response using cultured animal cells revealed premature anaphase onset (Gorbsky *et al.* 1998). More recently, it was found that the mitotic clock of unsynchronised rat basophilic leukaemia cells has a marked precision in which approximately 80% of cells complete mitosis in 32 ± 6 min, and that Mad2 inactivation in these cells consistently shortened mitosis (Jones *et al.* 2004). Furthermore, depletion of Mad2 by RNAi showed that HeLa cells exit mitosis prematurely (Meraldi *et al.* 2004). Our results are fully consistent with this data since depletion of Mad2 in S2 cells causes a severe reduction in the time from NEBD to anaphase onset. Interestingly, these same studies proposed a role of Mad2 in timing mitotic progression that is more complicated that previously expected. It was shown in HeLa cells that inactivation of kinetochore-bound Mad2 disrupts the SAC without significantly affecting the timing of mitotic progression. However, when the cytosolic pool of Mad2 present in these cells is depleted then both the SAC response is abnormal and the timing of NEBD to anaphase onset is severely reduced suggesting that Mad2 is also required to time mitotic progression in a kinetochore-independent manner. This contrasts with current models that propose that Mad2 plays an essential role in SAC activation and maintenance by providing a kinetochore-based signal that inhibits the APC/C (reviewed in Musacchio and Hardwick 2002; Kops *et al.* 2005b). Our observations suggest a much more subtle role for Mad2 in ensuring a SAC response. Surprisingly, we find that after a transient mitotic arrest, Mad2-depleted cells were able to respond to spindle damage and arrest in mitosis with cohesin still located at centromeres and high kinetochore levels of BubR1 suggesting that the SAC is fully functional. Thus, providing time in a checkpoint-independent and transient manner appears to be sufficient for Mad2-depleted cells to re-activate the SAC and respond correctly to microtubule depolymerization. Given that re-activation of the SAC cannot be observed after depletion of other SAC proteins like BubR1 we hypothesize that the APC/C inhibitory signal provided by Mad2 is specifically required during early stages of prometaphase to ensure maintenance of SAC activity. Subsequently, other checkpoint proteins such as BubR1, which strongly accumulate at kinetochores and are essential for APC/C inhibition, could ensure maintenance of SAC activity. These observations are in full accordance with previous studies in which biochemical studies showed that at the G2-M transition a multi-subunit complex, the Mitotic Checkpoint Complex (MCC) was identified (Sudakin *et al.* 2001). This complex contains the BubR1-Mad2-Bub3-Cdc20 proteins, and has been shown to be the most powerful APC/C inhibitor (Sudakin *et al.* 2001; Tang *et al.* 2001; Sudakin and Yen 2004). Furthermore, its formation does not

require unattached kinetochores given that it is present well before the NEBD. We propose that despite lacking Mad2, the additional time provided by the transient mitotic arrest is enough to accumulate other checkpoint proteins to such a level that the individual APC/C inhibitory complexes are now able to maintain a strong SAC response.

Taken together, these observations have suggested a “two-step” model for the activation and maintenance of SAC activity (Chan *et al.* 2005). This model proposes a first step involving the formation of the MCC as cells transit from G2 into mitosis, thus allowing accumulation of mitotic cyclins and consequent mitotic entry. Subsequently, in a second step after NEBD, SAC proteins can bind unattached kinetochores and produce additional inhibitory complexes that sustain SAC activity until all kinetochore pairs are properly attached and congression is achieved. Subsequent studies both in yeast (Fraschini *et al.* 2001) and *Drosophila* (Lopes *et al.* 2005) strongly support this model. Our results provide a further refinement of this model in that the second step can be separated into two events, one at the NEBD when cytoplasmic Mad2 might extend prometaphase and provide enough time so that in a second event, SAC proteins such as BubR1 and Bub3 can fully engage checkpoint activity. Further studies on the role of Mad2 and other SAC proteins in the inhibitory activity of the MCC before and during early stages of mitosis will be required to unravel how the different levels of regulation are organized. Nevertheless, our observations provide new insights into how the signals provided by different SAC proteins might contribute to a fully integrated and sustained checkpoint response.

Chapter 2

Mad2 kinetochore (in)dependent pathways of Spindle Assembly Checkpoint activation

1. Introduction

The SAC is an essential cell cycle control mechanism, which acts in mitosis to prevent anaphase onset in cells whose chromosomes have not yet achieved stable bipolar attachment and microtubule tension (reviewed in Musacchio and Salmon 2007). Most SAC components were originally identified in budding yeast, using genetic screens in the presence of spindle poisons. MAD1-3 (Li and Murray 1991) and BUB1-3 (Hoyt *et al.* 1991) were shown to be required for a mitotic arrest in the presence of spindle damage and further studies suggested that together with Mps1, the identified SAC proteins form distinct complexes at kinetochores, which are essential for APC/C inhibition (Malmanche *et al.* 2006; Musacchio and Salmon 2007; Kops 2008).

Subsequent studies identified two other proteins in *Drosophila*: Zeste-White 10 (Zw10) and Rough Deal (Rod), both of which are highly conserved amongst several multicellular organisms (Gatti and Baker 1989; Karess and Glover 1989; Williams *et al.* 1992; Scaerou *et al.* 1999; Scaerou *et al.* 2001). Further research yielded the identification of another protein, Zwilch (Williams *et al.* 2003), which forms a complex with Rod and Zw10 (RZZ complex) (Chan *et al.* 2005; Karess 2005). RZZ complex components display dynamic localization patterns throughout mitosis and have been proposed to play roles not only in chromosome motion (Li *et al.* 2007; Yang *et al.* 2007; Gassmann *et al.* 2010) but also in SAC control/maintenance (Basto *et al.* 2000; Chan *et al.* 2000; Savoian *et al.* 2000; Buffin *et al.* 2005; Vallee *et al.* 2006; Famulski *et al.* 2008). Both Zw10 and Rod display highly dynamic cell cycle localization patterns and accumulate strongly at kinetochores during prometaphase. By metaphase they are preferentially localized along kinetochore-microtubules and at anaphase onset these proteins re-localize to kinetochores and remain associated with kinetochores until telophase (Williams *et al.* 1992; Williams and Goldberg 1994; Scaerou *et al.* 1999; Wojcik *et al.* 2001; Basto *et al.* 2004). This dynamic localization pattern is consistent with a role of RZZ complex in kinetochore-microtubule attachment and SAC maintenance. However, several studies support the hypothesis that Zw10 is required during late mitosis, since chromosome segregation defects are observed in the absence of Zw10 (Karess and Glover 1989; Williams *et al.* 1992; Williams and Goldberg 1994).

During metaphase, the RZZ complex also plays a role in SAC silencing since it is required to target the microtubule motor dynein to kinetochores, possibly through direct interaction between Zw10 and the dynactin subunit p50-dynamitin (Starr *et al.* 1998; Chan *et al.* 2000; Wojcik *et al.* 2001; Buffin *et al.* 2005; Chan *et al.* 2005). The dynein/dynactin complex has been implicated in SAC control since its localization at

kinetochores is required for the stripping of Mad1, Mad2 and RZZ proteins from kinetochores (Howell *et al.* 2001; Wojcik *et al.* 2001). Nevertheless, studies on the roles of dynein in SAC silencing obtained through RZZ disruption, have yielded puzzling results since Zw10 and Rod have also been proposed to be required for SAC activity/maintenance (Basto *et al.* 2000; Chan *et al.* 2000; Savoian *et al.* 2000; Buffin *et al.* 2005).

During prometaphase, the prominent RZZ localization at kinetochores that have not yet achieved proper microtubule attachment and tension, is similar to what is observed for several of the previously identified SAC components (Chen *et al.* 1996; Taylor and McKeon 1997; Jablonski *et al.* 1998; Taylor *et al.* 1998; Martinez-Exposito *et al.* 1999; Logarinho *et al.* 2004). Accordingly, Zw10 or Rod mutant cells do not arrest in mitosis in response to spindle damage, providing further evidence to support a role for RZZ in SAC maintenance (Basto *et al.* 2000; Chan *et al.* 2000; Savoian *et al.* 2000; Buffin *et al.* 2005). Nevertheless, whether RZZ components are considered *bone-fide* SAC components remains a controversial issue. It has recently been shown that the RZZ complex promotes Mad2 recruitment to unattached kinetochores during prometaphase (Buffin *et al.* 2005; Kops *et al.* 2005a) suggesting that the RZZ complex contributes to SAC control by promoting Mad2 recruitment, and is involved in SAC silencing by recruiting dynein/dynactin that subsequently removes Mad2 from attached kinetochores. This hypothesis is consistent with an indirect role for RZZ in SAC maintenance, offering a simple explanation as to why Rod and Zw10 were characterized as the first SAC proteins that have no evident homologues in budding yeast (Basto *et al.* 2000). However, the kinetochore localization of other SAC proteins such as Bub1 and Bub3 is not Zw10-dependent, and neither is Zw10 kinetochore localization dependent on Bub1 or Bub3 (Basu *et al.* 1998; Basu *et al.* 1999), suggesting that Zw10 acts specifically on the Mad1/Mad2 pathway of SAC maintenance. This data is further confounded by the observation that while SAC activity is dependent on Mad2 and BubR1, the regulation of mitotic timing by BubR1 and Mad2 appears to be kinetochore-independent in fashion (Meraldi *et al.* 2004). The dissection of SAC maintenance and mitotic timing pathways is consistent with a “two-step” model, in which the kinetochore-independent pools of Mad2 and BubR1 (as part of the MCC) are essential for SAC signaling at early stages of mitosis when kinetochores are still assembling (Meraldi *et al.* 2004; Orr *et al.* 2007).

Therefore, to further study the contribution of cytoplasmic Mad2 in SAC maintenance and mitotic timing, we prevented Mad2 from reaching the kinetochore without affecting its cytoplasmic localization. We depleted Zw10 using RNAi in *Drosophila* S2 cells and confirm that in this cell type Zw10 is required for the

accumulation of Mad2 but not BubR1 to kinetochores, even in the absence of microtubules. Furthermore, we find that the kinetochore localization of Mad2 is not required for the regulation of mitotic timing since Zw10-depleted cells show a slow progression through mitosis. However, these cells display a weak mitotic arrest when treated with colchicine but accumulate in mitosis with high levels of Cyclin B. These observations indicate that the kinetochore localization of Mad2 is dispensable for regulating mitotic timing. Indeed, both mitotic timing and the mitotic index in the presence of spindle damage are strongly reduced if Zw10 is co-depleted with Mad2. In agreement, co-depletion of Zw10 and BubR1 yields similar effects in the regulation of mitotic timing and SAC maintenance indicating that in the absence of kinetochore bound BubR1, the cytoplasmic pool of Mad2 is not able to sustain even a low level of SAC activity. Collectively, our results provide further support for the refined version of the “two-step” SAC activation model (see Chapter 1) in which both BubR1 (at kinetochores and cytoplasm) and cytoplasmic Mad2, act together to provide a spatial-temporal regulation of mitotic timing and SAC activation.

2. Results

2.1 Zw10 depletion in *Drosophila* S2 cells

Recent reports have demonstrated that Zw10, a key component of the RZZ complex, is responsible for ensuring the correct targeting of Mad2 to kinetochores during early stages of prometaphase (Buffin *et al.* 2005; Kops *et al.* 2005a). Since we are interested in addressing the role of cytoplasmic Mad2 in SAC maintenance and mitotic timing, we aimed to specifically inhibit Mad2 localization at kinetochores and therefore performed RNAi against Zw10 in *Drosophila* S2 cells (Figure 2.1). To quantify the levels of Zw10 at kinetochores we treated control and Zw10 RNAi cells with MG132 to block mitotic exit and colchicine to depolymerise microtubules. Control cells display a strong Zw10 accumulation at unattached kinetochores, and we find that 120hr after dsRNA addition, Zw10 is efficiently depleted since protein 97% of kinetochores are negative by immunofluorescence (Figure 2.1A, B) and western blot analysis of total cell extracts reveals a 86% reduction by 120h. (Figure 2.1C). Since the highest level of depletion was obtained at 120h after the addition of the dsRNA, all of the following experimental analysis on Zw10-depeleted cells was conducted at this time-point.

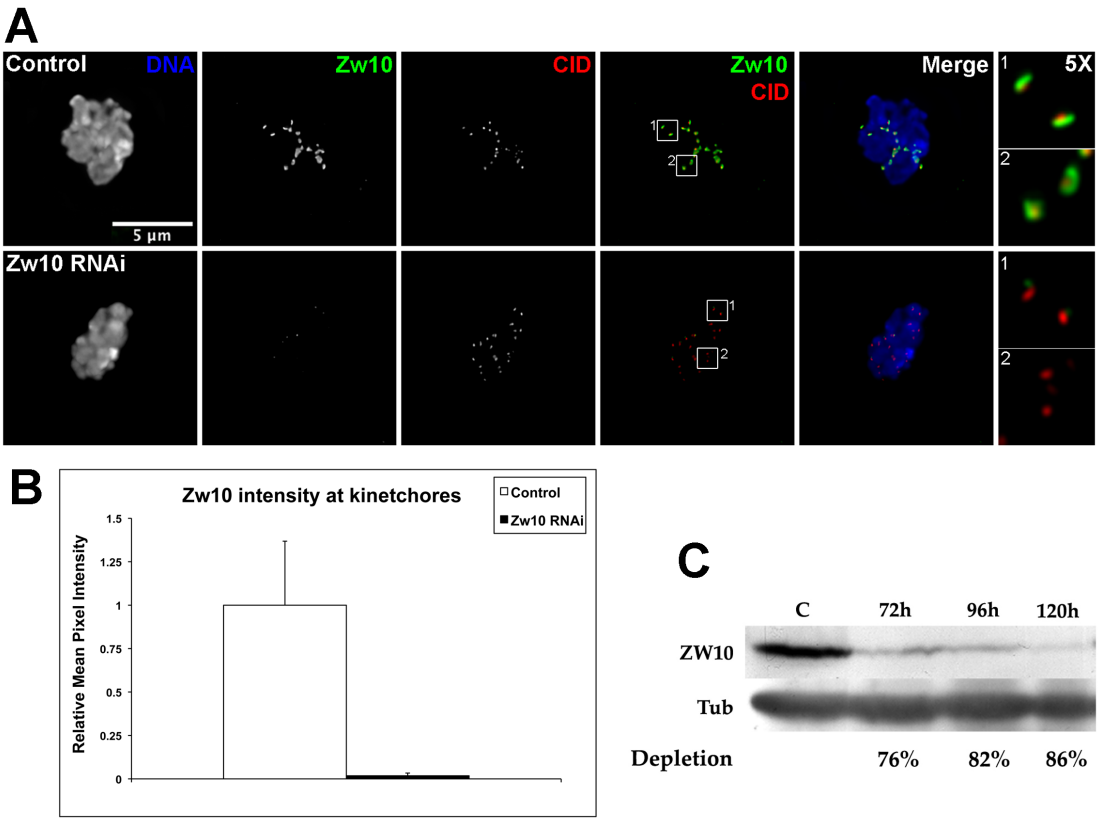


Figure 2.1 - Depletion of Zw10 by RNAi in S2 cells (A) Control and Zw10-RNAi treated S2 *Drosophila* S2 cells (120hr after dsRNA addition) were incubated in MG132 (2hr) and Colchicine (1hr further) prior to fixation and immunofluorescence staining to show DNA (blue), Zw10 (green) and CID (red). Insets 1 and 2 represent 5X magnifications of selected kinetochore regions (right). Scale bar is 5 µm. **(B)** Quantification of the relative mean pixel intensity of Zw10 signals (normalized to CID levels) at kinetochores, in both control and Zw10 RNAi cells 120h after dsRNA addition. Note that Zw10 is depleted in 97% of the kinetochores. **(C)** $1x10^6$ cells were processed for western blot analysis at different times after addition of Zw10 dsRNA. 'C' represents control cells. Below α-tubulin was used as a loading control and Zw10 levels were normalized to α-tubulin levels.

2.2 Zw10 is required for Mad2 accumulation at kinetochores

Previous studies have demonstrated that both Rod and Zw10 are required for proper Mad1-Mad2 complex accumulation at unattached kinetochores (Buffin *et al.* 2005; Karess 2005; Kops *et al.* 2005a). To test whether Mad2 is able to accumulate at unattached kinetochores in the absence of Zw10 we treated control and Zw10-depleted cells with MG132 to induce a mitotic block and generated unattached kinetochores

using colchicine. Cells were then fixed and stained to reveal Mad2 and CID (Figure 2.2A). Control cells display a prominent Mad2 accumulation at the outer region of all CID-labelled kinetochores but in the absence of Zw10, Mad2 is undetected at kinetochores while there is a significant amount scattered in the cytoplasm (Figure 2.2A). Quantification of the mean pixel intensity of Mad2 staining at kinetochores shows that Mad2 accumulation at kinetochores is reduced by more than 90% in Zw10-depleted cells, even in the absence of microtubules (Figure 2.2B). Mad2 mis-localization is specific to Zw10 depletion since accumulation of BubR1 is unaffected (Figure 2.2C). These conditions provide an experimental condition in which cells specifically lack the contribution of kinetochore-localized Mad2 without affecting the cytoplasmic pool of Mad2 or the kinetochore localization of other SAC proteins.

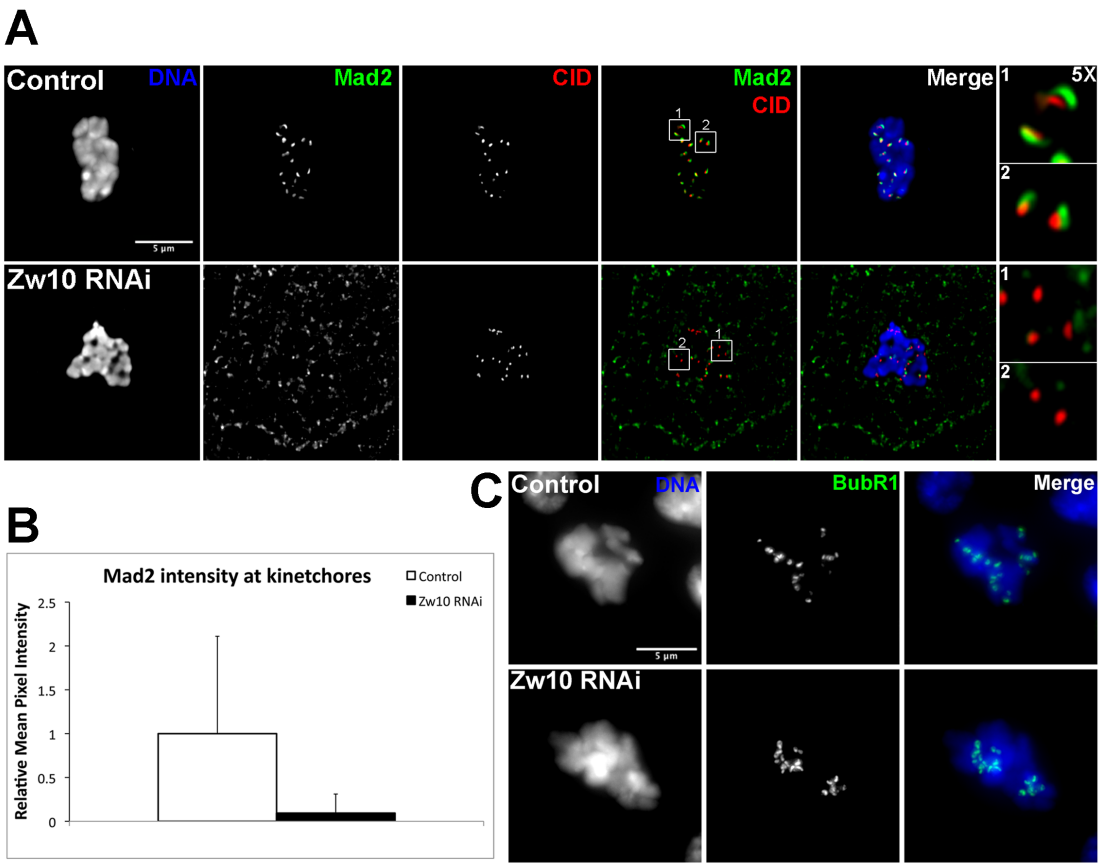


Figure 2.2 – Monitoring Mad2 accumulation at kinetochores (A) Control and Zw10-depleted cells were incubated in MG132 (2hr) and Colchicine (1hr further) prior to fixation and immunofluorescence staining to show DNA (blue), Mad2 (green) and CID (red). Insets 1 and 2 represent 5X magnifications of selected kinetochore regions (right). Note that in control cells Mad2 is found prominently associated with unattached kinetochores while in the Zw10-depleted cells Mad2 signals at kinetochores are strongly

reduced. Scale bar is 5 µm. **(B)** Quantification of the mean pixel intensity of Mad2 levels (normalized to CID levels) at kinetochores using images shown in **(A)**. Note that Mad2 levels are reduced by more than 90% at kinetochores in the absence of Zw10. **(C)** Cells were treated with MG132 and colchicine prior to fixation and immunofluorescence staining to reveal DNA (blue) and SAC protein BubR1 (green).

2.3 Cytoplasmic Mad2 is essential for regulating mitotic timing and SAC activation

Our data show that in Zw10-depleted cells, Mad2 levels are strongly reduced, even in the absence of microtubules. Since Mad2 is one of the first SAC proteins to leave the kinetochore upon microtubule attachment in prometaphase (Howell *et al.* 2000; Howell *et al.* 2004; Logarinho *et al.* 2004), it is probable that Mad2 kinetochore localization is essential only for the early stages of SAC maintenance, and that the kinetochore-based "wait-anaphase" signal may then be perpetuated by other SAC proteins such as BubR1, presumably in response to lack of microtubule tension. This "two-step" model of SAC maintenance is in full agreement with the results obtained from Mad2 depletion in the same cell type (Orr *et al.* 2007), and therefore, we expect that in the absence of Zw10 both the duration of mitotic timing and the efficiency of the mitotic response to colchicine are affected. To test this directly, we performed *in vivo* time-lapse imaging using S2 cells stably expressing GFP-Tubulin. We find that in the absence of Zw10, cells transit through mitosis with a mostly normal mitotic timing (Figure 2.3A, B; Supplementary Movies 3 - 5). Although most cells analysed formed bipolar spindles, a minor subset of cells displayed mild microtubule-associated phenotypes and therefore took longer to establish full spindle bi-polarity (Figure 2.3A). Quantification of mitotic timing shows that Zw10-depleted cells take slightly longer to complete mitosis (mean = 31 ± 10min; Supplementary Movie 4) than untreated controls (mean = 24 ± 8min; Supplementary Movie 3), presumably in response to some of the mild microtubule-associated phenotypes observed in a few Zw10-depleted cells (Figure 2.3B). Interestingly, we find that if Mad2 is prevented from accumulating at kinetochores, the mitotic schedule is mostly normal and cells appear to respond to minor microtubule-associated defects suggesting a fully functional SAC. When compared with the highly reduced mitotic timing observed in the Mad2 depletion in the same cell type (Figure 2.3B), the data suggest that the contribution of cytoplasmic Mad2 is sufficient for the previously described Mad2 kinetochore-independent function in the regulation of mitotic timing (Orr *et al.* 2007).

