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Kinetochore-Microtubule Interaction and Regulation

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SUMMARY

Kinetochores bind spindle microtubules and also act as signaling centers that monitor this interaction. Defects in kinetochore assembly lead to chromosome missegregation and aneuploidy. The interaction between microtubules and chromosomes involves a conserved super-complex of proteins, known as the KNL1Mis12Ndc80 (KMN) network, composed by the KNL1 (Spc105), Mis12, and Ndc80 complexes. Previous studies indicate that this network is necessary for kinetochore-microtubule attachment and recruitment of different kinetochore proteins, playing essential functions in chromosome movement, congression, biorientation, and segregation. Previous results had shown that CENP-E promotes kinetochore-microtubule instability, suggesting a role in the correction mechanism that monitors microtubule attachment. In the first experimental chapter of this thesis we address the role of CENP-E and Aurora B kinase activities and how they cooperate to promote efficient chromosome biorientation. Our results, obtained in HeLa cells, do not support a model in which CENP-E regulates Aurora B kinase activity. However, they strongly suggest that Aurora B kinase destabilizes spindle pole proximal kinetochore-microtubule interactions keeping the SAC active to allow CENP-E-mediated congression of monoriented chromosomes.

In the second chapter we investigate the dynamics during cell cycle of the *Drosophila* Mis12 kinetochore complex. We show that Mis12 is not a constitutive protein of the kinetochore since it fails to localize at the nucleus in a population of interphase cells indicating that is probably loaded either during the G1/S or the S/G2 transition. Moreover, our FRAP analysis showed no significant differences in Mis12 dynamics between interphase and mitosis, with identical mobile and immobile fractions and a high turnover of the protein levels in both phases.

Finally, in the third chapter we performed a detailed study addressing the role of the different KMN network components in microtubule attachment, chromosome movement and SAC signaling during mitosis. We use dsRNAi and *in vitro* and *in vivo* fluorescence microscopy in *Drosophila* S2 cells

allowing us to suggest that different KMN network components perform different roles in microtubule attachment and chromosome segregation. Depletion of different components results in mostly lateral kinetochore-microtubule attachments that are relatively stable revealing that Spc105 can sustain these interactions. *In vivo* analysis shows that lateral kinetochore-microtubule interactions in Mis12 and Ndc80 depleted cells are still functional allowing poleward kinetochore movement. We also find that different KMN network components affect differently the localization of spindle assembly checkpoint proteins. Taken together, our results suggest that Mis12 and Ndc80 complexes help to properly orient microtubule attachment, whereas Spc105 plays a predominant role in the kinetochore-microtubule attachment as well as in the poleward movement of chromosomes and cell viability.

Together, the work presented in this thesis through the study of specific network of kinetochore components provides further insights in the regulation of kinetochore-microtubule attachment, chromosome congression and segregation.

RESUMO

Os cinetocóros ligam-se aos microtúbulos do fuso mitótico funcionando igualmente como centros de sinalização que monitorizam essa interação. Problemas na construção do cinetocóro resulta na mal-segregação cromossómica e aneuploidia. A interação entre microtúbulos e cromossomas envolve um grupo conservado de proteínas designado por complexo KNL1Mis12Ndc80 (KMN). Este conjunto inclui a proteína KNL1 (Spc105), um conjunto de proteínas designado por complexo Mis12 e ainda outro conjunto de proteínas designado por complexo Ndc80. Estudos anteriores sugerem que este grupo de proteínas seja necessário quer para a ligação entre cinetocóros-microtúbulos bem como para o recrutamento de diversas proteínas cinetocorianas. Foi igualmente demonstrado que CENP-E, a proteína motora dos microtúbulos promove a instabilidade cinetocóro-microtúbulo, sugerindo um papel no mecanismo de correção que supervisiona a ligação dos microtúbulos aos cinetocóros. No primeiro capítulo do trabalho experimental desta tese, estudou-se o papel da actividade cinásica das proteínas CENP-E e Aurora B e como é que cooperam para promover uma eficiente biorientação cromossómica. Os resultados em células Hela por nós obtidos não suportam a teoria em que a proteína CENP-E regula a actividade cinásica da proteína Aurora B. Contudo, os nossos resultados sugerem que a atividade cinásica da proteína Aurora B destabilize as interações entre cinetocóros e microtúbulos nos polos do fuso mitótico, mantendo o ponto de controlo do fuso mitótico (SAC) ativo e permitindo deste modo que a proteína CENP-E promova a congressão dos cromossomas monorientados.

Na segundo capítulo do trabalho experimental estudou-se a dinâmica da proteína Mis12 durante o ciclo celular. Os nossos resultados mostram que a proteína Mis12 não é constitutiva nos cinetocóros; uma vez que não se encontra presente no núcleo de uma parte da população de células interfásicas. Esta deslocalização da proteína sugere um recrutamento da mesma para os cinetócoros durante a transição entre as fases G1/S ou S/G2. A análise da dinâmica da proteína por FRAP não revelou diferenças

significativas entre interfase e mitose, com idênticas frações móveis e imóveis e uma elevada rotação dos níveis da proteína em ambas as fases.

Por último, na terceiro capítulo do trabalho experimental, realizou-se um estudo detalhado sobre o papel das diferentes proteínas do complexo KMN durante a mitose, recorrendo ao uso de ARNi e microscopia de fluorescência *in vitro e in vivo*. Os nossos resultados sugerem que as diferentes proteínas que constituem o complexo proteico KMN desempenhem diferentes funções na ligação cinetocóros-microtúbulos e segregação cromossómica. A depleção das diferentes proteínas do complexo resulta maioritariamente em ligações laterais entre cinetocóros e microtúbulos. Estas ligações demostraram ser relativamente estáveis, com exceção para a proteína Spc105 que parece ser a responsável por a manutenção das mesmas. A análise *in vivo* revelou que as ligações laterais entre cinetocóro e microtúbulos nas células sem as proteínas Mis12 e Ndc80 são funcionais permitindo o movimento dos cinetocóros em direção aos polos. Os nossos dados mostraram-nos ainda que as diferentes proteínas do complexo KMN influenciam de modo distinto a localização das proteínas envolvidas no ponto de controlo do fuso mitótico. A integração destes resultados sugere-nos que os complexos proteicos Mis12 e Ndc80 ajudem a orientar corretamente a ligação dos microtúbulos aos cinetocóros. Por sua vez a proteína Spc105 assume um papel mais importante na ligação propriamente dita entre cinetocóro-microtúbulos bem como no movimento dos cromossomas em direção aos polos e na viabilidade celular.

Concluindo, o trabalho apresentado nesta tese, através do estudo de um complexo específico de proteínas abriu novas perspectivas na compreensão da regulação da ligação cinetocóro-microtúbulo, congressão e segregação cromossómica.

Part I

GENERAL INTRODUCTION

1. CELL CYCLE

1.1 OVERVIEW

In 1665, Robert Hooke took the first step towards the understanding of cell cycle; by visualizing at the microscope the honeycomb-like structure of cork that he termed as cells (Hooke, 1665). However only 200 years after, the cell was proposed to be the basic unit of all living organisms (Schwann, 1847). The first descriptions of the cell cycle were in 1880, when Walther Flemming described his observations in salamander cells. He identified a stainable material in the nucleus (chromatin) that during cell division suffered evident changes. These structures could undergo metamorphosis into threads (later called chromosomes) by shortening and thickening. More importantly, he described accurately the process of nuclear division where half of each chromosomes went to opposite directions of the dividing cell giving rise to two identical daughter nucleus. The threads became undistinguishable again resulting in two cells equal to the mother cell. He entitled this process *mitosis* (from the Greek word for thread) (reedited in Flemming, 1965). The essential features of cell cycle are conserved across species. This complex process has to be tightly regulated to ensure the fidelity of transmission of the genetic information. The cell cycle of eukaryotic organisms is divided in two major phases: interphase, the longest, in which cell duplicates its contents and grows and a shorter phase, M-phase, in which nuclear division takes place (mitosis) and two identical daughter cells are formed (cytokinesis) (Fig. 1).

1.2 INTERPHASE

Interphase is the period between the end of one M-phase and the beginning of the next and can be subdivided in three different stages. In the beginning of Synthesis phase (S phase), chromosomes start with a single chromatid and after DNA replication they end up with two sister chromatids. It is also during this phase that centrosomes, specialized structures associated with the nucleation of microtubules, are duplicated; but remain together until the beginning of mitosis. G1 and G2 are the two gap phases, where G1,

occurs before S phase and is particularly important since it is at this point that the cells become committed to either continue to divide or exit from cell cycle. When cells exit from cell cycle we say that enter G0, this situation may occur due to adverse growth conditions or inhibitory signal from other cells. G2 takes place before M phase (Fig. 1). The Gap phases are very important since they provide additional time for cell to grow and to prepare for a new round of nuclear division (reviewed in Morgan, 2007).

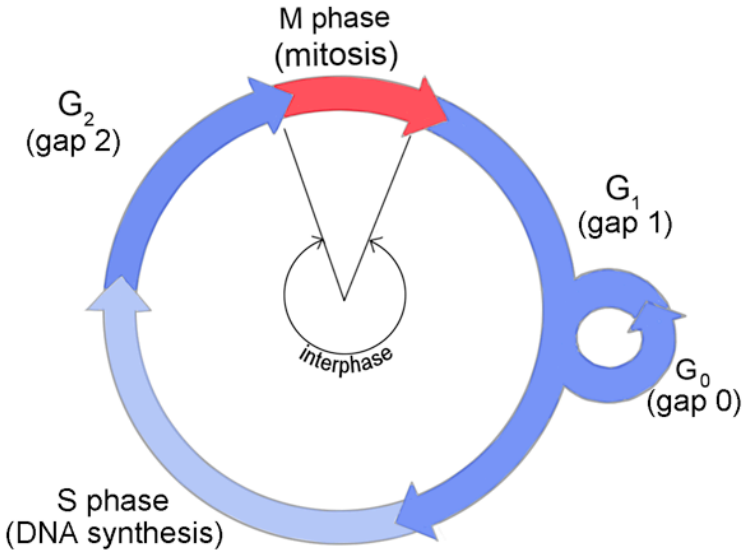


Figure 1. Schematic representation of eukaryotic cell cycle. There are two major phases, Interphase (blue) and M-phase (red). During interphase there are two GAP-phases G1 and G2, during which the cell grows by synthesizing components required for DNA synthesis or preparing for mitosis. During G1 cell can exit cell cycle (G0), if the cell continues to divide it has to replicate DNA and continues to the next phase S phase. After DNA replication the cell enter in G2 and the cell ultimately prepares for mitosis. During M-phase cell equally segregated the genetic content into two new identical daughter cells. (Adapted from http://cyberbridge.mcb.harvard.edu).

1.3 MITOSIS

Mitosis is a highly regulated process of eukaryotic cell division and is responsible for the distribution of the duplicated genome into two genetically identical daughter cells. During mitosis, the nuclear contents and the cell itself suffers a major structural transformation, based on these changes, mitosis

can be divided in five different stages: Prophase, Prometaphase, Metaphase, Anaphase and Telophase (Fig. 2).

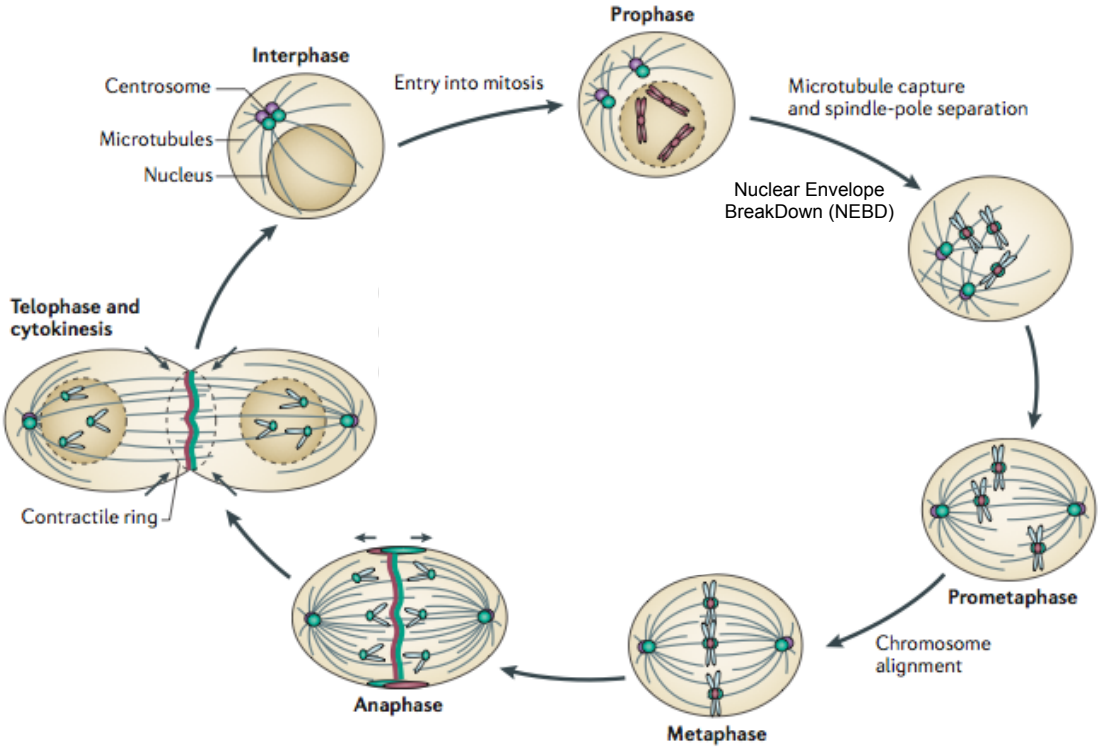


Figure 2. Schematic representation of mitosis and cytokinesis in a vertebrate cell. Mitosis starts at Prophase, DNA start to condense into well-defined chromosomes and the duplicate centrosomes migrate the opposite poles of the cell. After nuclear envelope breakdown (NEBD) the microtubules nucleated from centrosomes invade the nuclear space defining the beginning of Prometaphase. This phase is characterized by formation of mitotic spindle and capture of chromosomes by microtubules through out specialized structures built on chromosomes, the kinetochores. The mitotic spindle promotes the alignment of chromosomes at the equatorial plane of the cell and the cell is said to be in Metaphase. At Anaphase, the cohesin that holds sister chromatids together is degraded and they move to opposite poles of the cell. Mitosis is completed in Telophase, when the chromosomes decondense and the nuclear envelope re-forms around each group of daughter chromosomes. At same time, the cytoplasm is divided to give rise to two independent daughter cells by a process called Cytokinesis. (Adapted from Rath and Kozielski, 2012).

Prophase is characterized by the condensation of DNA into well individualized chromosomes, followed by separation of centrosomes, the major microtubule-organizing center (MTOC), that start to move to the opposite poles of the cell and begin to assemble the mitotic spindle. Prometaphase starts upon nuclear envelope breakdown (NEBD).

Microtubules invade the nuclear space and start to interact with chromosomes through a specialized structure that is assembled over the centromeric DNA, the kinetochore, also during this stage the mitotic spindle becomes fully organized. The kinetochore is responsible for the attachment of chromosomes to microtubules and also to recruit proteins that will monitor and correct erroneous attachments (Fig. 2). Eventually, chromosomes congress to the equatorial plane of the cell, align to form the metaphase plate and become bioriented so that each sister kinetochore is attached to microtubules emanating from opposite spindle poles. At this point the cell is in metaphase (Fig. 2). Anaphase starts with the degradation of cohesin, the structure that holds sister chromatids together, and subsequent movement of chromatis to the opposite poles of the spindle. Later due to spindle elongation, the poles move further apart. Finally, during telophase, the nuclear envelope reforms around each set of chromosomes. Concomitantly, the second stage of M-phase takes place, cytokinesis (Fig. 2). This phase consists in separation of the cell cytoplasm resulting in two genetically identical daughter cells (reviewed in Morgan, 2007).

1.4 CELL CYCLE CONTROL SYSTEM

To guarantee that all the events during the cell cycle occur properly and in the correct order, progression through cell cycle is tightly monitored and regulated. Errors during cell cycle can lead to cell death or cells can overpass these errors, accumulate genetic alterations leading to cell transformation and cancer. The cell monitors events mostly at three regulatory checkpoints: G1/S transition, G2/M transition and third the metaphase-to-anaphase transition. Once initiated, these transitions are irreversible, since that the proteins responsible for triggering them are degraded, making cell cycle progression unidirectional (King et al., 1996).

1.4.1 Cell cycle regulation

The central elements of the cell-cycle control system are a family of enzymes called cyclin-dependent kinases (Cdks). The level of the catalytic subunit of the enzymes is constant throughout cell cycle, however their activity oscillates triggering the initiation of cell cycle events. In order to be activated, Cdks have to bind to regulatory cell cycle specific Cyclins that are synthetized and degraded at different cell-cycle stages. Additionally, to be fully activated Cdks have to be phosphorylated at a threonine residue within the T-loop adjacent to the kinase active site. The enzymes responsible for this activation are known as Cdks-activating kinases (CAKs). Moreover, Cdk function can be negatively regulated by Wee1 phosphorylation at a T or Y residue within the ATP-biding site or desphosphorylated by Cdc25. As a result of these combined regulatory events, different Cyclins/Cdks complexes are formed at different cell-cycle stages. For example, in vertebrate cells, Cyclin D is essential for the cell to enter cell cycle and can be found associated to either Cdk4 or 6 kinases, in G1. Cyclin E is crucial for the initiation of DNA replication, forms a complex with Cdk2 in late G1 and falls during early S phase (Fig. 3).

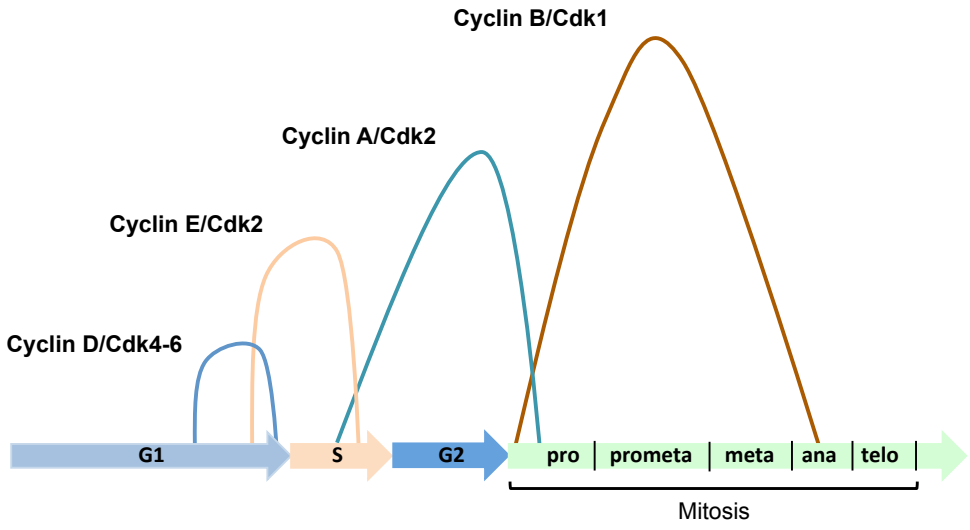


Figure 3. Cdk/Cyclin complexes during cell cycle. Cyclin D and E are present and responsible for several events during interphase. Cyclin A /Cdk2 complex starts to act during S phase and is involved in DNA replication and early

mitotic events. Cyclin B is the pivotal mitotic regulator, since its presence is essential for mitotic entry and exit.

Cyclin A plays distinct roles during cell cycle, starts to be synthesized in early S phase and remains high until the protein is degraded soon after NEBD, Cyclin A-Cdk2 complexes stimulate DNA replication and help to promote early mitotic events. Finally, Cyclin B levels start to rise during G2 and its association with Cdk1 increases dramatically during prophase. Cyclin B-Cdk1 is involved in the major mitotic events, centrosome separation, NEBD, and spindle formation, as well as mitotic exit that only happen after Cyclin B degradation (Fig. 3) (reviewed in Morgan, 2007).

Cell cycle progression depends as much on Cyclin degradation as on Cyclin synthesis. Cyclin degradation occurs via two distinct pathways: the SCF (Skp1/Cullin/F-box protein) and the Anaphase-Promoting Complex/Cyclosome (APC/C). Both proteolytic pathways rely on addition of ubiquitin-polymeric chains to specific cell cycle regulators, as are the cyclins. This ubiquitynilation is sufficient to target them to proteolysis by a protease complex the 26S proteasome. The SCF complex activity seems to be present during different stages of cell cycle, since its controls S phase and mitotic entry. The APC/C is essentially working in mitosis and is responsible for the degradation of cohesion between sister chromatids but it is also present in G1 where it keeps Cdk activity levels down allowing the reset of the cell cycle machinery (reviewed in Teixeira and Reed, 2013).

1.4.2. Cell Cycle Checkpoints

A true checkpoint must fulfill two different requirements; monitoring the process being tested and then transmitting an inhibitory signal to stop subsequent processes in case the previous events have not been completed successfully (Cooper, 2006).The goal of any checkpoint control is to prevent genetic alterations or situations that compromise cell survival (reviewed in Nojima, 1997). There are three major cell cycle checkpoints: Restriction point (R or G1-phase checkpoint), the DNA damage checkpoint and the third major

checkpoint, which monitors the metaphase-to-anaphase transition known as Spindle Assembly Checkpoint (SAC).

1.4.2.1 R- G1 phase and DNA damage checkpoints

When all the conditions are right for cell proliferation, G1/S cyclin-Cdk complexes are activated. After overcoming the restriction point the cell is committed to undergo a complete round of the cell cycle. However, if during G1, conditions are not right then, the G1-checkpoint is activated and the cell remains arrested in G1 (reviewed in Cooper, 2006).

However, DNA damage is monitored all through G1, S and G2 allowing the cells to stop at the G1/S transition or at G2/M transition. The DNA damage checkpoints facilitate repair or induce programmed cell death in the presence of irreversible DNA damage. p53 plays an active role during G1 and S since after DNA damage p53 is phosphorylated and activates the transcription of genes necessary for genotoxic stress response. However, the DNA damage can also occur during G2 and in this case the cell is prevented from entering mitosis. This mechanism is p53 independent and is though to be regulated through Cdk1-Cyclin B inhibition (reviewed in Niida and Nakanishi, 2006). ATM (Ataxia telangiectasia mutated) activates the kinases Chk1 and Chk2, which in turn inactivates Cdc25 preventing the cell to enter mitosis by upregulating Wee1 and Myt1 kinases. All these events have a final outcome, the inhibition of Cyclin B /Cdk1.

1.4.2.2 Spindle Assembly Checkpoint (SAC)

There is an additional checkpoint during cell cycle and it can be activated during mitosis. The spindle assembly checkpoint (SAC) monitors the defects in kinetochore-spindle-microtubule attachments and stops cell-cycle progression until all chromosomes are bioriented and properly attached to the spindle. The genes involved in this surveillance mechanism were identified for the first time in a screen performed in budding yeast where mutants were not able to arrest during mitosis in the presence of spindle poisons. The genes

identified in these screens are also conserved in eukaryotes and included the *MAD* (mitotic-arrest deficient) genes *MAD1*, *MAD2* and *MAD3* (*BubR1* in humans), the *Bub* (budding uninhibited by benzimidazole) gene *Bub1 and Bub3* (Hoyt et al., 1991; Li and Murray, 1991). SAC proteins delay precocious chromosome segregation by sequestration of cell division cycle 20 (Cdc20) protein (Hwang et al., 1998; Kim et al., 1998), a co-factor of the ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C) (Fang et al., 1998; Kramer et al., 1998). The APC/C complex targets cyclin B and securin to degradation by ubiquitylation through recognition of a destruction box (D-box) motif present in both of these proteins (Glotzer et al., 1991). To inhibit the APC/C, SAC proteins assemble into a mitotic checkpoint complex (MCC), a heterotetramer composed of Cdc20, Mad2, BubR1/Mad3 and Bub3. Incorporation of Cdc20 into the MCC prevents APC/C activation and subsequent cyclin B and securin degradation (Sudakin et al., 2001). After degradation, securin no longer inhibits the protease known as separase, which is responsible for the cleavage of the cohesion complex that keeps sister chromatids together. Concomitantly, the degradation of cyclin B inactivates the mitotic kinase, Cdk1 leading to mitotic exit (Fig. 4) (reviewed in Lénárt and Peters, 2006).

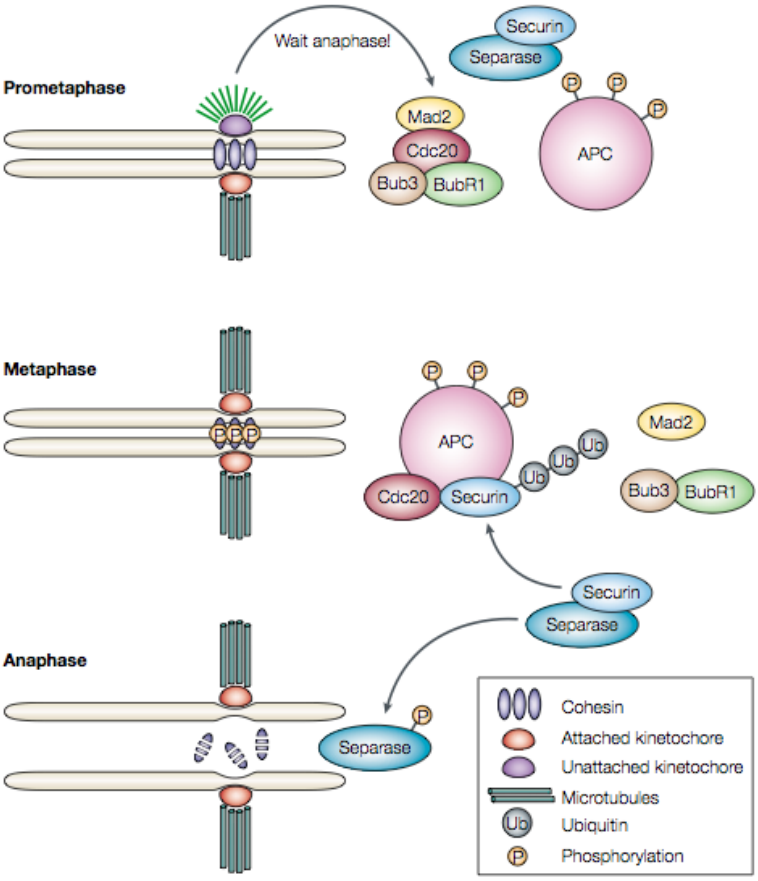


Figure 4. SAC components and mechanism. During prometaphase, Mad2 and BubR1-Bub3 together with Cdc20 are recruited to the unattached kinetochores to form MCC responsible for the "wait anaphase" signal. Cdc20 sequestration inhibits APC from ubiquitylating securin, which in turn prevents separase activation and loss of cohesion. The occupancy of kinetochores and chromosome biorientation turns off the "wait anaphase" signal. Cdc20 is able to activate APC/C causing the polyubiquitynilation of securin and subsequent activation of separase and cohesin degradation. Adapted from (Musacchio and Hardwick, 2002).

Recently, it has been shown that the recruitment of checkpoint proteins to unattached kinetochores is phosphoregulated. Moreover, Mps1 has been identified as one of the proteins responsible to recruit essential SAC components to kinetochores (Maciejowski et al., 2010; Santaguida et al., 2010; Hewitt et al., 2010).

Finally, there are other proteins that directly or indirectly influences SAC activity such as Rod (rough deal) - ZW10 (zeste white 10) - Zwilch (RZZ) complex, p31comet, mitogen-activated protein kinase (MAPK), Cdk1-cyclin B,

Nek2 and polo-like-kinase 1 (PLK1), CENP-E, dynein/dynactin and CLIP170 (reviewed in Musacchio and Salmon, 2007).

2. KINETOCHORE

2.1 OVERVIEW

During mitosis a special structure is built at the site of the primary constriction of condensed chromosomes that attaches to spindle microtubules. This region is called the centromere (from the Greek 'centro-', meaning 'central', and '-mere', meaning 'part') and later the **kinetochore** (from the Greek 'kineto-', meaning 'move', and '-chore', meaning 'means for distribution'). The kinetochore comprises a complex structure of proteins assembled on centromeric DNA. This interface interacts dynamically with microtubules, which is essential for faithful chromosome segregation during mitosis and meiosis.

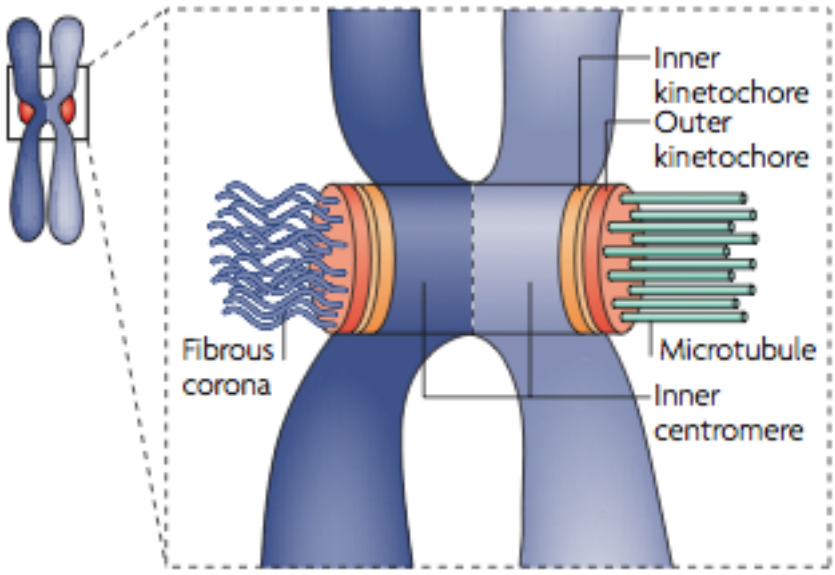


Figure 5. Schematic illustration of a mitotic chromosome. On the rigth it is represented an attached chromatid to microtubules and the chromatid on the left is unattached. The trilaminar layer structure, the inner kinetochore, the outer kinetochore, and the fibrous corona (unattached kinetochores), observed

by electron microscopy are represented here. Adapted from (Cheeseman and Desai, 2008).

The first observation of this structure was reported using electron microscopy (EM) in Chinese hamster cells, and revealed a trilaminar morphology, including electron-dense inner and outer plates of 200-300 Å in diameter, known as the inner and outer kinetochore respectively, surrounded by a less dense zone 200-600 Å wide (Brinkley and Stubblefield, 1966) (Fig. 5). When kinetochores are not attached to microtubules a meshwork of fibers, known as the fibrous corona, can be detected to extend from the surface of the outer kinetochore (Ris and Witt, 1981). Several proteins localize in this region, such as microtubules motors (CENP-E), or proteins involved in the mitotic checkpoint such as the Rod-ZW10-Zwilch (RZZ) complex (reviewed in Cheeseman and Desai, 2008). Recent biochemical and proteomic studies made possible the characterization of these super-complex protein structures. The players responsible for the attachment of chromosomes to microtubules localize at the outer kinetochore and form a network known as KMN (KNL1Mis12Ndc80) complex. The interaction established between kinetochores and microtubules by this network is tightly regulated by the Aurora B kinase, which localizes to centromeric chromatin before anaphase; (reviewed in Maresca and Salmon, 2010). It has been shown that microtubules penetrate into the outer kinetochore and sometimes reach the inner kinetochore (Rieder and Salmon, 1998). In budding yeast the interaction between kinetochores and microtubules is established by a single microtubule (O'Toole et al., 1999; Winey et al., 1995), however the number of microtubules interacting with kinetochores is not conserved across species. Fission yeast kinetochores have between 2-4 microtubules (Ding et al., 1993) and between 20–30 microtubules can be found in animal cells (McEwen et al., 1997). The inner kinetochore interacts directly with centromeric chromatin that is specified by the presence of the histone H3 variant CENP-A (Warburton et al., 1997).

2.2 CENTROMERE SPECIFICATION

The centromere is a specialized region on each chromosome required to specify the site at which the kinetochore will be assembled. Moreover, the centromeric region is also responsible for maintaining the attachment between sister chromatids through the accumulation of cohesion complexes.

Two seminal studies are in the basis of centromere research in vertebrate cells. First, the discovery at the base of an autoimmune sera from patients affected by calcinosis, Raynaud's syndrome, esophageal dysmotility, Sclerodactyly and Telangiectasia (CREST) syndrome that recognizes the centromere region (Moroi et al., 1980) and second the identification of the first set of three canonical human centromeric proteins: CENP-A, CENP-B, and CENP-C (Earnshaw and Rothfield, 1985).

Centromeric DNA is one of the most rapidly evolving parts of the chromosome (Murphy et al., 2005). It usually contains a repetitive sequence with a repeating unit, usually 160–180 bp that is smaller than the average spacing between nucleosomes on chromosomal arms. However, centromeres, except in some budding yeasts (e.g. Saccharomyces cerevisiae and Kluyveromyces lactis), are not defined by a specific DNA sequence. In fact, it has been shown that centromere identity is specified epigenetically. The histone H3 variant, CENP-A (called Cse4 in yeast, CID in flies, and CenH3 in plants) is the key to the epigenetic specification of centromeres (Black and Bassett, 2008) since all active centromeres, including natural or experimentally induced neocentromeres, contain CENP-A (Shang et al., 2010; Marshall et al., 2008). Structurally what differentiates centromeric chromatin from the rest of the chromosome is the presence of CENP-A or its homologue.

How epigenetic information encoded by chromatin at specific sites is retained during major chromosomal events, including DNA replication and transcription is poorly understood. One of the most challenging questions to be answer is how CENP-A that is already assembled into centromeric chromatin is kept at centromeres during replication, since during this process nucleosomes are disrupted by DNA polymerase and then reassembled onto each daughter centromere after replication. The deposition of newly

synthetized CENP-A at centromeres does not happens at same time as replication takes place. In human cells (Jansen et al., 2007) and fly embryos (Schuh et al., 2007) it has been reported that deposition of newly synthesized CENP-A onto centromeric DNA starts late in mitosis and extends through the G1 phase of the following cycle. The newly assembled histones primarily form complexes with other histones. To prevent promiscuous association of the highly basic proteins with highly acidic nucleic acids, the histone complexes are bound by “chaperones” (Ransom et al., 2010). The chaperone responsible for that process is called HJURP (Holiday JUnction Recognition Protein) in human cells (Foltz et al., 2009; Dunleavy et al., 2009) and Scm3 in yeast (Williams et al., 2009; Pidoux et al., 2009; Camahort et al., 2007; Mizuguchi et al., 2007; Stoler et al., 2007). HJURP binds CENP-A through the CENP-A Targeting Domain (CATD) to the CENP-A-H4 tetramer (Foltz et al., 2009). Different models have been proposed to explain the chromatin structure containing CENP-A. The most consensual is one that proposes an octameric structure with two copies of each histone, H2A, H2B, H4, and CENP-A (in place of H3) (Sekulic et al., 2010; Camahort et al., 2009; Conde e Silva et al., 2007; Foltz et al., 2006; Shelby et al., 1997; Palmer and Margolis, 1985) and DNA wrapping around the histones with a left- hand twist (Sekulic et al., 2010). However, several studies refute this model proposing additional structures for the CENP-A nucleosomes. There are six different models reported so far, the tetrasome, the hemisome, the octameric ‘‘reversome’’, the hexosome and the trisome model. Recently it has been proposed that all these structures can be found in centromeric DNA prior to the assembly of a final nucleosomal form. Moreover, prenucleosomal forms, nucleosomal forms (potentially including trisome/hexasome or tetrasome intermediates), nucleosomes are all stable structures, with the intrinsic properties of CENP-A leading this stability (Ben E Black and Cleveland, 2011).

Interestingly, the incorporation of CENP-A into chromosomes is not sufficient to define the chromatin as centromeric. Additional events have to take place in order to reinforce centromere identity. Some studies have reported a possible cooperative effect of CENP-A nucleosomes recognizing other CENP-A nucleosomes in higher-order chromatin folding (Ribeiro et al.,

2010; Blower et al., 2002); or direct recognition of CENP-A containing nucleosomes by other centromere components (Carroll et al., 2010; 2009). During mitosis when kinetochores play a predominant role the centromeres contain only half of maximal complement of CENP-A. The biological relevance of this is still elusive, however it has been reported that one tenth of the CENP-A levels is still sufficient to assemble a functional kinetochore (Liu et al., 2006). One possible explanation is that half the amount of CENP-A nucleosomes confers the right chromatin quality and the right balance between rigidness and elasticity, necessary for chromosome segregation and movement (reviewed in Perpelescu and Fukagawa, 2011).

Centromeres can be organized in three distinct structures: point centromere, regional centromere and halocentric centromere. In budding yeast centromeres are assemble upon a ~125-bp specific DNA sequence and are called point centromeres (Furuyama and Biggins 2007). Centromeres that are built along all chromosomes length can be found in C.elegans and are designated as halocentric (reviewed in Dernburg et al., 2001). Finally, centromeres built on tandem repeats of highly repetitive (satellite) elements, so-called regional centromeres, are characteristic of higher eukaryotes (Pluta et al., 1995).

2.3 KINETOCHORE STRUCTURE

2.3.1 Constitutive Centromere Associated Network (CCAN).

In vertebrates, the kinetochore is specified on centromeric chromatin by sequence-independent epigenetic mechanisms. However, although CENP-A deposition is necessary for kinetochore specification, it is not enough for the establishment of functional kinetochores in vertebrate cells (Gascoigne et al., 2011; Van Hooser et al., 2001). In *Drosophila* it has been shown, that expression of ectopic CID induces kinetochore formation (Heun et al., 2006). However, recently, it has been reported that *Drosophila* CENP-C N-terminal ectopically expressed on centrosomes is sufficient to recruit different kinetochore proteins (Przewloka et al., 2011). This suggests that there are additional proteins that are required to generate centromere-specific

chromatin and direct sequence-independent kinetochore assembly. In vertebrates and fission yeast a group of 16 chromatin-proximal proteins termed the constitutive centromere associated network (CCAN) has been characterized, which also associates with centromeric chromatin and have been described as playing a role in kinetochore assembly. The major function of CCAN is to link the KMN network in the kinetochore 'outer' domain to centromeric DNA within the inner domain (Nishino et al., 2013; Hori et al., 2008b; Saitoh et al., 1992) (Fig. 6). However, this network does not seem to be conserved across species, since in *Drosophila* and C. *elegans* only CENP-C has been identified so far (Cheeseman et al., 2008; Hori et al., 2008b).

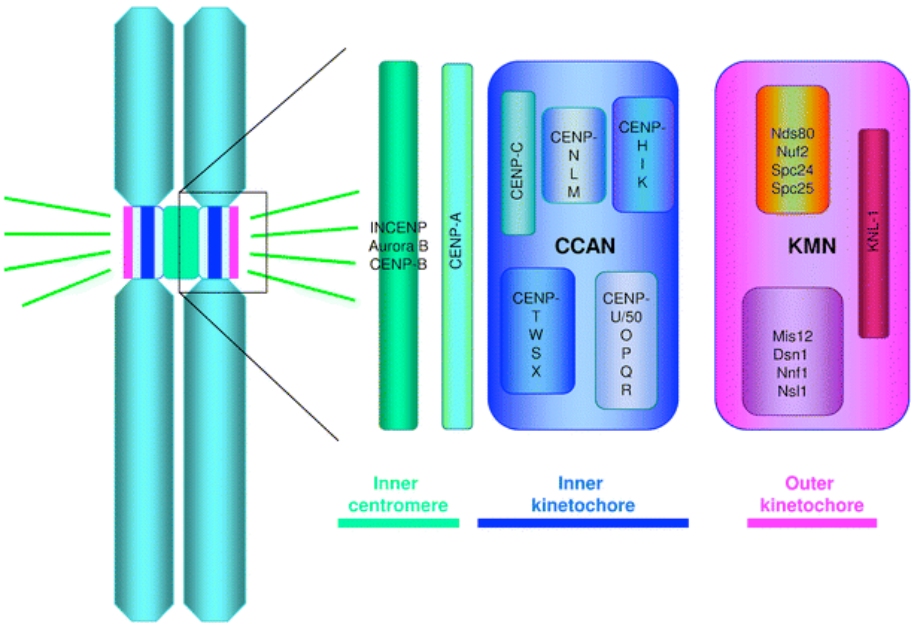


Figure 6. Schematic model of vertebrate kinetochore structure. Kinetochores are assembled onto duplicated sister chromatids starting G2/prophase and are disassembled after completion of mitosis/ meiosis. Within kinetochores we can find proteins that localize near the inner centromere and constitutes the inner kinetochore (blue). This group of proteins established the bridge between centromeric DNA and the proteins localized in the more distal part of Kinetechore, the outer kinetochore (pink) that are usually responsible to the kinetochore-microtubule attachment. (Adapted from Perpelescu and Fukagawa, 2011).

CCAN can be organized in distinct subgroups taking in account genetic and biochemical results. All components of the CCAN with the exception of CENP-

B protein and CENP-O complex are essential and necessary for proper chromosome congression and segregation (Kapoor et al., 1998; Perez-Castro et al., 1998). CENP-C is a constitutive centromere protein with DNA-binding ability (Yang et al., 1996; Saitoh et al., 1992). It was the first protein characterized by immunoelectron microscopy and later it was shown to be essential for kinetochore assembly by antibody microinjection (Tomkiel et al., 1994; Saitoh et al., 1992). More recently, several studies reported that upon CENP-C depletion severe defects in chromosome segregation and mitotic checkpoint were observed, confirming CENP-C fundamental role in the kinetochore integrity and function (Orr and Sunkel, 2010; Hori et al., 2008b; Przewloka et al., 2007; Kwon et al., 2007; Heeger et al., 2005; Moore and Roth, 2001). Several studies revealed that the N-terminal part of CENP-C is essential for the localization of proteins such as CENP-K, CENP-E, Mad2, Mis12, Dsn1, Nnf1, and KNL1 (Milks et al., 2009; Kwon et al., 2007; Liu et al., 2006) to kinetochores. Additionally, in *Drosophila* S2 cells it has been shown that CENP-C depletion leads to a kinetochore null phenotype and is necessary for the recruitment of CID, MEI-S332, and chromosomal passenger proteins suggesting that CENP-C could fulfill the structural roles of the human centromere-associated proteins not identified in *Drosophila* (Orr and Sunkel, 2010). More recently, in *Drosophila*, mass spectrometry and biochemical studies have demonstrated that the same region of CENP-C interacts with two subunits of the Mis12 complex (Nnf1 and more weakly with Nsl1) (Przewloka et al., 2011). With the exception of CENP-C, CENP-T/CENP-W complex acts upstream of other CCAN components. CENP-T and CENP-W are tightly associated and make a complex (Hori et al., 2008b). They interact with each other through the histone-fold domain (HFD) of both proteins. The HFD domain in CENP-W interacts directly with DNA (Gascoigne et al., 2011). CENP-T needs CENP-A to localize at centromeres despite of its intrinsic biding activity (Hori et al., 2008b). The N-terminal region of CENP-T interacts directly with NDC80 complex in the outer kinetochore and a motile structure in the middle region of the protein is thought to be able to stretch between the inner and outer kinetochore when tension is applied (Suzuki et al., 2011). Although CENP-C and CENP-T do not need CENP-A to localize at

centromeres, their presence is sufficient to generate ectopic kinetochore formation when associated to LacO arrays (Gascoigne et al., 2011).

CENP-C and CENP-T/W depletion results in distinct phenotypes. CENP-C deficient cells exit mitosis prematurely (Orr and Sunkel, 2010; Kwon et al., 2007); where CENP-T and CENP-W depleted cells exhibited a strong mitotic delay (Hori et al., 2008b). The data so far suggest that both CENP-C and CENP-T/W complexes works as mediators between the centromeric chromatin platform and outer kinetochore. The CENP-H complex, composed of CENP-H, -I and -K proteins appear to localize to centromeres downstream of CENP-T/W complex and are constitutively associated with centromeres (Okada et al., 2006; Sugata et al., 2000; 1999). CENP-H interacts with long sections of chromatin containing CENP-A without being directly associated to it (Hori et al., 2008b). However, the specific biological significance of CENP-H at the centromere still remains elusive. CENP-I is involved either in the recruitment of inner kinetochore proteins, such as CENP-C and CENP-H as well as in proteins from outer kinetochore as NDC80 and proteins involved in checkpoint such MAD1/2 (Liu et al., 2003; Measday, 2002; Nishihashi et al., 2002). More recently has been reported that CENP-H and CENP-I can be found preferentially associated to microtubules kinetochore plus ends regulating their turnover rate resulting in a correct chromosome alignment at the metaphase plate (Amaro et al., 2010).

CENP-L, CENP-M, and CENP-N were co-purified with CENP-H/I/K, the role-played by these sets of proteins in the assembly hierarchy is unclear, nevertheless, they depend on each other to localize at kinetochore. The depletion of each protein leads to severe mitotic defects (Okada et al., 2006). CENP-M depletion from kinetochores influences the assembly of CENP-H/I/K, which further directs CENP-O/P/Q/U/50 assembly (Hori et al., 2008a; b; Foltz et al., 2006; Okada et al., 2006; Izuta et al., 2006). After the depletion of this last group of proteins, Chicken DT40 cells are still viable but do not undergo prompt cycle progression after exit from nocodazole-induced mitotic block.

The CENP-S/X group of proteins causes mitotic defects in vertebrate cells after depletion and is necessary for the recruitment of outer kinetochores proteins such as KNL1 and Ndc80/Hec1 (Amano et al., 2009) it also depends on CENP-H/I/K to localize at kinetochores. It has been proposed that CENP-

S-X proteins together with CENP-T and W form a tetrameric nucleosome-like structure working as a scaffold for kinetochore formation (Nishino et al., 2013).

2.3.2 KMN network

2.3.2.1 Overall organization of the KMN network

The KMN network plays a central role in kinetochore–microtubule attachment and SAC signaling in all eukaryotes so far examined. Each microtubule-binding site may contain from six to eight KMN complexes (Johnston et al., 2010; Joglekar et al., 2008; 2006). This network is named after its components KNL1/Spc105, Mis12 and NDC80 sub-complexes (KMN) (Cheeseman et al., 2004). The KMN network starts to localize to the kinetochore just before mitosis and dissociates from kinetochores in telophase (reviewed in Santaguida and Musacchio, 2009). The recruitment of each component of the KMN network differs and will be address in detail later one. Importantly, in most of the organisms different KMN complexes are interdependent for their localization to kinetochores. In human and yeast it has been shown that the localization of Ndc80 does not depend on KNL1 (Liu et al., 2010; Cheeseman et al., 2008; Kiyomitsu et al., 2007). The same is not true in C.*elegans* and *Drosophila* where after depletion of KNL1/Spc105, Ndc80 complex localization at kinetochores is severely impaired (Feijão et al., 2013; Essex et al., 2009; Cheeseman and Desai, 2008). Interestingly, different studies have described an interdependence of both Mis12 and Spc105 for kinetochore localization (Venkei et al., 2012; Przewloka et al., 2007; Cheeseman et al., 2004), however recently it has been reported that both components depend on each other only partially (Feijão et al., 2013). Due to its localization in the outer part of kinetochore the depletion of Ndc80 from kinetochores has no impact on the structure of the inner kinetochore or the centromere. However, it has been described in S2 *Drosophila* cells it has been described a partial dependency of Mis12 protein on Ndc80/Nuf2 (Feijão et al., 2013). The localization of the Ndc80 complex is also affected upon

depletion of Mis12 complex (Feijão et al., 2013; Venkei et al., 2012; Przewloka et al., 2007; Kline et al., 2006; Cheeseman et al., 2004; Scharfenberger et al., 2003).

Despite being highly conserved in function, in budding yeast the structure appears to be more complex since as to interact with the point centromere and a single microtubule, when compared to higher eukaryotes with their regional centromeres and microtubule bundles (Westermann et al., 2007). However, it has been shown that due to rapid evolutionary divergence of kinetochore proteins the level of sequence homology across eukaryotic species is low (between 15%–30%) with the highest levels of divergence found within *Drosophila* kinetochore proteins (Meraldi et al., 2006).

2.3.2.2 KNL1/Spc105

KNL1/Spc105 was first identified in budding yeast in a highly enriched spindle pole preparation (Wigge et al., 1998). Later, also in budding yeast Spc105 was co-purified with the protein Ydr532 (Kerres et al., 2004; Nekrasov et al., 2003) and called Spc105 complex. The same study also found that Spc105p, and Ydr532p localize to the nuclear side of the spindle pole body and along short spindles. Interestingly, this complex is not conserved in in all higher eukaryotes, and can be found, separately as a single protein. Although KNL-1 was previously reported to lack homologs outside of nematodes (Desai et al., 2003), further studies revealed that it shares primary sequence features with F15q14 in human cells (Cheeseman et al., 2004; Obuse et al., 2004a), Spc7 in fission yeast (Kerres et al., 2004), KNL-1 in nematodes (Desai et al., 2003), dmSpc105R in *Drosophila* (Przewloka et al., 2007). It is a large protein of 2342 amino acids in human and 1959 in *Drosophila*, recruited to kinetochores during prophase and absent after telophase. This protein family has been shown to have a conserved N-terminal repeats [S/G]ILK and RRSVF motifs. In this region it is also localized a coiled-coil domain with divergent numbers of MELT repeats (Cheeseman et al., 2004; Desai et al., 2003; Nekrasov et al., 2003) (Fig. 7). In *Drosophila* only one MELT motif was identified (Przewloka et al., 2007) so far. Additionally, at the N-terminus of the

protein two helical motifs denominated KI motifs, which interact with the TPR domains of BUBR1 and BUB1 were identified. Recently, different studies demonstrated the important of this interaction in the maintenance of SAC response in human cells (Krenn et al., 2012; Kiyomitsu et al., 2011; Bolanos-Garcia et al., 2011).