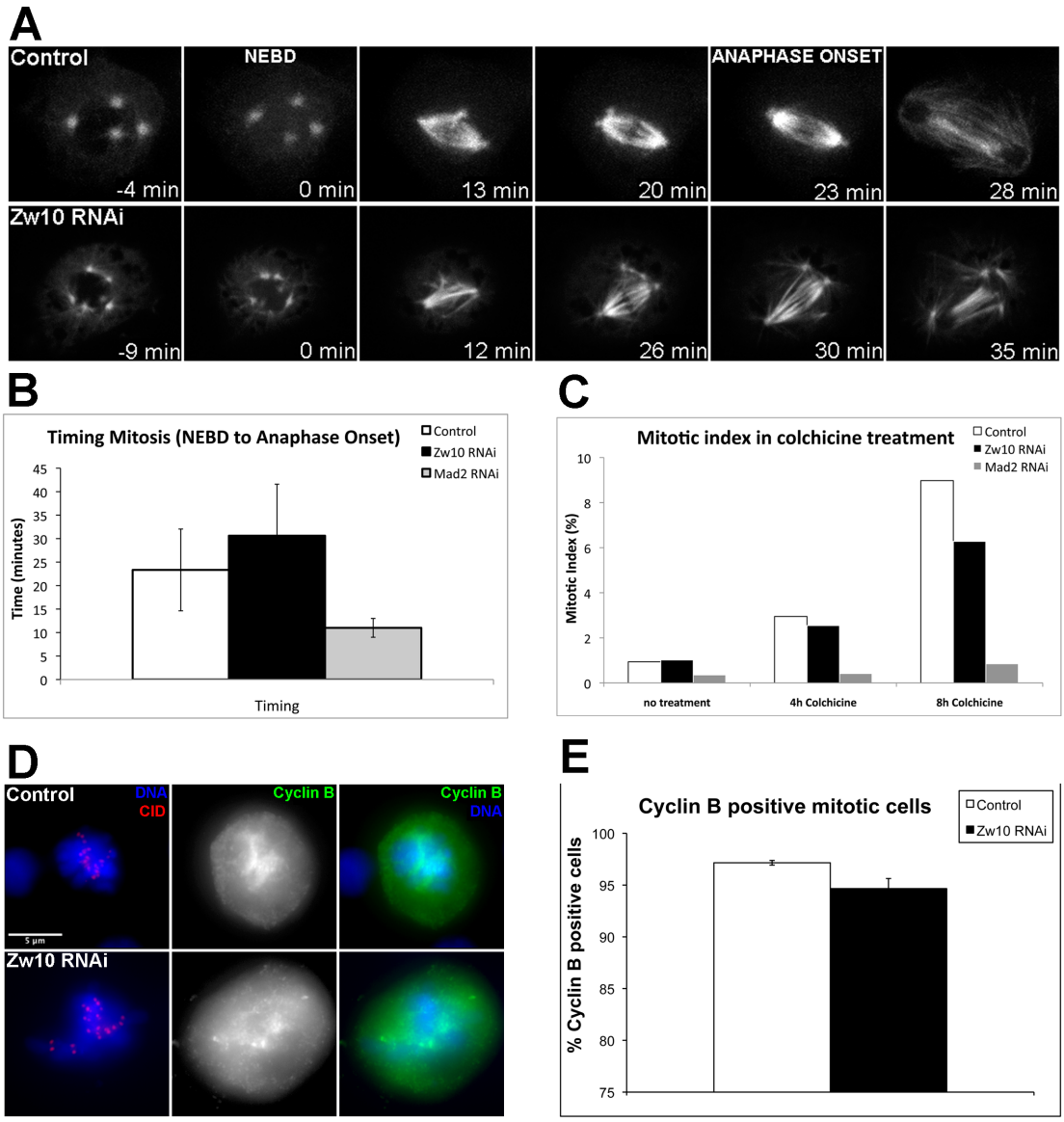


Figure 2.3 – Testing SAC activity in the absence of Zw10 (A) Selected frames from time-lapse movies (see Supplementary Movies 3 and 4) of either control or Zw10-depleted cells stably expressing GFP-Tubulin recorded every 30 seconds. Time=0 indicates NEBD. Cells were recorded until anaphase onset, characterized by rapid spindle elongation. Zw10-depleted cells display some microtubule-associated phenotypes but transit through mitosis with a regular mitotic timing. **(B)** Quantification of the time cells spend in mitosis (from NEBD to anaphase onset) reveals that in the absence of Zw10, cells take 31 ± 10min (n = 17 cells) to exit mitosis while control cells take 24 ± 8min (n = 28 cells). Results obtained from Mad2 depletion (13 ± 3min) in the same cell line used as control for loss of SAC function (Orr *et al.* 2007). **(C)** Control and Zw10-depleted cells were treated with 30μM of colchicine for 4hr or 8hr and then fixed and stained with antibodies against phospho-histone H3 and the resultant mitotic index was scored. Mitotic index in control cells was normalized to 1 (n= 5000 cells). Note that in shorter colchicine incubations (≤4hr), Zw10-depleted cells accumulate normally in mitosis in response to spindle damage. During longer incubation periods (≥8hr) the mitotic index scored was reduced by ~30%, when compared with control cells. **(D)** Cells treated with colchicine (≥8hr) were fixed and stained to reveal DNA (blue), Cyclin B

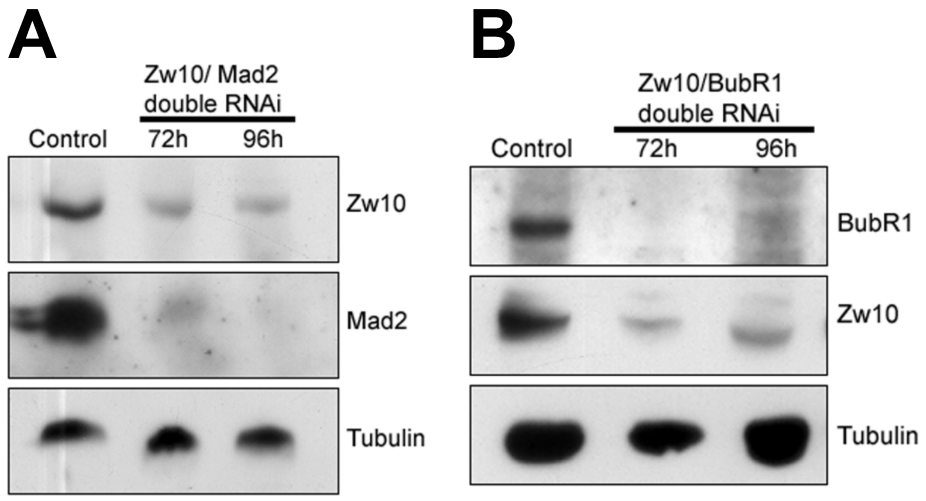
(green) and CID (red). Scale bar is 5 μm. **(E)** Quantification of the percentage of mitotic cells that stain positive for Cyclin B (≥8hr colchicine treatment), reveals that a similar proportion of control and Zw10-depleted cells display high levels of Cyclin B (n=150 mitotic cells).

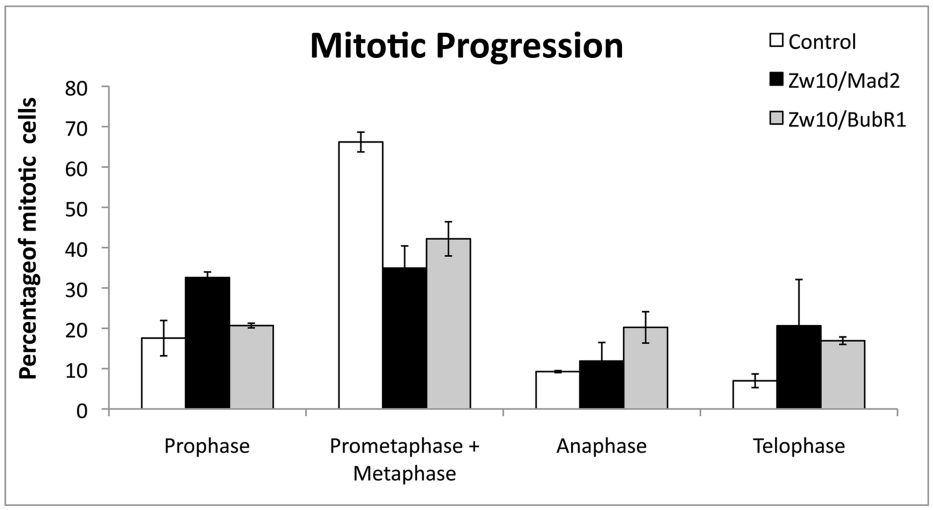
To determine the contribution of kinetochore Mad2 in SAC maintenance, cells depleted of Zw10 or Mad2 were challenged with microtubule poisons and the resultant mitotic index was scored (Figure 2.3C). We find that in shorter incubation times (≤4hr incubation), control and Zw10-depleted cells display similar mitotic index (Figure 2.3C). However, after longer periods of incubation in colchicine (≥8hr), Zw10-depleted cells display a 25-30% reduction in the number of mitotic cells suggesting a reduced efficiency in SAC maintenance (Figure 2.3C). To test whether depletion of Zw10 causes reduced SAC activity, we selected cells treated with colchicine for 8hr, and performed immunofluorescence analysis of fixed preparations stained for Cyclin B and CID (Figure 2.3D). Quantification of the percentage of mitotic cells staining positive for Cyclin B shows that 97% of control cells and 94% of Zw10-depleted cells stain positive for Cyclin B (Figure 2.3E). We find that this small difference does not account for the 25-30% reduction of mitotic cells observed in longer colchicine incubations (Figure 2.3C) suggesting that the efficiency of Cyclin B accumulation is unaffected. Taken together, we conclude that kinetochore Mad2 is dispensable for maintaining mitotic timing, however, it appears to be essential for cells to generate a prolonged mitotic arrest in response to spindle damage.

2.4 Cytoplasmic Mad2 is not sufficient for SAC maintenance in the absence of BubR1

Our data suggest that cytoplasmic Mad2 is sufficient to promote a normal mitotic timing and a weak SAC response to colchicine. To confirm whether the observed normal mitotic progression in the absence of Zw10 is dependent on cytoplasmic Mad2 we performed co-depletion of Zw10 and Mad2 (Zw10/Mad2) (Figure 2.4A). Additionally, to test whether the weak SAC response observed after Zw10 depletion was dependent on other SAC proteins such as BubR1, we co-depleted Zw10 and BubR1 (Zw10/BubR1) and analyzed SAC response in both Zw10/Mad2 and Zw10/BubR1 co-depleted cells (Figure 2.4B). The most efficient depletions were observed 96hr after the addition of the specific dsRNAs (Figure 2.4A, B) and therefore all of the following

analysis of Zw10 co-depleted cells was performed at this time-point. Fixed preparations were immunostained and cells were classified according to their mitotic phases (Figure 2.4C). The results show that both Zw10/Mad2 and Zw10/BubR1 RNAi treated cells show a significant decrease in the percentage of prometaphases and mostly accumulate at later stages of mitosis (Figure 2.4C), features consistent with loss of SAC function and accelerated mitotic timing. Consistently, after colchicine treatment, both Zw10/Mad2 and Zw10/BubR1 cells, fail to arrest in mitosis suggesting that the SAC is not functional under these conditions. The fast mitotic progression observed in the Zw10/Mad2 co-depletion confirms that cytoplasmic Mad2 is essential to prevent premature mitotic exit. Moreover, the data obtained from the Zw10/BubR1 double RNAi suggest that the slow mitotic progression and the weak SAC response observed in the absence of kinetochore-bound Mad2, is dependent on additional SAC proteins such as BubR1.


A
Zw10/ Mad2
double RNAi
Control
72h
96h
Zw10
Mad2
Tubulin
B
Zw10/BubR1
double RNAi
Control
72h
96h
BubR1
Zw10
Tubulin


C
Mitotic Progression
Control
Zw10/Mad2
Zw10/BubR1
Percentageof mitotic cells
80
70
60
50
40
30
20
10
0
Prophase
Prometaphase +
Metaphase
Anaphase
Telophase

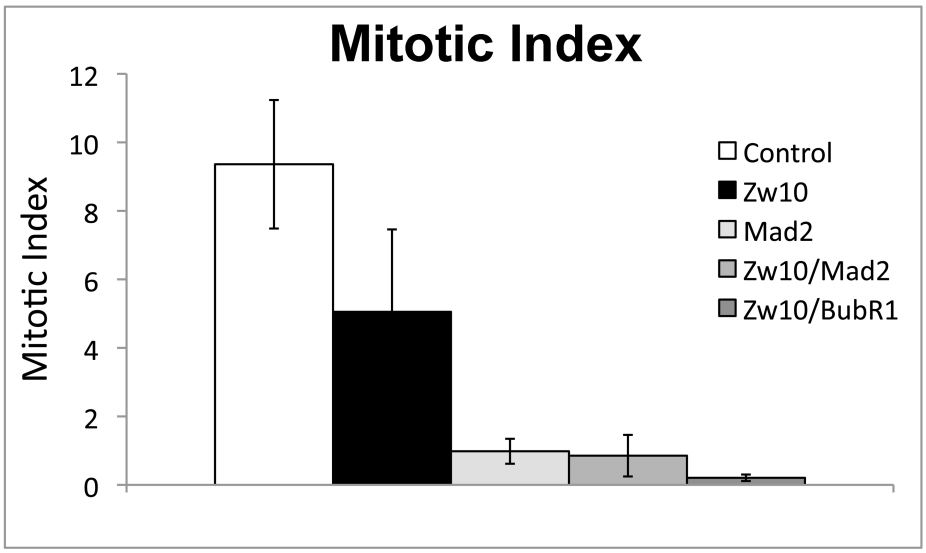
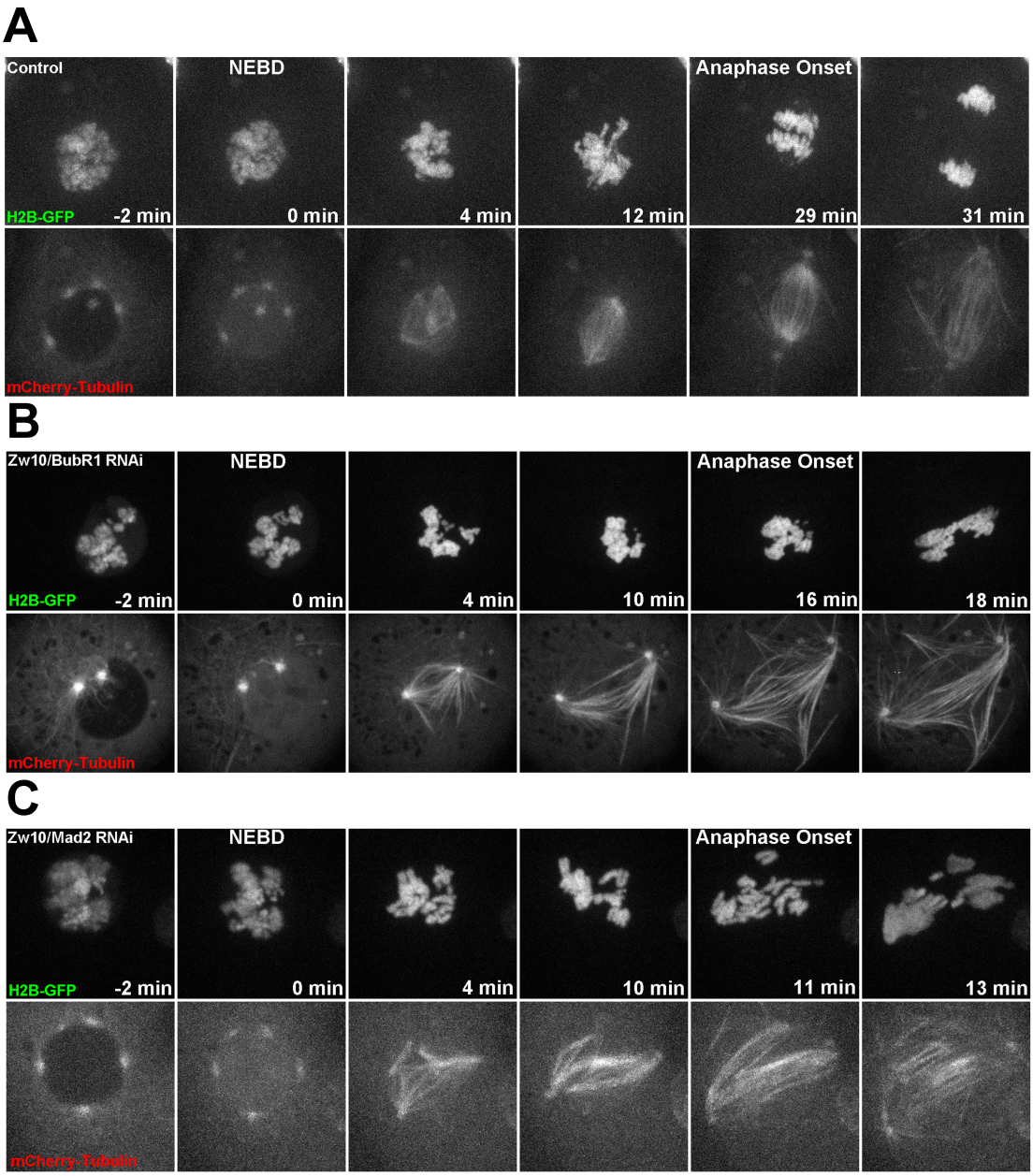

D
Mitotic Index
Mitotic Index
12
10
8
6
4
2
0
Control
Zw10
Mad2
Zw10/Mad2
Zw10/BubR1

Figure 2.4 – Performing Zw10 co-depletions with Mad2 or BubR1. Co-depletion experiments were designed to specifically target **(A)** Zw10 and Mad2 or **(B)** Zw10 and BubR1 for depletion in S2 cells. 10^6 cells (at 72hr and 96hr after the addition of the specific dsRNA) were processed for western blot analysis and probed using antibodies specific for **(A)** Zw10 and Mad2 or **(B)** Zw10 and BubR1. Below α-tubulin was used as a loading control. We find that in the case of Zw10/Mad2 and Zw10/BubR1 cells, the most efficient depletions were observed 96hr after the addition of the specific dsRNAs and therefore all of the following analyses were conducted at this time-point. **(C)** Control, Zw10/Mad2 and Zw10/BubR1 cells were grown for 96 hrs and then fixed and stained to reveal phospho-histone H3, α-tubulin and CID, allowing classification into distinct mitotic phases. **(D)** Cells depleted of Zw10, Mad2, Zw10/Mad2 or Zw10/BubR1 were treated with 30μM colchicine for 8hr and then fixed and stained to reveal phospho-histone H3 (PH3) and the resultant mitotic index was scored.

2.5 Cytoplasmic Mad2 acts together with BubR1 to time mitosis

Our results demonstrate that kinetochore Mad2 is dispensable for regulating mitotic timing but is required for a prolonged SAC response to spindle damage. Our data suggest that the contribution of cytoplasmic Mad2 *per se* is not sufficient for SAC maintenance, since cells co-depleted of Zw10 and BubR1 cells have a non-functional SAC. Therefore, to test whether Zw10/Mad2 or Zw10/BubR1 cells show an accelerated transit through mitosis, we used an S2 cell line stably expressing H2B-GFP; mCherry-Tubulin and performed *in vivo* time-lapse analysis of control (Figure 2.5A; Supplementary Movie 6), Zw10/BubR1 (Figure 2.5B; Supplementary Movie 7) and Zw10/Mad2 (Figure 2.5C; Supplementary Movie 8) cells. We find that Zw10/BubR1 cells display several of the Zw10-associated phenotypes and cells exit mitosis rapidly (16 ± 3min) even in the presence of misaligned or improperly attached chromosomes (Figure 2.5B, D). Similar phenotypes were observed in the Zw10/Mad2 cells (Figure 2.5C), although quantification of mitotic timing reveals that Zw10/Mad2 cells (14 ± 3min) transit through mitosis even faster than Zw10/BubR1 cells (Figure 2.5C, D). The time it takes for Zw10/Mad2 and Zw10/BubR1 cells to exit mitosis is highly reminiscent of what has been previously described for the single depletions of Mad2 (Orr *et al.* 2007) and BubR1 (Maia *et al.* 2007) respectively, in the same cell type. Altogether, the data suggest that cytoplasmic Mad2 plays an essential role in allowing time at early stages of mitosis, so that other SAC proteins, such as BubR1, can then relay the diffusible SAC signal through kinetochore-based APC/C inhibition.


A
Control
NEBD
Anaphase Onset
H2B-GFP
-2 min
0 min
4 min
12 min
29 min
31 min
mCherry-Tubulin
B
Zw10/BubR1 RNAi
NEBD
Anaphase Onset
H2B-GFP
-2 min
0 min
4 min
10 min
16 min
18 min
mCherry-Tubulin
C
Zw10/Mad2 RNAi
NEBD
Anaphase Onset
H2B-GFP
-2 min
0 min
4 min
10 min
11 min
13 min
mCherry-Tubulin

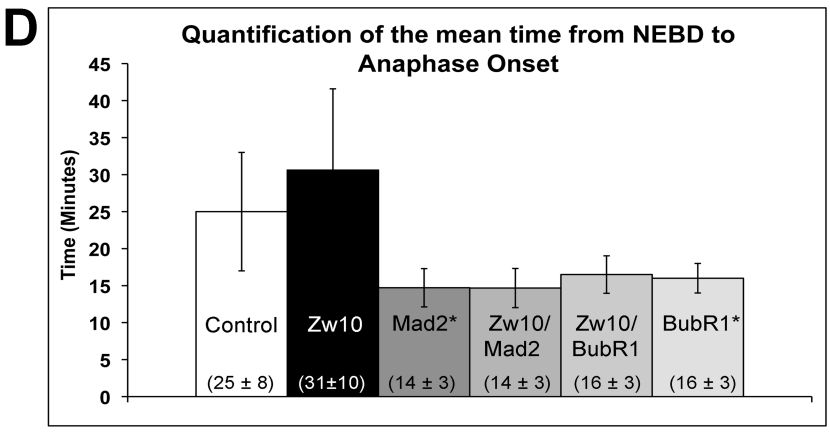

D
Quantification of the mean time from NEBD to Anaphase Onset
Time (Minutes)
45
40
35
30
25
20
15
10
5
0
Control
(25 ± 8)
Zw10
(31±10)
Mad2*
(14 ± 3)
Zw10/ Mad2
(14 ± 3)
Zw10/ BubR1
(16 ± 3)
BubR1*
(16 ± 3)

Figure 2.5 – Timing mitosis in Zw10/Mad2 or Zw10/BubR1 cells. Cells stably expressing H2B-GFP and mCherry-tubulin were harvested for 96hr and then visualized using time-lapse fluorescence microscopy. Images were collected at every 30s and selected frames from time-lapse movies (see Supplementary Movies 6 - 8) show mitotic progression in **(A)** control cells (Supplementary Movie 6), **(B)** cells depleted of Zw10 and BubR1 (Supplementary Movie 7) or **(C)** cells depleted of Zw10 and Mad2 (Supplementary Movie 8). **(A)** All control cells eventually achieved spindle bipolarity and faithful chromosome segregation was observed. Note that in the absence of **(B)** Zw10/BubR1 or **(C)** Zw10/Mad2, several spindle defects were observed and most cells displayed overall chromosome mis-segregation, and a fast mitotic schedule. **(D)** Quantification of the time cells spend in mitosis (from NEBD to anaphase onset) reveals that in the absence of Zw10/BubR1 (16 ± 3min; n = 14 cells) or Zw10/Mad2 (14 ± 3min; n = 19 cells) cells show an accelerated transit through mitosis consistent with lack of SAC activity. Asterisks represent results obtained from single depletions of Mad2 (Orr *et al.* 2007) or BubR1 (Maia *et al.* 2007) in the same cell line, included for comparison purposes.

3. Discussion

Although the precise role that the RZZ complex plays in SAC maintenance is still ambiguous (Basto *et al.* 2000; Chan *et al.* 2000; Savoian *et al.* 2000), it is clear that depletion of RZZ complex components leads to abnormal SAC response presumably due to mis-localization of the kinetochore-bound fraction of the Mad1/Mad2 complex (Buffin *et al.* 2005; Karess 2005; Kops *et al.* 2005a). Although the localization of Mad2 at kinetochores is Zw10-dependent, this is not the case for other SAC proteins such as Bub1 and Bub3 (Basu *et al.* 1998; Basu *et al.* 1999). This suggests that the requirement of Zw10 in SAC regulation specifically involves Mad1/Mad2 complex localization at unattached kinetochores. However, the RZZ complex has also been shown to target dynein/dynactin to kinetochores (Starr *et al.* 1998; Chan *et al.* 2000), which in turn has been shown to be essential for Mad1 and Mad2 stripping from kinetochores upon successful microtubule attachment (Howell *et al.* 2001; Wojcik *et al.* 2001) thereby silencing the SAC. Nevertheless, it is unlikely that the RZZ complex plays a direct role in SAC signaling during mitosis.

3.1 Zw10 is required for Mad2 recruitment to unattached kinetochores

Several studies have proposed that the RZZ complex specifically co-chaperones the Mad1/Mad2 complex to unattached kinetochores at early stages of mitosis (Buffin *et al.* 2005; Kops *et al.* 2005a). We show that *Drosophila* Zw10 is efficiently depleted 120h after the addition of the dsRNA and confirm that while Zw10 is required for the accumulation of Mad2 at unattached kinetochores, the localization of BubR1 is unperturbed. This experimental setup provides a method to specifically separate the functions of cytoplasmic Mad2 from that of kinetochore-bound Mad2, both in terms of SAC maintenance and mitotic timing.