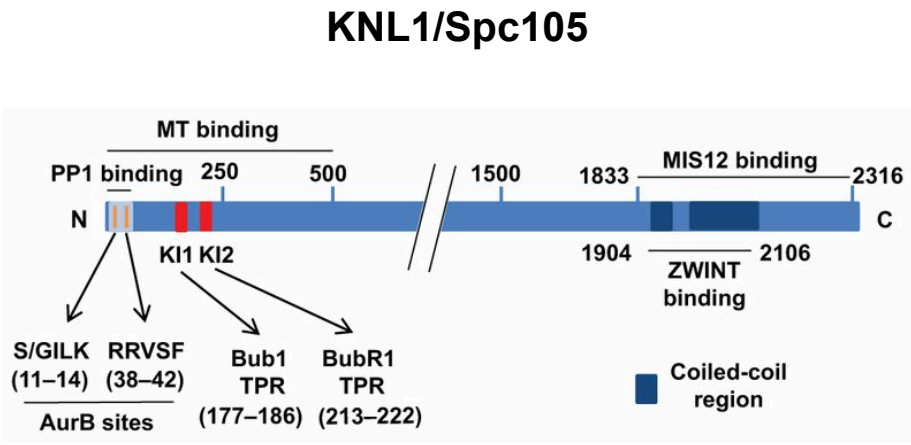


Figure 7. Schematic representation of KNL1/Spc105 structure. Human KNL1 is a large multi-domain protein with the known functional domains and motifs highlighted. (Adapted from Varma and Salmon, 2012).

Zwint, was recently shown to form a tight complex with the C-terminal coiled-coil region (Petrovic et al., 2010) of KNL1/Spc105, the same region which interacts with the Mis12 complex. Zwint has been described to play an important role in the recruitment of ZW10 to kinetochores (Starr et al., 2000). ZW10 is part of the RZZ complex (Rod-ZW10-Zwilch) that has been shown to recruit the adaptor protein Spindly, which serves as link between the dynein–dynactin motor complex and checkpoint proteins such as Mad1-Mad2 complex (Gassmann et al., 2008; Griffis et al., 2007).

In all organisms studied so far KNL1/Spc105 was shown to be essential for kinetochore function (Feijão et al., 2013; Cheeseman et al., 2008; Przewloka et al., 2007; Cheeseman et al., 2004; Nekrasov et al., 2003). In C. elegans depletion of KNL1/Spc105 leads to a kinetochore null phenotype (KNL) (Desai et al., 2003) similar to what was observed upon depletion of CENP-C or CENP-A. It localizes downstream of CENP-A and CENP-C in a linear assembly hierarchy and depends on both for kinetochore localization (Desai

et al., 2003). A similar phenotype was observed in *Drosophila*; with a dramatic decrease in stable kinetochore-microtubule interactions, severe chromosome misegragation, impaired SAC and lost of cell viability (Feijão et al., 2013; Schittenhelm et al., 2009; Przewloka et al., 2007). In human cells, depletion of KNL1 does not have such dramatic effects, however, it also causes a misegregation phenotype, affects SAC but the cell viability is not compromise (Cheeseman et al., 2008; Kiyomitsu et al., 2007). These differences in phenotypes could be explained by the fact that in C.elegans, Ndc80 (the primary binding protein to microtubules) fully depends on KNL1/Spc105 to localize to kinetochores. The same is not true in human cells where, Ndc80 binds to the CCAN protein CENP-T (Gascoine et al. 2011). This dependency of Ndc80 recruitment on KNL1/Spc105 is also not observed in budding and fission yeast (Kerres et al., 2007). KNL1/Spc105 interacts with the rest of the KMN network through its C-terminal domain that binds to one of the proteins of Mis12 complex (Nsl1). Recently, in *Drosophila* it has been shown that depletion of Spc105 also impairs kinetochore localization of Mad2 to kinetochores (Feijão et al., 2013). The microtubule binding activity found in the N-terminus of C. elegans KNL1/Spc105 (Espeut et al., 2012; Cheeseman et al., 2006) was shown to be necessary for SAC response but not needed to the establishment of proper kinetochore-microtubule (KT-MT) attachments or in chromosome segregation (Espeut et al., 2012). The extreme N-terminus of KNL1 has also been shown to be important for the recruitment of protein phosphatase 1 (PP1) to the outer kinetochore (Meadows et al., 2011; Rosenberg et al., 2011) as well as the docking site for Mps1 checkpoint kinase in yeast, which is in turn responsible for the recruitment of the Bub1–Bub3 checkpoint complex to kinetochores (London et al., 2012; Shepperd et al., 2012; Yamagishi et al., 2012).

2.3.2.3 Mis12 Complex

The Mis12 complex works as a major platform for outer kinetochore assembly (reviewed in Cheeseman and Desai, 2008). Mis12 protein was first described amongst 12 mis (minichromosome instability) genetic loci in fission yeast (Takahashi et al., 1994) and later the budding yeast homologue was identified and designated as Mtw1. Both proteins exhibit kinetochore localization and in mutant strains result in chromosome misegragation (Goshima and Yanagida, 2000; Goshima et al., 1999). Posterior studies showed similar defects in chromosome alignment, orientation, and segregation after depletion of Mis12 complex (Feijão et al., 2013; Venkei et al., 2011; Przewloka et al., 2007; Kline et al., 2006; Goshima et al., 2003; Euskirchen, 2002). Mis12 forms a complex with a number of other proteins including Nsl1, Nnf1 and Dsn1 (Euskirchen, 2002). In C.elegans depletion of Dsn1 results in a severe phenotype, similar to the one of KNL1 and was named as KNL-3 (kinetochore null 3) (Cheeseman et al., 2004). In human cells it has been shown that all the proteins in the Mis12 complex are interdependent for localization (Kline et al., 2006). However, Mis12 appears upstream to Nsl1 in the recruitment hierarchy in *Drosophila* cells (Venkei et al., 2011). Interestingly, the four subunits of Mis12 complex are not recruited to the kinetochore at the same time. Mis12 is recruited to centromeres during late G2 and leaves in early G1 (Kline et al. 2006). In *Drosophila*, Nnf1 and Mis12 can be detected at kinetochores during most of cell cycle, however, Nsl1 is recruited to kinetochores during prophase (Venkei et al., 2012; Przewloka et al., 2007). Moreover, it has been shown that Mis12/Nnf1 and Nsl1/Spc105 establish stronger pair-wise interactions (Venkei et al. 2012). Similar interactions were described in human and yeast Mis12 complex subunits (Hornung et al., 2011; Maskell et al., 2010; Petrovic et al., 2010).

The Mis12 complex is 22-nm long and has a rod-like shape, with the subunits linearly attached to each other in the order Nnf1, Mis12, Dsn1 and Nsl1 (Nekrasov et al., 2003; Euskirchen, 2002) (Fig. 8). The structure, however, is not conserved across species, in yeast, the Mis12 (Mtw1) complex has also been reported to associate directly with a Ctf19-containing complex called COMA (Hornung et al., 2011).

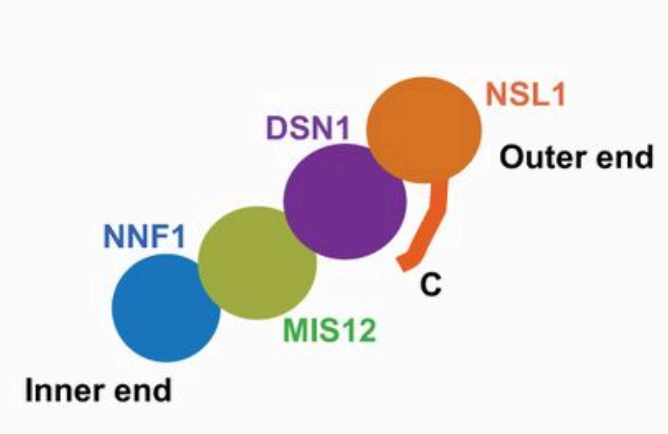


Figure 8. Structure of vertebrate Mis12 complex. The human Mis12 complex comprises four subunits Nnf1, Mis12, Dsn1 and Nsl1 that have been demonstrated to be disposed linearly, where Nnf1 interacts with the inner kinetochore and the C-terminal tail of Nsl1 with the two other components of KMN network. (Adapted from Varma and Salmon, 2012).

This association was not described for higher eukaryotes. Moreover, in *Drosophila* the homologue of Dsn1 was not yet identified (Schittenhelm et al., 2007; Przewloka et al., 2007). It has been proposed that the *Drosophila* KNL1 homologue, Spc105 had functionally replaced this subunit (Przewloka et al., 2009). Recently, an extensive study in human Mis12 complex structure revealed that the C-terminal tail of Nsl1 is necessary to interact with KNL1 and also with the subunits of the Ndc80 complex (Spc24-Spc25). However, the stretch of residues (257–281) and the PVIHL motif both present at the C-terminal region of Nsl1 may be not conserved outside vertebrates (Petrovic et al., 2010). Indeed, in yeast the Dsn1–Nsl1 heterodimer alone was not sufficient to bind the Ndc80 complex. Mis12 (Mtw1) and Nnf1 could play a significant role in this interface. The same study also proposed that Nnf1 and Nsl1 flank the Mis12 complex structure. Moreover, Mis12 protein establishes cross-links with residues within the predicted globular region of Dsn1 (K129MIS12 with K167DSN1 or K248DSN1), suggesting that it contacts directly with this subunit. It has also been proposed that Nnf1 was the responsible for the link between the inner kinetochore through interaction with

the N-terminal domain of CENP-C and the outer kinetochore (Screpanti et al., 2011; Przewloka et al., 2011). While Mis12 complex per se was never found to be associated directly to microtubules, the same study identified two distinct microtubule-binding activities within the KMN network. One associated with the Ndc80/Nuf2 subunits of the Ndc80 complex, and a second in KNL-1 while the presence of the Mis12 complex synergistically enhances microtubule-binding activity (Cheeseman et al., 2006). Later, it has been shown that microtubule-binding affinity of the KMN network is regulated by the Aurora B kinase through phosphorylation of one of Mis12 subunits (Dsn1) (Welburn et al., 2010). Mis12 protein has also been implicated in interactions with the chaperone complex Hsp90–Sgt1 (Davies and Kaplan, 2010) and interaction of Nls1 and the heterochromatic protein (HP1) were shown to be important for the assembly of the inner kinetochore (Kiyomitsu et al., 2010; Obuse et al., 2004b).

2.3.2.4 Ndc80 Complex

The Ndc80 complex appears to play a key microtubule-binding role within the kinetochore. The proteins of the complex were for the first time identified in budding yeast upon isolation of SPBs (Wigge et al., 1998; Osborne et al., 1994; Rout and Kilmartin, 1990). It was also in budding yeast that was shown that the four proteins of the complex Ndc80 (HEC1), Nuf2, Spc24 and Spc25 were associated (Janke et al., 2001). In human cells Ndc80 protein was for the first time identified in a yeast two-hybrid screen with the retinoblastoma tumor suppressor as bait and named HEC1 (highly expressed in cancer 1) (Chen et al., 1996a). The Ndc80 complex is a 57-nm-long heterotetrameric complex with a dumb-bell shape (Ciferri et al., 2008; Wei et al., 2005; Ciferri et al., 2005). Studies using atomic force and electron microscopy have shown the complex to have an elongated rod-like structure with globular domains at both ends (Fig. 9) (Wei et al., 2005). At the N-terminal region of the complex is localized the globular dimer Ndc80 (HEC1)/Nuf2. The globular region of Ndc80 and Nuf2 subunits fold as a calponin-homology (CH) domain (Alushin et al., 2010; Wilson-Kubalek et al., 2008; Ciferri et al., 2008; Wei et al., 2006;

Cheeseman et al., 2006). The presence of these domains in several proteins has been associated to actin or microtubule binding (Gimona et al., 2002; Korenbaum, 2002). At N-terminal region of Ndc80 it also can be found an unstructured tail that ranges from 80 to 112 amino acids (Fig. 9), which is involved in bidding to microtubules in vitro (Ciferri et al., 2008; Wei et al., 2006) and it is also required for stable kinetochore–microtubule attachment *in vivo* (Mattiuzzo et al., 2011; Guimaraes et al., 2008; Miller et al., 2008). It has been shown that the CH domain of Nuf2 does not interact directly with microtubules; nevertheless, it is required for generating normal microtubule-dependent kinetochore force and timely mitotic progression (Tooley et al., 2011; Sundin et al., 2011). The C-terminal region Ndc80 and Nuf2 interact with the N-terminus of Spc24/Spc25 dimer through an overlap of their α-helical coiled coil domains (Fig. 9) (Ciferri et al., 2008; Wei et al., 2006). Spc24/Spc25 dimer is responsible for anchoring the complex with to the inner kinetochore through CENP-T in human and budding yeast (Bock et al., 2012; Nishino et al., 2013; Schleiffer et al., 2012; Gascoigne et al., 2011); or with KMN network through Nsl1 subunit of Mis12 complex in humans and *Drosophila* (Przewloka et al., 2011; Petrovic et al., 2010; Ciferri et al., 2008). Moreover, within the α-helical coiled coil domain of Ndc80 protein a stretch of amino acids, which are not associated with the Nuf2 protein and were implicated in the recruitment of additional microtubule-binding proteins, was identified (Hsu and Toda, 2011; Maure et al., 2011).

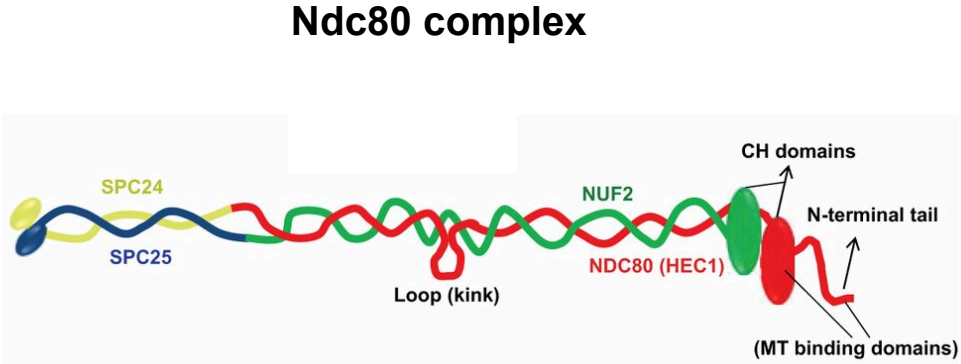


Figure 9. Schematic view of the structure of the Ndc80 complex. The human Ndc80 complex is composed of four subunits NDC80 (Hec1), Nuf2, Spc24 and Spc25, which use their coiled-coil regions to assemble the heterotetramer. The N-terminus of Ndc80 protein interacts directly with microtubules using the CH

domain and the charged unstructured tail regions, where globular C-terminal domains of Spc24 and Spc25 bind the complex to CENP-T or the Mis12 complex. (Adapted from Varma and Salmon, 2012).

The CH domain of Ndc80 is implicated in the recruitment of checkpoint proteins Mad1–Mad2 and Mps1 (Miller et al., 2008; Guimaraes et al., 2008; Ciferri et al., 2007; Hanisch et al., 2006; McCleland et al., 2003; DeLuca et al., 2003; Martin-Lluesma et al., 2002), as well as the kinase NEK2 (Wei et al., 2011).

In *Drosophila* the recruitment of Ndc80 occurs only after NEB (Venkei et al. 2012) whereas in vertebrate cells this protein localizes at centrosomes at interphase (Hori et al., 2003) and relocates to the kinetochore outer plate in late G2, where it remains stably bound at nearly constant levels until late anaphase (Liu et al., 2006; DeLuca et al., 2006; Bharadwaj et al., 2004; Hori et al., 2003).

The depletion of the complex leads to similar phenotypes in all the organisms studied so far, with disorganized metaphase plates, extensive chromosome misegregation, elongated mitotic spindles, checkpoint defective cells and impaired microtubule–kinetochore attachment (Feijão et al., 2013; Miller et al., 2008; Guimaraes et al., 2008; Wei et al., 2006; Przewloka et al., 2007; DeLuca et al., 2005; Kline-Smith et al., 2005; Janke et al., 2001).

2.3.3 The auxiliary proteins

The kinetochore is a highly complex structure with proteins that transiently localize to kinetochore in different stages of mitotic progression. Apart from the proteins already address in the previous sections there are innumerous proteins that localize to the outer kinetochore and fibrous corona and that are involved in distinct functions such as centromeric chromatin binding, kinetochore-microtubule attachment/correction and SAC control and maintenance. The KMN network is therefore consider to be a platform for the recruitment of a number of these proteins (reviewed in Santaguida and Musacchio, 2009). CENP-E also known as CENP-meta in *Drosophila* is a plus-end directed kinesin-7 motor protein (Wood et al., 1997), which localizes

at fibrous corona. CENP- E starts to be expressed during G2 and mitosis and localizes to kinetochores from early prometaphase through metaphase and it re-localizes to the antiparallel microtubules midzone at anaphase (Cooke et al., 1997; Brown et al., 1994; Yen et al., 1992).

CENP-E promotes slow processive microtubule plus-end-directed movement (Kim et al., 2008; Yardimci et al., 2008). Cenp-E has been shown to be responsible for chromosome congression (Kapoor, 2006). This observation is supported by different studies (Goshima, 2003; Putkey et al., 2002; Yucel et al., 2000; Wood et al., 1997). Additionally, it has been reported that AuroraB/PP1 phosphorylation/dephosphorylation switch regulates CENP-E motor activity, which is critical for chromosome congression and correct biorientation (Kim et al., 2010). Recently it was shown that CENP-E tip-tracks bi-directionally through a tethered motor mechanism contributing to the stability of attachments between kinetochores and dynamic microtubules ends (Gudimchuk et al., 2013). CENP-F also localizes to the fibrous corona with an expression and localization pattern similar to CENP-E (Liao et al., 1995; Rattner et al., 1993). CENP-F depleted cells exhibit problems in congression, segregation and cytokinesis as well as prolonged mitotic arrest followed by cell death (Bomont et al., 2005; Holt et al., 2005; Yang et al., 2005).

Dynein belongs to the group of Microtubule-Associated Proteins (MAPs) and is recruited to kinetochores during prometaphase (Pfarr et al., 1990; Steuer et al., 1990). It forms a tight complex with its co-factor dynactin and they appear to have a major role in SAC silencing by stripping the catalytic Mad1/Mad2 complex and other checkpoint proteins from kinetochores after microtubule attachment (Sivaram et al., 2009; Mische et al., 2008; Vergnolle and Taylor, 2007; Howell et al., 2001). The impact of kinetochore dynein/dynactin in the process of chromosome alignment is still elusive. It has been shown that when dynein/dynactin is inhibited after formation of a bipolar spindle, chromosome congression occurs normally (Vorozhko et al., 2007; Howell et al., 2001).

Interestingly, dynein/dynactin needs the Rod-Zw10-Zwilch (RZZ) complex to localize at kinetochores (Scaërou et al., 2001; Starr et al., 1998). The localization to kinetochores of RZZ complex is microtubule dependent and upon microtubule attachment this complex is removed from kinetochores

through the microtubules (Williams and Goldberg, 1994; Williams et al., 1992). Different studies have shown that RZZ complex is vital for the activity of the spindle checkpoint (Kops et al., 2005; Basto et al., 2000; Chan et al., 2000) most probably by promoting Mad2 accumulation at unattached kinetochores (Buffin et al., 2005). There is a third player in the interaction between dynein/dynactin and the RZZ complex characterized for the first time in *Drosophila* and called Spindly (Griffis et al., 2007). The depletion of Spindly in S2 cells impairs the recruitment of dynein to kinetochores where in C. elegans impairs the localization of both dynein and dynactin and needs the RZZ complex to be recruited to kinetochores (Gassmann et al., 2008). Interestingly, the same study reported that the co-depletion of Spindly and RZZ reversed the severe phenotype observed upon depletion of RZZ alone. Recently, it was proposed that the kinetochore dynein could inhibit initial Ndc80 mediated kinetochore-microtubule attachments via an Aurora B independent pathway. This regulation is mediated by RZZ and is necessary for faithful chromosome segregation (Cheerambathur et al., 2013).

The *bona fide* SAC proteins Bub1, Bub3, Mad1, Mad2, BubR1 and Mps1 are also recruited to the outer kinetochore and fibrous corona (Abrieu et al., 2001; Martinez-Exposito et al., 1999; Chan et al., 1999; Basu et al., 1999; Jablonski et al., 1998; 1998; Basu et al., 1998; 1998; Chen et al., 1998; 1998; Taylor et al., 1998; 1998; Li and Benezra, 1996; 1996; Chen et al., 1996b). Although there are slight differences in kinetochores composition and structure across species, the basic kinetochore complexes appear to have a highly conserved function that has been maintained during evolution (reviewed in Cheeseman and Desai, 2008).

3. LINKING KINETOCHORE STRUCTURE TO FUNCTION

Kinetochores plays a main function of attach chromosomes to the mitotic spindle. This attachment is responsible to produce and/or transduce forces that are required for chromosome segregation. The fidelity of chromosome segregation is achieved by the stabilization of properly attached in a bi-oriented conformation so that each sister chromatids attaches to opposite

spindle poles so that if kinetochores fail to attach properly, SAC activation can take place and this way prevent premature exit from mitosis and allow time for correction mechanisms to act. How these functions are integrated in one structure will be discussed in this chapter.

3.1 KMN NETWORK AS THE CORE MICROTUBULE ATTACHMENT SITE

The first's Biochemical studies identified two distinct microtubule-binding activities within KMN network. The first was shown to be associated with the Ndc80/Nuf2 subunits of the Ndc80 complex and the second with KNL1 (Cheeseman et al., 2006) (Fig. 10). It now generally accepted that the Ndc80 complex is the first key component for microtubule-binding (reviewed in DeLuca and Musacchio, 2012). Integrated biochemical, cell biology, and structural studies support a model in which both the Ndc80 CH domain and N-unstructured tail play an important role in the formation of stable kinetochore-microtubule attachments (reviewed in Tooley and Stukenberg, 2011). In turn, the CH domain of Nuf2 seems to be essential for producing normal microtubule-dependent kinetochore force and timely mitotic progression (Sundin et al., 2011). Additionally, it has been shown using Cryo-EM that Ndc80 complex binds tubulin monomers at both the inter-tubulin and intra-tubulin dimer interfaces through a CH domain within Ndc80/Hec1, named the 'toe', (Alushin et al., 2010; Wilson-Kubalek et al., 2008). This particular form of biding to microtubules allows Ndc80 complex to bind microtubules every 4nm, where other microtubule associated proteins only bind every 8nm, promoting the oligomerization of Ndc80 complex on microtubules. Furthermore, it has also been reported that the N-terminal tail of NDC80 could interact with the C-terminal tails of tubulin monomers (known as E-hooks) (Tooley et al., 2011; Alushin et al., 2010; Ciferri et al., 2008). The 'toe print' located within the CH domain acts as a sensor of the tubulin conformation allowing Ndc80 complex to swop between high affinities biding state in straight microtubules protofilaments (polymerizing microtubules) and low affinity biding state in curled microtubules protofilaments (depolymerizing microtubules) (Alushin et

al., 2010; Wilson-Kubalek et al., 2008). The N-unstructured tail Ndc80/Hec1 was described as being essential for driving cooperativity binding of Ndc80 complex to microtubules (Alushin et al., 2012; 2010), this property is achieved either by tail-tail interaction or by coordinating the interactions between dual CHDs of adjacent complexes (Alushin et al., 2010). However these functions do not seem to be conserved in yeast, where the tail is necessary to enhance the microtubule binding but not for the cooperative binding of the Ndc80 complex. Nevertheless in both systems the Ndc80 complex can couple microtubule depolymerization to bead movement (Powers et al., 2009; Ciferri et al., 2008; Wei et al., 2006). Moreover, *in vivo* yeast experiments show that the tail seems to be dispensable for kinetochore function (Kemmler et al., 2009). Therefore, the N-unstructured tail of metazoan Ndc80/Hec1 seems to have two distinct roles increasing affinity for tubulin and modulate cooperativity.