3.2 Cytoplasmic Mad2 is essential for timing mitosis

SAC activation/maintenance is thought to be mediated by a signal continuously generated at unattached or improperly attached kinetochores during prometaphase (Rieder *et al.* 1995; Chan and Yen 2003). Studies in primary spermatocytes demonstrated that not only microtubule occupancy but also tension across kinetochore

pairs is required in order to satisfy the SAC (Nicklas *et al.* 1995). Mad2 and BubR1 are two essential SAC proteins that localize at kinetochores and have been proposed to contribute to SAC activity by monitoring microtubule attachment and tension, respectively (Logarinho *et al.* 2004). Despite localizing at kinetochores, Mad2 and BubR1 are found mostly at the cytoplasm, and together with Bub3 and Cdc20 form the MCC, a far more potent kinetochore-independent APC/C inhibitor (Sudakin *et al.* 2001; Tang *et al.* 2001; Sudakin and Yen 2004). Accordingly, in human cells depleted of Mad2 with disrupted kinetochores, sister chromatid separation follows very shortly after NEBD (Meraldi *et al.* 2004). However, if these kinetochore-deficient cells now contain cytoplasmic Mad2, prometaphase is extended significantly, although these cells still fail to accumulate in mitosis in response to spindle damage, suggesting a Mad2 kinetochore-independent role in timing mitotic progression (Meraldi *et al.* 2004).

In order to further study the contribution of cytoplasmic Mad2 in mitotic progression we used an experimental condition in which Mad2 was selectively prevented from accumulating at kinetochores without disrupting the kinetochore. We find that in the absence of Zw10, mitotic timing is normal and cells show a weak SAC activity in response to spindle damage. Our results suggest that the cytoplasmic pool of Mad2 is essential for maintaining mitotic timing and for providing a weak, yet significant SAC response to spindle damage. Results obtained from Mad2 depletion indicate that the transient mitotic arrest caused by cytoplasmic Mad2 is sufficient to allow accumulation other SAC proteins at kinetochores to the levels required for ensuring the observed SAC response, suggesting that Mad2 is a mitotic timer that participates in SAC activation at early stages of mitosis (Orr *et al.* 2007). Together with the results presented here, our data is consistent with a model in which the cytoplasmic pool of Mad2 is enough to restrain anaphase onset at early stages of mitosis. We propose that MCC-dependent APC/C inhibition is the first step of the “two-step” SAC activation model at early stages of mitosis when kinetochores are still assembling.

3.3 Cytoplasmic Mad2 acts together with BubR1 to time mitosis

We show that in the absence of kinetochore Mad2, cells are still able to generate a weak SAC response and transit through mitosis with a normal mitotic schedule. Nevertheless, it is widely accepted that SAC activation/maintenance takes place through multiple, converging pathways that often involve several proteins. The results obtained from co-depletion of Zw10 and Mad2 suggest that the normal mitotic timing observed in the absence of Zw10, is strictly dependent on the cytoplasmic pool of

Mad2 since Zw10/Mad2 cells display an accelerated transit through mitosis (similar timing to Mad2 RNAi cells) and are unable to accumulate in mitosis in response to spindle damage.

Interestingly, we show that cytoplasmic Mad2 is necessary for the regulation of mitotic timing, however, co-depletion of Zw10 and Mad2 causes mis-localization of Mad2 at kinetochores but also affects MCC complex formation. In these cells, both mitotic timing and the SAC response to microtubule poisons are severely affected, suggesting that cytoplasmic Mad2 is required but not sufficient, for a fully integrated SAC response. Furthermore, we find that in the absence of kinetochore Mad2, SAC maintenance is BubR1-dependent. However, given that in Zw10/BubR1 cells, MCC formation is impaired due to loss of BubR1, we cannot dissociate between the specific roles of cytoplasmic and kinetochore BubR1 in SAC activity. However, the data does not allow us to directly address whether the MCC *per se* is enough to sustain SAC activity, since we lack the molecular tools to specifically disrupt BubR1 at kinetochores without affecting MCC complex formation. However, the results presented here are consistent with a model in which BubR1 (either kinetochore or cytoplasmic) and cytoplasmic Mad2, act together to maintain mitotic timing and prolong SAC activity during prometaphase.

3.4 "Two-step" SAC maintenance model

The "two-step" model proposes that the SAC control is initially controlled by the MCC, a kinetochore-independent APC/C inhibitor that is present interphase before entry into mitosis (Sudakin *et al.* 2001; Tang *et al.* 2001). From prophase onwards, the MCC appears to be required for early stages of APC/C inhibition, while unaligned kinetochores appear to play a role in the second step by sensitizing the APC/C for MCC-mediated inhibition at later stages of mitotic progression (Chan *et al.* 2005). A further refinement of the model suggests that the second step is separated into two events: one at the NEBD when cytoplasmic Mad2 might extend prometaphase and provide enough time so that in a second event, SAC proteins such as BubR1 and Bub3 can fully engage checkpoint activity (Orr *et al.* 2007). This model proposes that Mad2 kinetochore localization is not required for the second step of SAC maintenance since Mad2 plays an important kinetochore-independent role in regulating mitotic timing. The data we present here is in full agreement with the "two-step" SAC activation model and we propose that the localization of Mad2 at kinetochores is dispensable for SAC maintenance during early stages of prometaphase. However, we find that the

localization of Mad2 at kinetochores is required for cells to ensure a prolonged mitotic arrest in response to spindle damage. In the context of the MCC complex, it appears that cytoplasmic Mad2 is required but not sufficient, for competent APC/C inhibition before kinetochores are fully assembled. We hypothesize that the time provided by the MCC at NEBD, allows for the proper localization of other SAC proteins at fully assembled kinetochores, which in turn is required for relaying the SAC signal responsible for prolonging the mitotic arrest in the presence of unaligned chromosomes. Moreover, our results are also consistent with a role for kinetochore-bound Mad2 in ensuring the sustainability required for a prolonged mitotic arrest.

Chapter 3

Drosophila CENP-C is essential for centromere identity

1. Introduction

The choice for centromere locus on chromosomal domains is an epigenetically defined process thought to promote kinetochore assembly during early mitosis. Centromeres can be highly variable in shape and size, and *Drosophila* chromosomes have been characterized as bearing large regional centromeres, located within non-conserved repeated DNA (reviewed in Cleveland *et al.* 2003; Przewloka and Glover 2009; Torras-Llort *et al.* 2009). Nevertheless, it has been recognized for some time that while centromeric DNA is not conserved either between different species or even between different chromosomes of the same species, the kinetochore which forms at the centromere and plays an essential role in chromosome segregation and cell cycle progression, is likely to be conserved (Sunkel and Coelho 1995; Joglekar *et al.* 2009). Eukaryotic centromeres are characterized by the presence of the centromere-specific histone H3 variant originally identified in humans as CENP-A, which is necessary to maintain both centromere identity and kinetochore organization (Earnshaw and Migeon 1985; Van Hooser *et al.* 2001; Heun *et al.* 2006). Recent data suggests that neocentromere formation requires CID (*Drosophila* CENP-A homologue) accumulation (Heun *et al.* 2006), consistent with a role for CID in determining centromere identity and thus lay the foundation for the kinetochore (Carroll and Straight 2006). However, CID inactivation in *Drosophila* has been shown to cause mis-localization of kinetochore components, accompanied by a cytoplasmic BubR1-dependent mitotic delay, which suggests that centromere inactivation is compatible with a functional SAC (Blower *et al.* 2006). Consistently, CENP-A inactivation in chicken cells has been shown to cause a mitotic delay in the absence of proper kinetochore assembly (Regnier *et al.* 2005) while in human cells, CENP-A overexpression is not sufficient to drive full kinetochore assembly (Van Hooser *et al.* 2001).

Functional centromeres are also characterized by the presence of CENP-C, identified as a human autoantigen that localizes to the inner kinetochore plate (Earnshaw and Rothfield 1985; Saitoh *et al.* 1992) and shown to bind alpha-satellite DNA (Yang *et al.* 1996; Politi *et al.* 2002; Trazzi *et al.* 2002). Unlike CENP-A, CENP-C has been reported to be partly dispensable for kinetochore assembly in both budding yeast and human cells (Saitoh *et al.* 1992; Ando *et al.* 2002; Westermann *et al.* 2003; Foltz *et al.* 2006). Despite this, CENP-C inactivation is thought to severely compromise kinetochore function. In mammalian tissue culture cells depletion of CENP-C by RNA interference (RNAi) or inactivation by microinjection of specific antibodies causes mitotic delay, chromosome missegregation, aneuploidy and apoptosis (Tomkiel *et al.* 1994;

Fukagawa and Brown 1997; Fukagawa *et al.* 1999). In chicken cells, the extensive prometaphase delay observed after depletion of CENP-C, occurs despite having weak signal for SAC protein Mad2 but not BubR1 at kinetochores (Kwon *et al.* 2007). In *C.elegans* loss of HCP-4 (CENP-C homologue) results in failure of sister centromere resolution and affects kinetochore geometry (Moore and Roth 2001; Moore *et al.* 2005) while in fission yeast CENP-C has been shown to serve as a scaffold for the recruitment of specific factors essential for kinetochore construction (Tanaka *et al.* 2009). Although CENP-C roles in kinetochore assembly are conserved amongst several systems, CENP-C inactivation in *C.elegans* has been shown to cause the most severe kinetochore phenotypes (Oegema *et al.* 2001; Cheeseman *et al.* 2004). In agreement, recent results have shown that in *Drosophila*, CENP-C appears to also play a fundamental role in kinetochore organization (Przewloka *et al.* 2007; Schittenhelm *et al.* 2007; Erhardt *et al.* 2008).

The overall kinetochore assembly pathway in different cell types suggests that kinetochore and centromere structure may vary from one organism to another and that the exact protein composition of kinetochores may to some extent be species specific. For example it has been shown that in human cells a large number of proteins included in the Constitutive Centromere-Associated Network (CCAN) complex that are involved in the organization of the inner kinetochore layers (reviewed in Cheeseman and Desai 2008; Przewloka and Glover 2009), have thus far not been identified in *Drosophila* (Goshima *et al.* 2007). It appears that the protein composition of the *Drosophila* centromere-kinetochore interface is simpler than that of vertebrate cells as the only inner centromere proteins identified thus far are CID and CENP-C (Moroi *et al.* 1980; Earnshaw and Rothfield 1985; Cheeseman and Desai 2008; Przewloka and Glover 2009), making *Drosophila* an excellent model organism to study centromere function.

Given the discrepancies in the results obtained in different systems we analyzed the role of CENP-C in centromere and kinetochore organization and mitotic progression in *Drosophila* by depleting CENP-C in S2 cells. We find that depletion of CENP-C strongly affects kinetochore organization and function resulting in a true kinetochore-null phenotype. More importantly, we observed that CENP-C depletion causes loss of centromere identity in mitosis. We suggest that in *Drosophila*, CENP-C plays an essential role in maintaining centromere identity, which is required to form the platform onto which the kinetochore modules involved in microtubule attachment and SAC signalling may then be assembled.

2. Results

2.1 CENP-C is required for CID localization

BLAST searches and amino acid sequence alignments show that CENP-C is highly conserved amongst several *Drosophila* species, yet when compared to higher eukaryotes, there is little homology both in terms of protein size and amino acid conservation (Figure 3.1A, B).

A

Species	Amino Acid number	Amino Acid Sequence
Mus Musculus	698	-------PNV RRSNRIRLKP LEYWRGERVD YQESSSGQLV LEIISPSSV-
Monodelphis domestica	735	-------LPV RRTTRIKIKP LEYWRGERFD YENMLLDGFT VSGTISNDRV
Rattus novegicus	709	-------PNV RRSNRIRLKP LEYWRGERID YQESSSGRLV LEIVSPASE-
Homo Sapiens	735	-------PNV RRTKRTRLKP LEYWRGERID YQGRPSGGFV ISGVLSPDTI
Branchiostoma floristae	3	-------PGV RRSRRNRIRP LAWWEHERVV YERRNSGRVM VDVQKPEIKD
Branchiostoma floristae	894	-------PGV RRSRRNRIRP LAWWEHERVV YERRNSGRVM VDVQKPEIKD
Lottia gigantea	1192	iypapapDGL RRSSRVRVQR LEGYKNERII YDRRRKSGVH VLAIQPSNEa
Cannis familiaris	737	-------PNV RRTKRTRLKP LEYWRGERID YHGRPSGGFV IGGILSPDTV
Strongylocentrotus purpuratus	1204	-------DGV RRTRRQRVRP LEYWRNERPL YERRKSGGLA LAGIISPgap
Gallus gallus	653	-------PNV RRTKRIRLKP LEYWRGERVT YTLKPSGRLL ISGIagaeae
Drosophila Melanogaster	1094	-------TGI RRSKRGQVPL QMSWCHtmd- ----PSKFNF MSGFIEPRSK

B

Species	Amino Acid number	Amino Acid Sequence
Mus Musculus	866	-ETPYKLTTG DSFYVPSGNH YNIKNLLNVE SSLLFTQIKR ----------
Monodelphis domestica	898	-ETNYNLNSG DTFYVPSGNF YNIKNLIDER SILLFTQIKt ----------
Rattus novegicus	877	-ETPYMLTTG DSFYVPSGNH YNIKNLLNVE SCLLFTQIKR ----------
Homo Sapiens	905	-ETPYILSTG DSFYVPSGNY YNIKNLRNEE SVLLFTQIKR ----------
Branchiostoma floristae	180	-RSTVMMQSG DMFLIPAGNT YNIKNLRKDR ARLCFCQIKP SAQ-------
Branchiostoma floristae	1077	-RSTVMMQSG DMFLIPAGNT YNIKNLRKDR ARLCFCQIKP SAQ-------
Lottia gigantea	1769	-RKVSVLETG DVFFVPSGAV YKLKNLRNEE AKLfymnivr vedseseret
Cannis familiaris	907	-ETSYIITAG DSFYVPSGKY VSIWIILNIQ NSvlh----- ----------
Strongylocentrotus purpuratus	1377	-ETSQILQSG DWFFVPKGNV YNITNLRRDE AKLTFFQLKS S---------
Gallus gallus	812	-KTSYYLTSG DYFYVPAGNG YNIRNLLNEE SVLHFTQLkn drapvgaelc
Drosophila Melanogaster	1374	kEVHSVLRVG DMIEIDRGTR YSIQNAIDKV SVLMcirs-- ----------

Figure 3.1 - CENP-C is poorly conserved in *Drosophila*. Amino acid sequence alignment of CENP-C from various species shows that the *Drosophila* protein bears very little homology with CENP-C protein in other species. **(A, B)** The two most conserved domains of CENP-C fail to show significant homology with the *Drosophila* protein. Note that the *Drosophila* CENP-C protein is also significantly larger than in other species.

Therefore, to explore the role of *Drosophila* CENP-C in kinetochore assembly we depleted CENP-C by performing double-stranded RNA (dsRNA) interference in *Drosophila* S2 cells (Figure 3.2). We find that CENP-C is partially depleted 48hr after the addition of the specific double stranded RNA (dsRNA) but after 96hr more than 99% of the protein is absent as can be seen both by immunofluorescence and western blot analysis (Figure 3.2A, B). Interestingly, depletion of CENP-C has a dramatic effect upon the localization of CID (Figure 3.2A and C). Quantitative immunofluorescence reveals that at 48h after the addition of the dsRNA, CID and CENP-C levels are reduced in most kinetochores, however by 96hr CID levels are strongly reduced at all kinetochores and the amount of CENP-C was found to be below detectable levels (Figure 3.2D, E). Moreover, western blot analysis of total protein extracts from CENP-C depleted cells shows that total CID protein levels were unaffected (Figure 3.2B) suggesting a failure in CID loading/maintenance rather than a down-regulation in CID gene expression or degradation of the protein. We find that the amount of detectable CID correlates linearly with the amount of detectable CENP-C at both 48h and 96h after the addition of the specific dsRNA (Figure 3.2D, E). At 96hr most kinetochores with low CID levels either have none or display very low CENP-C levels (Figure 3.2E) and therefore all further analysis was conducted at this time point using both CID and CENP-C antibodies as markers to identify CENP-C depleted kinetochores. It has been previously shown that CID localization at centromeres is partly CENP-C-dependent, although it was also reported that other proteins such as Cal1 and RCA1 also play a role in this process (Erhardt *et al.* 2008). Although we have not explored the roles of Cal1 and RCA1 in this process, quantification of CENP-C/CID levels at individual kinetochores (Figure 3.2D, E) shows a clear correlation between the amount of detectable CENP-C and CID at each kinetochore, suggesting that in *Drosophila*, there is a clear dependency between CID localization/maintenance and the levels of CENP-C at kinetochores.

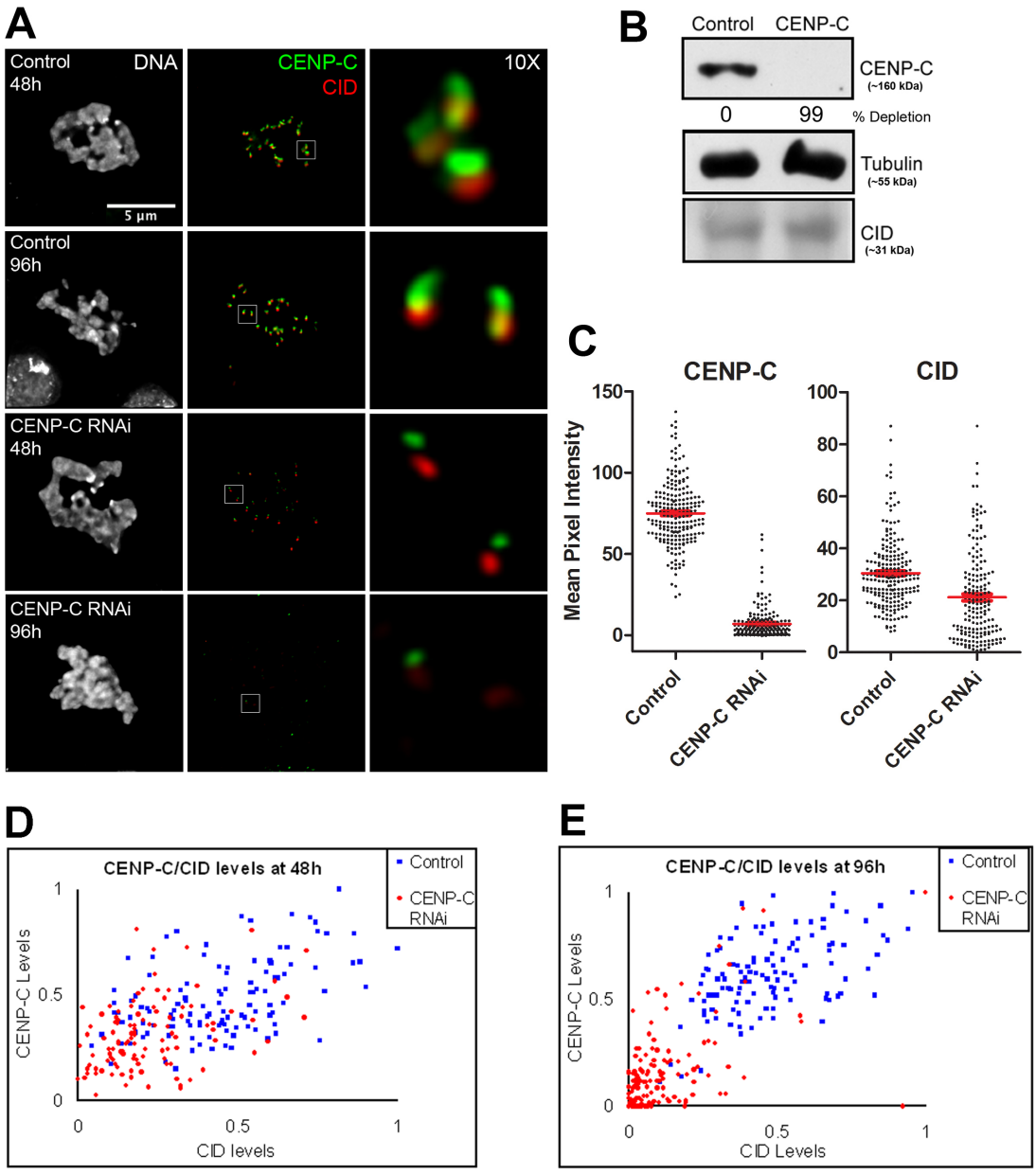


Figure 3.2 - CENP-C is required for CID localization at centromeres. (A) Control and CENP-C RNAi treated S2 *Drosophila* cells were incubated in MG132 (2hr) and Colchicine (1hr further) prior to fixation and immunofluorescence staining to show DNA (grey), CENP-C (green) and CID (red). Efficient CENP-C and CID depletion observed 96hr after the addition of specific CENP-C dsRNA. 10X magnifications of selected regions are shown on the right. **(B)** Total protein extracts from 10^6 cells were separated by SDS-PAGE and the protein levels of CENP-C and CID were monitored using western blotting techniques. Quantification of CENP-C depletion levels from total protein extracts was performed by densitometric analysis using ImageJ software and α-tubulin was used as a loading control. Quantification of the mean pixel intensity of **(C)** CENP-C and CID levels at kinetochores at 96hr using immunofluorescence images shown in **(A)** where each dot represents an individual kinetochore (n= more than 200 kinetochores from 15 different cells). The red lines in **(C)** represent the mean average of all quantified kinetochores. Relative levels of CID and CENP-C were plotted to determine the relationship between both proteins at **(D)** 48hr and **(E)** 96hr after the

addition of the dsRNA. Dots represent individual kinetochores and all quantifications were performed using Image J software with a previously defined ROI.

2.2 CENP-C depletion causes a kinetochore-null phenotype

Previous reports have shown that CENP-C inactivation causes mitotic delay during prometaphase, chromosome missegregation, aneuploidy and apoptosis (Tomkiel *et al.* 1994; Fukagawa and Brown 1997; Fukagawa *et al.* 1999). However, in chicken DT40 cells, the extensive prometaphase delay observed after CENP-C depletion, occurs despite having weak signals for Mad2 but not BubR1 at kinetochores (Kwon *et al.* 2007) suggesting that overall kinetochore assembly is not fully dependent on CENP-C. This is controversial since contradicting reports in human cells demonstrate that Mad1, Mad2, Bub1, BubR1 and microtubule motor protein CENP-E all depend on CENP-C for proper kinetochore localization (Liu *et al.* 2006), suggesting that the role of CENP-C in kinetochore structure may be species-specific. Therefore, to determine the role of CENP-C on the organization of the *Drosophila* kinetochore, we stained CENP-C depleted cells for the presence of SAC proteins that normally accumulate at kinetochores in the absence of microtubules. Control and CENP-C depleted cells were prevented from exiting mitosis in a checkpoint-independent manner (treated with MG132) and spindle microtubules were depolymerised before fixing and staining. We find that in control cells, SAC proteins are enriched at kinetochores in the absence of microtubules, but in the absence of CENP-C none of the SAC proteins Mad2, Bub1, BubR1 and Bub3, the mitotic regulator Polo, or the microtubule-associated motor protein CENP-meta (*Drosophila* CENP-E homologue) are able to localize to kinetochores (Figure 3.3A-D and Figure 3.4 for quantification of intensity levels). However, in the case of CENP-meta, while levels at kinetochores drop by 85% (Figure 3.4A, B) we observe a strong accumulation of this protein at centrosomes as can be seen by the co-localization with γ-tubulin (Figure 3.3C). In control cells, CENP-meta is strongly associated with unattached kinetochores and yet has a weak, low-turnover association with centrosomes during mitosis (Maffini *et al.* 2009). The observed centrosome enrichment of CENP-meta might reflect the requirement of a functional centrosomal-kinetochore linkage for CENP-meta dissociation from centrosomes to kinetochores along spindle microtubules during prometaphase.