The difference observed between the two systems could be explained by the presence of the Dam1 complex, which has only been identified in yeast. The complex is built around microtubules and can form a closed stable ring-like structure or open spirals around the MT lattice (Westermann et al., 2005; Miranda et al., 2005). *In vitro*, it has been shown that it is able to slide along microtubules in response to force (Westermann et al., 2007), increasing the processivity of the Ndc80 complex when attached to depolymerizing microtubules (reviewed in Lampert and Westermann, 2011). In fact, recently in yeast it has been shown that the Ndc80–Dam1 interaction is critical for cell cycle progression, sharing an essential function with the N-unstructured tail of Ndc80 (Lampert et al., 2013). Interestingly, no structural homologs of the Dam/Dash complex components have been identified in higher eukaryotes. Only recently, a complex, which associate with the KMN network has been described (Ska complex) that together can regulate the kinetochore microtubule interaction. This complex was first characterized in human cells (Hanisch et al., 2006) and it comprises three proteins Ska1, Ska2, Ska3 (RAMA1) (Raaijmakers et al., 2009). All the three components localize at spindle microtubules and kinetochores and depletion of any of the three proteins results in impairment of kinetochore-microtubule attachment, severe chromosome alignment defects, checkpoint-dependent mitotic arrest and

defects during anaphase (Raaijmakers et al., 2009; Welburn et al., 2009; Gaitanos et al., 2009; Daum et al., 2009; Hanisch et al., 2006). *In vitro* reconstitution of the complex has been shown to bind microtubules in a cooperative manner, as well as assemble oligomeric structures capable of diffusing along microtubules. The same complex is also able to bind to depolymerizing microtubules plus-ends along both straight and curved microtubules protofilaments (Schmidt et al., 2012; Welburn et al., 2009).

Similarly to what is observed for the DAM/Dash complex the association of Ska complex with the Ndc80 complex increases the ability of the Ndc80 complex to bind microtubules in a cooperative manner (Schmidt et al., 2012). The Ndc80 complex is not the only component of KMN network that has been described to associate with Ska complex. Mis12 complex and KNL1 interact with the Ska complex and all of the three components of the KMN network are required to recruit Ska complex to kinetochores (Chan et al., 2012). All these findings support the idea that the Ska complex is indeed the functional homologue of DAM/Dash complex in higher eukaryotes. Recently, the prominent kink or bend (the loop domain) in the structure of the Ndc80 complex, which localizes approximately 16 nm apart from the CH domain of Ndc80 protein, has been reported to contribute to stabilize kinetochore-microtubule attachments (Zhang et al., 2012; Matson and Stukenberg, 2012; Varma et al., 2012). Two different studies in yeast have shown that the loop domain is necessary for the recruitment of the Dam/Dash complex (Maure et al., 2011) and MAP Dis1 (TOG or XMAP215) to kinetochores (Hsu and Toda, 2011) In the absence of this loop domain the conversion of lateral to load-bearing kinetochore-microtubule attachments is abnormal and kinetochore bi-orientation fails (Maure et al., 2011). Mutations in the loop domain in vertebrate cells inhibit the association of SKA complex with the Ndc80 complex. This study showed that the delocalization of the SKA complex from kinetochores due to mutation in loop domain resulted in defects in kinetochore-microtubule attachment (Zhang et al., 2012). Finally, in vertebrate cells, a DNA replication licensing protein CDT1 was identified as a novel kinetochore protein that interacts with the Ndc80 complex and promotes robust kinetochore-microtubule interaction (Varma et al., 2012).

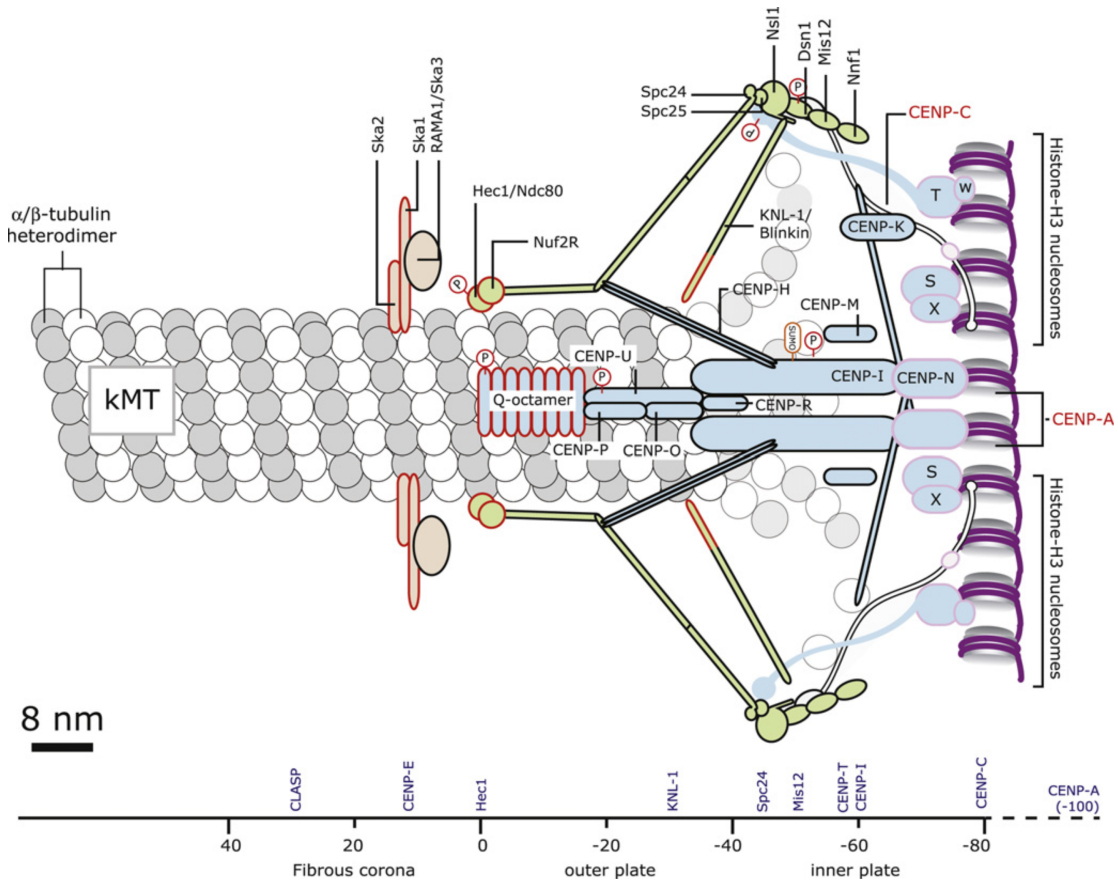


Figure 10. Human kinetochore–microtubule attachment site. Structure and spatial arrangement of the KMN network (colored green) and Ska complex (colored orange). In blue is depicted the structure of CCAN, which links nucleosomes to microtubules, outlined in pink those that bind to DNA/histones. CENP-C bridges CENP-A, the CCAN and the KMN network. The proteins involved in direct binding to microtubules are outlined in red. Spatial distribution of kinetochore constituents within the kinetochore is based on super-resolution imaging and adapted from (Wan et al., 2009). (Adapted from McAinsh and Meraldi, 2011).

Unequivocally, the Ndc80 complex plays a central role in kinetochore-microtubule biding but is not alone in mediating this interaction. Within the KMN network, the N-terminal region KNL1/Spc105 has been described as the second microtubule-binding domain (Welburn et al., 2010; Pagliuca et al., 2009; Cheeseman et al., 2006). At same time, KNL1/Spc105 is required for recruiting to kinetochores ZWINT, BubR1 and Bub1, as well as the RZZ complex in C. elegans (Gassmann et al., 2008). In agreement with the role played by KNL1/Spc105 in recruiting proteins involved in SAC response to kinetochore a recent study suggested that the microtubule binding motif of KNL1/Spc105 is likely to be a key sensor for controlling checkpoint activity

(Espeut et al., 2012). Although some of the functions of C- and N-terminus of KNL1 have been already identified, the function of central region of the protein is still unknown and detailed analyses could shed some light to additional involvement in the kinetochore-microtubule interaction. In C. elegans and *Drosophila* the phenotype caused by depletion of KNL1/Spc105 results in a severe phenotype with a decrease in cell viability and impairment of kinetochore-microtubule interaction (Feijão et al., 2013; Przewloka et al., 2007; Desai et al., 2003). The phenotype cannot be explained only taking in account the dependency of Ndc80 for kinetochores recruitment on KNL1/Spc105, since depletion of Ndc80 complex does not have the same out come. Since kinetochores have evolved rapidly (Meraldi et al., 2006), and some of the components responsible for kinetochore-microtubule attachment are not conserved across species, it is possible that KNL1/Spc105 could have a more active and direct function in kinetochore-microtubule attachment.

3.2 SWITCH ON/OFF OF SAC

The Spindle-Assemble Checkpoint (SAC) is a surveillance mechanism, conserved across eukaryotes, that senses unattached kinetochores and prevents premature entry to anaphase, assuring the fidelity of chromosome segregation in mitosis. This is a subject of extensive research and it is now well established that both SAC activation and silencing are intrinsically linked to kinetochores. The proteins that are directly involved either in detecting the state of attachment of kinetochore to spindle microtubules or in transmitting the nature of attachment to the cell-cycle machinery are known as SAC proteins (reviewed in Musacchio and Salmon, 2007) and it includes Mps1, Bub1, Mad1, Mad2, Bub3 and BubR1 (Abrieu et al., 2001; Basu et al., 1998; Weiss and Winey, 1996; Chen et al., 1996b; Hoyt et al., 1991; Li and Murray, 1991). The major questions in the field are; why SAC proteins are only recruited in presence of unattached kinetochores; how is this inhibitory signal produced and how is this process regulated. Specific kinetochores complexes play a major role in these processes since it has been shown that KMN network impairs recruitment of SAC proteins to the kinetochore and/or SAC

activity (Feijão et al., 2013; Kiyomitsu et al., 2007; McCleland et al., 2003; Martin-Lluesma et al., 2002; Janke et al., 2001; Wigge and Kilmartin, 2001).

Some studies have demonstrated that KNL1/Spc105 is the docking site for Bub1 and BubR1, through interaction with KI motifs in KNL1/Spc105 and TPR sequences in Bub1 and BubR1 (Krenn et al., 2012; Kiyomitsu et al., 2011). Previously, it was already described that a point mutation in TPRs sequence of Bub1 impaired its kinetochore localization and in the same study it was shown that after depletion of KNL1/Spc105, Bub1 and BubR1 were not able to localize at kinetochores (Kiyomitsu et al., 2007). However, there is conflicting data on this point. Previous studies have shown that the TPR region was unnecessary for kinetochore localization and that Bub3 binding domain of Bub1 was sufficient for kinetochore localization (Taylor et al., 1998). Mutations in the Bub3 binding domain impaired kinetochore localization of Bub1 and BubR1, as well as affecting the function of BubR1 in checkpoint and chromosome congression (Elowe et al., 2010; Klebig et al., 2009; Taylor et al., 1998). These results are supported by recent results in human cells, where it has been show hat the Bub3-binding region of Bub1, rather than the TPRs, was essential for kinetochore recruitment of Bub1 and the interaction between Bub1 and Bub3 was essential to promote the interaction of Bub1 and BubR1 (Krenn et al., 2012). KI motifs within KNL1/Spc105 are not sufficient to recruit Bub1 to kinetochores and additional pathways must be involved in this regulation. Recent data shed some light on this process since phosphoregulation by Mps1 appears to be involved. In fission yeast and human cells it has been shown that phosphorylation of KNL1/Spc105 at different Thr residues within MELT repeats sequence by Mps1 recruits Bub1 to kinetochores and is negatively regulated by protein phosphatase 1 (PP1) (London et al., 2012; Shepperd et al., 2012; Yamagishi et al., 2012). It was also demonstrated that *in vitro* this phosphorylation promotes the assembly of the Bub1-Bub3 complex (Yamagishi et al., 2012). Previous studies had already described that Bub1 is required and sufficient to recruit other SAC proteins to kinetochores (Vanoosthuyse et al., 2004; Sharp-Baker and Chen, 2001). Moreover, it has been shown to be essential for kinetochore-based SAC activation and chromosome alignment (Sharp-Baker and Chen, 2001). KNL1 seems to be involved in an additional pathway of SAC activation.

ZWINT and KNL1/Spc105 interact through the C-terminal domain of KNL1/Spc105. This interaction is responsible to the recruitment of ZWINT to kinetochores. It has been suggested that ZWINT phosphorylation by Aurora B is necessary to recruit the RZZ complex and dynein to kinetochores and this regulation has been shown to be important for chromosome movement and SAC signalling (Kasuboski et al., 2011; Famulski and Chan, 2007). At the same time it has been shown that the RZZ complex also needs Mps1 phosphorylation to be targeted to kinetochores (Santaguida et al., 2010; Hewitt et al., 2010; Maciejowski et al., 2010).

So far, the evidences points in the direction that phosphorylations play an important role in SAC activation. It is logical to assume that a phosphatase can negatively regulate the process contributing to SAC extinction. Recent studies showed that KNL1 also plays a role in SAC silencing, in fact PP1 is recruited to the kinetochore through a PP1-binding motif present in KNL1. In yeast and C. elegans when this interaction is perturbed, SAC silencing is compromised (Espeut et al., 2012; Rosenberg et al., 2011; Meadows et al., 2011). PP1 is also involved in regulating Aurora B activity, which is responsible to destabilize kinetochore-microtubule interactions through phosphorylation of multiple residues within the KMN network (Welburn et al., 2010; Liu et al., 2010). Moreover, at the N-terminus of KNL-1/Spc105 a second microtubule binding site was identified (Cheeseman et al., 2006). In C. elegans, this site was shown not to be necessary for kinetochore-microtubule attachment *in vivo*, but rather participates in SAC silencing (Espeut et al., 2012). The same study also demonstrates that the recruitment of PP1 by KNL1 also contributes to SAC inactivation, suggesting that are two independent routes to SAC extinction. In human cells, PP1 activity has only been demonstrated to participate in the stabilization of kinetochore–microtubule attachments (Liu et al., 2010). Overlapping KNL1-independent mechanisms that contribute to SAC silencing at kinetochores may mask the role of PP1 in SAC extinction.

3.3 ERROR CORRECTION MECHANISM

Accurate chromosome segregation implies that each pair of sister kinetochores attaches to microtubules from opposite spindle poles reaching biorientation. Prior to the establishment of biorientation incorrect attachments occur, for example when only one kinetochore is attached to microtubules is defined as monotelic attachment (Fig. 11), if both sister kinetochores are attached to microtubules of the same spindle pole is called syntelic attachment (Fig. 11) and when the same kinetochore is attached to microtubules from both spindle poles and is called merotelic attachment (Fig. 11).

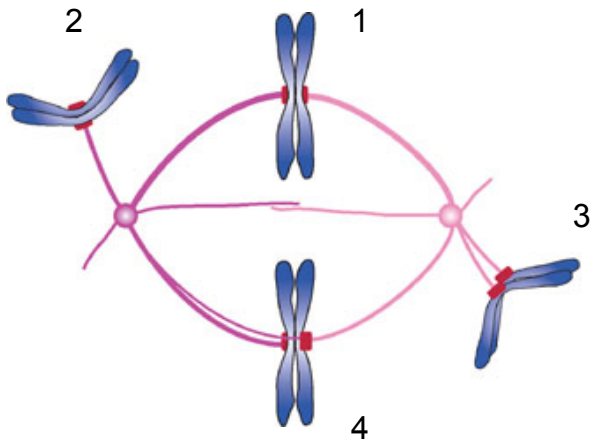


Figure 11. Interactions between kinetochores and microtubules. (1) Amphitelic attachment, (2) monotelic attachment, (3) syntelic attachment and (4) merotelic attachment. (Adapted from Tanaka, 2012).

In order to ensure faithful chromosome segregation chromosomes have to be bioriented by converting erroneous attachments into a corrected amphitelic orientation (Fig. 11). The studies so far have demonstrated that Aurora B plays a central role in this error correction process (reviewed in Lampson and Cheeseman, 2011). Aurora B is a serine/threonine protein kinase (Kimura et al., 1997) that phosphorylates preferentially sequences with a consensus motif as [RK]x[TS][ILV] (Cheeseman et al., 2002). Perturbing the activity of Aurora B has different outcomes taking into account the organism studied. In yeast, the homolog of Aurora B promotes kinetochore microtubules turnover,

which leads to a faster biorientation process (Tanaka et al., 2002). In vertebrate cells, an increased of incorrect attachments is observed when Aurora B activity is inhibited or the protein depleted (Hauf, 2003; Ditchfield, 2003). Similar to what was observed in yeast, Aurora B levels also promote the turnover of kinetochore microtubules preventing segregation errors (Cimini et al., 2006). Aurora B has been described to phosphorylate preferentially a group of proteins intimately involved in kinetochore-microtubule attachment, such as the KMN network (Welburn et al., 2010; DeLuca et al., 2006; Cheeseman et al., 2006), the Dam1 complex (Cheeseman et al., 2002) and more recently, the Ska complex (Chan et al., 2012). The activity of Aurora B can act directly in the affinity of these proteins for microtubules (Cheng et al., 2011; Welburn et al., 2010; Cheeseman et al., 2006) or indirectly, by perturbing the interaction between them, for example between Ndc80 and the Dam1 complex (Lampert et al., 2010) or the Ska complex (Chan et al., 2012). However, the first substrate of Aurora B to be identified and claimed to be involved in attachment error correction was a microtubule depolymerase protein, MCAK (Knowlton et al., 2006). Interestingly it has been demonstrated that phosphorylation of MCAK by Aurora B inhibits its depolymerase activity (Ohi et al., 2004; Lan et al., 2004; Andrews et al., 2004).

There are different models that try to explain by which mechanism tension and/or microtubule attachment status is detected and how these states regulate Aurora B kinase activity. The model that is more consensual takes into consideration the distance between active Aurora B at the inner centromere and its kinetochore substrates. As bi-orientation of chromosomes is achieved and tension exerted upon sister chromatids the accessibility of Aurora B to kinetochores substrates would be severely impaired (Liu et al., 2009) (Fig. 12). It was also described that the total levels of Aurora B are increased in misaligned chromosomes (Salimian et al., 2011). Also during prometaphase, intermediates levels of Aurora B are under tight regulation of B56-PP2A phosphatase that regulates negatively the phosphorylation levels of different kinetochore proteins and more specifically KMN network components (Foley et al., 2011). Recently, it was proposed a possible regulatory mechanism for kinetochore-microtubule attachment, where the phosphorylation of BubR1 by PLK1 was responsible to recruit PP2A-B56

phosphatase to kinetochores during prometaphase mediating dephosphorylation of Aurora B substrates at the kinetochore-microtubule interface (Suijkerbuijk et al., 2012).

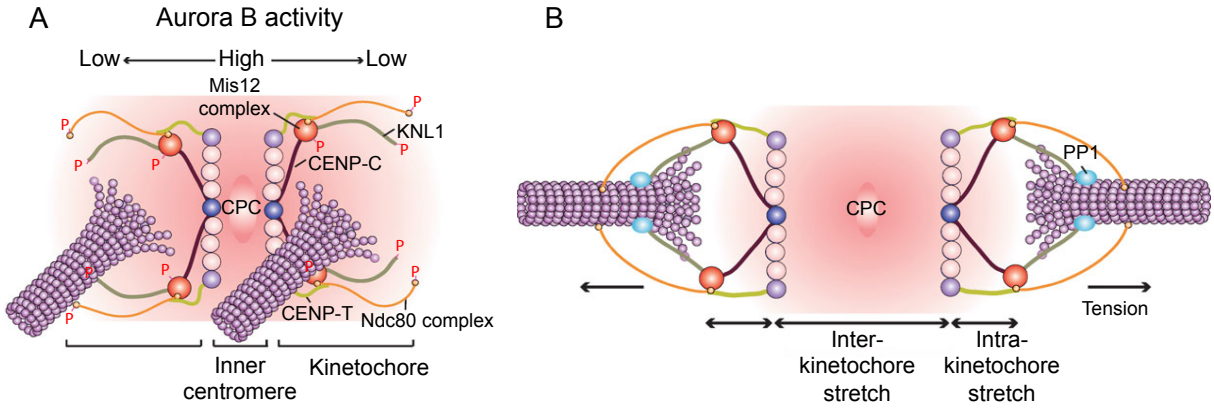


Figure 12. Regulation of kinetochore-microtubule attachment by Aurora B. (A) schematic representation illustrating the 'spatial separation model': In a situation of monotelic or syntelic attachment, sister kinetochores since there is no tension sister chromatids are not pull apart. This proximity of the kinetochores to the inner centromere leads to phosphorylation of KMN network components by Aurora B kinase (shown as 'P'), which in turn results in kinetochore-microtubule attachment destabilization. (B) After biorientation, chromosomes are now under tension, and kinetochores are stretched both internally and externally. Aurora B is no longer able to phosphorylate the KMN components and kinetochore-microtubule interactions are stabilized. PP1 also plays an important role in this stabilization, since upon KNL1 desphosphorylation, PP1 is recruited to KNL1 and helps to keeps the unphosphorylated state of KMN network components. (Adapted from Tanaka, 2012).

It is unclear how the first kinetochore-microtubule interactions are stabilized when the access of Aurora B to kinetochore substrates is expected to be highest at this stage. In fact, in early prometaphase when tension is low, the phosphorylation of KMN network components is increased but not saturated when compared with cells treated with microtubule poisons or with no tension (Welburn et al., 2010) (Fig. 12). Moreover, kinetochores initially interact laterally with microtubules walls (Magidson et al., 2011). KMN network does not seem mediate lateral attachments (Magidson et al., 2011; Cai et al., 2009) and therefore Aurora B and B56-PP2A cannot disturb the initial attachment, giving time the cell to switch from lateral to end-on attachment. As microtubule occupancy increases, tension is exerted across sister chromatids (Uchida et

al., 2009; Maresca et al., 2009) and phosphorylation of Aurora B substrates decreases and simultaneously B56-PP2A phosphatase is removed from kinetochores. This process is counter acted by the action of protein phosphatase 1 (PP1) in attached kinetochores (Liu et al., 2010; Posch et al., 2010) keeping phosphorylated levels low on bi-oriented kinetochore pairs (Fig. 12).

4. CHROMOSOME MOVEMENT AND CONGRESSION

The main energy source for chromosome oscillation is centred in microtubule depolymerization at the leading kinetochore (Khodjakov et al., 1996). The coordination between movement and attachment is a subject of extensive debate. In this section we will try to address some crucial points of this process.

4.1 MICROTUBULES - STRUCTURE AND ORGANIZATION

Microtubules can be found in all types of cells and are involved in a wide range of processes; for example, in cell morphogenesis, as intracellular transport tracks, and play a pivotal role in distinct cellular structures as axonemes and mitotic spindles. Accurate chromosome segregation is dependent on the assembly of a dynamic arrangement of microtubule structure called spindle. The spindle is responsible for the generation of forces that physically separate chromosomes to daughter cells and additionally establish the position of the cytokinetic furrow (Glotzer, 2003).

The structural unit of the spindle is the microtubule. Microtubules are composed by heterodimers of α and β tubulin that bind head to tail to form polarized protofilaments. Approximately 13 of these protofilaments associates laterally to form a cylindrical wall of a 25 nm wide hollow polymer: the microtubule (Tilney et al., 1973). Since protofilaments are polarized and oriented in the same direction, β -tubulin is exposed at one end (plus-end) and α-tubulin at the other end (minus-end) (Desai and Mitchison, 1997). α- and β- tubulin heterodimers co-purify with two moles of guanine nucleotide

per mole αβ dimer (Weisenberg, 1972), although, α-tubulin binds GTP irreversibly and does not hydrolyse it (N-site for Non-exchangeable), β-tubulin binds GTP reversibly and can hydrolyse it to GDP, since GTP and GDP can swap, this site is called E-site (for Exchangeable) (Downing and Nogales, 1998). When the α- and β-tubulin are associated to GTP, polymerization takes place and the α-tubulin subunit binds to β-tubulin at the microtubule plus-end, where it is stabilized by the β-tubulin - GTP at the plus end known as GTP-cap. Microtubules can switch between states of polymerization or depolymerisation, when the GTP-cap is maintained at the plus-end, the microtubule continues to polymerize assuming a more “straight” and stable conformation. However, if hydrolysis of GTP is faster that polymerization, the GTP-cap will be lost and this allows GDP-tubulin dimmers to acquire their natural curved conformation, peeling away from the microtubule lattice and acquire a ram-horn-shaped structure at the microtubule end (Tran et al., 1997; Desai and Mitchison, 1997; Mandelkow et al., 1991) (Fig. 13). These two states of polymerization and depolymerisation are abrupt and stochastic and are known as "rescue" for the transition from shortening to growth and as "catastrophe" for the shift from growth to shortening. In living cells it was also observed a third behavior where microtubules are neither polymerizing nor depolymerizing which is referred as pause state (Walker et al., 1988; Schulze and Kirschner, 1988). This intrinsic behavior of microtubules is known as dynamic instability (Mitchison and Kirschner, 1984).