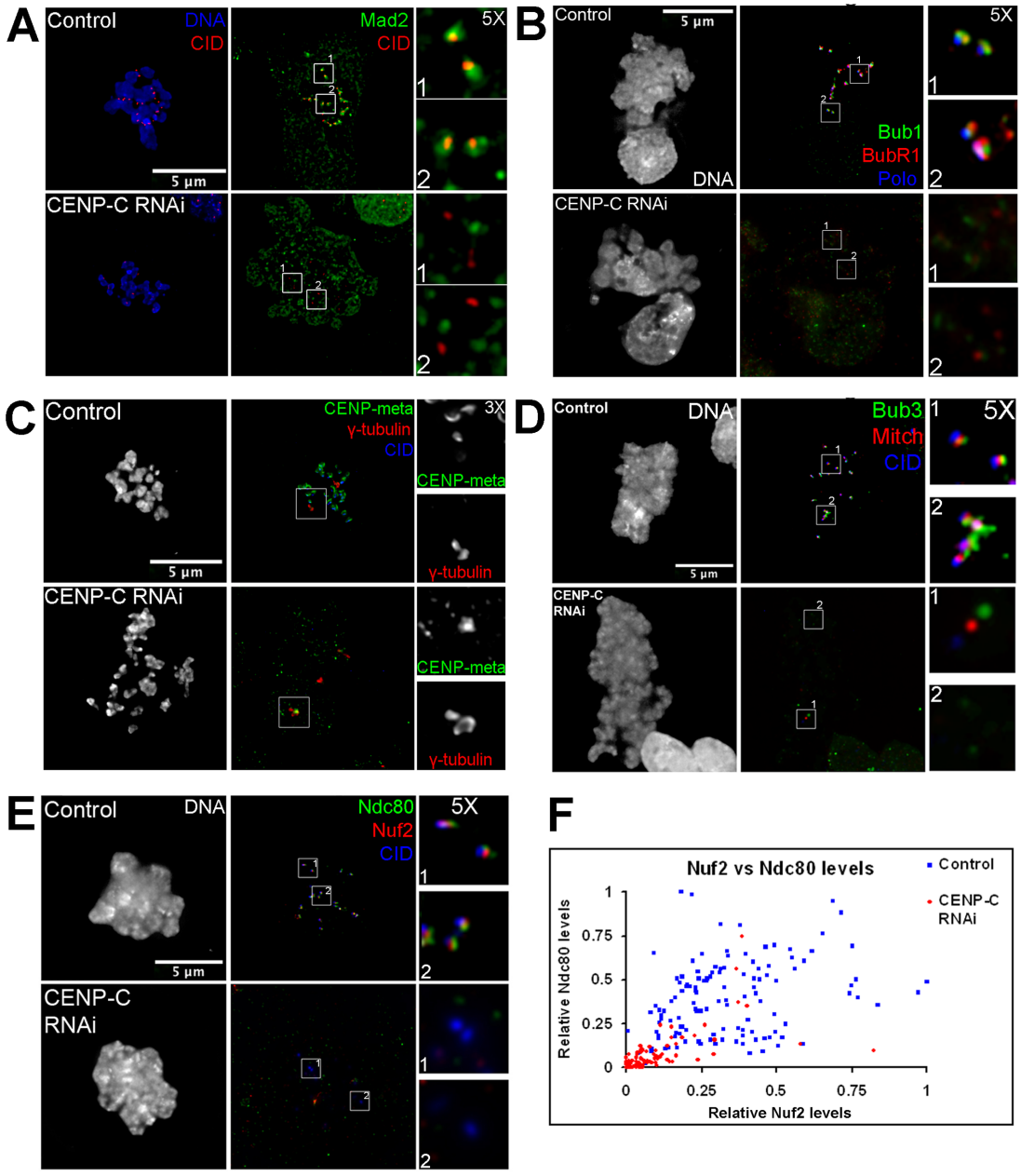


Figure 3.3 - CENP-C disruption causes a kinetochore-null phenotype. Control or CENP-C RNAi treated S2 *Drosophila* cells were incubated in MG132 (2hr) and Colchicine (1hr further) prior to fixation and immunofluorescence staining for all co-localization experiments. **(A)** Immunofluorescence staining to reveal DNA (blue), Mad2 (green) and CID (red). **(B)** Immunofluorescence staining to reveal DNA (grey), Polo kinase (blue), and SAC proteins Bub1 (green) and BubR1 (red). For immunofluorescence panels **(A)** and **(B)** insets 1 and 2 represent 5X magnifications of selected kinetochore regions. **(C)** Immunofluorescence staining to reveal DNA (grey), CID (blue), microtubule motor protein CENP-meta (green) and γ-tubulin (red); insets 1 and 2 represent 3X magnifications of selected γ-tubulin positive regions. **(D)** Immunofluorescence staining to reveal DNA (grey), CID (blue), SAC protein Bub3 (green) and Ndc80 complex component Mitch/Spc25 (red); insets correspond to 5X magnifications of selected kinetochore regions shown in merged images. **(E)** Cells

incubated in MG132 (2hr) and Colchicine for (1hr further) prior to fixation were immunostained to reveal DNA (grey), CID (blue), Ndc80 (green) and Nuf2 (red); insets 1 and 2 represent 5X magnifications of selected kinetochore regions. **(F)** For each individual kinetochore, relative levels of Ndc80 and Nuf2 were plotted against each other in both control and CENP-C depleted cells showing that both proteins maintain a linear dependency upon CENP-C levels. Ndc80 and Nuf2 quantifications performed using Image J software with a previously defined ROI.

The Ndc80 complex has the ability to bind microtubules *in vitro* (Wei *et al.* 2007; Cheeseman and Desai 2008) and previous studies have shown that the KNL-1/Mis12/Ndc80 (KMN) complex localization is independent of CENP-A and CENP-C in human cells (Goshima *et al.* 2003; Fujita *et al.* 2007) but not in *Drosophila* (Przewloka *et al.* 2007). However, this is a controversial issue since reports in various systems have shown that KMN localization to be dependent on CENP-C (Liu *et al.* 2006; Kwon *et al.* 2007; Milks *et al.* 2009). To determine whether CENP-C is required for the kinetochore localization of KMN proteins, CENP-C was depleted as previous described and cells were fixed and stained to reveal Ndc80 complex proteins (Figure 3.3D, E). In control cells we find that both Ndc80 and Nuf2 localize to the outer kinetochore when compared to the centromeric CID staining (Figure 3.3E) while in the absence of CENP-C, kinetochores failed to accumulate significant levels of either Nuf2 or Ndc80 since their levels drop by 75% and 83% respectively (Figure 3.3E; Figure 3.4C-E). Similar results were obtained for the localization of Mitch (Spc25 subunit of Ndc80 complex) (Figure 3.3D) and Mis12, a central component of the Mis12 complex (unpublished data), suggesting that in *Drosophila,* KMN localization is fully dependent on CENP-C for proper targeting to kinetochores (Tomkiel *et al.* 1994; Fukagawa and Brown 1997; Fukagawa *et al.* 1999; Goshima *et al.* 2003; Fujita *et al.* 2007; Kwon *et al.* 2007; Tanaka *et al.* 2009).

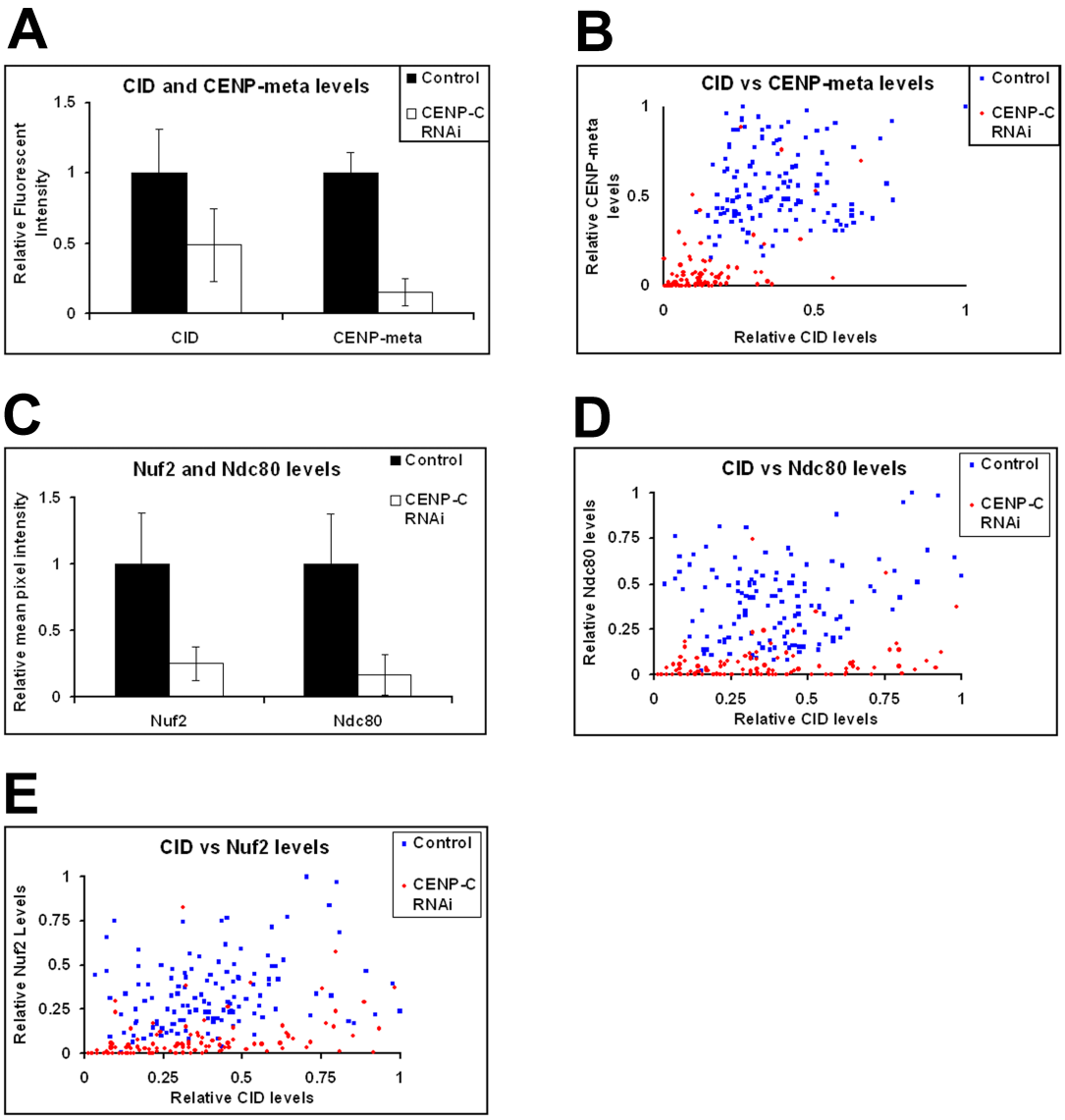


Figure 3.4 - CENP-C is required for kinetochore organization. (A) Quantification of the mean pixel intensity of CID and CENP-meta levels at kinetochores of control and CENP-C depleted cells obtained from cells shown in (Fig. 2C). Control levels of CID and CENP-meta have been normalized to 1 (n= more than 200 kinetochores from 15 different cells). **(B)** Relative levels of CID versus CENP-meta were plotted to determine the relationship between both proteins 96hr after the addition of the dsRNA, where each dot represents one kinetochore. Note that there is a linear relationship between CENP-meta and CID levels. **(C)** Quantification of the relative mean pixel intensity of Nuf2 and Ndc80 levels at kinetochores of both control and CENP-C depleted cells obtained from cells shown in (Fig. 2E). Control levels of Nuf2 and Ndc80 have been normalized to 1 (n=more than 150 kinetochores from 15 different cells). Relative levels of CID were plotted against **(D)** Ndc80 or **(E)** Nuf2 where each dot represents a single kinetochore. Note that there is a population of kinetochores with high levels of CID and low levels of Ndc80 and Nuf2 suggesting that Ndc80 complex mis-localization at kinetochores is specific to CENP-C depletion. All quantifications were performed using Image J software with a previously defined ROI spanning the entire kinetochore region.

2.3 Kinetochore-null chromosomes fail to interact with microtubules

Our data is consistent with a model in which *Drosophila* CENP-C plays an essential role in kinetochore organization and expectedly also on kinetochore-microtubule attachments. Therefore to address whether are any interactions between chromosomes and the mitotic spindle in the absence of functional kinetochores, cells were prevented from exiting mitosis using MG132 and treated with Taxol to promote spindle collapse into a monopolar structure (see Materials and Methods). This MG132-Taxol assay allows an easy readout of kinetochore-microtubule attachment and orientation at the time of spindle collapse. Chromosomes that are strongly attached maintain their attachment after spindle collapse and localize around the periphery of the monoaster (Figure 3.5A). In untreated cells at the time of spindle collapse all chromosomes localize around the monopolar spindle and quantification of kinetochore-microtubule attachment shows that only 3% of kinetochores were found to be unattached (Figure 3.5A, B). However, in the absence of CENP-C, 71% of detectable kinetochores (as shown by residual CID staining) were found to be unattached and in most cases chromosomes were scattered and failed to align at the periphery of the monoaster. Consistently, these scattered chromosomes had very little or no detectable CENP-C staining (Figure 3.5A, B). To further test whether there are any interactions between chromosomes and microtubules, control and CENP-C depleted cells were prevented from exiting mitosis and chromosome congression was scored (Figure 3.5C, D). We find that even after long periods of SAC-independent mitotic arrest, chromosome congression was not achieved in CENP-C depleted cells. Furthermore and consistent with the lack of observable metaphases, we found no cold-stable microtubule fibres interacting with chromosomes in the absence of CENP-C (Figure 3.5C).

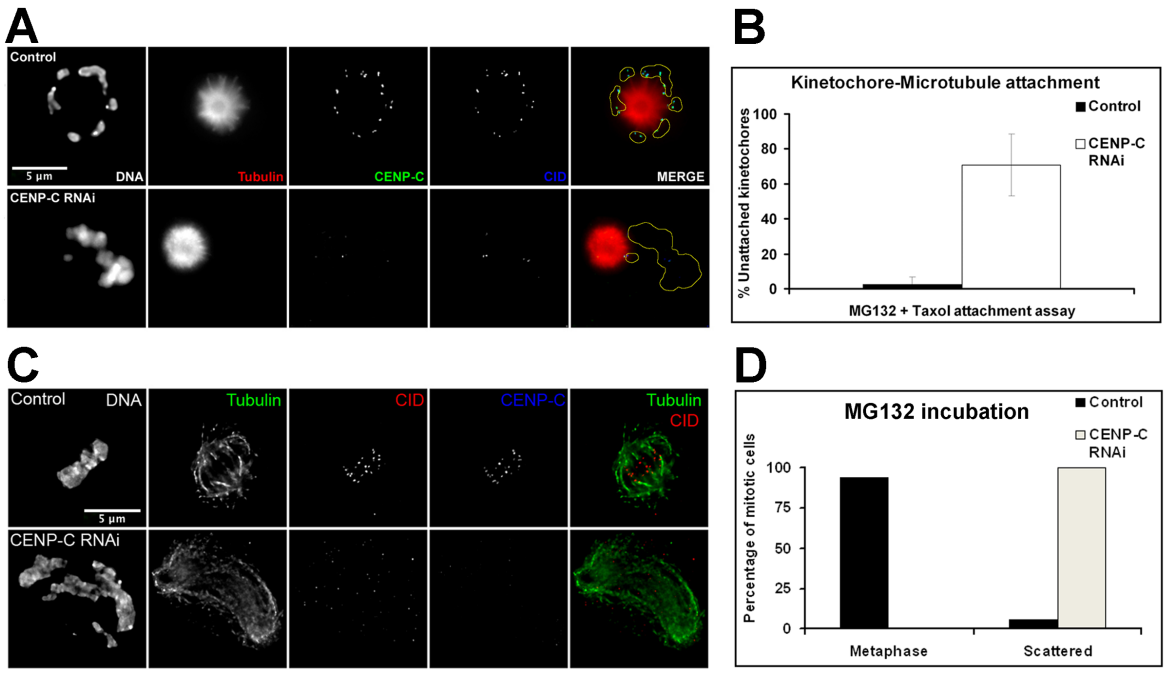


Figure 3.5 – Kinetochore-null chromosomes fail to interact with microtubules. (A) Immunofluorescence images showing DNA (shown in grey in single channels and represented as yellow contours on merged images), α-tubulin (red), CENP-C (green) and CID (blue) of either control or CENP-C depleted cells after treatment with MG132+Taxol (kinetochore-microtubule interaction assay: see materials and methods). In control cells, kinetochores are arranged around the periphery of the monoaster suggesting that kinetochore-microtubule interaction was of high affinity at the time of spindle collapse. Note that in CENP-C depleted cells chromosomes were scattered and failed to align at the periphery of the monoaster consistent with loss of kinetochore-microtubule interactions. **(B)** Quantification of the percentage of unattached kinetochores in both control and CENP-C depleted cells obtained from cells subjected to the MG132 + Taxol kinetochore-microtubule interaction assay depicted in **(A)**. **(C)** Control and CENP-C depleted cells were treated with MG132 (2hr) to block mitotic exit and then placed on ice for 12min prior to fixation to reveal only cold-stable microtubules. Immunofluorescence shows DNA (grey), α-tubulin (green), CID (red) and CENP-C (blue). **(D)** Quantification of the percentage of mitotic cells in both control and CENP-C depleted cells which have chromosomes arranged in metaphase or scattered configurations (obtained from images shown in **(C)**) shows that in the absence of CENP-C, microtubules do not interact with chromosomes.

2.4 CENP-C is required for SAC maintenance and regulation of spindle length

Our results show that loss of CENP-C causes a kinetochore-null phenotype as can be seen by the absence of essential kinetochore proteins involved in kinetochore-microtubule binding and SAC activity, even in the absence of microtubules. We

hypothesize that under these conditions cells should rapidly exit mitosis with severe metaphase alignment defects due to loss of SAC activity and impaired kinetochore-microtubule interactions. However, previous reports on CENP-C inactivation have shown that loss of CENP-C causes a strong mitotic delay (Tomkiel *et al.* 1994; Fukagawa and Brown 1997; Fukagawa *et al.* 1999; Kwon *et al.* 2007) and that kinetochore-microtubule interactions appear to be unaffected under these conditions (Kwon *et al.* 2007). Therefore, to explore the mitotic relevance of a kinetochore-null phenotype, control and RNAi treated cells were fixed, stained and classified according to their distinct mitotic phases. Mitotic cells were identified as being anti-Phospho-Histone H3 (PH3) positive, and immunolocalization of α-tubulin, CID and DNA were used to define successive mitotic stages. Overall quantification in fixed cells suggests that CENP-C depleted cells undergo normal prophase, spend little time in prometaphase, none in metaphase and accumulate during telophase (Figure 3.6A). Analysis of DNA content by Fluorescence-Activated Cell Sorting (FACS) shows that by 48hr cells become progressively more aneuploid and by 96hr after the addition of the dsRNA cells display a highly variable DNA content and FACS profiles consistent with an S-phase delay (Figure 3.6B). The data suggest that CENP-C is required for normal mitotic progression as can be seen by the low percentage of cells in prometaphase and the absence of metaphases.

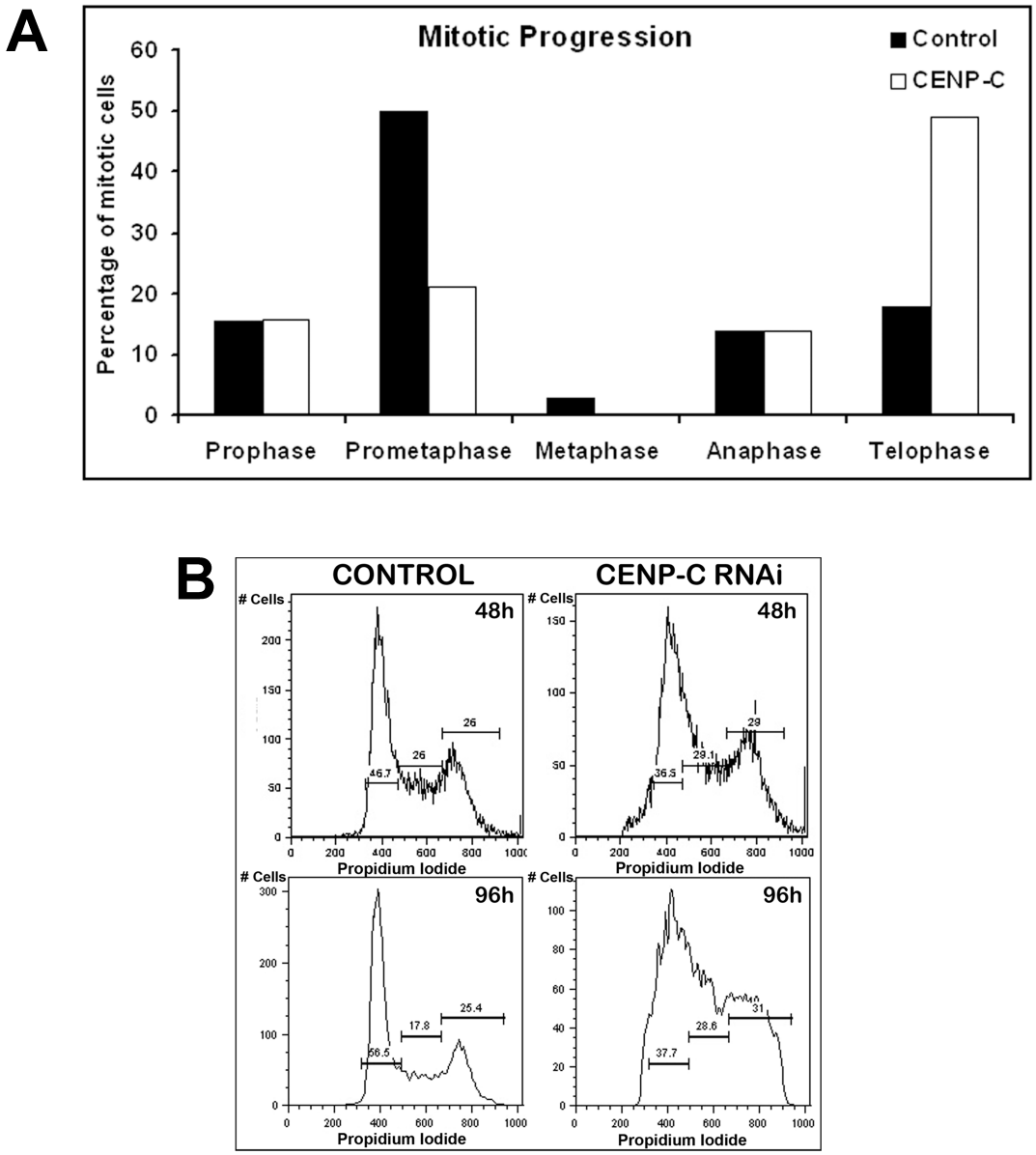


Figure 3.6 - CENP-C depletion causes failure in chromosome congression and aneuploidy. (A) Control and CENP-C RNAi treated S2 cells were grown for 96 hrs and then fixed and stained to reveal phospho-histone H3, α-tubulin and CID allowing classification into distinct mitotic phases. The quantification of the different mitotic phases shows that in the absence of CENP-C cells spend little time in prometaphase, none in metaphase but accumulate at telophase. **(B)** Analysis of DNA content by Fluorescence Activated Cell Sorting (FACS) at 48h and 96h after the addition of the dsRNA and results were analysed using Cell Quest data acquisition software.

Further characterization of the kinetochore-null phenotype induced by CENP-C depletion was performed *in vivo* using cells stably expressing H2B-GFP and mCherry-α-tubulin, that were followed by time-lapse confocal microscopy (Figure 3.7A,

Supplementary Movies 9 - 11). We find that in the absence of CENP-C, cells exhibit multiple mitotic errors including mis-regulated spindle organization and consequent failure in chromosome alignment and segregation. Additionally, sister chromatids were found to separate soon after NEBD and quantification revealed that in the absence of CENP-C the time from NEBD to anaphase onset was significantly shortened (12 ± 3min; Supplementary Movies 10 and 11) when compared with control cells (34 ± 11min) (Figure 3.7B; Supplementary Movie 9). The time these CENP-C depleted cells take from NEBD to anaphase onset is highly reminiscent of what has been previously described when Mad2 was depleted in the same cell type (Orr *et al.* 2007) suggesting that in the absence of CENP-C, APC/C activation takes place soon after NEBD. CENP-C depleted cells were unable to achieve metaphase chromosome configurations and rapidly exited mitosis in the presence of improperly attached chromosomes. Accordingly, when we challenged these cells with microtubule poisons and scored the resultant mitotic index we find that in the absence of CENP-C, cells fail to accumulate in mitosis in response to spindle damage (Figure 3.7C), demonstrating further evidence to support that the SAC is not maintained after NEBD.

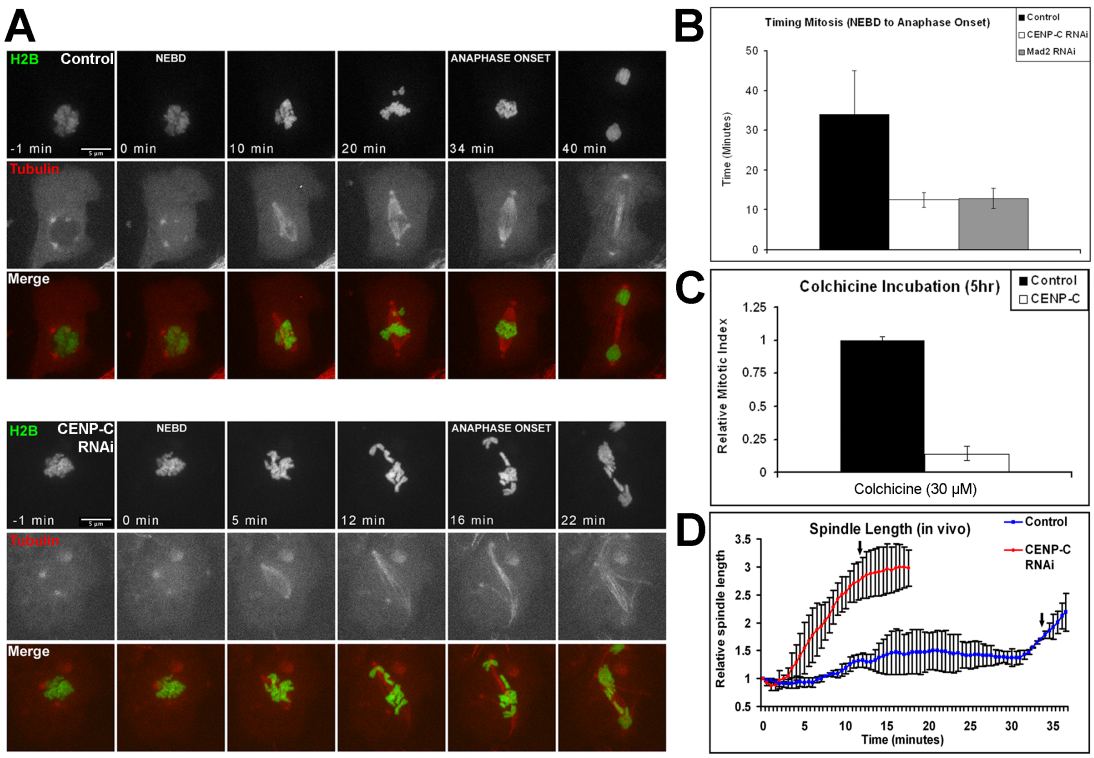
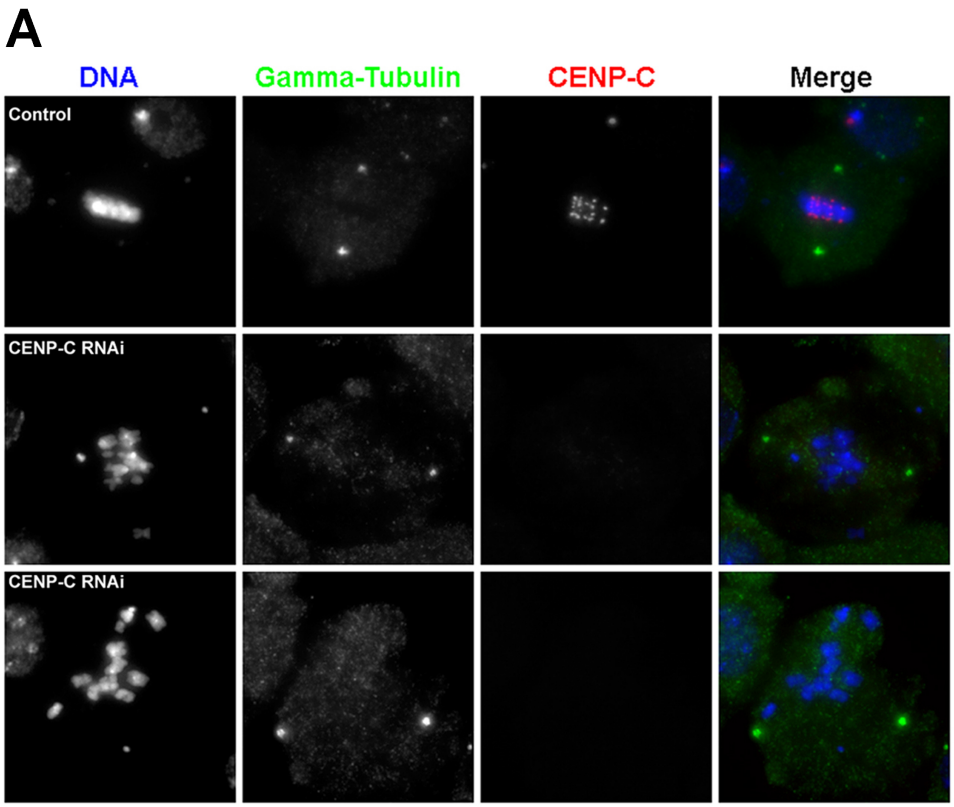


Figure 3.7 - Kinetochores are essential for SAC maintenance and regulation of spindle length. (A) Selected frames from time-lapse movies (see Supplementary

Movies 9 - 11) of either control or CENP-C depleted cells stably expressing H2B-GFP;mCherry-Tubulin recorded every 30 seconds. Time = 0 indicates NEBD and cells were recorded until anaphase onset in control cells or sister chromatid separation in the case of CENP-C RNAi cells. CENP-C depleted cells failed to align chromosomes into a metaphase configuration and exited mitosis prematurely with several missegregation errors and displaying long mitotic spindles. **(B)** Quantification of the time cells spend in mitosis (from NEBD to anaphase onset) reveals that CENP-C depleted cells exit mitosis in 12 ± 3min while control cells take 34 ± 11min. Asterisk represents results obtained from Mad2 depletion in the same cell line included for comparison (Orr *et al.* 2007). **(C)** Control and CENP-C depleted cells were treated with 30μM of Colchicine for 5h prior to fixation, then stained to reveal phospho-histone H3 and the resultant mitotic index was scored. Mitotic index in control cells was normalized to 1 (n= more than 4000 cells). Note that in the absence of CENP-C cells are unable to accumulate in mitosis in response to spindle damage suggesting that these cells have no SAC activity. **(D)** Using *in vivo* time-lapse images (see Figure 3.7A) spindle length was measured at each time frame and in the absence of CENP-C, quantification shows that spindle length increases at a fast and steady rate soon after NEBD. Arrows indicate anaphase onset in both control and CENP-C depleted cells (determined by sister chromatid separation in CENP-C depleted cells) and all quantifications of spindle length were performed using Image J software. For *in vivo* measurements, spindle length was set to 1 at the time of NEBD.

Previous studies have proposed that proper metaphase spindle length is regulated by the presence of KMN network proteins (Goshima *et al.* 1999; Goshima *et al.* 2003; Przewloka *et al.* 2007) and failure to sustain normal kinetochore-microtubule attachment may cause mis-regulated addition of tubulin subunits at microtubule plus-ends leading to abnormally large spindles. Interestingly, and confirming the kinetochore-null phenotype, quantification of spindle length *in vivo* shows that control cells begin spindle elongation very shortly after NEBD at a slow but steady rate (Figure 3.7D). Metaphase is achieved after ~15min when the spindle size remains constant until cells begin anaphase which is characterized by a fast and steady spindle elongation. However, in cells lacking CENP-C, rapid spindle elongation is observed immediately after NEBD and continues at the same rate until late stages of mitosis, frequently until spindle poles reach the cell cortex causing the spindle poles to move along the cortex and the whole spindle to bend (Figure 3.7A, D). Further analysis in which spindle length was measured in control and CENP-C depleted cells (arrested in mitosis with MG132 and stained for γ-tubulin to identify centrosomes) confirms that in the absence of CENP-C spindles elongate significantly (Figure 3.8A, B). Taken together, these results demonstrate that in kinetochore-null cells, SAC activity is severely compromised even in the absence of microtubules. Furthermore, the data indicate that metaphase spindle length is regulated by kinetochore-dependent mechanisms that may act to inhibit spindle growth when proper kinetochore-microtubule attachment is established.



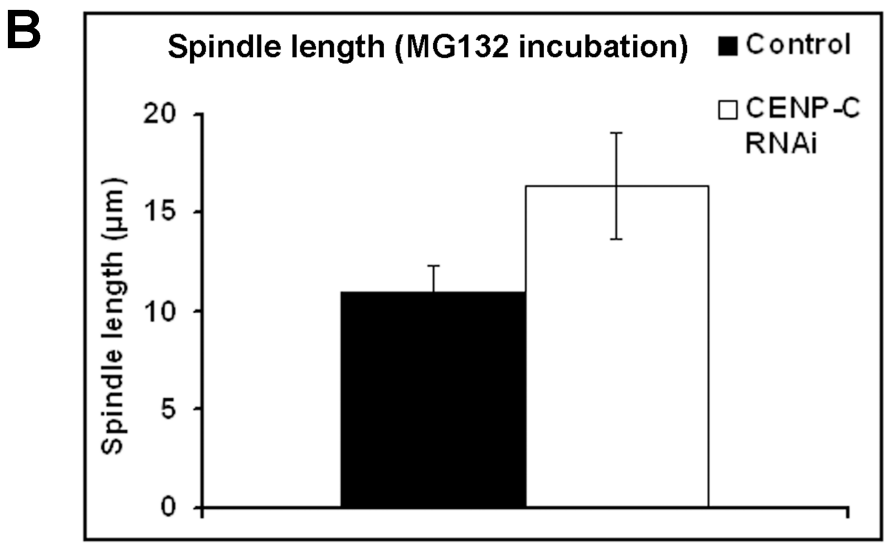


Figure 3.8 – Kinetochore-null cells display increased spindle length. (A) Control and CENP-C depleted cells were incubated in MG132 (2hr) prior to fixation and stained to show DNA (blue), γ-tubulin (green) to reveal spindle poles and CENP-C (red). Note that CENP-C depleted cells fail to align their chromosomes at the spindle equator and exhibit longer mitotic spindles than those observed in control cells. **(B)** Quantification of pole-to-pole distance (length between each γ-tubulin positive signal) in MG132-arrested cells shows that in the absence of CENP-C spindles elongate 1.5-fold more than they do in control cells.

2.5 CENP-C is required for the centromere localization of MEI-S332 and CPC components

Previous reports have suggested a strict hierarchical model of kinetochore assembly in *Drosophila* with CID at the top of the hierarchy (Przewloka *et al.* 2007; Schittenhelm *et al.* 2007; Erhardt *et al.* 2008). However, while our results confirm that *Drosophila* CENP-C plays a major role in CID localization/maintenance, the observed CID and CENP-C interdependency suggests that centromere organization may be impaired in the absence of these proteins. Nevertheless, *Drosophila* CID mutants retain an intact SAC response to spindle disruption despite the inability of many kinetochore proteins, including SAC components, to target to kinetochores, suggesting that the observed mitotic phenotypes in the CENP-C RNAi cannot be a consequence of impaired CID localization (Blower *et al.* 2006). Thus, to further explore the role of CENP-C in centromere structure we determined the localization of other known centromere markers in the absence of CENP-C, including MEI-S332, INCENP and Aurora B (Figure 3.9). MEI-S332 is the *Drosophila* homologue of hShugoshin that has been shown to localize to both meiotic and mitotic centromeres (Moore *et al.* 1998). Shugoshin has been shown to prevent premature degradation of cohesin before the metaphase-anaphase transition (Watanabe and Kitajima 2005). INCENP tightly co-operates with Aurora B and other Chromosomal Passenger Complex (CPC) proteins at mitotic centromeres, to successfully regulate chromosome alignment and SAC activity (Adams *et al.* 2001). We find that in untreated control cells, MEI-S332 localizes as two dots between CID innermost regions and INCENP shows a single perpendicular staining in between CID pairs (Figure 3.9A). In the absence of CENP-C, both MEI-S332 and INCENP fail to accumulate to clearly defined centromeric axes and are found mostly associated with non-centromeric DNA, suggesting that these proteins require CENP-C for correct targeting to inner centromere regions (Figure 3.9A).

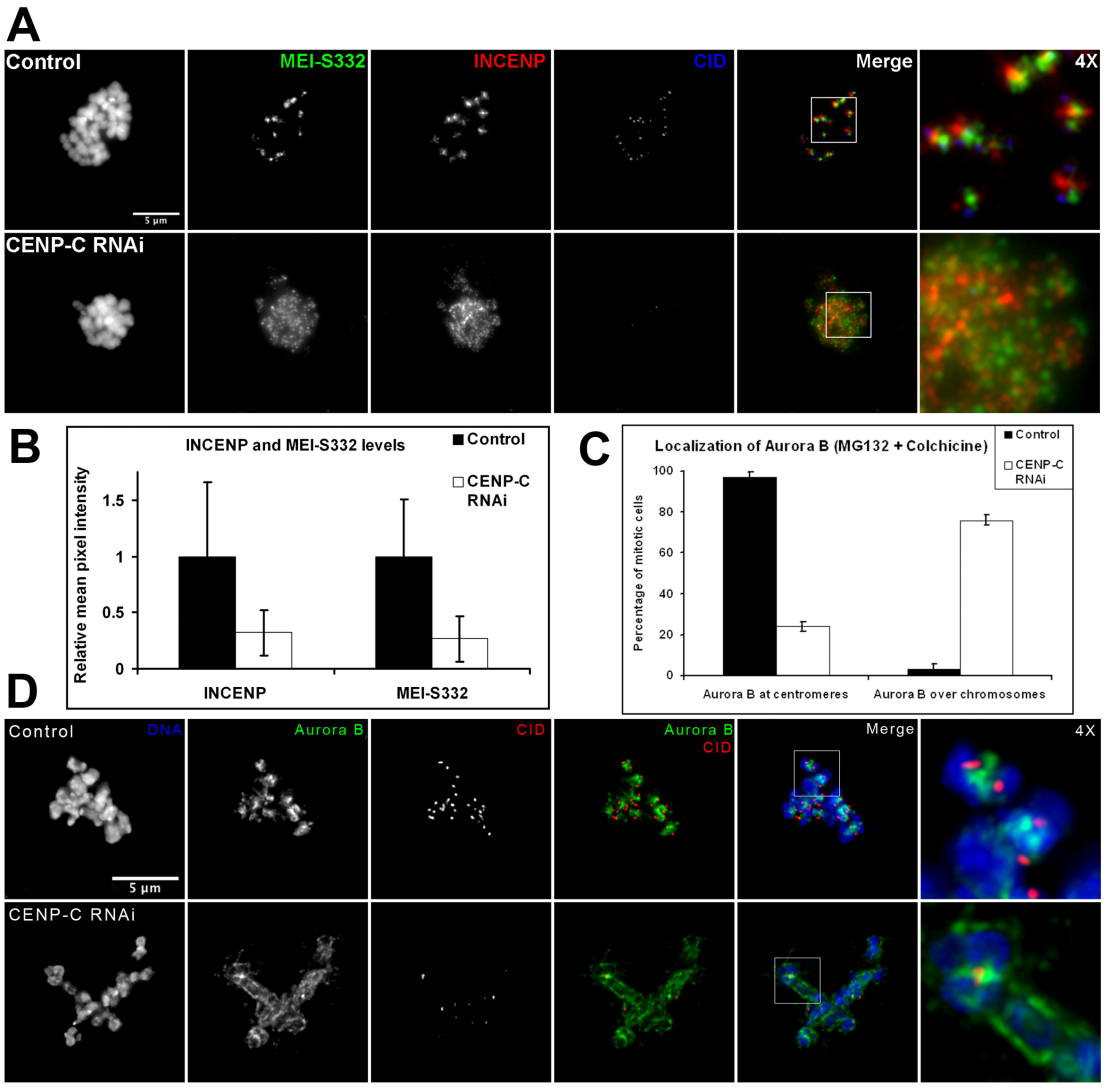


Figure 3.9 - CENP-C is required for maintaining centromere identity. Control and CENP-C depleted cells were incubated in MG132 (2hr) and Colchicine for (1hr further) prior to fixation and immunofluorescence staining. **(A)** Fixed cells were stained to reveal DNA (grey), MEI-S332 (green), INCENP (red) and CID (blue). In the absence of CENP-C both MEI-S332 and INCENP fail to localize at defined centromeric axes and display a diffuse staining (see 4X magnifications). **(B)** Quantification of the mean pixel intensity of MEI-S332 and INCENP levels at centromeres at 96hr using immunofluorescence images obtained from **(A)**. Control levels of MEI-S332 and INCENP have been normalized to 1 (n= more than 100 kinetochore pairs from 15 different cells). **(C)** Quantification of the percentage of mitotic cells with Aurora B localized at centromeres or over chromosomes in cells treated with MG132 and Colchicine. **(D)** Cells treated as described in **(A)** and then stained to reveal DNA (blue), Aurora B kinase (green) and CID (red). Note that in CENP-C depleted cells, Aurora B fails to localize at clearly defined centromeric regions and is found mostly associated with non-centromeric chromatin. All quantifications were performed using Image J software with a previously defined ROI. Immunofluorescence panels **(A)** and **(D)** include 4X magnifications of selected regions.

Quantification of signal intensity shows that both INCENP and MEI-S332 levels are reduced to 31% and 26% respectively (Figure 3.9B), and in most cases showing a highly diffuse pattern of staining. We also identified a small population of kinetochores with normal CID levels at kinetochores but very low or entirely absent MEI-S332 or INCENP signals (Figure 3.10A, B) suggesting that the centromeric localization of MEI-S332 and INCENP is CENP-C-dependent rather than specifically dependent on CID localization. Additionally, we find that Aurora B is also delocalized from centromeric regions but remains strongly associated with non-centromeric chromatin (Figure 3.9C, D). Quantification indicates that 77% of CENP-C depleted mitotic cells show abnormal Aurora B localization (Figure 3.9C). The association of Aurora B with non-centromeric chromatin has never been observed in fully condensed mitotic chromosomes, although a study in *Drosophila* S2 cells, demonstrates that Aurora B displays a punctuate distribution throughout all regions of condensing chromosomes of prophase cells (Giet and Glover 2001). Aurora B mis-localization in the absence of CENP-C could be a consequence of failing to define centromere identity at early stages of mitosis, which could explain why Aurora B is unable to concentrate at centromeres. It also suggests that Aurora B function in chromosome condensation is achieved independently of its specific localization to centromeres. The same study in *Drosophila* demonstrated that Aurora B is required for histone H3 phosphorylation and for recruitment of condensins to condensing chromosomes (Giet and Glover 2001). Concurrently, we also find that Aurora B does not need to be confined to centromeres to ensure proper phosphorylation of histone H3 in mitotic chromosomes (Figure 3.10A-C). These results indicate that CENP-C is essential for the localization and/or maintenance of MEI-S332, INCENP and Aurora B at clearly defined centromeric axes during mitosis suggesting that communication between inner kinetochore proteins and the centromere is essential for proper centromere organization.

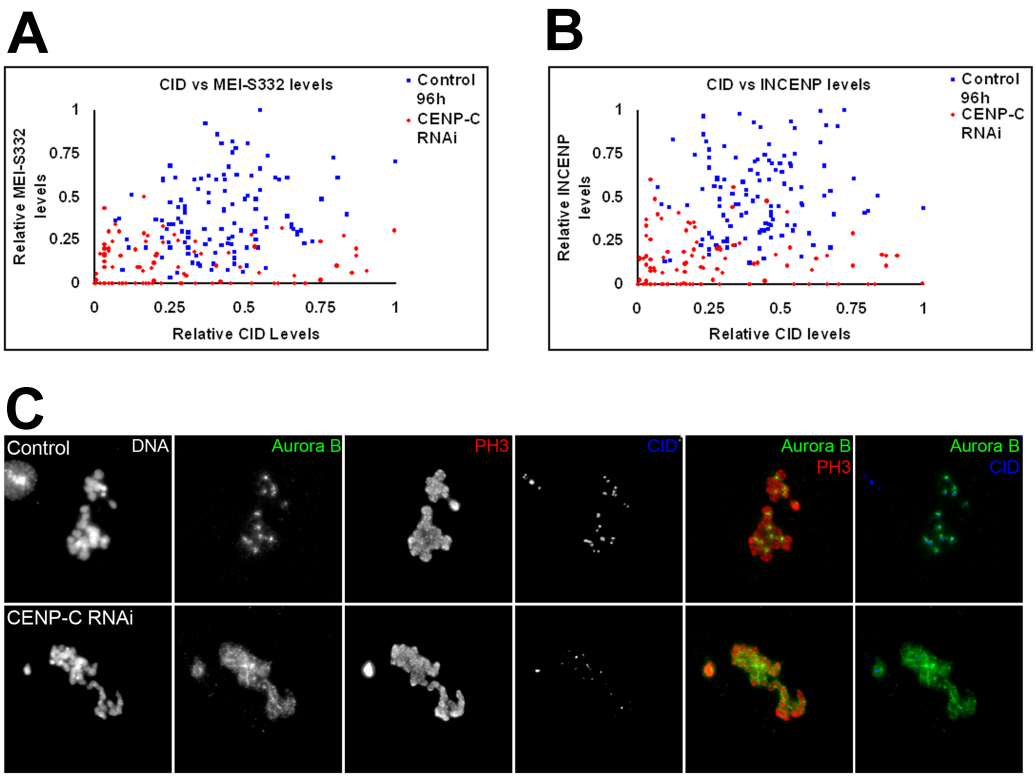


Figure 3.10 - Centromere localization of MEI-S332 and CPC proteins is CENP-C dependent. Control and CENP-C depleted cells were incubated in MG132 (2hr) and Colchicine for (1hr further) prior to fixation and immunofluorescence staining with specific antibodies. Relative levels of CID were plotted against **(A)** MEI-S332 or **(B)** INCENP where each dot represents a single centromere/kinetochore pair. Note that there is a population of kinetochores with high levels of CID and low levels of MEI-S332 and INCENP suggesting that centromeric localization of MEI-S332 and INCENP is CENP-C-dependent rather than dependent on the prior localization of CID. All quantifications were performed using Image J software with a previously defined ROI. **(C)** Cells treated with MG132 (2hr) and Colchicine (1hr further), then fixed and stained to reveal DNA (grey), Aurora B (green), phospho-histone H3 (PH3) (red) and CID (blue). Note that Aurora B mis-localization does not affect PH3 staining.

3. Discussion

Kinetochores are assembled at the centromere of each replicated chromatid and provide an essential protein interface to allow binding of spindle microtubules and consequent chromosome segregation during mitosis. We find that in *Drosophila*, CENP-C plays a major role not only in kinetochore organization but also in the proper assembly/maintenance of important centromere components suggesting that communication between the inner kinetochore and the centromere is an essential step in determining centromere identity.

CENP-C inactivation in vertebrate cells has been performed by antibody microinjection in HeLa cells (Tomkiel *et al.* 1994), using CENP-C knockout mice (Kalitsis *et al.* 1998) or by tetracyclin-inducible knockouts in DT40 cells (Fukagawa and Brown 1997; Fukagawa *et al.* 1999; Kwon *et al.* 2007) and all studies concluded that CENP-C is essential for cell viability and mitotic progression. Detailed immunofluorescence analysis in CENP-C-deficient DT40 cells revealed a partial disruption of the inner kinetochore accompanied by a BubR1-dependent mitotic delay (Kwon *et al.* 2007). While CENP-C inactivation in vertebrate cells causes partial disruption of the inner kinetochore, in *Drosophila*, CENP-C appears to perform more important roles (Przewloka *et al.* 2007; Schittenhelm *et al.* 2007). Consistently, bioinformatic approaches directed at evaluating CENP-C conservation between species reveals that while CENP-C is highly conserved amongst other *Drosophila* species, it bears very limited homology with its counterparts in higher eukaryotes. These differences may reflect different functions for the *Drosophila* CENP-C homologue and argue in favour of a different centromere-kinetochore interface specific to *Drosophila* chromosomes.