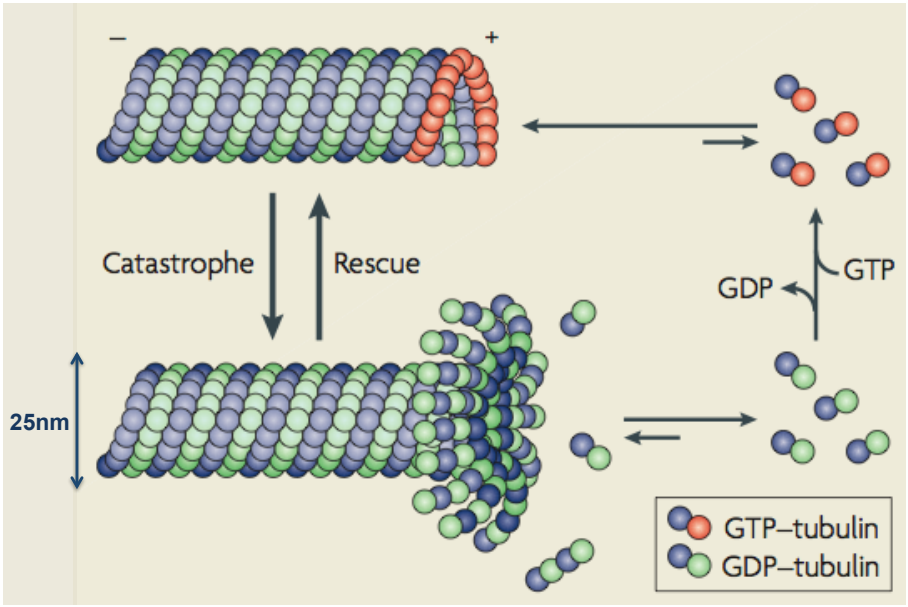


Figure 13. Microtubule dynamics. 12-15 parallel protofilaments of αβ-tubulin heterodimers associate laterally to form a 25 nm hollow cylindrical structure (microtubule). Dynamic instability is characterized by the coexistence of polymerizing and depolymerizing microtubules. GTP-tubulin is incorporated at polymerizing MT ends, the bound GTP is hydrolyzed during or soon after polymerization, and Pi is subsequently released. The switch from polymerization to depolymerization called catastrophe, and the reverse transition is defined as a rescue. (Adapted from Cheeseman and Desai, 2008).

4.2 NUCLEATION AND ORIGIN OF MICROTUBULES IN THE SPINDLE

The interaction between the 92 human kinetochore cells and microtubules has to be accomplished efficiently in a relatively short period of time (10-15min). There are distinct spindle assembly pathways that ensure the proficiency of this process. The most widely accept mechanism is based on the nucleation of microtubules from centrosomes and the dynamic instability of microtubules, which increases the opportunity of kinetochores to encounter microtubules. The initial interaction with kinetochores leads to its stabilization and promotes spindle formation. This model called 'search-and-capture' model and has been extensively validated in many systems (Fig. 14) (Alexander and Rieder, 1991; Hayden et al., 1990; Kirschner and Mitchison, 1986). However this model has been criticized mostly from the pit of view of modelling. According to mathematical modelling of kinetochore capture using the 'search-and-capture' hypothesis a human cell would take several hours

before all kinetochores had been properly captured by microtubules (Wollman et al., 2005).

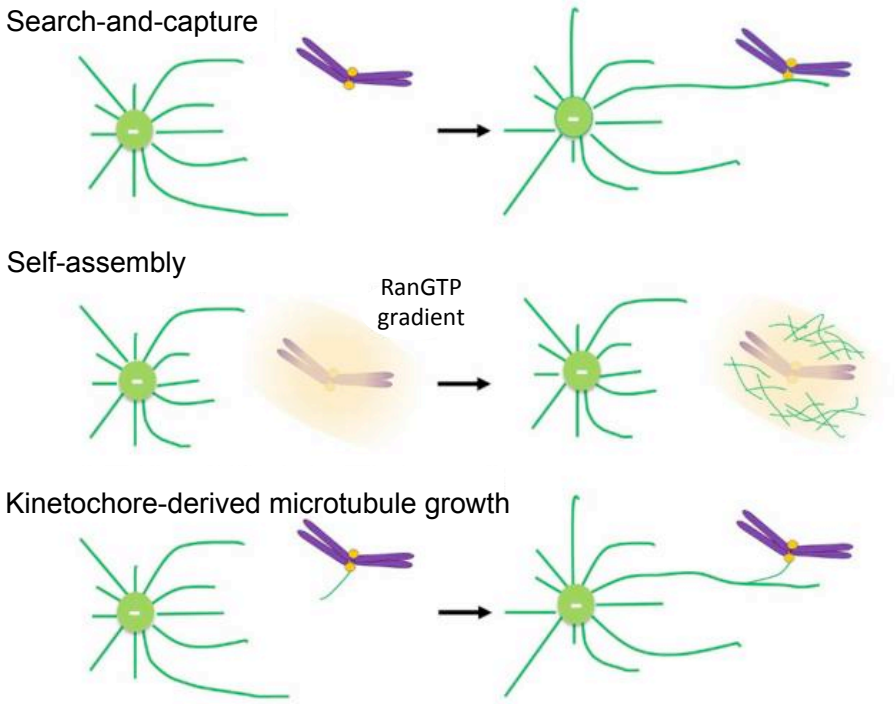


Figure 14. Nucleation and origin of microtubule in the spindle. Search-and-capture" model. Microtubules are nucleated from centrosomes. This model is also based in the intrinsic dynamic instability of microtubules, which undergo in alternative cycles of polymerization and depolymerisation and eventually are captured and stabilized by kinetochores. A Ran-GTP gradient dependent "self-assembly" model is based in the capacity that this gradient has to promote centrosome independent microtubule nucleation. Finally there is the Kinetochore-derived microtubule growth model, where microtubules are nucleated at kinetochores or near them and subsequently start to interact with other spindle microtubules and then are integrated into the mitotic spindle. (Adapted from Guo et al., 2013).

Recently, *in vivo* observations demonstrated that chromosomes and microtubules are spatially positioned at early stages of spindle assembly promoting chromosome-microtubule interactions, justifying the hypothetical parameters necessary for rapid spindle assembly posited in simulation (Magidson et al., 2011). Moreover the 'search-and-capture' model is not applicable to cells, which lack centrosomes such as those of higher plants and many meiotic eggs. In these organisms, spindle formation is promoted by a RanGTP gradient around chromosomes (Fig. 14) (reviewed in Clarke and

Zhang, 2008). Ran belongs to the family of regulatory GTPases that combines the binding and hydrolysis of GTP to specific cellular processes. This mechanism leads to spindle formation by facilitating the release of SAFs (spindle assembly factors) from importin a/b around chromosomes, which results in microtubule formation around chromosomes. Minus-end-directed motors are the responsible for regulating the formation of a bipolar spindle, additionally RanGTP gradient is also engaged in attracting microtubules to elongate preferentially towards chromosomes by enabling microtubule rescue, as is shown in Xenopus (Carazo-Salas and Karsenti, 2003).

Apart from these two mechanisms, microtubules are also able to nucleate from kinetochores (Fig. 14) (Maiato et al., 2004; Khodjakov, 2003; Pepper and Brinkley, 1979; Snyder and McIntosh, 1975). The molecular mechanism beyond microtubule nucleation from kinetochores is still elusive. It was reported that small GTPase Ran could be involved in the process (Torosantucci et al., 2008; Tulu et al., 2006). Further studies, demonstrated that GTPase Ran around chromosomes could induce nucleation from centrosomes but on its own was not able to induce spindle formation (O'Connell et al., 2009). Other proteins were also describes as being involved in in kinetochore nucleation as TPX2 and NUP 106-170 complex (Mishra et al., 2010; Tulu et al., 2006). This population of microtubules is often seen growing rapidly after the use of depolymerizing drugs thereby helping spindle formation through bundling with microtubules from different kinetochores or spindle poles. In *Drosophila* such microtubules were observed even in the presence of microtubules derived from centrosomes and together promote an efficient kinetochore capture (Maiato et al., 2004).

The recruitment of γ-tubulin onto preexisting microtubules constitutes an additional mechanism for microtubule generation known as microtubule-branching nucleation (Mahoney et al., 2006). Also, in *Drosophila* was identified for the first time, a complex of proteins named Augmin responsible to recruit γ-tubulin to spindle microtubules (Goshima et al., 2008). Augmin was shown to be tightly associated with one component of γ-tubulin the γ-TURC, this association was also reported in human cells (Uehara et al., 2009) predicting a model where Augmin binds to pre-existing microtubules recruiting γ-TURC to nucleate new microtubules. Together, this data suggest that

spindle formation is under regulation from different pathways, so that after NEBD, microtubule nucleation occurs at centrosomes and in the vicinity of chromosomes. These two processes are crucial to generate the first set of mitotic microtubules enabling the assembly of the mitotic spindle. However, the microtubule-branching nucleation mediated by Augmin can have a synergistic effect in the spindle formation since it can rapidly amplify the number of microtubules, which promotes chromosome capture and k-fiber formation.

4.3. SPINDLE MICROTUBULES AND BIPOLARITY

The bipolar, antiparallel arrangement of spindle microtubules is what certifies faithful chromosome segregation (Fig. 15). The highest density of microtubules minus-ends it's localized at opposite ends of the spindle known as spindle poles. At spindle poles we can find microtubules organizing centers (MTOC) or centrosomes (Fig. 15), these two structures are responsible for microtubule nucleation and for ensuring that microtubule minus-ends are kept attached to spindle pole or near by.

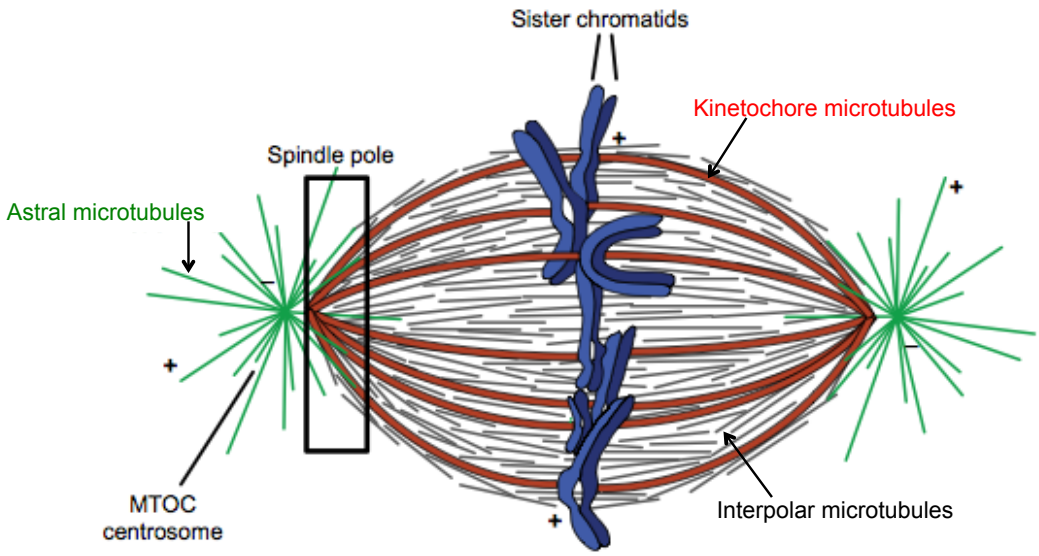


Figure 15. Metaphase spindle architecture and microtubules populations. Microtubules are organized in an antiparallel arrangement with their plus-ends oriented toward the center of the spindle and their minus-ends toward the

poles. Three different classes of microtubules compose mitotic spindle: kinetochore-microtubules, interpolar-microtubules and astral-microtubules. (Adapted from Helmke et al., 2013).

The microtubules minus-ends are always oriented towards the spindle pole and the plus-ends towards chromosomes or cell cortex (Telzer and Haimo, 1981; Heidemann et al., 1980). Within the mitotic spindle we can find three different classes of microtubules. Microtubules that are attached to kinetochores at the plus end and the minus ends to spindle poles and are designated as kinetochore-microtubules, which bundle and eventually form k-fibers (kinetochores-fibers) (Fig. 15) (Merdes and De Mey, 1990; Hayden et al., 1990; Rieder and Alexander, 1990). When all sister chromatids are properly attached to opposite poles, oscillations in sister chromatids occur. This dynamic movement is due to the growth and shrinking of k-fibers, promoting the congression and alignment of chromosomes at the metaphase plate. This population is present in all cell types and is essential for a correct spindle function (reviewed in Guo et al., 2013).

Interpolar-microtubules (ipMT) constitute the second population of microtubules. These microtubules cross over at the center of the spindle and contact with microtubules in the opposite side of the spindle, which are oriented in an antiparallel manner (Fig. 15). In contrast to kMTs, most of minus-ends of ipMTs are not near the spindle poles but instead, distributed along the half of spindle length. These interactions are present during mitosis and are one of the key factors for establishing and maintaining spindle bipolarity and to ensure spindle poles separation by antiparallel microtubule sliding (Mastronarde et al., 1993; Sharp et al., 2000a). Finally, there is a third class of microtubules, the astral microtubules, whose plus-ends are oriented towards the cell cortex with their minus-ends connected to MTOC, centrosomes or near the spindle poles (Fig. 15). They are present in almost spindle types, however they are absent from female meiotic spindles and plant spindles without centrosomes. Since in many cell types they frequently contact the cell cortex, they can play a pivotal rule in orienting and position the metaphase spindle, which is essential for asymmetric cell divisions, tissue development and organization (reviewed in Noatynska et al., 2012).

There are special features that distinguish the different microtubule populations. K-fibers are resistant to treatments that depolymerize other spindle microtubules, such as cold temperature or low doses of MT-depolymerizing drugs, however are less dynamic than interpolar or astral microtubules (Kirschner and Mitchison, 1986; Rieder, 1981; Salmon and Begg, 1980).

4.4 MICROTUBULE DYNAMICS

Despite *in vitro* studies that have shown the presence of polymerization and depolymerisation events in both microtubule ends, *in vivo* polymerization at minus-end has never been reported, perhaps due to a conserved mechanism that concomitantly inhibits polymerization and promotes depolymerisation (Dammermann et al., 2003; Walker et al., 1988). The poleward movement of tubulin subunits at a constant rate from microtubule plus end to microtubule minus end is called "treadmilling". So, a microtubule undergoing “treadmilling” is constantly assembling tubulin subunits at one end, with a balanced loss at the opposite end (Margolis and Wilson, 1981) causing a phenomenon known as flux.

An association of microtubule speckling and kinetochore labelling techniques revealed that chromosome movements during metaphase and anaphase A are the result of two different mechanisms: poleward microtubule flux and “Pac-Man” motility. The poleward microtubule flux is bases on the movement of tubulin subunits from microtubule plus-ends to the microtubule minus-ends at the spindle pole (Mitchison, 1989). This activity is present either in microtubules associated to kinetochores as well as in interpolar microtubules. Interestingly, the flux velocity is more heterogeneous within a spindle that between spindles, denoting that this is a process spatial and temporally regulated with contributions from different proteins (Cameron et al., 2006).

This poleward movement of tubulin subunits is achieved by polymerization at microtubules plus-ends, shown in *Drosophila* S2 cells to be dependent on Mast protein (Maiato et al., 2005) and by minus-ends depolymerisation at

spindle poles under regulation of proteins from kinesin-13 family (Kif2a, KLP10A) (Ganem et al., 2005; Goshima et al., 2005). There is a third activity responsible for poleward microtubule flux, the sliding of antiparallel microtubules towards their minus-ends known as microtubule poleward translocation where, due to its antiparallel microtubule-sliding activity, kinesin-5 is believed to be the central player (Cameron et al., 2006; Goshima et al., 2005; Mitchison et al., 2004; Miyamoto et al., 2004).

The “Pac-Man” activity is characterized by a coupled depolymerisation of the plus ends of kinetochore microtubules that maintain the attachment site at the kinetochore (Gorbsky et al., 1987; Mitchison et al., 1986). How the process is regulated is still unknown. However it is described that *Drosophila* kinesin-13 (KLP59C) is involved in the process (Rogers et al., 2004) since upon KLP59C inhibition anaphase chromatid movement is severely impaired with normal values of microtubule flux. The minus-end directed motor protein dynein (Yang et al., 2007; Savoian et al., 2000; Sharp et al., 2000b) and the plus-end directed motor protein CENP-E (McEwen et al., 2001; Lombillo et al., 1995) have also been described to be involved in “Pac-Man” activity.

The impact of these processes in chromosome movement during anaphase differs between model organisms. Where “Pac-Man” input in budding yeast and vertebrate tissue culture cells is 100% and 70% respectively. In meiotic oocyte spindles and early embryonic spindles, microtubule flux seems to be the major mechanisms contributing for microtubule dynamics and anaphase movement. The same is also valid for grasshopper and crane fly meiosis I spermatocytes where apparently microtubule flux is the only mechanism observed so far (reviewed in Maiato et al., 2004).

4.5 FROM LATERAL BINDING TO END-ON ATTACHMENT

The initial interaction between kinetochores and microtubules is mediated through lateral attachment. Lateral binding provides a much larger contact surface than through microtubule ends resulting in a more efficient kinetochore capture. The proteins involved in the initial interaction may differ

between organisms. In budding yeast has been reported that Ndc80, Mis12 complex and Ctf19 complex are involved in this process (Tanaka et al., 2005), however in metazoans the process is not so well characterized. Nevertheless, different studies demonstrated that the motor protein minus end directed, dynein could be a key protein mediating this interaction (Vorozhko et al., 2007). Dynein is also responsible to poleward movement of chromosomes during lateral attachment in metazoans (Yang et al., 2007; Alexander and Rieder, 1991). The protein responsible for this movement also differs in budding yeast where Kar3 mediates this transport, however it is not involved in establishing the initial lateral attachment (reviewed in Tanaka et al., 2005). Since Kar3 belongs to the family of kinesin-14 and its processivity is much slower that dynein and slower than the shrinking rate of microtubules, it was also demonstrated in the same system that Stu2 promotes microtubule rescue and therefore prevents kinetochores from falling from microtubules (Gandhi et al., 2011). In *Drosophila* it was also shown that Ncd a member of kinesin-14 protein family, is also involved in this lateral sliding since it showed a high mobility along microtubules *in vitro* and has been implicated in kinetochore-microtubule attachment (Endow and Komma, 1996; McDonald et al., 1990). CENP-E is also responsible for congression of mono-oriented chromosomes toward the metaphase plate via lateral attachments with existing k-fibers prior biorientation (Kapoor, 2006). However, it has argued that this congression mediated by CENP-E, once thought to be only possible in presence of k-fibers (Kapoor, 2006), also occurs along any microtubule bundle (Cai et al., 2009). The chromokinesin Kid was also described to be important in congression through the interaction between chromosomes arms and microtubules (Levesque and Compton, 2001; Tokai et al., 1996).

Eventually, lateral attachments are converted to end-on attachments. In C.elegans it was proposed that the RZZ complex could prevent the formation of load-bearing attachments and this could be under the control of dynein/dynactin, by helping the conversion from the initial lateral attachment to an end-on attachment (Gassmann et al., 2008). Moreover, kinetochore dynein could also directly regulated Ndc80, in an Aurora B-independent manner, inhibiting initial microtubule binding by the Ndc80 complex (Cheerambathur et al., 2013). In budding yeast this transition occurs

spontaneously and irreversibly by microtubule depolymerisation until reaching the kinetochore attachment site (Tanaka et al., 2007). In metazoan cells, the conversion from lateral to end-on attachment is a process where different proteins have been shown to be involved. It has been shown that Bub1 and Bub3 are involved in this process (Logarinho et al., 2008; Meraldi and Sorger, 2005). There are however, some circumstances where the initial interaction established between kinetochores and microtubules is end-on. Such events were reported in *Drosophila* and human cells and take place during k-fiber formation through a centrosome-independent microtubule generation mechanism promoted by the Augmin complex (Lawo et al., 2009; Uehara et al., 2009; Goshima et al., 2008). Recently, a combined analysis with electron microscopy, molecular perturbations and immunofluorescence has shown that lateral interactions between kinetochores and microtubules are the major microtubule population in early prometaphases responsible for the disposition of chromosomes in an equatorial ring around the nascent spindle. This chromosome arrangement promotes kinetochore interaction with microtubules from both spindle poles facilitating amphitelic attachment (Magidson et al., 2011). In the light of these recent findings, lateral attachments turn out to be the predominant attachment in early prometaphase and seem to play an important role towards achieving biorientation.

The end-on attachments established upon biorientation are characterized to be stable and highly dynamic. These dynamic interactions between kinetochores and microtubules plus-ends lead to chromosome movement coupled with microtubule polymerization and depolymerisation. How this process is regulated is subject of intensive research and different models have been proposed to explain it. It is, however, well established that different proteins contribute for this dynamic and stable attachment, as well as the processive microtubule plus-end tracking.

The first model proposed was called the sleeve model (Hill, 1985). The kinetochore proteins involved in kinetochore-microtubule attachment would surround microtubules nearby the plus-ends and form a rigid sleeve at the outer face of microtubules. This model assumes that the proteins responsible for the attachment bind weakly in multiple sites in outer microtubule wall, allowing the sleeve to slide along polymerizing or depolymerizing

microtubules without dissociating. Further studies identified in yeast a oligomeric complex Dam1/DASH that forms a ring-like structure around microtubules making the Hill-Sleeve model more attractive (Westermann et al., 2005; Miranda et al., 2005). However this structure was never observed *in vivo* and no homologs of the Dam1/DASH complex have been identified in vertebrates. More recently, it has been proposed a biased-diffusion mechanism for force generation, where Ndc80 proteins play a key role (Wan et al., 2009; Powers et al., 2009; Ciferri et al., 2008). The Ndc80 complex structure and the weak affinity for microtubules make it the perfect candidate for biased diffusion (Cheeseman et al., 2006). It was also demonstrated that the Ndc80 complex binds to microtubules by recognizing α- and β- tubulin at both intra- and inter- tubulin interfaces. Due to these features the Ndc80 complex is able to distinguish different tubulin conformations and still remain attached, as well as diffuse along them (Alushin et al., 2010). Additionally, *in vitro*, when the Ndc80 complex is associated with Dam1/DASH complex, its affinity to form load-bearing attachments is drastically increased promoting a permanent association of Ndc80 with dynamic microtubules plus ends (Lampert et al., 2010). The Ska complex, which has been described as being the functional vertebrate “homologue” of Dam1/DASH complex, needs Ndc80 to be recruited to Kinetochores. All these results support the idea that Ndc80 acts as the force generator in a biased diffusion model. The last two models are based in the assumption that kinetochores bind to microtubule sides near the plus ends. There is however a third and alternative model that proposes that kinetochores can bind to the luminal side of peeling protofilaments through fibril-like attachments. The fibril-like attachments were described for the first time in Ptk1 cells using electron microscopy (McIntosh et al., 2008). In this study both polymerizing and depolymerising microtubules plus-ends appeared to be curved where a fibril-like structure from the luminal side of microtubules interact with the inner kinetochores (McIntosh et al., 2013). These structures were not present in non-kinetochores microtubules. Later, the same authors preformed an integrated study analysing different organisms showing that the slender fibrils were present in all interactions between kinetochore microtubules protofilaments and the nearby chromatin (McIntosh et al., 2013). The molecular composition of these structures is still

unknown, as several of the kinetochore-associated proteins are filamentous, there are some candidates suggested to play this function. The kinetochore-associated kinesin-like motor protein CENP-E is a good candidate, since its structure has a flexible coiled coil region 200nm long (Kim et al., 2008) that looks like the fibrils structure identified by electron microscopy. Other proteins such as Ndc80 and XMAP212 could be good candidates to play this function, however all these three proteins have been shown to bind to microtubule lattice. Finally, an alternative possibility is the 400 kDa kinetochore protein, CENP-F; whose structure is predicted to have a central region flanked by two coiled-coil regions (Rattner et al., 1993).

Part II

EXPERIMENTAL WORK

Chapter 1

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

1. INTRODUCTION

For the correct segregation of the DNA content during cell division, chromosomes need to be bioriented on the mitotic spindle. Before reaching biorientation, during prometaphase, chromosomes interact randomly with microtubules emanating from centrosomes that poduce chromosome movement that finally result in alignment at the metaphase plate. There are several players that contribute to these movements including the action of kinetochore and microtubule-associated motor proteins, as well as the balance between microtubule polymerizing and depolymerizing factors (McIntosh et al., 2002). In this study we will focus our attention on two particular proteins; centromere associated protein E (CENP-E) and one of the components of chromosomal passenger complex (CPC), Aurora B kinase. CENP-E, also known as plus-end-directed kinesin-7 motor protein has been described as responsible for mediating the sliding of mono-oriented sister-chromatids along the kinetochore fibers of already bioriented sister-chromatid pairs, as well as, along microtubule bundle without needing of the losing kinetochore attachment (Cai et al., 2009). Aurora B kinase, plays a critical function in chromosome biorientation (Tanaka et al., 2002). This kinase is known to promote destabilization of incorrectly kinetochore-microtubule interactions via the microtubule deploymerase, mitotic centromere-associated kinesin (MCAK) (Andrews et al., 2004; Lan et al., 2004). More recently, it has been implicated in kinetochore function by the phosphorylation of KMN network and the Ska complex (Jeyaprakash et al., 2012; Welburn et al., 2010).

Here, our purpose was to determine if CENP-E and Aurora B cooperate to promote efficient chromosome biorientation and timely anaphase onset. To address these questions we initially characterized by time-lapse microscopy, the phenotype of Hela cells stably expressing YFP-H2B after depletion of CENP-E by small interfering RNA (siRNA). We find that in all cells (n=182) analysed, chromosomes close to spindle poles (polar chromosomes) were observed, allowing us to identify two distinct phenotypes. In phenotype (a): 54% of the cells aligned their chromosomes into a metaphase plate after a

prolonged delay (time spent in mitosis (TIM) 238 ± 123 min vs. 50 ± 18 min for control cells) and anaphase onset started after the last chromosome congressed into the plate. In phenotype (b): In 37% of the cells, mitosis was also significantly delayed (TIM 306 ± 121min), but anaphase onset started while a few chromosomes still resided near the spindle poles and failed to align. The remaining 9% of the cells were delayed in mitosis for more than 480 min of continuous filming and were therefore denominated as “stop”.

2. RESULTS

2.1 Aurora B kinase is highly active on polar chromosomes

The mitotic delay observed in CENP-E mitotic depleted cells is suggestive of an active spindle assembly checkpoint (Hemmerich et al., 2008; Tanudji et al., 2004). Indeed, publish data show that CENP-E depleted cells arrest in mitosis when treated with the spindle poisons nocodazole, colcemid or taxol; (Weaver et al., 2003). Moreover, previous studies already demonstrated that in CENP-E depleted cells polar chromosomes stain positive in immunofluorescence for SAC protein Mad1 (Chen et al., 1998) and of CLIP-170, a microtubule “plus-end-tracking” protein that leaves the kinetochores upon microtubule attachment (Dujardin et al., 1998), confirming that these chromosomes were unattached or poorly attached and explaining why mitotic progression is delayed in CENP-E knockdown cells.

We then asked why do kinetochores of the polar chromosomes remained unattached or poorly attached for such a long time. For this we follow CENP-E depleted cells by time-lapse microscopy and observed that the polar chromosomes are not static but show random movements away and towards the poles suggesting that microtubules are indeed able to interact with these chromosomes but these attachments might not be properly stabilized. Aurora B is responsible for destabilizing incorrect kinetochore-microtubule attachments through phosphorylation of Hec1/Ndc80 and MCAK (Cheeseman et al., 2006; DeLuca et al., 2006; Andrews et al., 2004) . To test if the failure

to stabilize pole-proximal kinetochore-microtubule attachments was due to local Aurora B activity, auto-phosphorylation of the kinase was monitored using phospho-specific antibodies designed to identified the T232 epitope in the kinase (Yasui et al., 2004). Strikingly, the levels of T232 phosphorylation were significantly increased on the polar chromosomes of CENP-E knockdown cells when compared to aligned chromosomes, suggesting high levels of active Aurora B kinase at the polar chromosomes (Fig. 1.1 A and B). This is most probably due to the increased protein levels of Aurora B as measured by antibodies that recognize the protein irrespective of its phosphorylated state (Fig. 1.2). The specificity of the phosphorylation signal was confirmed by inhibition of Aurora B kinase activity with the small-molecule inhibitor ZM447439 (Ditchfield et al., 2003) (Fig. 1.1 A and B).

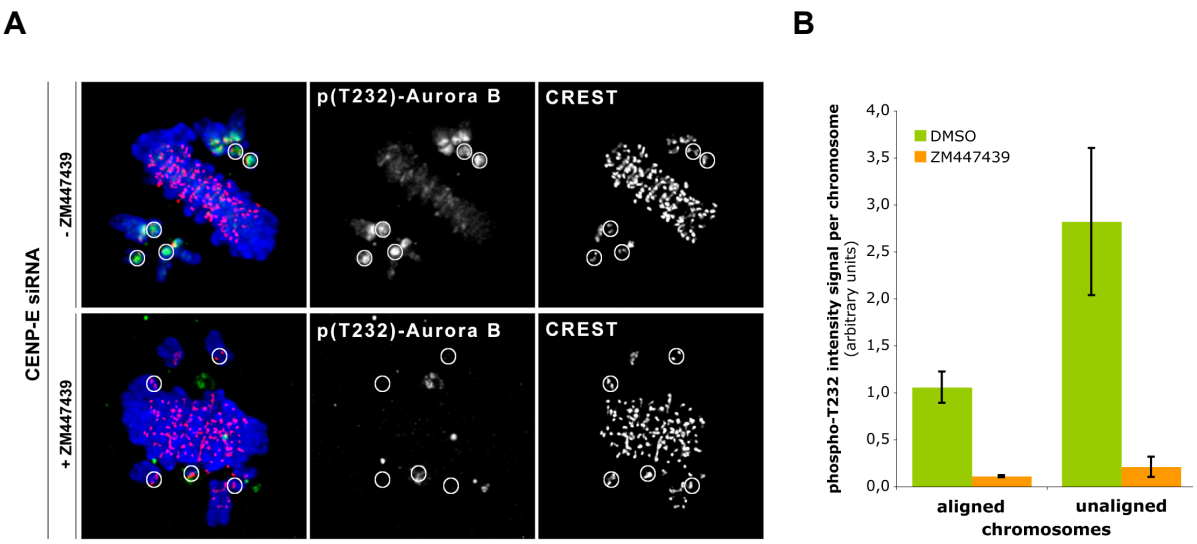


Figure 1.1 Aurora B kinase activity in CENP-E-depleted cells. (A) Immunofluorescence images after CENP-E depletion (-ZM447439) for T232 autophosphorylation of Aurora B in green, DNA in blue and Crest in red. Specificity of the phosphorylation signal is shown by decrease in the IF signal of pT232 upon inhibition of Aurora B kinase activity (+ZM447439). White circles indicate representative kinetochore pairs used for the quantification of the pT232 fluorescent signal (B). Error bars represent SD of three independent experiments.

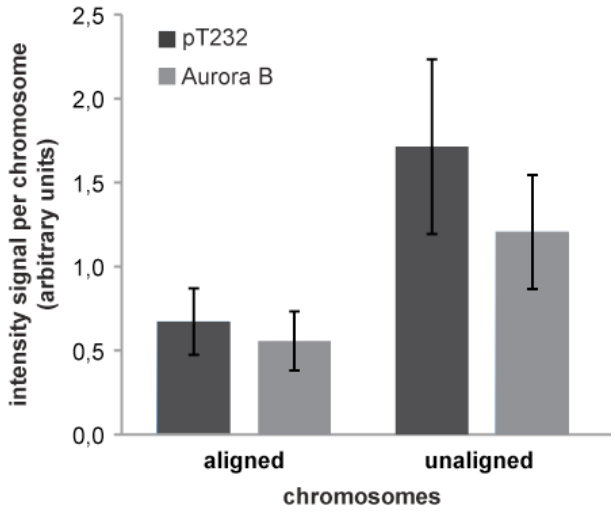


Figure 1.2 Aurora B kinase protein levels and activity in CENP-E-depleted cells. Graphic representation of the quantification of pT232 and total Aurora B intensity levels in the aligned and unaligned chromosomes of CENP-E-depleted cells. See materials and methods for further details on the quantification. Error bars represent SD

To determine if the polar chromosomes in CENP-E depleted cells fail to establish stable kinetochore attachments due to high local activity of Aurora B kinase, CENP-E depleted cells were treated with a small molecule inhibitor of Aurora B kinase activity. Thus, ZM447439 was used to inhibit Aurora B and Mad1 was used to indirectly monitor kinetochore attachment status (Fig 1.3 A and B). In the absence of ZM447439, immunofluorescence analysis shows that most cells accumulate Mad1 in one or both kinetochores indicating that attachment is severely impaired. However, after treatment with ZM447439, almost all the polar chromosomes failed to accumulate Mad1 at kinetochores suggesting that these kinetochores had established stable microtubule attachments (Fig. 1.3 A and B).

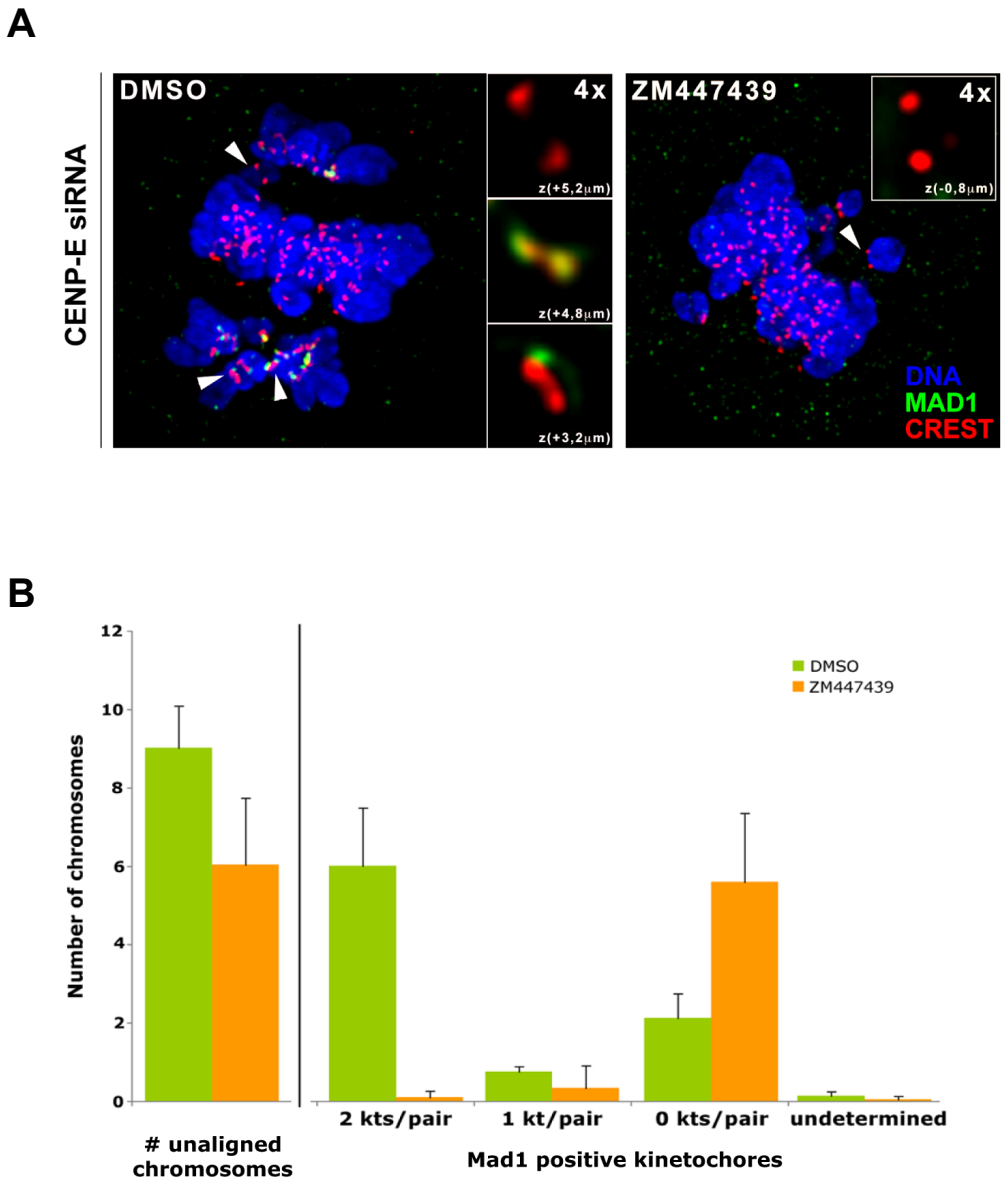


Figure 1.3 Kinetochore-microtubule attachments in CENP-E depleted cells. (A) Immunofluorescence of Mad1 as an indirect marker for the attachment status of the kinetochore. Insets show higher magnifications of a single optical Z-stack of an unaligned chromosome with attached kinetochores (no Mad1, inset 1), with unattached kinetochores (Mad1 labels both kinetochores, inset 2) or with mono-attached kinetochores (Mad1 labels one kinetochore, inset 3). Mad1 is not present in all of the unaligned chromosomes when cells are treated with ZM447439 (inset shows higher magnification of a representative chromosome). (B) Graphic representations of quantification of the number of unaligned chromosomes in CENP-E depleted cells with or without ZM447439 (left two bars). From these the number of chromosomes with unattached, mono-attached or attached kinetochores was scored. Error bars represent SD of three independent experiments (n=27, DMSO; n=22, ZM447439).

As a control, CENP-E depleted cells treated with ZM447439 were treated with nocodazole to depolymerize microtubules and the levels of Mad1 at kinetochores were similar to those cells not treated with the Aurora B inhibitor (Fig. 1.4 A and B). Our results show that Aurora B kinase activity destabilizes kinetochore attachments of polar chromosomes in CENP-E depleted cells and demonstrates that phenotype (a) is caused by transient activation of the SAC due to destabilization of kinetochore microtubule attachment of polar chromosomes by the activity of Aurora B.

Figure 1.4 Mad1 localization before and after Aurora B kinase inhibition. (A) Immunofluorescence images of control CENP-E depleted cells showing Mad1 localization (green) DNA is shown in blue and CREST in red with or without 35 min. treatment with ZM447439. Nocodazole (microtubule depolymerising drug) was also added to the cells. (B) Graphic representation of immunofluorescence ratio of Mad1 to CREST signal under control of (DMSO) or ZM447439 treatment. Error bars represent SD

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

CENP-E depletion causes also a second phenotype in which cells exit mitosis without proper chromosome alignment, which could be due either to mitotic slippage (Andreassen and Margolis, 1994) or by satisfying the SAC (reviewed in Musacchio and Salmon, 2007). Indeed, as indicate above, that 37% of the cells have prometaphase delay that extends more than a five-hour

delay and then undergo anaphase with a few misaligned chromosomes (data not shown) (Maia et al. 2010). Interestingly, cells that bypass the SAC are known to retain checkpoint proteins Mad1, Mad2, and BubR1 at kinetochores in telophase and do not degrade the APC/C substrate Tpx2 while cells that satisfy the SAC or overcome the checkpoint do not exit mitosis with SAC proteins at the kinetochores (Brito and Rieder, 2006). Therefore, we used immunofluorescence with antibodies against Mad1 and Tpx2 on CENP-E depleted cells associated with the phenotype (b) that had been arrested in mitosis for at least 5 hours. We found that while in mitotic cells Mad1 and Tpx2 was detected at kinetochores (Fig. 1.5 A, inset 1 and C), cells that exited mitosis and formed micronuclei show no positive signal (Fig 1.5 B, inset 2-3 and C). The absence of both Mad1 and Tpx2 in the (micro) nuclei of CENP-E depleted cells indicates that exit from mitosis was associated with SAC silencing and was not due to mitotic slippage.

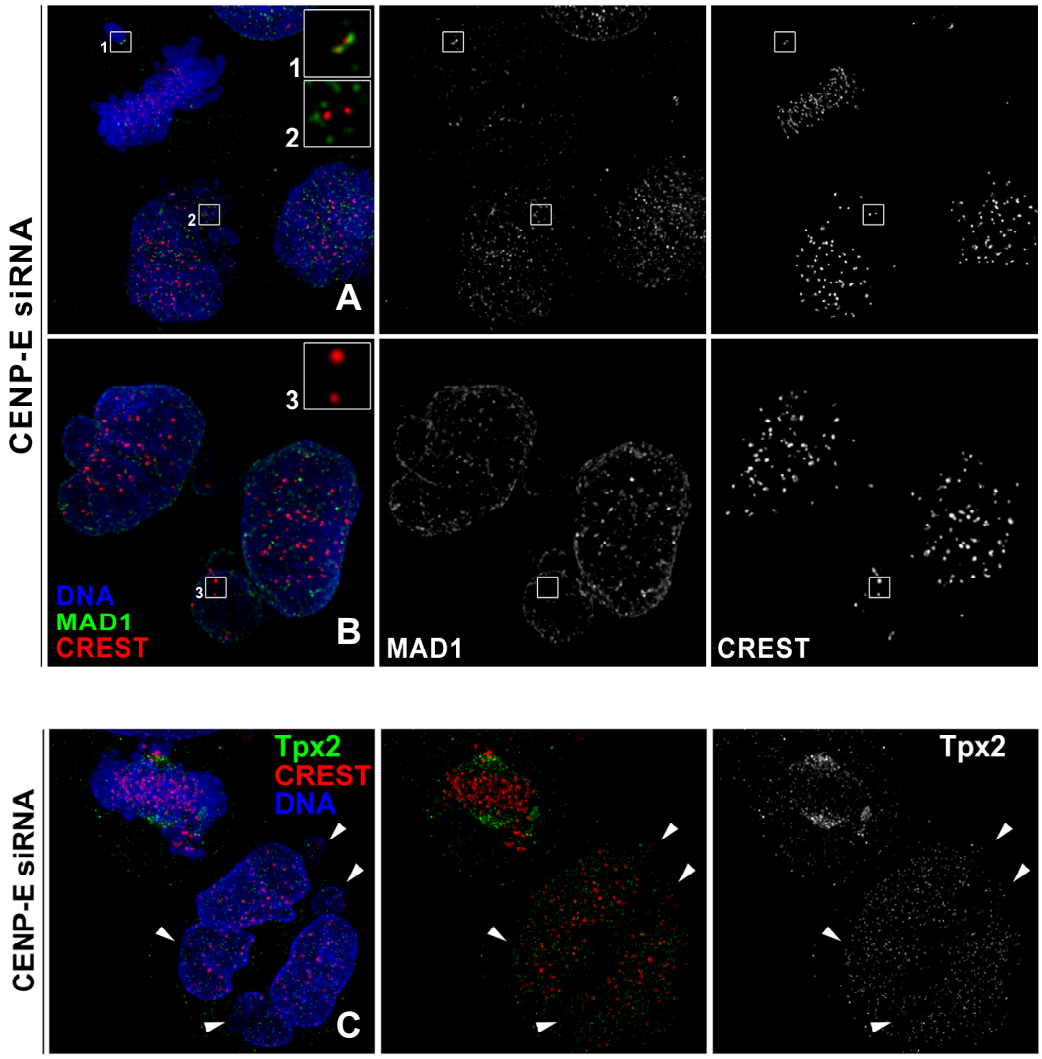


Figure 1.5. CENP-E depleted cells silence the SAC. Immunofluorescence images for the SAC protein Mad1 (A and B) and the APC/C substrate Tpx2 (C). Note the presence of Mad1 on the kinetochores of the polar chromosomes (inset 1) and absence of Mad1 in telophase (inset 2-3). (C) Tpx2 localization in a CENP-E-depleted mitotic cell and absence of Tpx2 in micronuclei (arrowheads).

3. DISCUSSION

Our data shows that CENP-E facilitates efficient and timely chromosome congression but is not absolutely essential for full chromosome alignment in most cells. In the absence of CENP-E a few chromosomes fail to congress and stay close to the spindle poles. Although, kinetochores of these polar chromosomes can interact with microtubules (a few polar chromosomes

negative for Mad1 are always found, Fig. 1.2 A and B), these microtubule attachments are not stabilized because of high local Aurora B kinase activity. Instead, these pole-proximal kinetochore-microtubule attachments appear to be destabilized by Aurora B, resulting in a SAC-dependent mitotic delay. In the majority of the CENP-E-knockdown cells this allows extra time for chromosome congression by CENP-E independent mechanisms (McEwen et al., 2001), thus reducing chromosome segregation errors. Yet, in a subset of CENP-E depleted cells that arrest for long periods and that exit mitosis with misaligned chromosome the SAC was eventually silenced and the question remains; how do these cells with clearly miss-attached kinetochore manage to silence the SAC?. Studies in *Xenopus* extracts showed a direct link between CENP-E and the mitotic checkpoint protein BubR1 suggesting that depletion of CENP-E could directly weaken the checkpoint (Mao et al., 2003; Yao et al., 2000). However, in human cells CENP-E knockdown does not shorten the mitotic delay induced by the microtubule poisons taxol and nocodazole (Tanudji et al., 2004); (data not shown), indicating that CENP-E is not required for a robust SAC response in these cells. In addition, it was found that inhibition of Aurora B only silenced the SAC in CENP-E-depleted cells when microtubules were present. In the presence of the microtubule depolymerizing drug nocodazole, co-inhibition of Aurora B and CENP-E did not further compromise the mitotic checkpoint. This indicates that in the CENP-E-depleted cells the mitotic delay is due to the microtubule destabilizing activity of Aurora B (Pinsky et al., 2005) and silencing of the SAC in a subset of the cells is most likely due to acquisition of microtubule attachments. So, how do the kinetochores of these polar chromosomes eventually become attached?

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

activity is high. This idea is supported by experiments that show that error correction can be attenuated over time while Aurora B is active, if microtubules are stabilized by high taxol concentrations (Yang et al., 2008). However, mitotic exit in cells of phenotype (b) could be a result of cohesion fatigue (Daum et al., 2011).

Finally, although a cooperative function for Aurora B in CENP-E dependent congression has been implied earlier (Kapoor, 2006), our work suggest that in normal cells high local Aurora B kinase might release polar kinetochores from end-on attachments by destabilizing pole-proximal syntelic attachments. In this way, Aurora B could allow lateral attachment and CENP-E dependent sliding along microtubule bundles to promote efficient chromosome congression and timely anaphase onset.

4. MATERIAL AND METHODS

4.1 Cell Culture and siRNA

HeLa cells were cultured in DMEM with 6% FCS and antibiotics, at 37ºC in a humidified atmosphere with 5% CO2. For cell synchronization at the G1/S transition, cells were incubated with 2.5 mM thymidine for 24 hours. siRNA transfection was done with HiPerFect (Qiagen) according to the manufacturer's protocol. The CENP-E siRNA duplex (GAACUAAGAAGAAGCGUAU) and the Luciferase siRNA duplex (CGUACGCGGAAUACUUCGA) were from Dharmacon®. Drugs were used at the following concentrations: nocodazole, 0.25 µg/mL; MG132, 5 µM; ZM447439 (Tocris Bioscience, Bristol, UK), 2µM. Incubation with ZM447439 was done for 35 minutes unless stated otherwise. To determine the mitotic index, RNAi-treated cells were released from a thymidine block to DMSO or nocodazole for 16 hours. Before fixation incubation with DMSO/ZM447439 was performed for 1 hour. Cells were fixed for 5 minutes in 8% formaldehyde, washed once with PBS, followed by 5 minutes incubation in ice-cold methanol. After washes with PBS, cells were counterstained with DAPI. Determination of the mitotic index was done in a Thermo Scientific

Cellomics® ArrayScan® VTI, software version x.5. >104 cells were counted per condition.

4.2 Antibodies and Reagents

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

4.3 Immunoblotting and Immunofluorescence

Immunoblotting was performed as described (Smits et al., 2000). Prior to IF coverslips were washed with PEM buffer (100 mM Pipes, 10 mM EGTA, 1 mM MgCl2), fixed for 10 minutes in 4% paraformaldehyde/0.2% sucrose, then washed once with PEM and permeabilized with 0.5% Triton X-100/PEM for 15 minutes. For IF, cells were washed with DPBS and incubated with the appropriate primary/secondary antibody combinations diluted in DPBS/3% BSA. Cells were counterstained with DAPI to visualize the DNA. Images were acquired using a Zeiss Axio Imager microscope (Carl Zeiss, Germany) using an Axiocam (Carl Zeiss, Germany). Data stacks were deconvolved using the Huygens Professional software (Scientific Volume Imaging BV, The Netherlands).

4.4 Quantification of Active Aurora B

For quantification of Aurora B centromere activity, cells were stained with pT232-Aurora B and CREST antibodies. Images with multiple z-planes were

captured and the integrated density of the T232 signal was quantified in individualized kinetochore pairs with ImageJ software (NIH) after deconvolution of the data stacks. Normalization for the background signal was performed. The average integrated density for pT232 was set in relation to average integrated density of the CREST signal. Five randomly chosen individual kinetochore pairs were analysed per cell in five different cells, in 3 independent experiments.

Chapter 2

Characterization of Mis12 protein in *Drosophila* S2 cell

1. INTRODUCTION

The kinetochore provides the interface between the chromosomes and spindle microtubules, is also required for sister chromatid segregation and is a signalling center associated with the Spindle Assembly Checkpoint. The size and complexity of kinetochores vary considerably among different species (Chan et al., 2005). The interface responsible for the interaction between microtubules and chromosomes involves a conserved super-complex of proteins, known as the KNL1/Mis12/Ndc80 (KMN) network.

The Mis12 complex is composed of 4 subunits: Mis12, Nnf1, Nsl1 and Dsn1 that localize at the inner plate of the Kinetochore (reviewed in Chan et al., 2005). The *Drosophila* Mis12 gene product was first reported by Meraldi and co-workers, and initially characterized in two different studies (Meraldi et al., 2006). Mis12 proteins show low similarity among eukaryotes (approximately 15%-30%) with the highest levels of divergence found in the *Drosophila* protein. Nevertheless protein sequence alignments of Mis12 homologues of fungal, plant and metazoan revealed that sequence similarity is confined to 30 to 100 residues interspersed by stretches of low-homology, many of which correspond to coiled coil domains (Meraldi et al., 2006). In *Drosophila* S2 cell line Mis12 has been reported to localize at the kinetochores throughout the cell cycle similar to what was observed in fission yeast and humans; (Kline et al., 2006; Goshima et al., 2003; 1999). However in *Drosophila* transgenic embryos expressing EGFP-Mis12, the centromeric signal observed was mitosis-specific (Schittenhelm et al., 2007).