3.1 CENP-C depletion causes a kinetochore-null phenotype

We show that CENP-C is required for the loading/maintenance of all kinetochore proteins tested including SAC proteins (Mad2, Bub1, BubR1 and Bub3), mitotic regulator Polo kinase, microtubule motor protein CENP-meta and KMN proteins (Ndc80, Nuf2, and Mitch). Interestingly, the kinetochore-null phenotype observed after CENP-C depletion appears to be specific to *Drosophila* and *C.elegans* chromosomes (Oegema *et al.* 2001; Cheeseman *et al.* 2004), since CENP-C has been shown not to be required for full kinetochore organization in higher eukaryotes (Tomkiel *et al.* 1994;

Fukagawa and Brown 1997; Fukagawa *et al.* 1999). Similar to *Drosophila*, no CCAN homologues have yet been identified in *C.elegans* (Przewloka and Glover 2009), which suggests that in systems lacking CCAN, centromere function relies uniquely on the structural role of CENP-C. Different to what as been reported in vertebrate cells, our results are consistent with a model in which CENP-C is required to lay the foundation for all components essential for kinetochore assembly.

3.2 Mitotic timing and SAC activation in kinetochore-null cells

Previous reports have shown that loss of CENP-C in mammalian cells causes a mitotic delay (Tomkiel *et al.* 1994). In chicken cells this mitotic delay is BubR1-dependent and associated to a 3-fold increase in the overall duration of mitosis (Kwon *et al.* 2007). We demonstrate that in the absence of CENP-C, *Drosophila* kinetochores are unable to recruit essential SAC proteins Mad2, Bub1, BubR1 and Bub3, even if mitotic exit is prevented and microtubules removed. Nevertheless, consistent with the observed loss of SAC proteins, these cells are insensitive to microtubule poisons and rapidly exit mitosis in the presence of spindle damage. As expected when analysing SAC-deficient phenotypes, these cells undergo fast mitotic exit accompanied by premature sister chromatid separation. CENP-C depleted cells exit mitosis with a mitotic timing similar to what has been observed after Mad2 depletion in the same cell line (Orr *et al.* 2007). Two possible hypotheses could explain why CENP-C inactivation in other systems causes cells to block in mitosis. Either CENP-C inactivation was not as efficient in other species as it is in *Drosophila* S2 cells or these discrepancies could reflect structural differences in kinetochore organization specific to *Drosophila* chromosomes. Interestingly, *Drosophila* CID mutants display mis-localization of several kinetochore components accompanied by a BubR1-dependent mitotic delay (Blower *et al.* 2006) which suggests that CID inactivation cannot account for the loss of SAC maintenance observed when disrupting *Drosophila* CENP-C. However, in the case of CID mutants that were analyzed during early embryonic development, the maternally contributed CID protein might have occluded phenotypes that may explain the SAC-dependent mitotic delay. However, in tissue culture cells we show that kinetochore-null cells fail to maintain SAC activity even in the presence of microtubule poisons, which suggests that kinetochore inactivation is not compatible with a functional SAC. Taken together, our data demonstrate that CENP-C is essential for full kinetochore assembly, a pre-requisite for efficient SAC maintenance.

3.3 *Drosophila* CENP-C is required for centromere identity

In *Drosophila*, the localization of all outer kinetochore proteins appears to be dependent on CENP-C, as shown by our data and previous studies(Przewloka *et al.* 2007; Schittenhelm *et al.* 2007). Moreover, together with a previous study (Erhardt *et al.* 2008), our results show that CENP-C is an essential factor for CID assembly at *Drosophila* centromeres. In accordance, it was recently proposed that CCAN protein copy number at kinetochores varies between vertebrates and yeast (Johnston *et al.* 2010) suggesting that although specific centromere/kinetochore assembly modules appear to be conserved, differences in protein copy number may affect phenotypic analyses.

Our data also demonstrates that in *Drosophila*, CENP-C is essential for the proper localization of other centromere-specific proteins including the cohesion protector MEI-S332 and the CPC components INCENP and Aurora B. Taken together, these results are consistent with the proposal that *Drosophila* CENP-C is essential for maintaining normal centromeric architecture and identity, which appears to be species-specific. In vertebrates, however, a large cluster of constitutive centromere-associated proteins (CENP-C, -H, -I, and -K to -U, and -X) was identified as the CCAN which associates with CENP-A throughout the cell cycle (Foltz *et al.* 2006; Liu *et al.* 2006; Okada *et al.* 2006; Cheeseman and Desai 2008; Amano *et al.* 2009; Santaguida and Musacchio 2009), although a recent report also identified CENP-W, that forms a DNA-binding complex together with CENP-T (Hori *et al.* 2008), all of which have no identified *Drosophila* orthologues. However, similarly to CENP-C, many of the CCAN proteins may have failed to be detected in the *Drosophila* genome because they lack significant conservation. At this point we cannot rule out this possibility, although it is clear that in *Drosophila*, CENP-C plays an essential role in overall centromere and kinetochore organization, a role that might be shared with the CCAN protein complexes in other systems.

Together with the cumulated published evidence on the functional analyses of CID and CENP-C, our data suggest that the *Drosophila* centromere/kinetochore interface is simpler than that of higher eukaryotes. We propose that CENP-C plays a direct role in maintaining centromere identity and may fulfil many of the structural roles of CCAN complex proteins present in other organisms.

Chapter 4

Spatio-temporal control of mitosis by the conserved spindle matrix protein Megator

1. Introduction

The mitotic spindle that assembles soon after NEBD is composed of highly dynamic microtubules and is essential for powering chromosome movement and segregation during mitosis. However, due to several incompletely understood features of mitotic spindle dynamics, it has long been proposed that mitotic cells may require an additional structure, such as a spindle matrix, ensuring that microtubule associated proteins required for driving chromosome motion are localized in the vicinity of the mitotic spindle (Pickett-Heaps *et al.* 1984). Although a matrix-like structure has been observed in fixed cell samples, there is no direct evidence supporting whether it plays a role in mitosis or even whether such a structure exists in living cells.

Nevertheless, a spindle matrix would be expected to (a) form a fusiform structure coalescent with spindle microtubules, (b) persist even in the absence of microtubules, (c) be resilient in response to changes of spindle shape and length, and (d) affect spindle assembly and/or function if one or more of its components are perturbed. In *Drosophila melanogaster*, a complex of at least four nuclear proteins, Skeletor, Megator (Mtor), Chromator, and EAST (Enhanced Adult Sensory Threshold), have been found to form a putative spindle matrix that persists in the absence of microtubules in fixed preparations (Johansen and Johansen 2007). However, its biological relevance has been questioned since is has never been observed in living cells, suggesting that the observed matrix-like structure was an artifact produced by fixation artifact. From the four-protein complex, Mtor is the only protein that shows clear sequence conservation in other organisms, ranging from mammals to plants. Mtor homologues include the nuclear pore complex (NPC) protein translocated promoter region (Tpr) in mammals (Cordes *et al.* 1997; Zimowska *et al.* 1997), its respective counterparts Mlp1 and Mlp2 in yeast (Strambio-de-Castillia *et al.* 1999), and nuclear pore anchor in plants (Xu *et al.* 2007b). Interestingly, NPC proteins including Mtor/Tpr orthologues in yeast, were shown to functionally interact with SAC proteins (Iouk *et al.* 2002; Scott *et al.* 2005). The SAC is essential for ensuring correct chromosome segregation by providing the time required for proper kinetochore-microtubule attachments while inhibiting APC/C activity (Musacchio and Salmon 2007). This functional interaction between Mtor/Tpr orthologues and SAC proteins suggests that the spindle matrix could provide an essential medium involved in the direct targeting of both SAC proteins and microtubule-associated proteins during mitosis.

If critical functions performed by the spindle matrix are widely conserved, then it would be possible to uncover the mitotic role of Mtor in living *Drosophila* somatic cells. The results obtained provide a new conceptual view of a spindle matrix not as a rigid structural scaffold but as a spatial determinant of key mitotic regulators that play essential roles in chromosome motion and SAC maintenance (Lince-Faria *et al.* 2009). In this report, Lince-Faria *et al.* (2009) show that Megator (Mtor), the *Drosophila* counterpart of the human nuclear pore complex protein translocated promoter region (Tpr), and the SAC protein Mad2 form a complex that localizes to a nuclear derived spindle matrix in living cells. Fluorescence recovery after photo-bleaching (FRAP) experiments supports that Mtor is retained around spindle microtubules, where it shows distinct dynamic properties. Mtor/Tpr promotes the recruitment of Mad2 and Mps1 but not Mad1 to unattached kinetochores, thus mediating normal mitotic duration and SAC response. During anaphase, Mtor plays a role in spindle elongation, thereby affecting normal chromosome movement. However, Mtor/Tpr also appears to function as a spatial regulator of the SAC, which efficiently directs Mad2 to unattached kinetochores at the onset of mitosis allowing time for proper spindle maturation. Consistently, enrichment of Mad2 in a spindle matrix helps confine the action of a rapidly-triggered, diffusible “wait-anaphase” signal to the vicinity of the spindle. My contribution to this report involved the analysis of all the *Drosophila* Mad2-associated phenotypes presented in this manuscript (Lince-Faria *et al.* 2009).

2. Results

2.1 Megator is required for proper mitotic timing and SAC response

To address the mitotic role of Megator (Mtor), we performed RNAi against Mtor in *Drosophila* S2 cells (Figure 4.1F) stably co-expressing GFP–tubulin and the kinetochore marker mCherry–CID and followed mitotic progression (see Supplementary Movies 12 – 14). Mtor-depleted cells show no major spindle defects but typically form a poorly defined metaphase plate, presumably as the result of a 15% acceleration in the time it takes from NEBD to anaphase onset (median = 23.5 min, range = 14–50 min; Figure 4.1B and D; Supplementary Movie 13) when compared with controls (median = 28 min, range = 13.5–70 min; Figure 4.1A and D; Supplementary Movie 12). Such problems in completing chromosome congression are corrected if anaphase onset is delayed by proteasome inhibition with MG132 (data not shown). Similar to what is observed in Mtor RNAi, S2 cells depleted of the SAC protein Mad2 undergo a faster mitosis (median = 11.5 min, range = 8–21 min; Figure 4.1C and D; Supplementary Movie 14). Moreover, when compared to control cells, Mtor-depleted cells show a lower mitotic index as well as a weakened response to MT depolymerization (Figure 4.1E), suggesting that Mtor is required for proper SAC activity.

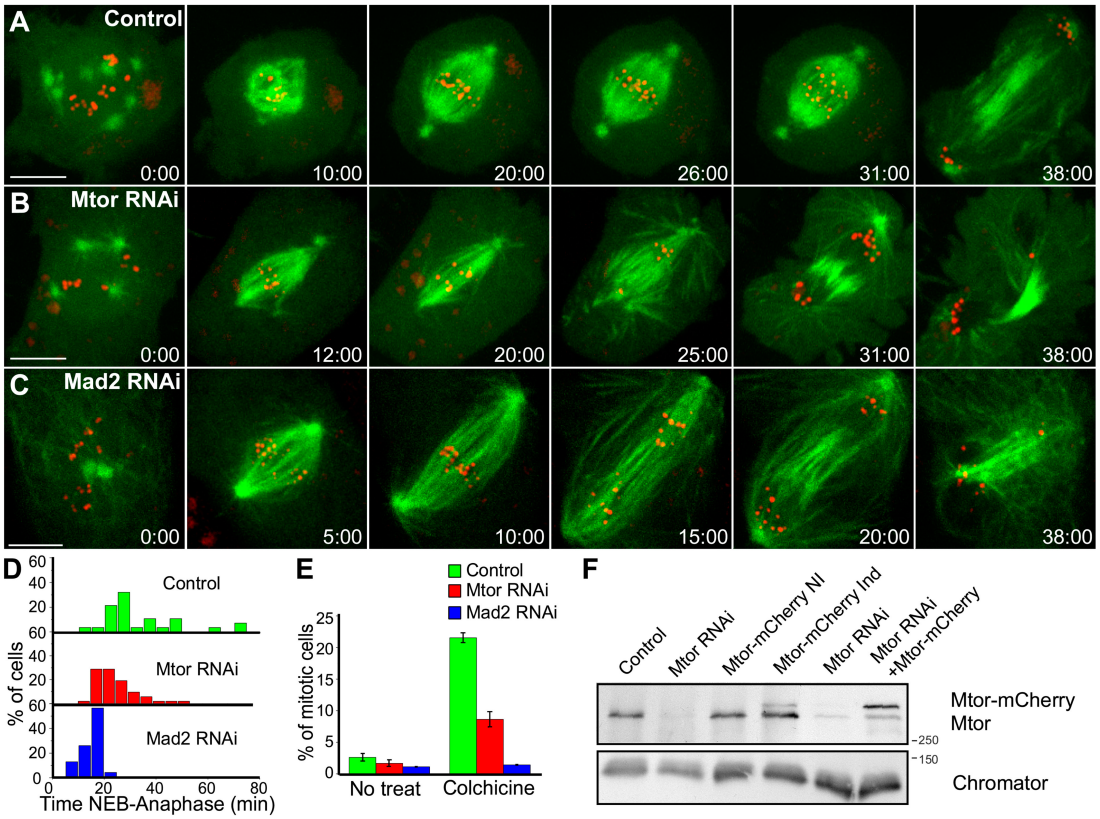


Figure 4.1 - Megator is required for proper mitotic timing and SAC response. **(A–C)** S2 cells stably expressing GFP-tubulin (green) and mCherry-CID (red) were used for live imaging of mitotic progression in **(A)** control (n = 28 cells; Supplementary Movie 12), **(B)** Mtor RNAi (n = 52 cells; Supplementary Movie 13) and **(C)** Mad2 RNAi (n = 23 cells; Supplementary Movie 14) cells. **(D)** Respective quantification of the time from NEBD to anaphase onset. Mtor and Mad2 RNAi are statistically different from controls. Mtor is also statistically different from controls in a pairwise comparison. **(E)** Mitotic index under physiological conditions or after colchicine treatment. Error bars represent SD from the mean obtained from three independent experiments. **(F)** Western blot analysis of Mtor. (left to right) Control, Mtor RNAi (75% depletion), stable expression of Mtor-mCherry without induction, stable expression of Mtor-mCherry after induction, RNAi using the 3' UTR region of Mtor as target (86% depletion), and stable expression of Mtor-mCherry after induction and RNAi using the 3' UTR region of Mtor as target. Chromator was used as loading control.

2.2 Megator is required for allowing the time required for proper spindle maturation

Quantitative analysis of anaphase revealed a significant attenuation in the velocity of chromosome separation in Mtor-depleted cells by affecting spindle

elongation (half-spindle elongation rate in controls = 0.9 ± 0.2 µm/min, range = 0.5–1.4 µm/min, n = 28 cells; Mtor RNAi = 0.6 ± 0.3 µm/min, range = 0.1–1.2 µm/min, n = 70 cells; Figure 4.2A–C). These results could be accounted for if Mtor is part of a structural scaffold where motor proteins assemble to generate force (Pickett-Heaps *et al.* 1984). However, an alternative hypothesis is that Mtor may function to provide the necessary time for proper maturation of a competent spindle. To test this, we delayed anaphase onset by treating Mtor-depleted cells with MG132 and measured half-spindle elongation velocity after drug washout. We found no difference in half-spindle elongation velocity between Mtor RNAi (0.7 ± 0.1 µm/min; Figure 4.2B'') and control cells (0.7 ± 0.2 µm/min; Figure 4.2A'') treated with MG132 (mean ± SD; range in Mtor RNAi = 0.5–0.8 µm/ min; range in controls = 0.5–1.1 µm/min; n = 7 cells/condition). Consistently, half-spindle elongation in Mad2-depleted cells (Figure 4.2C), which progress faster through mitosis (Figure 4.1C and D), was similar to half-spindle elongation values of Mtor-depleted cells, namely 0.5 ± 0.2 µm/min (mean ± SD, range = 0–1.2, n = 19 cells; Figure 4.2C' and C''), thus supporting the spindle maturation hypothesis.

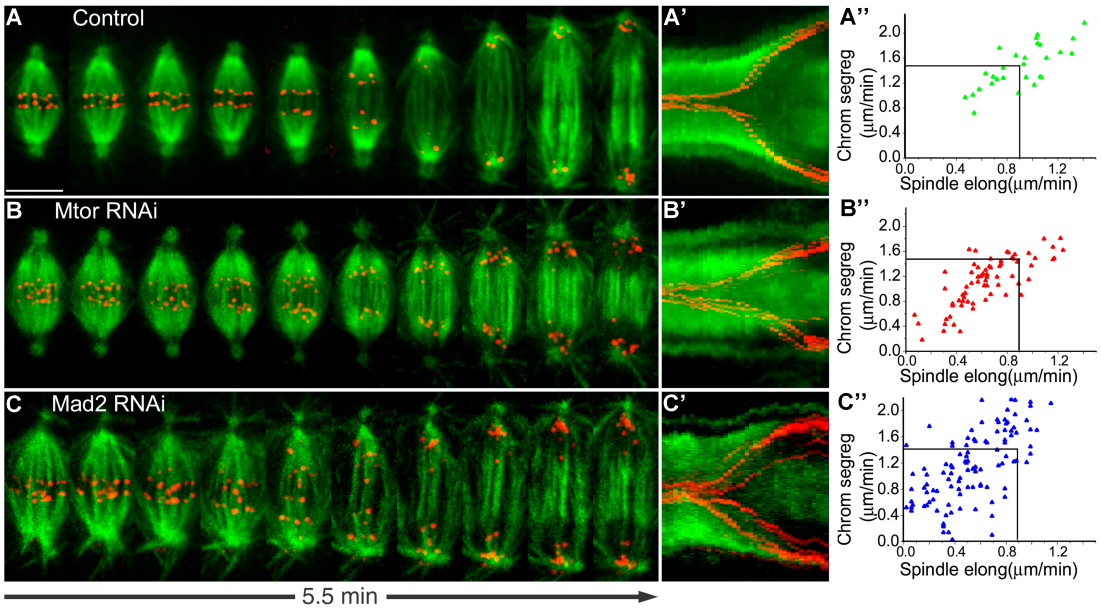


Figure 4.2 – Measuring the half-spindle elongation velocity in Megator and Mad2 depleted cells. (A–C) Analysis of chromosome and spindle dynamics during anaphase in **(A)** control, **(B)** Mtor RNAi and **(C)** Mad2 RNAi cells. **(A'–C')** Corresponding kymo- graph analyses are shown. **(A''–C'')** Quantification of half-spindle elongation (spindle elong) and chromosome segregation (chrom segreg) velocities in **(A'')** control, **(B'')** Mtor RNAi and **(C'')** Mad2 RNAi cells treated with

MG132. Black lines indicate reference mean values for control cells. Spindle elongation in Mtor and Mad2 RNAi is statistically different from controls. Time is shown in minutes/seconds. Bars correspond to 5 μm.

3. Discussion

Overall, the results support a model in which Mtor/Tpr acts as a spatial regulator of the SAC, ensuring a timely and effective recruitment of Mad2 and Mps1 to unattached kinetochores as cells enter mitosis. In budding yeast, Mps1 phosphorylates Mad1 (Hardwick *et al.* 1996), which is continuously recycled to kinetochores from Mlps (Mtor yeast homologues) at NPCs, but N-terminal deletion mutants of Mad1 lacking the Mlp-binding domain still retain a functional SAC (Scott *et al.* 2005). In humans and *Drosophila*, Mps1 regulates Mad2 but not Mad1 accumulation at kinetochores. Because Mad1 kinetochore localization does not depend on Mlps/Mtor/Tpr and Mps1 kinase activity, the residual Mad2 at kinetochores after Mtor/Tpr RNAi possibly corresponds to the stable Mad1-bound fraction. One possibility is that Mps1 phosphorylation of Mad1 regulates the recruitment of a fast-exchanging pool of Mad2 to kinetochores (Chung and Chen 2002; Musacchio and Salmon 2007). However, Mtor/Tpr may spatially regulate Mps1 autophosphorylation, which is important for its proper kinetochore accumulation, together with Mad2 (Xu *et al.* 2007b; Xu and Meier 2008). The presence of Mad2 in the complex may act as a positive feedback mechanism to ensure continuous Mps1 kinase activity upon SAC activation.

SAC proteins evolved from systems with a closed mitosis like budding yeast, where the spindle assembles inside an intact nuclear envelope into more complex systems like animals and plants, where the nuclear envelope is thought to fully or partially disintegrate during spindle formation, justifying the requirement of a nuclear derived spindle matrix for an effective SAC response. Nevertheless, what maintains matrix components around the spindle in systems where there is an open mitosis, still remains an intriguing question. In this regard, lamin B was proposed to tether several factors that mediate spindle assembly in Xenopus laevis egg extracts and possibly in human cells (Tsai *et al.* 2006; Zheng and Tsai 2006). Additionally, a continuous endoplasmic reticulum surrounding the mitotic spindle is thought to be recycled from the nuclear envelope after its disassembly and has been observed in several systems undergoing an open mitosis, including humans (Ellenberg *et al.* 1997; McCullough and Lucocq 2005). Although such fenestrated membranous systems cannot work as diffusion barriers, it is possible that they indirectly help to generate local gradients or concentrate matrix-associated substrates.

The enrichment of Mad2 in the spindle matrix provides an explanation for an unsolved SAC paradigm in which the “wait-anaphase” signal emanating from

unattached kinetochores must be diffusible to prevent premature anaphase onset of already bioriented chromosomes but at the same time is known to be restricted to the vicinity of the spindle (Rieder and Khodjakov 1997; Rieder *et al.* 1997). The proposed role of Mtor/Tpr further supports the necessity of spindle maturation for proper kinetochore-microtubule attachments and anaphase spindle elongation in which the spindle matrix may help extend the duration of mitosis for the assembly of a competent chromosome segregation machinery. Mtor/Tpr-depleted cells display a weakened SAC response that, as opposed to complete checkpoint loss (observed in Mad2-depleted cells), may be compatible with cell viability and ultimately lead to cancer (Michel *et al.* 2001). Importantly, the involvement of Tpr in the activation of several oncogenes (Park *et al.* 1986; Ishikawa *et al.* 1987; Greco *et al.* 1992) may translate into an unfavorable combination that facilitates transformation and tumorigenesis in humans.

Part III

General Discussion

1. General Discussion

Since the cell theory was formulated, it is known that all cells arise from the division of pre-existing cells and that every living organism is descended from a single ancestral cell that may have lived up to 4 billion years ago. Throughout this vast period of evolution, the proliferation of cells and organisms has depended heavily on the capacity to faithfully transmit genetic information during millions of rounds of cell division. The accurate segregation of DNA is achieved during the M-phase of the cell cycle and is fundamental for promoting the proliferation and propagation of all cells and organisms. However, how a machine as complex as a cell can reproduce itself with extreme precision after countless generations, still remains a subject of intense investigation.

One of the most fascinating processes of cell division is the faithful segregation of chromosomes that takes place during mitosis. The accuracy of this process is essential for the faithful transmission of genetic material and therefore, the metaphase-anaphase transition must be tightly regulated to ensure that cells complete chromosome segregation with high precision. Accordingly, cells are equipped with a quality control surveillance mechanism, also known as the mitotic checkpoint or SAC, which is responsible for preventing mitotic exit in the presence of improperly attached or unattached kinetochores. The SAC significantly reduces the frequency of abnormal chromosome segregation thus contributing to the sustained maintenance of genomic stability. Although this process is under intense regulation, some cells acquire errors during mitosis that commonly cause overall loss or gain of chromosome number, a condition known as aneuploidy that is considered to be a major hallmark of cancer cells. It is currently clear that aneuploidy is a common characteristic of solid tumors, however, whether aneuploidy is the cause or consequence of malignant transformation is still a highly controversial issue. Whilst some tumors are stably aneuploid, in most cases the observed aneuploidy is the result of chromosomal instability (CIN), characterized by increased rates of gain or loss of whole chromosomes during cell division. Although the molecular mechanisms underlying CIN are currently unclear, cells displaying CIN were originally reported to fail to accumulate in mitosis in response to spindle damage, leading to the proposal that errors in SAC function could be the primary cause of CIN.

The SAC has been extensively studied in a number of systems and is in part regulated by kinetochores. In agreement, both Mad2 and BubR1 have been implicated in SAC control by localizing to kinetochores in the absence of microtubule

attachment and tension, respectively. Accordingly, their dynamic localization at kinetochores is thought to provide a diffusible 'wait-anaphase' signal through sequestration of Cdc20, the principal activator of the APC/C. However, these proteins are also present in the cytoplasm where they associate with Bub3 and Cdc20 to form the MCC, a powerful, kinetochore-independent APC/C inhibitor that is present in interphase, even before kinetochores begin to assemble. Importantly, Mad and Bub proteins are thought to act as SAC sensors of microtubule attachment status by ensuring that chromosome segregation only takes place when all chromosomes are correctly attached and under tension.