Mis12 was first described in fission yeast where its inactivation leads to extensive DNA missegregation (Goshima et al., 1999). 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 depletion of different subunits leads to similar phenotypes including defects in chromosome alignment, orientation, and segregation (Venkei et al., 2011; Przewloka et al., 2007; Kline et al., 2006; McAinsh et al., 2006; Obuse et al., 2004b; Goshima et al., 2003). Nevertheless, human Nnf1 was found not to be required for chromosome attachment *per se*, but rather for the metaphase alignment of chromosomes and for the correct generation of inter-kinetochore forces (McAinsh et al., 2006).

Moreover, some data indicates that depleting hMis12 by RNAi leads to a mitotic delay (Kline et al., 2006). Previous studies only reported a mitotic delay after hDsn1 depletion (Obuse et al., 2004b) but not after hMis12 depletion (Goshima et al., 2003). Recently, a mitotic delay has been reported in a study performed in Mis12 and Nsl1 *Drosophila* mutants (Venkei et al., 2011).

Depletion of Mis12 components in yeast strongly inhibit recruitment of several outer kinetochore proteins, including Ndc80 and the DAM–DASH complex (Kline et al., 2006; Scharfenberger et al., 2003), and may also affect the localization of some inner centromere proteins such as CENP-A, although there are conflicting data on their interdependence. The data so far support a model in which the Mis12 complex is functionally downstream and physically poleward of the inner kinetochore proteins and is necessary for correct localization of the outer microtubule binding proteins, as well as some checkpoint components such as Bub1, BubR1, Mad1, and Mad2 (Venkei et al., 2011; Kline et al., 2006; McAinsh et al., 2006).

Therefore, it is crucial to study the function of each individual component of the KMN network in *Drosophila* in order to understand the evolutionary adaptations that may have occurred in its structure. In this section we will focus on the characterization of *Drosophila* Mis12 protein, its dynamics during the cell cycle and the mitotic phenotype resulting from its depletion.

2. RESULTS

2.1 Mis12 antibody production

An antibody against DmMis12 was produced. We used the full-length cDNA clone RE19545 from the *Drosophila* Genomics Resource Center. The fragment was amplified in order to create two restriction sites EcoRV and EcoRI, digested and then cloned into pET-30 (A) expression vector using the same restriction sites (EcoRV and EcoRI) (Fig. 2.1). The positive recombinants as determined by restriction mapping were then sequenced to confirm if the Mis12 cDNA was cloned in frame (see Appendix 3).

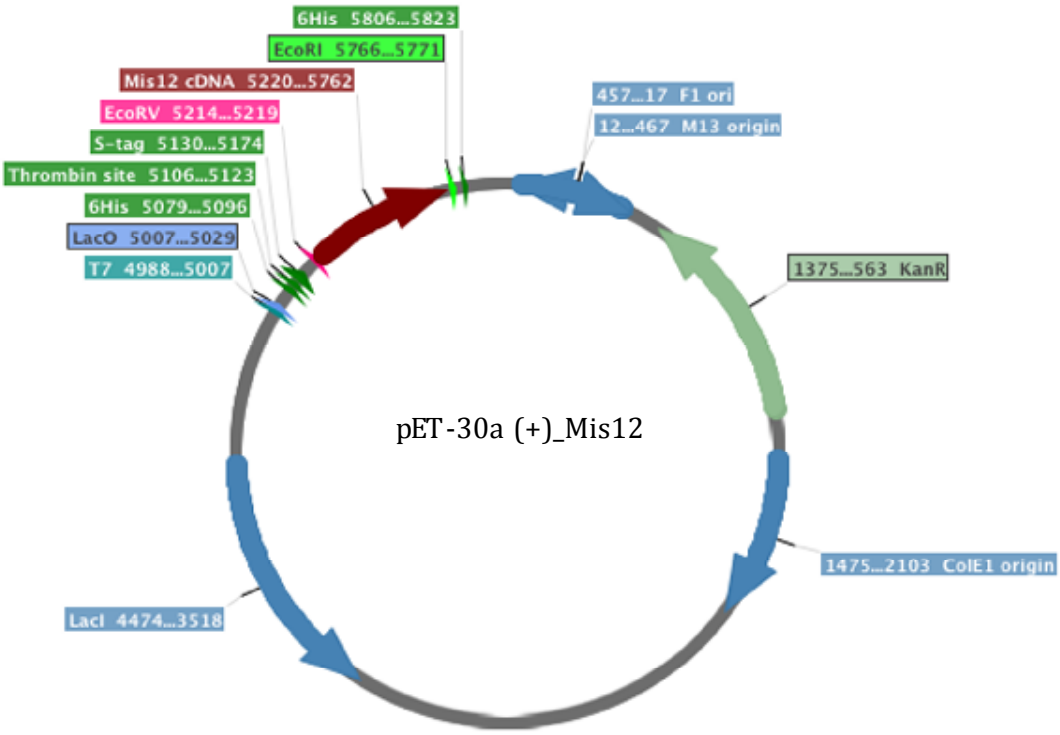


Figure 2.1 Schematic representation expression plasmid pET-30a (+)_Mis12. Mis12 cDNA was amplified and cloned into the expression vector using EcoRV and EcoRI sites.

E.coli BL21 cells were transformed and protein synthesis was induced by the addition of 1 mM IPTG. After a 3 hours induction the bacterial suspension was sonicated and two different fractions were obtained, soluble fraction and insoluble fraction (inclusion bodies). The different samples were run in a SDS-PAGE gel. The SDS-PAGE analysis showed an efficient induction with most of the protein retained in the inclusion bodies (Fig. 2.2). The extraction of the inclusion bodies and solubilisation with guanidine hydrochloride was performed and the protein was purified using affinity chromatography. The chromatogram graph showed that the protein was enriched mainly in 3 of the 8 fractions obtained. The result was then confirmed in a 12% SDS-PAGE gel showing a high level of purification where the Mis12 protein was present in fraction 3,4 and 5.

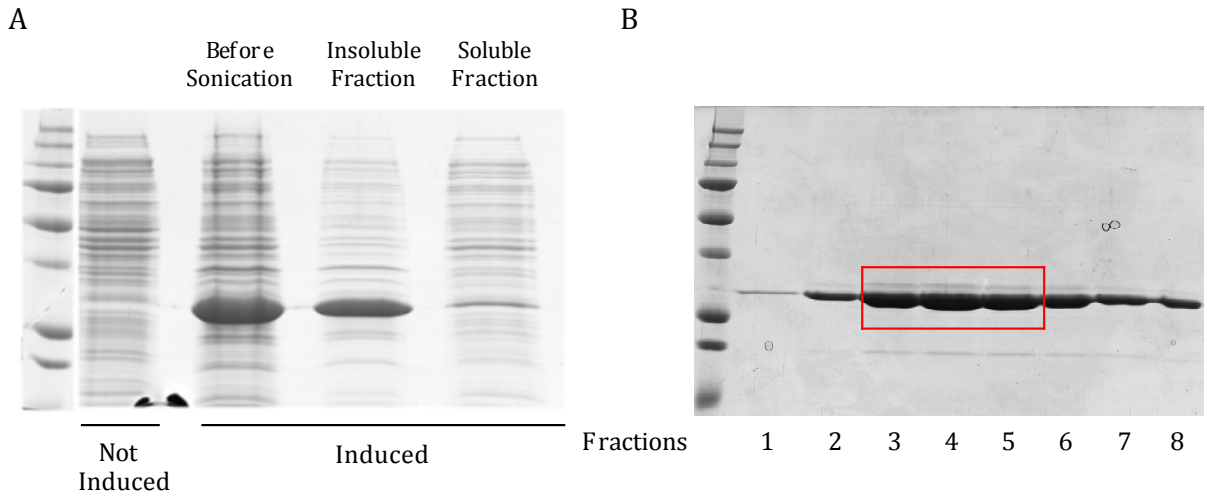


Figure 2.2 SDS-PAGE gel. A - Analysis of the different bacterial fractions before and after induction with 1mM IPTG. The induced fractions where subjected to sonication and different fractions where obtained (soluble fraction; insoluble fraction – inclusion bodies) B - Purified protein fractions obtained by affinity chromatography. Highlight in red injected fractions for immunization.

These fractions were precipitated with Trichloroacetic acid (TCA) and ressuspended in PBS to a final concentration of 1µg/µl. The protein suspension was injected in two rats and one rabbit. Immunofluorescence was performed in *Drosophila* S2 fixed cells to determine specificity and cellular localization of the antibody. A final dilution of 1:5000 either for rat and rabbit was established. A co-staining with the centromeric marker CENP-C and CID was used confirming that *Drosophila* Mis12 protein localizes at the external region of the centromeres in mitotic cells. Specific signals were also present in interphase cells. The antibodies were also tested by western blot using a dilution of 1:1000 (Fig. 2.3).

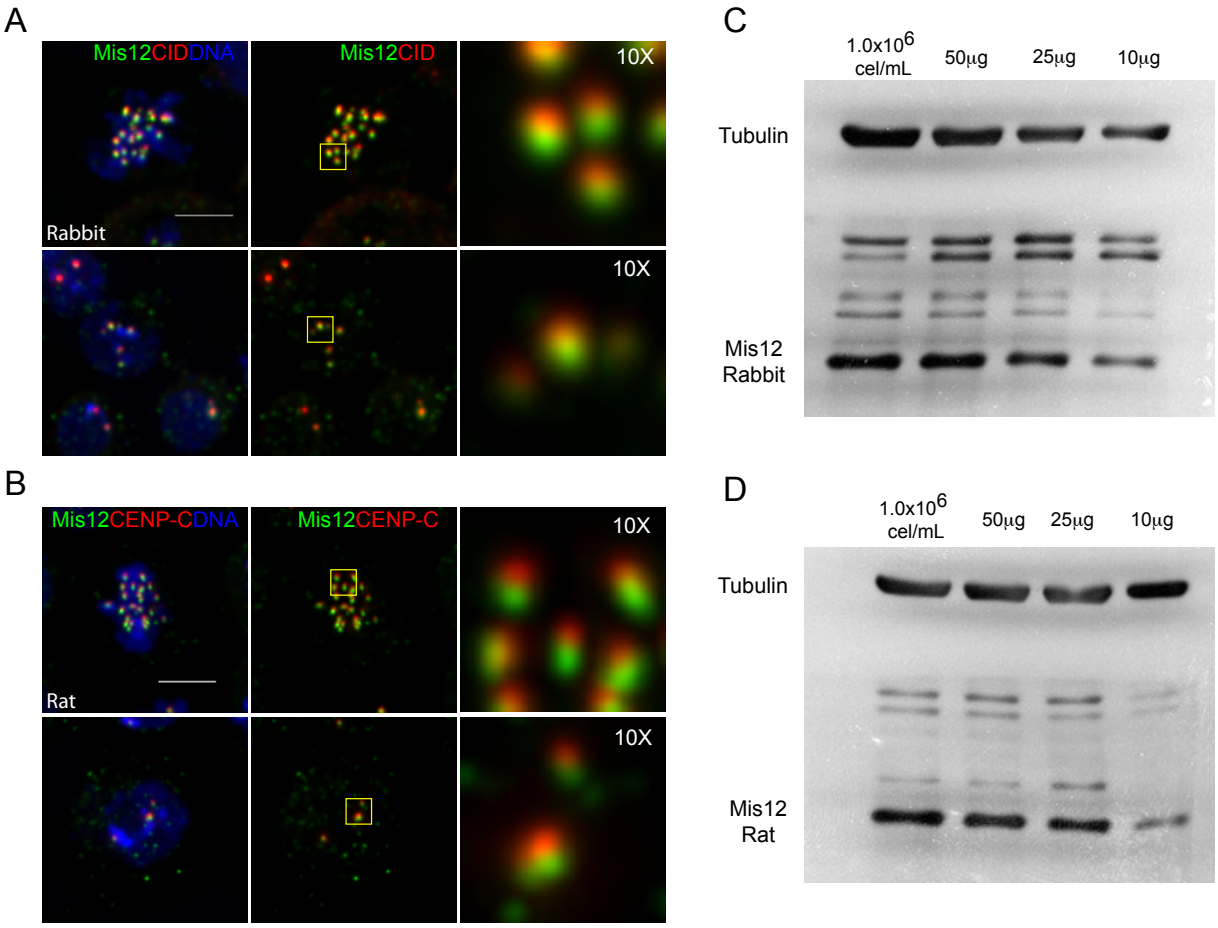


Figure 2.3 Specificity of Mis12 antibodies by immunofluorescence and western-blot. (A, B) Anti-rabbit and anti-rat Mis12 antibodies were used 1:5000 in immunofluorescence. Specific signals of Mis12 (green) were detected co-localizing with CID and CENP-C (red) centromere markers in mitotic and cells in interphase. Scale bar is 5µm. (C, D) Antibody titration of anti-rabbit and anti-rat Mis12 antibodies where 1:1000 of diluted antibody was used. Total protein extracts from 1x10^6 cell/mL, 50µg, 25µg and 10µg cells were separated by SDS-PAGE and the protein level of Mis12 was monitored using western blotting techniques. α-tubulin was used as a loading control.

2.2 Establishment of S2 cell line stably expressing Mis12-GFP.

In order to study the dynamic behavior of Mis12 during cell cycle, a cell line stably expressing Mis12 tagged with GFP was constructed using the S2 *Drosophila* cell line derived from a macrophage-like lineage. The cloning of Mis12 tagged with GFP was achieved using pENTR Directional TOPO Cloning Kit (Invitrogen). This technique allows the insertion of the cDNA in the correct orientation with efficiencies equal to or greater than 90%. The full-length cDNA of

Mis12 was amplified by PCR using a forward primer with a CACC sequence in 5´ and then inserted in the entry vector. One Shot chemically competent *E.Coli* were transformed and restriction analysis with Acc1+EcoRV, Acc1 and EcoRV was performed to confirm positive recombinants. To transfer our gene of interest (Mis12) from the entry vector to the destination vector we used a LR recombination reaction using Gateway LR Clonase II enzyme mix. The positive recombinants as determined by restriction mapping were then sequenced to confirm if the Mis12 cDNA was cloned in frame with GFP (see Appendix 3). Two destination vectors from the *Drosophila* Gateway Vector collection (Invitrogen) tagged with GFP (C-Terminal and N-terminal) under the control of an Hsp70 (heat shock protein) promoter were used.

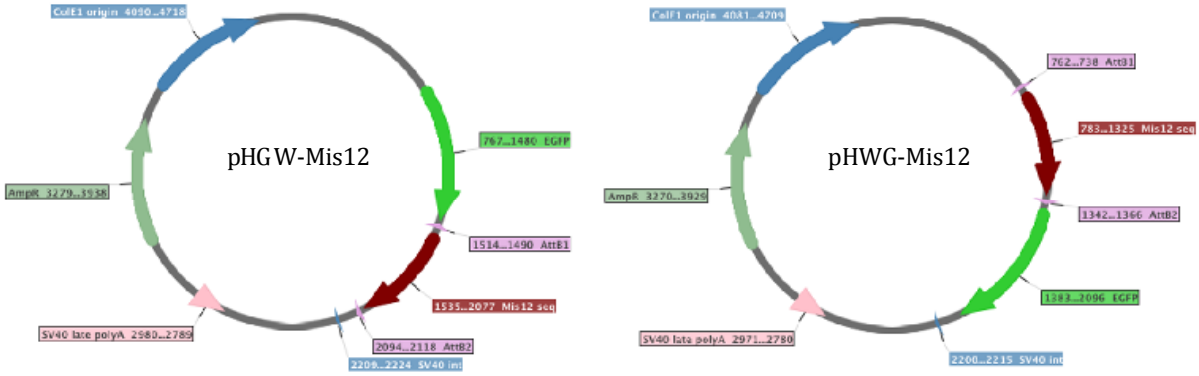


Figure 2.4 Schematic representation of pHGW-Mis12 and pHWG-Mis12 plasmid.

S2 cells were co-transfected with pHGW-Mis12, pHWG-Mis12 and a G418 selection containing pyCOBlast (Invitrogen). Both plasmids were successfully transfected, however, the cells expressing the pHWG-Mis12 clone were used preferentially because a higher basal expression was observed and therefore there was not need for heat-shock induction, preventing oscillations in the protein expression levels that could affect our studies. Since the Mis12 protein was fused to GFP, we were able to monitoring the protein throughout the cell cycle. Cells were then plated in incubation chambers treated with concanavalin A and the S2 stably expressing Mis12-GFP was monitored using *in vivo* fluorescent microscopy.

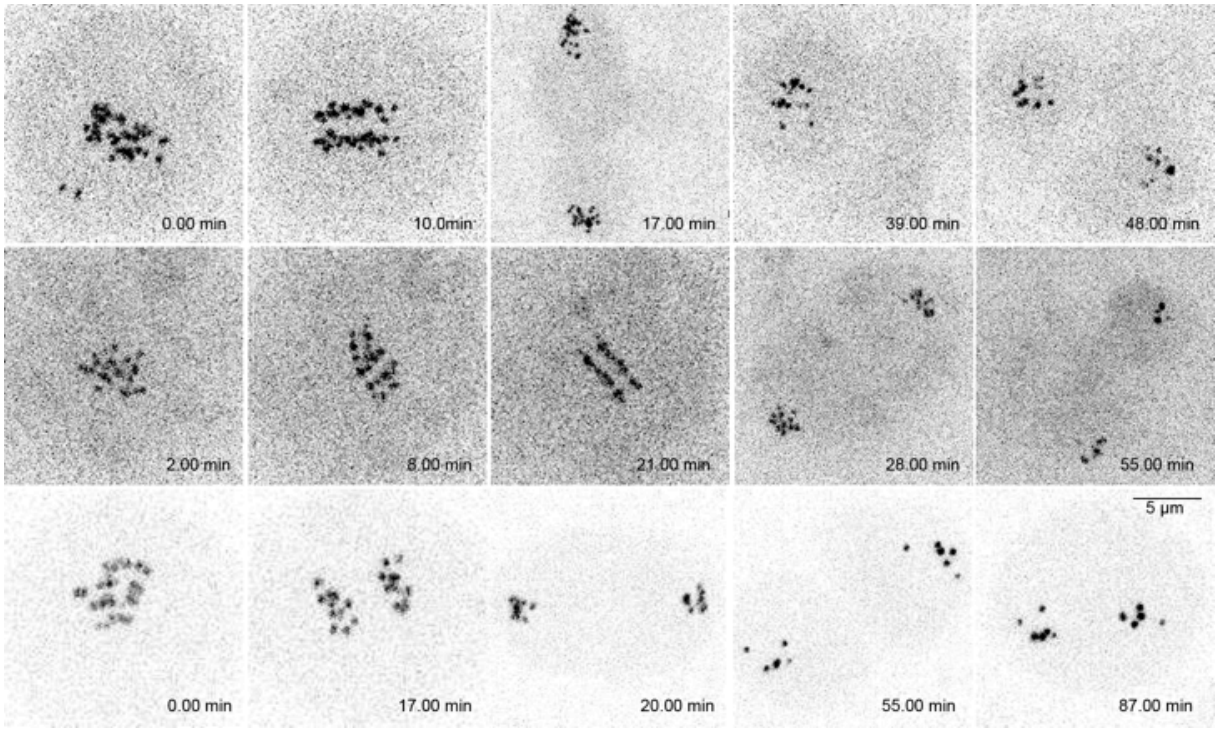


Figure 2.5 *In vivo* analysis of pHWG-Mis12 during mitosis. pHWG-Mis12 S2 cells were placed in an appropriate chamber and images were taken every 60 sec using time-lapse microscopy. This figure shows still images from movies of mitotic cells between prometaphase and telophase.

2.2.1 Subcellular localization of Mis12 during the cell cycle.

The time-lapse image analysis suggests that Mis12 is present throughout mitosis including telophase. Given that the incubation chambers were treated with concanavalin A, preventing cells undergo cytokinesis, complete cell division and proceed to G1, it was not possible to follow these cells into subsequent cycles. Additionally the presence of markers indicating mitotic entry was required. We also observed by immunofluorescence in S2 fixed cells that while Mis12 is clearly visible in all mitotic cells, a proportion of interphase cells do not stain with anti-Mis12 antibodies suggesting that the protein may not be present during a specific time in interphase (Fig. 2.6).

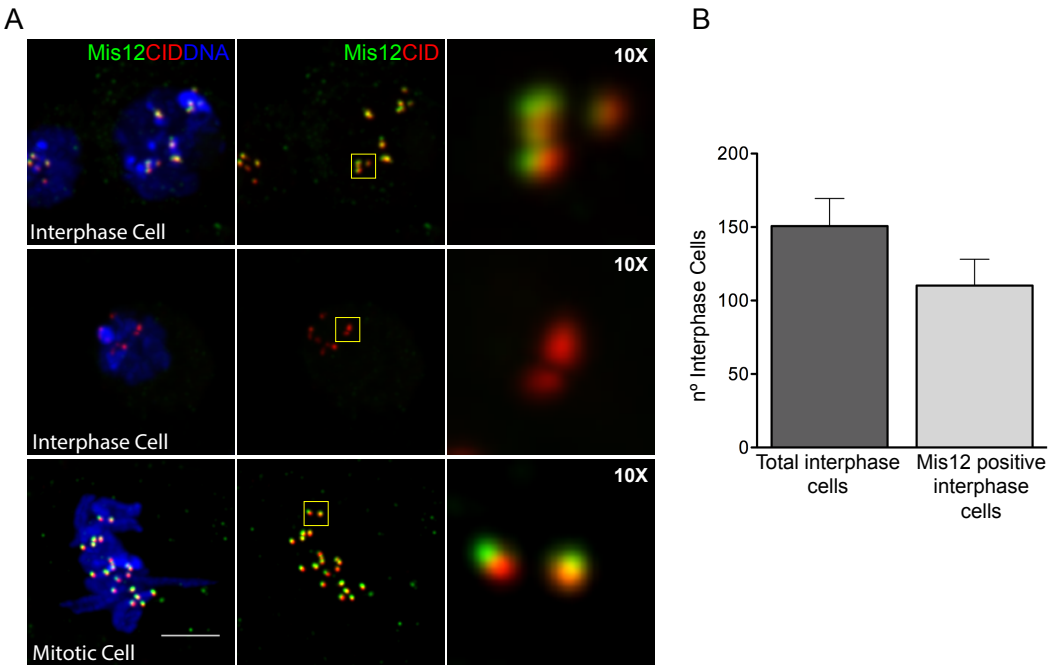


Figure 2.6 (A) Immunofluorescence was performed in S2 fixed cells, Mis12 (green), CID (red) and DNA (blue). Scale bar 5μm. (B) Quantification of Mis12-interphase positive cells. Errors Bars represent SD.

The images and further quantification show that in mitosis Mis12 is always present at kinetochores. However, during interphase Mis12 fails to localize in 23% of cells. The low percentage of cells that do not show Mis12 signal suggest that Mis12 do not localize in the kinetochores at a specific stage and for a restricted period of time during the cell cycle.

In *Drosophila* embryos, S phase is the longest among the three stages of interphase. We found a low proportion of the S2 interphase cells that do not stain for Mis12, suggesting that Mis12 is not at kinetochores during a short interphase period (see Fig. 2.6). In order to test if this period correspond to S phase we perform BrdU incorporation assay to label S phase cells (Soames et al., 1994) followed by staining with Mis12 antibody (Fig. 2.7).