1.1 Work objectives

The work presented in this thesis aims to provide further understanding on the mechanisms of SAC maintenance and kinetochore assembly in *Drosophila*. One of the main objectives was to understand how SAC components interact at different stages of mitosis to provide a spatial and temporal regulation of the mitotic checkpoint. Specifically, using molecular tools designed for generating different experimental conditions of Mad2 localization, this work is mainly focused on dissecting the kinetochore-dependent and -independent roles of Mad2 in SAC activation.

1.2 Mad2-independent SAC activation

Recent studies have provided significant understanding on the molecular mechanisms involving Mad2 in SAC activation, however little data has been obtained characterizing mitotic progression in cells lacking this essential SAC component. Moreover, since individual depletions of other SAC proteins such as BubR1 and Bub3 yield different phenotypes (Basu *et al.* 1998; Basu *et al.* 1999; Lopes *et al.* 2005; Maia *et al.* 2007), it is likely that SAC proteins work through parallel signaling pathways that are mutually required to sustain full SAC activity.

In the first chapter of this study, we provide the first detailed analysis of Mad2-depleted cells as they progress and eventually exit mitosis. We find that cells lacking Mad2 transit through mitosis with an accelerated mitotic schedule resulting in extensive chromatin bridges during anaphase and telophase. These phenotypes are

exclusively Mad2-dependent since we show that Mad2 is not required for the localization of other SAC proteins at unattached kinetochores. Surprisingly, we find that all Mad2-associated phenotypes can be reverted and the SAC effectively re-activated by a transient mitotic arrest. Thus, contrary to current models which view Mad2 at the centre of the inhibition of the APC/C by the SAC, we hypothesize that Mad2 is only required for proper timing of mitotic progression during early stages prometaphase, allowing cells to fully engage the SAC through kinetochore accumulation of other checkpoint proteins. Our results are consistent with a model in which Mad2 acts as a mitotic timer responsible for allowing the completion of the early stages of mitosis so that other SAC proteins can be activated, thus allowing a controlled metaphase-anaphase transition.

1.3 Mad2 acts as a mitotic timer

Early studies on Mad2 function revealed that disruption of Mad2 causes cells to exit mitosis prematurely with a significantly shortened mitotic schedule (Gorbsky *et al.* 1998; Meraldi *et al.* 2004). However, the role of Mad2 in mitotic timing appears to be more complex than previously expected since inactivation of the kinetochore-bound fraction of Mad2 disrupts the SAC without significantly affecting the timing of mitotic progression (Meraldi *et al.* 2004). Accordingly, our results show that Mad2 depletion causes cells to progress from NEBD to anaphase onset with a highly accelerated mitotic schedule. Together with the data presented by Meraldi and co-workers, our results suggest that cytosolic Mad2 (in the context of the MCC), is essential for regulating mitotic timing as cells enter mitosis to prevent premature APC/C activation soon after NEBD. The Mad2-dependent inhibition of APC/C activity during prophase is clearly kinetochore-independent since full kinetochore maturation only occurs at the time of NEBD. However, the molecular nature of the Mad2 inhibitor present during late G2 and prophase still remains unknown.

1.4 The ‘two-step’ model of SAC activation

The data presented in the first chapter of the thesis is in accordance with a ‘two-step’ model of SAC activation in which Mad2 is required in the context of the MCC. This model proposes that in a first step, active MCC prevents premature activation of the APC/C allowing cells to accumulate mitotic cyclins and to enter

mitosis so that in a second step, other SAC proteins can bind unattached kinetochores and produce additional inhibitory complexes that amplify the signal and that are required for a sustained SAC activity. The results presented here provide strong evidence for this model and show that even in the absence of Mad2, other SAC proteins such as Bub3 and BubR1 can fully engage SAC activity if APC/C activation is prevented during early stages of mitosis.

1.5 RZZ-dependent SAC regulation

The precise role that the RZZ complex plays in SAC maintenance is currently unclear (Basto *et al.* 2000; Chan *et al.* 2000; Savoian *et al.* 2000) and whether RZZ components are *bona-fide* checkpoint proteins is still a matter of much debate. Despite this, disruption of RZZ components causes an abnormal SAC response to spindle poisons, presumably due to mis-localization of the kinetochore-bound fraction of Mad1/Mad2 complex, suggesting that RZZ is indirectly involved in SAC maintenance (Buffin *et al.* 2005; Karess 2005; Kops *et al.* 2005a). However, the RZZ complex has also been implicated in SAC silencing by ensuring the localization of dynein/dynactin to kinetochores (Starr *et al.* 1998; Chan *et al.* 2000), which in turn has been shown to be essential for the stripping of Mad1 and Mad2 from kinetochores upon successful microtubule attachment (Howell *et al.* 2001; Wojcik *et al.* 2001). Consistently, the results presented in Chapter 2 show that *Drosophila* Zw10 is specifically required for Mad2 accumulation at unattached kinetochores and that under these conditions, the localization of other SAC proteins such as BubR1 is unperturbed. Importantly, this experimental setup provides a method to specifically separate the functions of cytoplasmic Mad2 from that of kinetochore-bound Mad2, both in terms of SAC maintenance and mitotic timing.

1.6 Kinetochore-bound Mad2 is dispensable for SAC maintenance

It has long been proposed that SAC activation/maintenance is mediated by a signal continuously generated at unattached or improperly attached kinetochores during prometaphase (Rieder *et al.* 1995). Studies in primary spermatocytes fully corroborate this data and show that not only microtubule occupancy but also tension across kinetochore pairs is required in order to satisfy the SAC (Nicklas *et al.* 1995). Despite this, human cells with disrupted kinetochores progress through mitosis with a

normal mitotic schedule suggesting a kinetochore-independent role for SAC proteins in regulating mitotic timing (Meraldi *et al.* 2004). Accordingly, despite localizing prominently at unattached kinetochores, Mad2 and BubR1 are found mostly at the cytoplasm, and together with Bub3 and Cdc20 form the MCC, a far more potent kinetochore-independent APC/C inhibitor (Sudakin *et al.* 2001; Tang *et al.* 2001).

We show that if Mad2 is prevented from localizing at kinetochores, cells display weak SAC activity in response to colchicine but the time from NEBD to anaphase onset is mostly normal. Furthermore, the results obtained from single depletions of Zw10 or Mad2 or co-depletion suggest that mitotic timing is dependent on cytoplasmic Mad2, since Zw10/Mad2 RNAi treated cells display an accelerated transit through mitosis (similar timing to Mad2 RNAi cells) and are unable to accumulate in mitosis in response to spindle damage. Accordingly, Zw10 and BubR1 co-depletion also causes loss of SAC function, suggesting that cytoplasmic Mad2 is required but not sufficient for sustained SAC activity. The data presented indicate that in the absence of kinetochore-bound Mad2, SAC maintenance is BubR1-dependent. Although we cannot dissociate between the cytoplasmic and kinetochore roles of BubR1 in SAC activity, it seems probable that the observed loss of SAC activity in Zw10/BubR1 cells is caused by impaired MCC formation. Significantly, our results are consistent with a model in which BubR1 (either kinetochore or cytoplasmic) and cytoplasmic Mad2, act together to maintain mitotic timing and prolong SAC activity during prometaphase.

1.7 Cytoplasmic Mad2 and the ‘two-step’ model

Consistent with the “two-step” model of SAC activation our results are consistent with a kinetochore-independent role for Mad2 in mitotic timing. Interestingly, the results presented in Chapter 1 provide a further refinement of the model in that the second step may be conceptually separated into two events: one at NEBD when Mad2 might extend prometaphase and provide enough time so that in a second event, other SAC proteins such as BubR1 and Bub3 can bind kinetochores and fully engage checkpoint activity (Orr *et al.* 2007). The data we present here fully supports the refined version of the “two-step” model and additionally, we show that Mad2 localization at kinetochores is dispensable for regulating mitotic timing. However, since Zw10-depleted cells display a weakened SAC response to longer colchicine incubations, our results show that Mad2 localization at kinetochores is required for ensuring a prolonged mitotic arrest in response to spindle damage. In the

context of the MCC complex, it appears that cytoplasmic Mad2 is required but not sufficient, for competent APC/C inhibition before kinetochores are fully assembled. Our results suggest that MCC-dependent APC/C inhibition during early stages of mitosis, is required for proper accumulation of other SAC proteins such as BubR1 at fully assembled kinetochores. In conditions where Mad2 is prevented from accumulating at kinetochores, the presence of BubR1 is essential for prolonging SAC activity thus providing a mechanism for promoting a spatial-temporal regulation of SAC activity.

1.8 Kinetochore-dependent SAC maintenance

Experimental analyses in a number of systems have shown that kinetochores are required to sustain SAC activity. In Chapter 3 of the thesis we present evidence to support that this is also the case in *Drosophila* (Orr and Sunkel 2010). Previous reports have shown that CENP-C inactivation causes mitotic delay, chromosome segregation errors, aneuploidy and apoptosis (Tomkiel *et al.* 1994; Fukagawa and Brown 1997; Fukagawa *et al.* 1999) and in chicken cells, the observed mitotic delay occurs despite having weak signals for Mad2 but not BubR1 at kinetochores (Kwon *et al.* 2007). However, contradicting reports using human cells show that Mad1, Mad2, Bub1, BubR1 and microtubule motor protein CENP-E all depend on CENP-C for proper localization at kinetochores (Liu *et al.* 2006). Therefore, to disrupt kinetochore assembly we depleted CENP-C from *Drosophila* cells and analysed mitotic timing and SAC response. Our results show that CENP-C depletion causes a kinetochore-null phenotype, a feature specific to *Drosophila* and *C.elegans* chromosomes (Oegema *et al.* 2001; Cheeseman *et al.* 2004), since CENP-C is not required for full kinetochore organization in higher eukaryotes. We show that under these conditions, essential SAC proteins Mad2, Bub1, BubR1 and Bub3 fail to accumulate at kinetochores, even if mitotic exit is prevented by proteasome inhibition and microtubules are depolymerised. Previous studies in mammalian cells demonstrated that loss of CENP-C causes a mitotic delay (Tomkiel *et al.* 1994) that in chicken cells was shown to be BubR1-dependent and associated to a 3-fold increase in the length of mitosis (Kwon *et al.* 2007). However, our results demonstrate that in the absence of functional kinetochores, premature sister chromatid separation is observed and cells quickly exit mitosis with a timing similar to what has been previously reported for Mad2 depletion in the same cell type (Orr *et al.* 2007). This observation fully supports the refined version of the ‘two-step’

model since the prediction of this model is that cells lacking functional kinetochores fail to engage in the second step (kinetochore-dependent) of SAC maintenance. Therefore, if Mad2-dependent APC/C inhibition takes place at early stages of prometaphase, we would expect mitotic timing in the absence of Mad2 to be very similar to that observed for CENP-C depletion. Collectively, our results support the 'two-step' model and suggest that even if MCC formation is unperturbed, kinetochore inactivation is incompatible with a functional SAC.

1.9 Defining centromere identity in *Drosophila*

The depletion of CENP-C also allowed us to address an important question relating to the previously proposed hierarchical model of kinetochore assembly in *Drosophila*, with CID at the top of the hierarchy (Przewloka *et al.* 2007; Schittenhelm *et al.* 2007; Erhardt *et al.* 2008). Our results confirm that *Drosophila* CENP-C plays a major role in CID localization/maintenance and suggest that the observed CID and CENP-C interdependency may be a consequence of impaired centromere organization. In agreement, our results show that CENP-C is essential for the proper localization of other centromere-specific proteins in *Drosophila*. These results are consistent with an essential role for *Drosophila* CENP-C in maintaining normal centromere identity, which appears to be species-specific. Interestingly, similar to *Drosophila*, no CCAN homologues have yet been identified in *C.elegans* (reviewed in Przewloka and Glover 2009), suggesting that in systems lacking CCAN, centromere function relies heavily on the structural role of CENP-C. Different to what as been reported in vertebrate cells, our results are consistent with a model in which CENP-C is required to lay the foundation for all components essential for kinetochore assembly. We hypothesize that tight communication between inner kinetochore proteins and the centromere, is an essential step in determining centromere identity.

1.10 *Drosophila* centromere-kinetochore interface

CENP-C inactivation has been performed in several systems and while all studies concluded that CENP-C is essential for cell viability and mitotic progression, several inconsistencies in phenotypic analyses were observed (Fukagawa and Brown 1997; Fukagawa *et al.* 1999; Kwon *et al.* 2007). Two possible hypotheses

could explain why CENP-C phenotypic analyses yield variable results in different systems. Either CENP-C inactivation was not as efficient in other species as it is in *Drosophila* S2 cells or these discrepancies could reflect structural differences in kinetochore organization specific to *Drosophila* chromosomes.

It was recently proposed that CCAN protein copy number at kinetochores varies amongst species (Johnston *et al.* 2010) suggesting that although specific centromere/kinetochore assembly modules appear to be mostly conserved, differences in protein copy number may affect phenotypic analyses. Furthermore, bioinformatic approaches directed at evaluating CENP-C conservation between species reveals that while CENP-C is highly conserved amongst other *Drosophila* species, it bears very limited homology with its counterparts in higher eukaryotes. Similarly to CENP-C, many of the CCAN proteins may have failed to be detected in the *Drosophila* genome because they lack significant conservation. While at this point we cannot rule out this possibility, it is also possible that CCAN proteins are not present in *Drosophila*. Given that *Drosophila* CENP-C plays an essential role in overall centromere/kinetochore organization, which is shared with CCAN components in other eukaryotes, our results are consistent with the idea that in *Drosophila*, CENP-C fulfils many of the functional roles of CCAN proteins. Importantly, our results argue in favour of a simpler centromere-kinetochore interface specific to *Drosophila* chromosomes.

1.11 SAC regulation by the spindle matrix

The data presented in Chapter 4 demonstrate that Mtor is part of a dynamic, nuclear-derived spindle matrix with mobility properties distinct from microtubules and associated proteins. Similar to what is observed for Mtor, retention of Mad2 at the spindle matrix is also shown to be resistant to microtubule depolymerisation suggesting that Mad2 itself may also be involved in spindle matrix function. Indeed, we show that Mad2 and Mtor/Tpr interact directly both in *Drosophila* and human cells and accordingly, depletion of Mtor/Tpr leads to reduced Mad2 accumulation at kinetochores in both systems demonstrating functional conservation. Although the localization of Mad1, BubR1, Ndc80 and Rod (component of the RZZ) at unattached kinetochores is unaltered, the levels of Mps1 are strongly reduced in the absence of Mtor. However, since Mps1 kinase activity is required for proper Mad2 accumulation, the results are more consistent with an indirect regulatory role of Mtor in Mad2 accumulation that may be catalyzed by Mps1. Nevertheless, we show that

Mtor ensures a timely and effective recruitment of Mad1 and Mad2 to unattached kinetochores thus providing a plausible explanation for a long-lasting SAC paradigm: the wait-anaphase signal generated continuously at unattached kinetochores must be diffusible to prevent anaphase onset, but at the same time must be restricted to the vicinity of the spindle.

1.12 The spindle matrix and the 'two-step' model

The data presented here show that depletion of spindle matrix component Mtor causes mis-localization of Mad2 and Mps1 at unattached kinetochores and consistently, Mtor-depleted cells transit through mitosis slightly faster than control cells and also display a weakened SAC response to microtubule depolymerisation. Furthermore, Mtor depletion results in significant attenuation of chromosome segregation velocities by affecting spindle elongation. Interestingly, this phenotype is reverted if cells are artificially provided with time prior to anaphase, suggesting that the role of Mtor in SAC maintenance is to provide enough time for proper spindle maturation. Collectively, the results support a model in which the spindle matrix is involved in SAC maintenance by ensuring that Mad2 and Mps1 are efficiently recruited to unattached kinetochores. Moreover, Mtor is also proposed to play a role in compartmentalizing Mad2 to the vicinity of the mitotic spindle, even in the absence of microtubules.

Affecting the proper recruitment of Mad2 and Mps1 to unattached kinetochores results in decreased mitotic timing and cells display a weakened SAC response to spindle damage. Whether MCC formation is affected under these conditions is currently unclear, but in light of the 'two-step' model of SAC activation, it seems unlikely that the observed interaction between Mtor and Mad2 is required for MCC-dependent APC/C inhibition. Furthermore, Mad2 and Mps1 levels are reduced and not absent at unattached kinetochores, which could explain why mitotic progression in the absence of Mtor is significantly slower than that observed for Mad2 and CENP-C single depletions in S2 cells. Nevertheless, the collective results presented here are in full agreement with the predictions formulated by the 'two-step' model of SAC maintenance.

1.13 Final comments

In conclusion, the results presented in this thesis provide further insight on the mechanisms of Mad2-dependent SAC activation and on overall centromere-kinetochore assembly in *Drosophila*. Collectively, the results fully support the pathways involved in regulating SAC maintenance predicted by the refined version of 'two-step' model **(Figure 1)**. Our results demonstrate that Mad2 localization at kinetochores is dispensable for the initial stages of SAC activation (stages I and II) and that cytoplasmic Mad2 is required, but not sufficient for sustained APC/C inhibition. Cytoplasmic Mad2 can prevent activation of the APC/C before NEBD, thus allowing other SAC proteins such as Mps1, Bub3 and BubR1 to reach unattached kinetochores in early prometaphase. However, under these conditions cells can produce a sustained, but not fully effective SAC response to microtubule damage. Accordingly, since kinetochore-null cells show a similar mitotic timing to Mad2-depeleted cells, it is probable that BubR1 kinetochore localization is essential for ensuring this partial SAC maintenance in conditions when Mad2 is prevented from localizing at kinetochores. Consistently, our results also show that if Mad2 and Mps1 levels are decreased at kinetochores, cells transit faster through mitosis and show a weakened SAC response to microtubule poisons.

Anti-mitotic chemotherapeutics are known to induce apoptosis in cancer cells by causing prolonged SAC activation. Precisely how prolonging mitosis contributes to cancer treatment is not completely understood. Our results shed light on the molecular players involved in sustained mitotic arrest and provide functional data on SAC maintenance that may be exploited in the design of more efficient cancer therapeutics. Identifying the mitotic lesions that co-operate with aneuploidy, polyploidy and DNA damage to promote tumourigenesis might provide novel therapeutic strategies that increase the efficiency of cancer treatment.

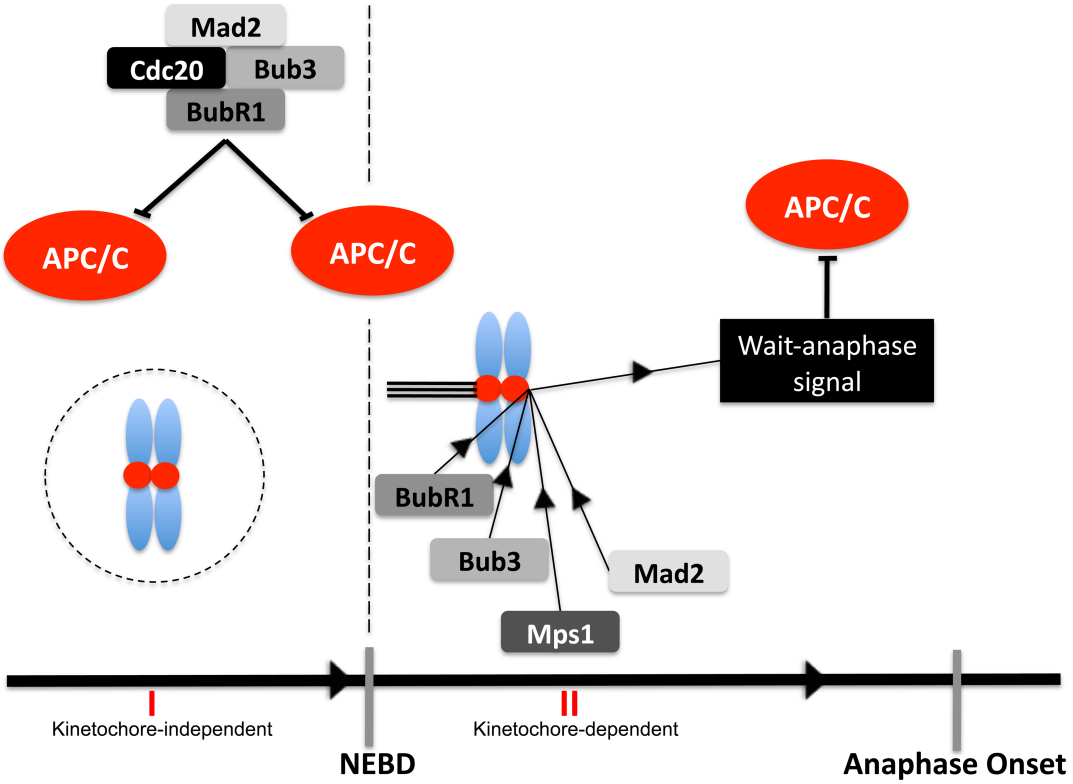


Figure 1 – Schematic representation of the refined version of the 'two-step' model of SAC maintenance in *Drosophila*. Roman numbers shown in red represent the different stages of SAC maintenance. In a first event (stage I), MCC (composed of Mad2, Bub3, BubR1 and Cdc20) formation specifically promotes APC/C inhibition before nuclear envelope breakdown (NEBD) and kinetochore assembly take place, thus allowing cells to accumulate mitotic cyclins and enter prometaphase. In the second, kinetochore-dependent step (stage II) predicted by this model, sustained APC/C inhibition is achieved soon after NEBD through the concerted action of the localization of SAC proteins such as Mps1, Bub3, BubR1 and Mad2 at unattached kinetochores.

Part IV

Materials and Methods

1. Materials and Methods

1.1 Double-stranded RNA interference in *Drosophila* S2 cells

To deplete Mad2, S2 cells were transfected with dsRNA corresponding to a fragment of Mad2 defined by the primers (Forward) TAA TAC GAC TCA CTA TAG GGA ATA GCG GCA ATT TAGC and (Reverse) TAA TAC GAC TCA CTA TAG GGA GAA GCG CAG CTG GA. The PCR product was purified (QIAGEN) and used as a template for the synthesis of the dsRNA using the MEGAscript T7 kit (Ambion) and following the manufacturer's instructions. For Zw10 depletion, S2 cells were transfected with dsRNA corresponding to a fragment of Zw10 defined by the primers (Forward) TAA TAC GAC TCA CTA TAG GGT GGC ACC TAC GTT CGA TT and (Reverse) TAA TAC GAC TCA CTA TAG GGA TCA TGC AGC GTG GGA AG. The PCR product was purified and used a template for dsRNA synthesis using MEGAscript T7 kit (Ambion) and following the manufacturer's instructions. For CENP-C depletion, *Drosophila* S2 cells were transfected with double-stranded RNA (dsRNA) corresponding to a fragment of CENP-C spanning the 5'UTR and including the ATG initiation codon of CENP-C cDNA (CG11746). CENP-C cDNA construct was cloned into pSPT18 and pSPT19 expression vectors (Roche) and dsRNA was synthesized using the MEGAscript T7 kit (Ambion) and following the manufacturer's instructions. For BubR1 depletion, a fragment of BubR1 cDNA was cloned into pSPT18 and pSPT19 expression vectors and the RNA synthesized as done for Mad2, Zw10 and CENP-C (for further details see Maia *et al.* 2007). RNAi experiments were performed as previously described (Maiato *et al.* 2003) by adding 15-30 µg of dsRNA to 10^6 cells. For Mad2 and BubR1 RNAi experiments 15 µg were used; for Zw10 RNAi experiments, 25 µg were used; for CENP-C depletion 30 µg were used. At 24 hour time-points after the addition of the dsRNA, cells were collected and processed for immunofluorescence, western blot and FACS analysis.

1.2 Cell cultures and drug-induced treatments

Drosophila cultured S2 cells were used for antibody staining in fixed material. In fixed cell analysis, for depolymerizing microtubules 30 µM of Colchicine (Sigma-Aldrich) was added to cells for 5hr. To block proteasome activity, 20 µM of MG132 (Calbiochem) was added to cells for 4hr. MG132+Taxol kinetochore-

microtubule interaction assay was performed using 20 µM of MG132 (Calbiochem) and 100 nm Taxol. For the cold treatment assay, cells were incubated for 10-12 min on ice prior to cell fixation (PHEM; see Appendix 2) to promote the disassembly of unstable microtubules. Cells were then fixed and stained for image processing.

1.3 Immunofluorescence in S2 cells

Cells were centrifuged onto slides (5 minutes at 1000 rpm) and processed for simultaneous fixation and extraction in 3.7% methanol-free formaldehyde, 0.5% Triton X-100 in 1X PBS for 10 minutes followed by 3 washes in 1X PBS, 0.05% Tween 20. For separate fixation/extraction protocol (used to reveal spindle morphology) the fixation solution was prepared using 3.7% methanol-free formaldehyde in 1X PBS for 10 minutes and then extraction was performed 2 x 5 minutes using 1X PBS, 0.5% Triton X-100. For Mad2 antibody detection, slides were fixed using 4% paraformaldehyde in 1X PBS for 12 minutes and further extracted for 8 minutes in 1X PBS, 0.1% Triton X-100. Blocking was performed in 1X PBS, 10% FBS, 0.05% Tween 20 for 30 minutes. Primary antibody incubations were prepared in Blocking solution for at least 1 hour at room temperature or overnight at 4°C, followed by 3 x 10 min washes in 1X PBS, 0.05% Tween. Secondary antibody incubations performed as described for the primary antibodies, including the 3 x 10 minutes washes at the end. Slides were then mounted using Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories, Inc. Burlingame, CA 94010). Calcium treatment was performed as previously described (Kapoor *et al.* 2000). Z-series optical sections were collected using the Zeiss Axiovert 200M microscope (Carl Zeiss, Germany) using an AxioCam. Data stacks were deconvolved using either the Axiovision AxioVs40 V 4.2.0.0 (Carl Zeiss, Germany) or the Huygens Essential version 3.0.2p1 (Scientific Volume Imaging B.V., The Netherlands). Images treated using Adobe Photoshop CS (Adobe Microsystems, CA).

1.4 Primary Antibodies

The primary antibodies used were anti-Mad2 (Rb 1223) rabbit, anti-BubR1 (Rb 666) rabbit, anti-Bub1 (Rb 1112) rabbit, and anti-Bub3 (Rb 730) rabbit (Logarinho *et al.* 2004). Anti-phospho Histone H3 rabbit (Polyclonal, Upstate), anti-

a-tubulin clone B-5-1-2 mouse (Monoclonal, Sigma), anti-Barren rabbit (Bhat *et al.* 1996), anti-Topo II (P2G3) mouse (Swedlow *et al.* 1993), anti-Polo (MABMA294) mouse monoclonal (Llamazares *et al.* 1991), anti-DRad21 (Rb 735) rabbit (Warren *et al.* 2000), anti-CID chicken (Blower and Karpen 2001) and anti-CID rabbit (Henikoff *et al.* 2000). Other primary antibodies used were anti-CENP-C (Rb1) rabbit (Heeger *et al.* 2005); anti-BubR1 rat (Maia *et al.* 2007); anti-α-tubulin mouse DM1A (Sigma-Aldrich); anti-DRad21 guinea pig (Heidmann); anti-MEIS332 guinea pig (Orr-Weaver). Anti-Aurora B rabbit and anti-INCENP rabbit antibodies were used as previously described (Adams *et al.* 2001). Anti-CENP-meta rabbit, anti-Zw10 rabbit, anti-Ndc80 rabbit and anti-Nuf2 guinea pig were made by Byron Williams and Michael Goldberg. Anti-γ-tubulin mouse (GTU88, Sigma-Aldrich) and anti-CID rat antibody (Steffensen and Sunkel unpublished).

1.5 Secondary Antibodies

For western blot analysis, the secondary antibodies used were anti-rabbit HRP (Amersham); anti-mouse HRP (Amersham) and anti-rat HRP (Amersham) using the manufacturer's recommended working dilutions. For immunofluorescence analysis, the secondary antibodies used were Alexa 488, 568 or 647 (either anti-rabbit, -rat, -mouse, -guinea pig or -chicken) (Invitrogen) depending on the immunofluorescence analysis and using manufacturer's recommended working dilutions.

1.6 Image Processing and quantifications

Immunofluorescence Z-stack optical sections acquired using an AxioImager Z1 microscope (Carl Zeiss, Germany) connected to an Axiocam MR ver.3.0 (Carl Zeiss, Germany). All images were collected using a 100X objective. Data stacks were deconvolved using the Huygens Essential version 3.0.2p1 (Scientific Volume Imaging B.V., The Netherlands). Image projections performed using ImageJ 1.3v software (http://rsb.info.nih.gov/ij/) and Adobe Photoshop CS (Adobe Microsystems, CA). Quantification of fluorescence intensity at kinetochores was performed using the ImageJ 1.3v software on raw images after maximal intensity projections using a previously defined region of interest (ROI) that includes the whole kinetochore.

1.7 In vivo time-lapse fluorescence imaging

In vivo timing of mitotic progression was performed using S2 cells stably expressing either GFP-α-Tubulin (Rodgers 2002); H_2B-GFP;mCherry-α-Tubulin (provided by G.Goshima) or GFP-α-Tubulin;mCherry-CID. Control (72h, 96h or 120h), Mad2 RNAi cells (72h), Zw10 RNAi cells (120h), Zw10/Mad2 co-depleted cells (96h), Zw10/BubR1 co-depleted cells (96h) or CENP-C depleted cells (96h) were plated onto glass coverslips previously treated with 100 mg/ml concanavalin A (Sigma). Images were collected at 25°C with a spinning disc confocal system (Revolution; Andor) equipped with an electron multiplying charge-coupled device camera (iXonEM; Andor) and a CSU-22 unit (Yokogawa) based on an inverted microscope (IX81; Olympus). Two laser lines (488 and 561 nm) were used for near-simultaneous excitation of GFP and mCherry/mRFP, and the system was driven by iQ software (Andor). Time-lapse imaging of z stacks with 0.2-0.4µm steps spanning the entire volume of the mitotic apparatus were collected at every 30 seconds and image treatment was performed using AxioVision 4.3 Software (Carl Zeiss, Germany).

1.8 FACS analysis

Control, Mad2 and CENP-C depleted cells were centrifuged at 2000 rpm for 5 minutes, re-suspended in 200 ml PBS and fixed using 2ml 70% ice cold ethanol in PBS, added drop by drop whilst vortexing. Samples were kept on ice for 30 minutes before centrifugation at 1000 rpm for 5 minutes. The pellet was re-suspended in 200 ml PBS with 100 mg/ml RNAse and 100 mg/mL of propidium iodide. Samples were incubated for a further 30 minutes at 37°C. For DNA content analysis, we used a FACSCalibur (Becton Dickinson) flow cytometer and data from 10000 cells was obtained. Results were analysed using Cell Quest data acquisition software.

1.9 Transient mitotic arrest with MG132

Cells were collected every at various time points and fixed onto slides for analysis. Time 0 min corresponds to 72h after the addition of the dsRNA. At this time point cells were incubated with a low dose of MG132 (2 µM), the sufficient

concentration to induce a mitotic arrest in *Drosophila* S2 cells. After 120 min incubation, the cell culture was diluted 3-fold either with fresh media, or media containing the microtubule poison colchicine. After the MG132 washout, cells were collected at different time-points for immunofluorescence or western blot analysis of cell cycle progression after a transient mitotic arrest. Slides were fixed and stained accordingly using the various antibody incubations described in the results section.

1.10 Kinetochore-Microtubule interaction (MG132-Taxol) assay

Cells were incubated with 20 µM of MG132 (Calbiochem) during 1h and then with 100 nM of taxol (Sigma-Aldrich) for 3h. This treatment blocks mitotic exit and induces the collapse of the mitotic spindle into a monopolar structure with all chromosomes localized around the periphery of the mono-aster. This chromosome organization allows an easy read-out of the kinetochore-microtubule attachments. Note that this assay does not allow us to discriminate between amphitelic and syntelic attachment, however, mono-oriented and unattached chromosomes can be easily scored (Maia *et al.* 2007).

1.11 Western Blot analysis

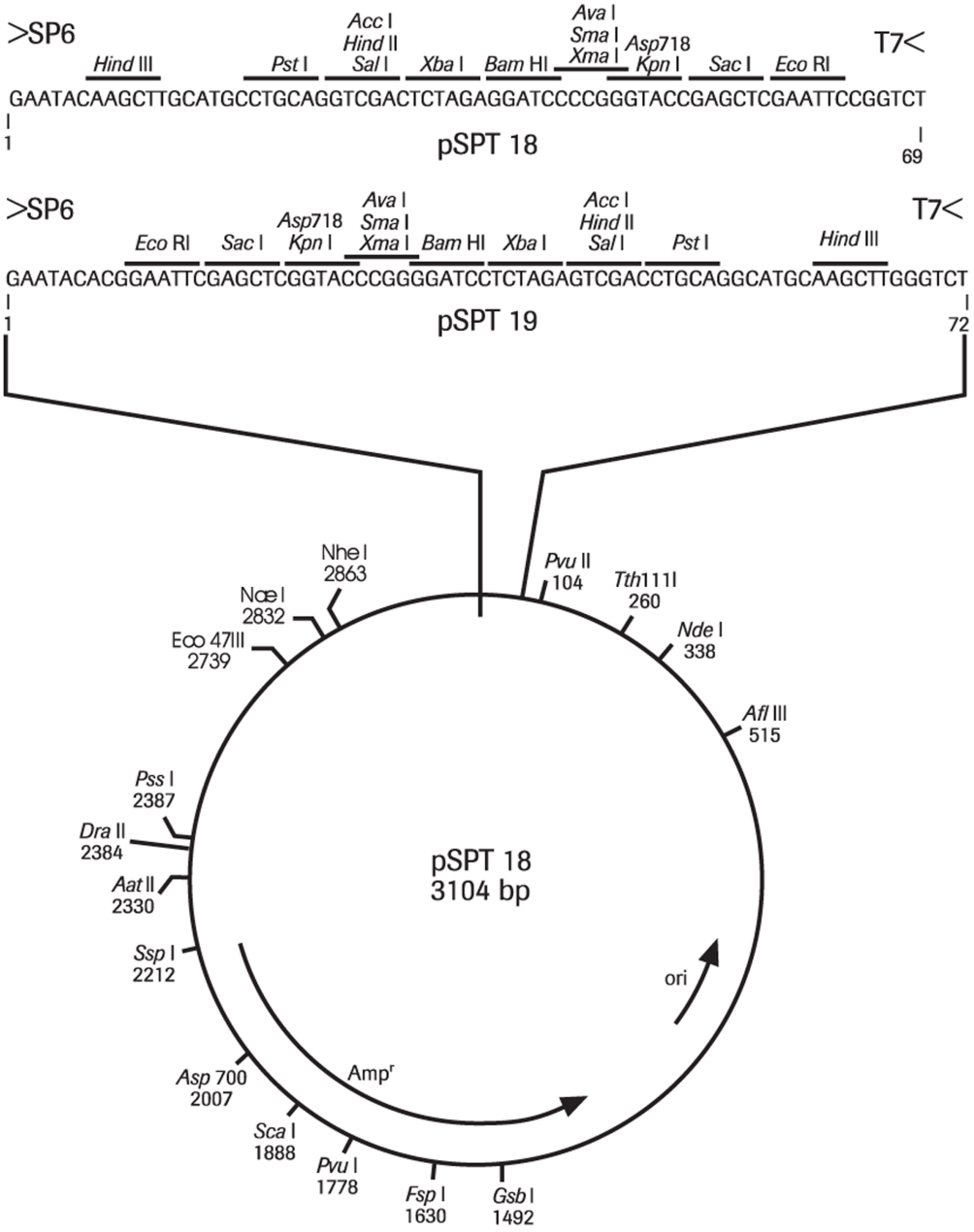
For cell sample preparation, $1x10^6$ control and RNAi-treated cells were harvested and centrifuged at 13,000 rpm for 10 min at 4ºC. Pellets were re-suspended and washed in 1X PBS with protease inhibitors and re-centrifuged at 13,000 rpm for 10 min at 4ºC. Cell pellets were re-suspended in 20 µl of 1X Laemmli Buffer, incubated at 95ºC for 5 min and loaded into a 7.5-12% polyacrylamide gel, depending on the size of the protein of interest. Gels ran at 100V and upon completion protein contents were transferred onto a nitrocellulose membrane (Schleicher & Schuell) using a wet transfer system (Bio-Rad) at 100V for 90 min at 4ºC. To access transfer efficiency, the nitrocellulose membrane was incubated for 5 min with Ponceau S solution and the excess of dye was then washed out with ddH2O. Membranes containing protein extracts were then incubated in blocking solution (5% powdered milk in 1X PBS, 0.05% Tween 20) for at least 1hr at RT and then incubated with specific primary antibodies (diluted in 1%BSA,1XPBS, 0.05% Tween 20) overnight at 4ºC. Membrane was then washed 3 X 10 min in 1XPBS, 0.1% Triton X-100 and incubated with specific secondary antibodies (diluted in

1%BSA,1XPBS, 0.05% Tween 20) for at least 1-3h at RT. Membrane is washed again 3 X 10 min in 1XPBS, 0.1% Triton X-100 and incubated in ECL detection solution for 2-5 min before detection with X-ray films (Fuji Medical X-Ray Film).

1.12 Plasmids

For dsRNA preparation, fragments corresponding to full-length or truncated versions of the proteins of interest (BubR1 or CENP-C) were sub-cloned into pSPT18 and pSPT19 vectors (Roche) before transcription using MEGAscript T7 kit (Ambion).

pSPT18 and pSPT19 vectors (Roche)



Part V

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Part VI

Appendixes

Appendix 1

Abbreviations

ACA: Anti-Centromere Antibodies

APC/C: Anaphase-Promoting Complex/Cyclosome

ATM: Ataxia Telangiectasia Mutated

ATR: Ataxia Telangiectasia and Rad-3-related

BSA: Bovine Serum Albumin

Bub: Budding uninhibited by benzimidazole

CATD: CENP-A Targeting Domain

CCAN: Constitutive Centromere-Associated Network

Cdks: Cyclin-dependent kinases

cDNA: complementary DNA

C.elegans: Caenorhabditis elegans

CENP: Centromere-associated Protein

CH: Calponin-homology

CID: Centromere Identifier

CIN: Chromosomal Instability

CLIP: Cytoplasmic Linker Protein

CPC: Chromosomal Passenger Complex

CREST: Calcinosis, Raynaud's phenomenon, Esophageal dysmotility, Sclerodactyly and Telangiectasia.

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

DNA: DeoxyriboNucleic Acid

DSBs: Double-Strand Breaks

dsRNA: double-stranded RNA

DUB: de-ubiquitylating protein

EAST: Enhanced Adult Sensory Threshold

ECL: Enhanced ChemiLuminescence

EGFP: Enhanced Green Fluorescent Protein

FACS: Fluorescence-Activated Cell Sorting

FBS: Fetal Bovine Serum

FRAP: Fluorescence Recovery After Photobleaching

GDP: Guanosine Di-Phosphate

GFP: Green Fluoresent Protein

GTP: Guanosine Tri-Phosphate

G1: Gap phase 1

G2: Gap phase 2

HeLa: Human immortal cell line

h(r): hour

kDa: kiloDalton

k-fibre: kinetochore fibre

KMN: KNL1/Mis12 complex/Ndc80 complex

KT: Kinetochore

Mad: Mitotic-arrest deficient

MAPs: Microtubule-Associated Proteins

MCC: Mitotic Checkpoint Complex

mg: milligram

ml: milliliter

min: minutes

MPF: Maturation/Mitosis-Promoting Factor

mRFP: monomeric Red Fluorescent Protein

MT: Microtubule

MTOC: Microtubule Organizing Centre

Mtor: Megator

n: number of samples in experiment

n.d.: not determined

NEBD: Nuclear Envelope Break-Down

NPC: Nuclear Pore Complex

nm: nanometer

nM: nanoMolar

PBS: Phosphate-Buffered Saline

PCR: Polymerase Chain Reaction

PH3: Phospho-histone H3

Plk(s): Polo-like kinase(s)

PSCS: Precocious Sister Chromatid Segregation

RNA: RiboNucleic Acid

RNAi: RNA interference

RZZ: Rod-Zw10-Zwilch

ROI: Region Of Interest

rpm: rotations per minute

RT: room temperature

SAC: Spindle Assembly Checkpoint

S.cerevisiae: Saccharomyces cerevisiae

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

SDS: Sodium Dodecyl Sulfate

SDS-PAGE: Sodium Dodecyl Sulfate-PolyAcrilamide Gel Electrophoresis

S.pombe: Saccharomyces pombe

S phase: DNA Synthesis phase

ssDNA: Single-Stranded DNA

S2: Schneider 2

Topo II: Topoisomerase II

Tpr: Translocated Promoter Region

UTR: UnTranslated Region

UV: UltraViolet

V: volts

YFP: Yellow Fluorescent Protein

µg: microgram

µl: microliter

µm: micrometer

µM: microMolar

ºC: degrees Celsius

Appendix 2

Recipes

LB medium:

1% Tryptone

0.5% Yeast Extract

1% NaCl

LB Agar:

1.5% (w/v) in LB medium

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

PHEM:

60 mM PIPES

25 mM HEPES pH 7.0

10 mM EGTA

4 mM $MgSO_4$

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% acrylamide; 125 mM Tris-HCl, pH 6.8; 0.1% SDS;

Separating gel: 7.5%-12% acrylamide; 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

Protein Transfer Buffer:

25 mM Glycine

192 mM Tris pH 8.3

20% (w/v) Methanol

Enhanced ChemiLuminescence (ECL) detection:

Solution A: 10 ml Tris 100 mM pH 8.5, 44 µl Coumaric 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 for 2-5 min with the membrane at the time of ECL detection.

Schneider's Insect Medium:

Schneider's Insect Medium, with L-glutamine and sodium bicarbonate, (Invitrogen) 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

Supplementary Movie Legends

Movie 1 - Control cell at 72h expressing GFP-α-Tubulin. Animated time-lapse images from control S2 *Drosophila* cells stably expressing GFP-Tubulin. From these images it is possible to calculate the time that cells spend in prometaphase, from NEBD (indicated by the rapid entry of GFP-tubulin into the nuclear space) to the initiation of anaphase (as the spindle elongates and chromatids start to move to the poles). Images collected at every 30 seconds.

Movie 2 - Mad2 RNAi cell at 72h expressing GFP-α-Tubulin. Animated time-lapse images from S2 *Drosophila* cells stably expressing GFP-Tubulin, previously treated for 72 h with dsRNA against Mad2. From these images it is possible to calculate the time that cells spend in prometaphase, from NEBD (indicated by the rapid entry of GFP-tubulin into the nuclear space) to the initiation of anaphase (as the spindle elongates and chromatids start to move to the poles). Depletion of Mad2 causes a rapid exit from mitosis soon after NEBD. Images collected at every 30 seconds.

Movie 3 - Control cell at 120h expressing GFP-α-Tubulin. Animated time-lapse images from control S2 *Drosophila* cells stably expressing GFP-Tubulin. From these images it is possible to calculate the time that cells spend in prometaphase (mitotic timing). NEBD is indicated by the rapid entry of GFP-tubulin into the nuclear space and anaphase onset is characterized by rapid spindle elongation. Images collected at every 30 seconds.

Movie 4 – Zw10 RNAi cell at 120h expressing GFP-α-Tubulin. Animated time-lapse images from Zw10-depleted S2 *Drosophila* cells stably expressing GFP-Tubulin. From these images it is possible to calculate the time that cells spend in prometaphase (mitotic timing). NEBD is indicated by the rapid entry of GFP-tubulin into the nuclear space and anaphase onset is characterized by rapid spindle elongation. In the absence of Zw10 extra centrosomes are commonly observed, however, most cells normally form bi-polar spindles and transit through mitosis with a mitotic schedule similar to untreated cells. Images collected at every 30 seconds.

Movie 5 – Zw10 RNAi cell at 120h expressing GFP-α-Tubulin. Animated time-lapse images from Zw10-depleted S2 *Drosophila* cells stably expressing GFP-Tubulin. In the absence of Zw10 extra centrosomes are commonly observed and but cells transit through mitosis with a similar mitotic timing as untreated cells. In the absence of Zw10 extra centrosomes are commonly observed. Despite several spindle defects, multi-polar cells delay in mitosis and exit in the presence of mis-aligned chromosomes, a feature that is consistent with a weakened SAC. Images collected at every 30 seconds.

Movie 6 - Control cell at 96h expressing H2B-GFP;mCherry-Tubulin. Untreated S2 cell stably expressing H2B-GFP (green);mCherry-Tubulin (red) at 96h. Images were collected using a spinning disc confocal system at intervals of 30 seconds. NEBD is indicated by the rapid entry of mCherry-Tubulin into the nuclear space and anaphase onset takes place when chromatid separation is observed. Note that even in the presence of multilple centrosomes, untreated cells form robust bi-polar spindles and segregate normally.

Movie 7 – Zw10 and BubR1 co-depleted cell at 96h expressing H2B-GFP;mCherry-Tubulin. S2 cells stably expressing H2B-GFP (green);mCherry-Tubulin (red) previously treated for 96hr with specific dsRNA against Zw10 and BubR1. Images were collected using a spinning disc confocal system at intervals of 30 seconds. NEBD is indicated by the rapid entry of mCherry-Tubulin into the nuclear space and anaphase onset takes place when chromatid separation is observed. In the absence of Zw10 and BubR1, cells transit through mitosis with an accelerated mitotic schedule, even in the presence of mis-aligned chromosomes.

Movie 8 – Zw10 and Mad2 co-depleted cell at 96h expressing H2B-GFP;mCherry-Tubulin. S2 cells stably expressing H2B-GFP (green);mCherry-Tubulin (red) previously treated for 96hr with specific dsRNA against Zw10 and Mad2. Images were collected using a spinning disc confocal system at intervals of 30 seconds. NEBD is indicated by the rapid entry of mCherry-Tubulin into the nuclear space and anaphase onset takes place when chromatid separation is observed. In the absence of Zw10 and Mad2, cells transit through mitosis with a highly accelerated mitotic schedule similar to what is observed for Mad2 RNAi cells, resulting in extensive chromatin bridges.

Movie 9 – Control cell at 96h expressing H2B-GFP;mCherry-Tubulin. Untreated S2 cells stably expressing H2B-GFP (green);mCherry-Tubulin (red). Images were collected using a spinning disc confocal system at intervals of 30 seconds. NEBD is indicated by the rapid entry of mCherry-Tubulin into the nuclear space and anaphase onset takes place when chromatid separation is observed. Anaphase onset only takes place when all chromosomes are correctly positioned at the spindle equator.

Movie 10 - CENP-C depleted cell at 96h expressing H2B-GFP;mCherry-Tubulin. S2 cell stably expressing H2B-GFP (green);mCherry-Tubulin (red) previously treated for 96hr with specific dsRNA against CENP-C. Images were collected using a spinning disc confocal system at intervals of 30 seconds. Note that in the absence of CENP-C the time from NEBD to anaphase onset is severely shortened and random chromosome missegregation is observed. Anaphase onset is characterized by sister chromatid separation rather than chromatid migration to opposite poles since CENP-C disruption strongly affects kinetochore-microtubule attachment.

Movie 11 - CENP-C depleted cell at 96h expressing H2B-GFP;mCherry-Tubulin. S2 cell stably expressing H2B-GFP (green);mCherry-Tubulin (red) previously treated for 96hr with specific dsRNA against CENP-C. Images were collected using a spinning disc confocal system at intervals of 30 seconds. Note that in the absence of CENP-C the time from NEBD to anaphase onset is severely shortened and random chromosome missegregation is observed.

Movie 12 – Control cell expressing GFP-Tubulin;mCherry-CID. Untreated S2 cell stably expressing GFP-Tubulin (green);mCherry-CID (red). Images were collected using a spinning disc confocal system at intervals of 30 seconds. Greyscale images correspond to CID signal. NEBD is indicated by the rapid entry of GFP-Tubulin into the nuclear space and anaphase onset takes place when sister kinetochore separation is observed.

Movie 13 – Megator-depleted cell expressing GFP-Tubulin;mCherry-CID. Megator-depleted cell stably expressing GFP-Tubulin (green);mCherry-CID (red). Images were

collected using a spinning disc confocal system at intervals of 30 seconds. Greyscale images correspond to CID signal. Note that Mtor RNAi cells form normal bi-polar spindles but transit through mitosis 15% faster than untreated cells.

Movie 14 – Mad2-depleted cell expressing GFP-Tubulin;mCherry-CID. Mad2-depleted cell stably expressing GFP-Tubulin (green);mCherry-CID (red). Images were collected using a spinning disc confocal system at intervals of 30 seconds. Greyscale images correspond to CID signal. Note that in the absence of Mad2, mitotic timing is severely shortened and cells exit mitosis in the presence of mis-aligned chromosomes.