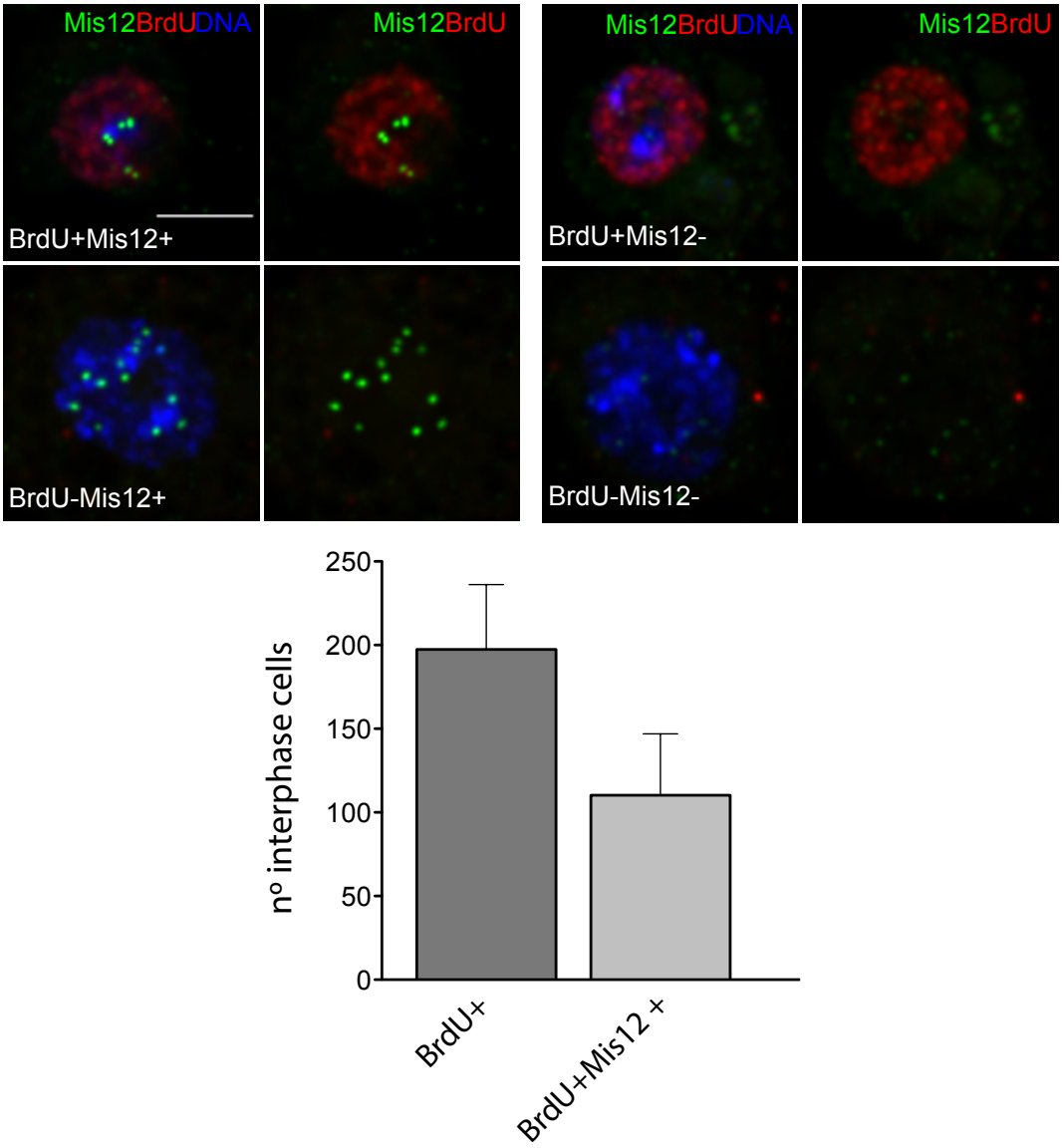


Figure 2.7 BrdU incorporation and Mis12 staining in cells in interphase. BrdU was added to S2 cells and incubated for 30 minutes. (A) Immunofluorescence was performed after fixation showing Mis12 in green, BrdU in red and DNA in blue. Scale bar 5μm. (B) Quantification of Mis12-interphase positive cells in the positive BrdU cell population. Errors Bars represent SD.

Our results show that only 55% of the cells that incorporate BrdU are also positive for the Mis12 protein suggesting that Mis12 is present at kinetochores even when DNA is being replicated in approximately half of the cells. We were unable to discriminate cells in G1 or G2 and therefore the exact period in which Mis12 is absent from kinetochores is not yet identified.

2.3 Dynamic behavior of Mis12 protein

To study the dynamic behavior of Mis12 at kinetochore during interphase and mitosis we performed FRAP on the stably transfected Mis12-GFP cell line. FRAP (Fluorescence Recovery After Photobleaching) was used to determine the turnover of Mis12 protein during different stages of cell cycle. Previous studies in human cells had shown that hMis12 has a fast turnover during interphase while in mitosis it is much more stable (Hemmerich et al., 2008). Similar results had been achieved while studying the orthologue Mtw1p in *S. cerevisiae* (Joglekar et al., 2006). These observations suggest that hMis12 is loosely attached to kinetochores in interphase whereas during mitosis it is stabilized supporting the idea that Mis12 is not a constitutive kinetochore protein (Hemmerich et al., 2008). To test these hypotheses in *Drosophila melanogaster* we perform FRAP analysis during interphase and mitosis (Fig. 2.8).

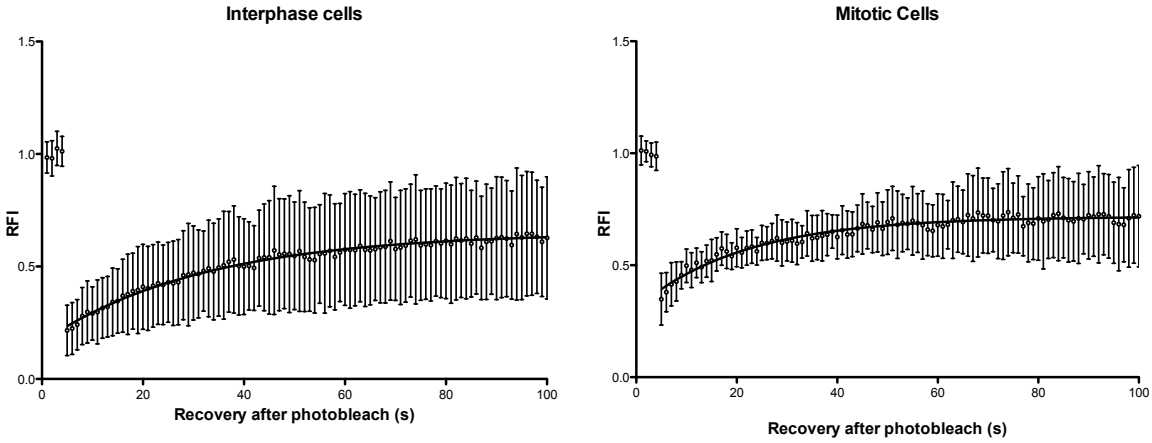


Figure 2.8 Dynamics of Mis12 during interphase and mitosis. Short-term FRAP experiments were performed on interphase S2 cells expressing Mis12-GFP. Images of GFP fluorescence were captured as single confocal sections before (pre), immediately after (post) bleaching at different time points as indicated in the graphs. Graphs on the top display quantification of FRAP measurements from at least 10 cells each (±SD). Data could be fitted to exponential functions (one-phase association) from which the mobile fraction and residence time were determined.

No differences in Mis12 dynamics were observed between interphase and mitosis on *Drosophila* S2 cells. The mobile fraction is similar in the two phases with a value of approximately 60%. A faster turnover of 21.34s was observed in

mitotic cells, when compared with 31.97s in cells in interphase, however both values correspond to a high turnover of the protein at kinetochores. Moreover, both in interphase and mitosis we found that there is 40% immobile fraction of the protein at the kinetochores. Regarding interphase, a more detailed FRAP analysis is required in order to distinguish different phases in interphase and draw more accurate conclusions regarding the dynamic behavior of Mis12 during this stage of the cell cycle. Nevertheless, our results in S2 *Drosophila* cells indicate that Mis12 has an opposite behavior from that described in both yeast and human cells. The reasons for these discrepancies are not yet know.

2.4 Mitotic progression is affected in Mis12 depleted cells.

To investigate the role of Mis12 protein during mitosis, it was depleted by double-stranded RNA (dsRNA) in *Drosophila* S2 cells. After 120h transfection, we achieved a depletion of more than 90% of the protein as determined by immunofluorescence and western blot (Fig. 2.9).

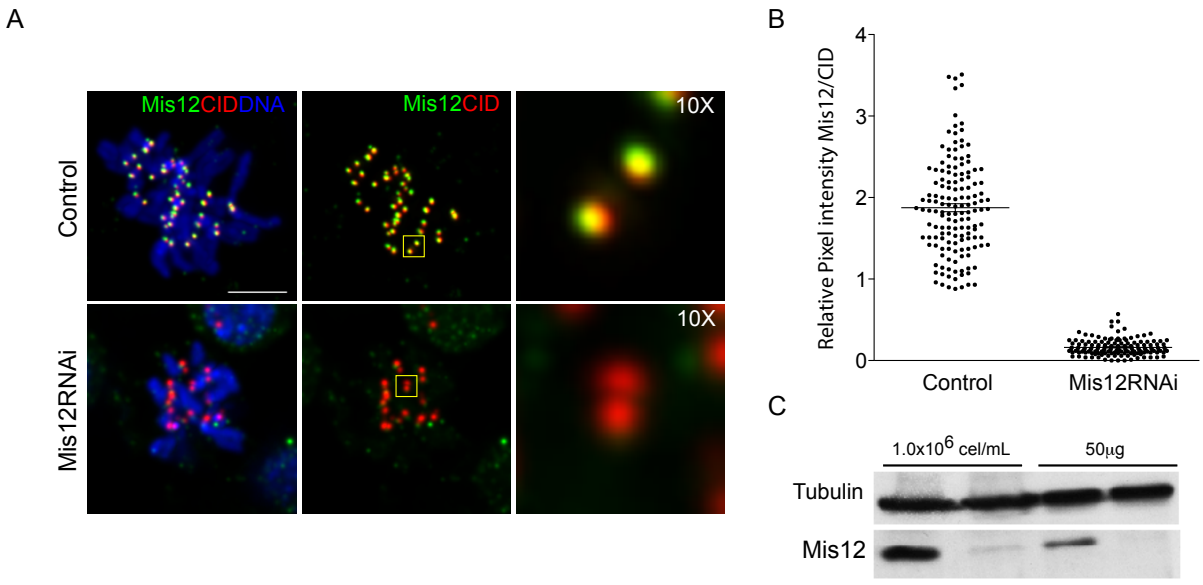


Figure 2.9 Depletion of *Drosophila* Mis12 protein by dsRNA in S2 cells. (A) After 120h treatment, control and Mis12 dsRNA S2 cells were fixed and immunostained showing Mis12 (green), DNA (blue) and CID (red). 10X magnifications of selected regions are shown on the right. Scale bar is 5µm. (B) Quantification of the mean pixel intensity of Mis12 at kinetochores using immunofluorescence images shown in (A) where each dot represents an individual kinetochore (n>100). (C) Total protein extracts from 1x10^6 cell/mL and 50µg cells were separated by SDS-PAGE

and the protein level of Mis12 was monitored using western blotting techniques. Quantification of Mis12 depletion levels from total protein extracts was performed by densitometry analysis using BioRad software and α-tubulin was used as a loading control.

We then characterized the mitotic phenotype of Mis12 depleted cells. Previous studies have already reported that Mis12 depleted cells showed problems in chromosome congression, segregation, as well as impaired biorientation and spindle formation (Przewloka et al., 2007; Kline et al., 2006; McAinsh et al., 2006; Cheeseman et al., 2004). Depletion of Mis12 in *Drosophila* displayed similar phenotypes; spindle elongation, problems in chromosome biorientation and segregation with anaphase figures containing lagging chromosomes (Fig. 2.10 A and C). Interestingly, incubation of Mis12 depleted cells in MG132 to allow more time for congression did not revert the alignment phenotype (Fig. 2.10 B and D).

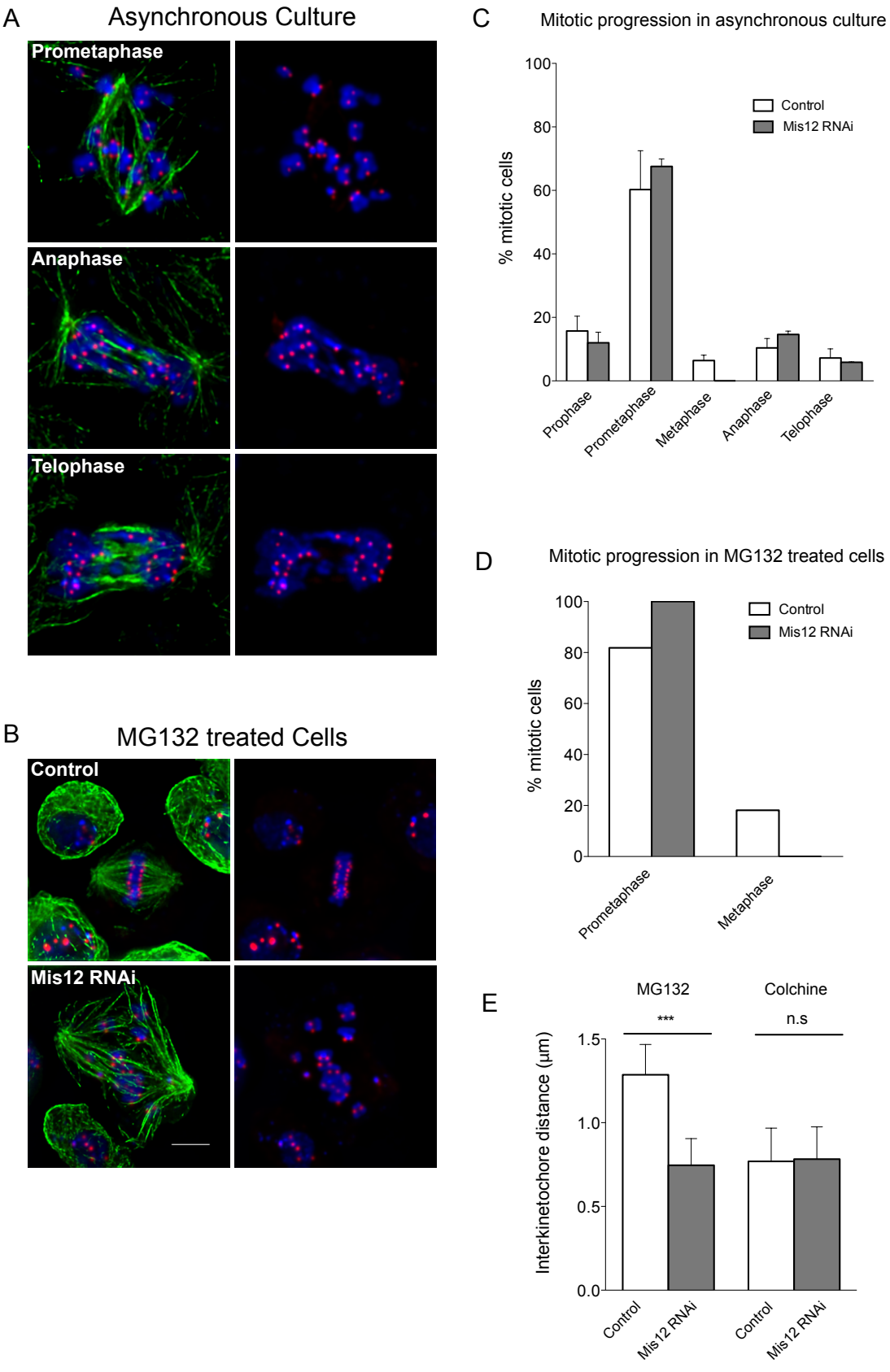


Figure 2.10 Mitotic characterization of Mis12 depleted cells. (A) Mis12 dsRNA treated S2 *Drosophila* cells were fixed and immunofluorescence staining was performed showing DNA (blue), tubulin (green) and CID (red). Images of different mitotic figures were taken. (B) Immunofluorescence in S2 cells blocked in mitosis

with MG132 treatment showing DNA (blue), tubulin (green) and CID (red). (C) Mitotic progression of Mis12 depleted cells in asynchronous culture (D) Mitotic progression in cells incubated with MG132. (E) Interkinetochore distance in MG132 and colchicine treated cells.

Nevertheless, Mis12 depleted cells not incubated with MG132 are ultimately able to exit mitosis (Fig. 2.10 A and C). To analyse the impact of Mis12 in generating tension across sister kinetochores (Waters et al., 1996), we mesured the distance between CID signals in metaphase control cells and in cells lacking Mis12. The interkinetochore distance was measured in a condition where tension can be applied by treating cells with MG132 or with colchicine where no tension at kinetochores is exerted. We found that in MG132 treated cells the mean interkinetochore distance decreased from 1.25±0.18μm in control metaphase cells to 0.74±0.16μm in Mis12 depleted cells where no metaphase figures were observed. After colchicine treatment, control and Mis12 depleted cells exhibited no differences in interkinetochore distances, with 0.77±0.19μm in control cells and 0.78±0.19μm in Mis12 depleted cells. Altogether the data suggests that the net force produced by kinetochore microtubule attachments is diminished upon depletion of Mis12 (Fig. 2.10 A and E).

3. DISCUSSION

The mitotic localization and recruitment of Mis12 during mitosis has been widely studied and data reported so far is consensual, despite the small differences observed across species (Venkei et al., 2012; 2011; Przewloka et al., 2007; Kline et al., 2006). What concerns the interphase localization of the protein, in mammalian cells it has been reported that the Mis12 complex subunits recruit together to the centromere in late G2 and dissociate in early G1 (Mcainsh et al., 2006; Kline et al., 2006), however previous work in *Drosophila* reported that, Mis12 and Nnf1 subunits are constitutive at centromere during all the cycle (Venkei et al., 2012, 2011). Nevertheless, ours results differ from the reported so far. Using both fixed and *in vivo* analysis we observed that there is percentage of interphase cells (23%) that lacks Mis12 protein (Fig. 2.6). To further understand in what stage of interphase Mis12 was not at centromeres we co-stained cells with

Mis12 antibodies after a pulse of BrdU and observed that half of the BrdU positive cells fails to localize Mis12. Our results suggest that Mis12 is not at kinetochores during G1 and then is incorporated in S phase or leaves the kinetochore during S phase and is incorporated again in G2. Since S phase in these cells lasts about 10 hours and our cells were exposed to the thymidine analogous only for 30 minutes, it is unlikely that Mis12 is loaded after S phase is completed. Therefore, we favour the interpretation that Mis12 protein is loaded either during the G1/S or the S/G2 transition. However, further experiments using specific markers for G1 and G2 will be necessary to determine the exact moment of unloading and loading of Mis12 on kinetochores.

We also analysed the dynamic behavior of Mis12 in mitosis and interphase using FRAP. Surprisingly, our data are not in accordance with previously published work for the mammalian and yeast homologues (Hemmerich et al., 2008; Joglekar et al., 2006). These studies reported differences in the percentage of mobile and immobile fraction between mitosis and interphase with Mis12 being highly mobile during interphase and vary stable during mitosis. We found the turnover of *Drosophila* Mis12 in mitosis to be slightly higher that in interphase but in both stages showed a 60% of mobile fraction.

The production of Mis12 antibodies allowed us to further study the phenotype caused by the depletion of the protein. The phenotype observed is very similar to what has been reported in previous studies (Venkei et al., 2011; Przewloka et al., 2007), where cells fail to align their chromosomes even when blocked in mitosis by the addition of the proteasome inhibitor (MG132). Cells exhibited problems in biorientation and in anaphase, lagging chromosomes and chromatin is observed (Fig 2.10). The reduced interkinetochore distance also suggests that chromosomes are not under tension. Despite the problems observed in congression and biorientation, Mis12 depleted cells are not blocked in mitosis. This preliminary characterization will be addressed further in the next section, integrated in the context of other components of the KMN network.

4. MATERIAL AND METHODS

4.1. Mis12 antibody production

In order to generate the antibody that recognizes DmMis12, the full-length cDNA clone (RE19545) from the *Drosophila* Genomics Resource Center was cloned into an expression vector pET-30a (+) (Novagen).

4.2. Construction of pET-30a –Mis12

The insert Mis12 was amplified by PCR using Mis12 cDNA (RE19545) as template, the primers *EcoRVforward* - 5' GATATCATGGACTTCAATAGCCTAGCC 3' (creates a EcoRV site) and *EcoRI reverse - 5'* GAATTCCAGTTAGTTAGTTTATTTAATC *3'* (creates a EcoRI site), and the enzyme FidelTaq polymerase (USB) with proof reading activity, using the following program:

95ºC 5min	-	
95ºC 45sec	-	30 cycles
58ºC 45sec	-	
72ºC 1min	-	
72ºC 7min	-	

PCR product was digested with EcoRV and EcoRI and cloned into pET-30a (+) digested with the same restriction enzymes.

4.3 Mis12 protein expression and purification.

Expression

E.coli BL21 cells were transformed with the plasmid pET-30a (+) - Mis12 and protein synthesis was induced by the addition of 1 mM IPTG (Sigma-Aldrich) at 37ºC for 3h. The soluble and insoluble fractions were obtained by sonication, centrifuged and ressuspended in Tris-HCL 20mM pH7.5. The fractions were loaded in 12% polyacrylamide gel and stained with Comassie blue for protein expression detection.

Inclusion bodies extraction and solubilisation

Inclusion bodies were extracted by sonication (4ºC); cell lysates were ressuspended in Isolation buffer (2M urea, 20mM Tris-HCl, pH 8, and 1mg/mL of lysozyme) and solubilized in buffer A (6M Guanidine Hydrochloride, 20mM Tris-HCl, 0,5M NaCl, 5mM imidazole, 1mM 2-mercaptoethanol, pH 8).

Purification

Affinity Chromatography was performed using high-resolution purification of histidine-tagged protein column (BIO RAD). The purified fractions were collected solubilized in buffer B (8M urea, 20mM Tris-HCl, 0,5M NaCl, 1mM 2-mercaptoethanol, pH 8).

4.4 Protein precipitation and Immunization

Protein precipitation with TCA (Sigma-Aldrich) was carried out using the enriched fractions, recovered in PBS, sonicated and injected in two different rats and one rabbit. After the third immunization the serums were collected and the specificity was confirmed by immunofluorescence and western blot analysis.

4.5. Stable S2 cell line expressing Mis12-GFP

4.5.1 Construction of pHGW/ pHWG – Mis12

Gateway technology was used in order to construct the pHGFPMis12 (pGWMis12) and pHMis12GFP (pWGMis12) expressing plasmids. The cDNA (RE19545) was used as template to amplify the complete coding region of Mis12.

The forward primer 5'CACCATGGACTTCAATAGCCTAG 3' (with an additional CACC sequence for the proper entry in the vector) and a reverse primer 5'-ATCAGTCTCCTTCTTTATCTGCAG 3 where used together with the enzyme FidelTaq (USB) with proof reading activity using a specific PCR program:

95ºC	-	
5min		
95ºC	-	
45sec		
55ºC	-	35
45sec		cycles
72ºC	-	
1min		
72ºC	-	
10min		

pENTR Directional TOPO cloning Kit from Gateway System (Invitrogen) was used to introduce the Mis12 coding sequence to the entry vector which use the properties of Topoisomerase I from *Vaccinia* virus for efficiently cloning of blunt-end PCR products. Topoisomerase I bind to double-strand DNA and cleaves the phosphodiester backbone in one strand at the specific sequence CCCTT. During this cleavage, the energy released is conserved in the covalent bond that forms between the 3′ phosphate of the cleaved strand and the tyrosyl residue (Tyr-274) of topoisomerase I. The directional joining of our blunt-end cDNA with the vector is possibly because of the sequence (CACC) that is added at the forward primer used to amplify our gene of interest. This sequence is the complement of the overhang in the cloning vector created by Topoisomerase I cleavage allowing the hybridization of these two sequences and the directional cloning of Mis12 cDNA.

The Mis12 cDNA fragment and the TOPO vector (Gateway System-Invitrogen) were mixed with a salt solution ($MgCl_2$ and NaCl) and incubated for 30 min at room temperature (RT) and then incubated on ice to proceed to transformation.

Transformation

One Shot chemically competent *E.Coli* (Invitrogen) were used. The construct and the cells were incubated on ice for 30 min and then heat shocked at 42ºC for

30 sec and put on ice for 3 min. 400µL of S.O.C. medium (Invitrogen) were added and cells incubated for 1h at 37ºC. The transformation reaction was plated on LB kanamycin (50µg/mL) (Sigma-Aldrich) plates and incubated overnight at 37ºC.

Construction of Mis12- GFP

This technology uses lambda integrase in order to recombine the ORF of the entry vector, flanked by attL1 and attL2 recombination sites, with the attR1 and attR2 recombination sites of the destination vector. Recombination was performed using Gateway LR Clonase II Enzyme mix (Invitrogen). The entry, destination vector and LR Clonase II were incubated for 2h at 25ºC in TE buffer (pH=8) and Proteinase K (Invitrogen) was added to stop the recombination. *E.coli* DH5α (Invitrogen) was transformed with LR reaction and as mentioned above. The transformation was plated on LB ampicillin (100µg/mL) (Sigma-Aldrich) plates and incubated overnight at 37ºC. The transformants were analysed by restriction analysis using ACCI (Biolabs) and EcoRV (Biolabs). DNA from positive colonies was then confirmed by sequencing analysis.

4.5.2 Stable Transfection of S2 *Drosophila* culture cells

Stable Transfections were performed using Cellfectin reagent (Invitrogen). 1x 10^6 cells/mL of S2 cells were seeded in a 6 well plate in 2 mL Schneider's medium (Sigma-Aldrich) supplemented with 10% of foetal bovine serum (FBS-GBICO) overnight at 25ºC. Solution A (1µg of pHWG-Mis12-GFP plasmid or pHGW-Mis12, 0.2µg of the selection marker plasmid - pyCOBLAST (Invitrogen) and 25µL of Schneider's medium without FBS) and solution B (5µL of Cellfectin and 25µL of Schneider's medium without FBS) were prepared and incubated for 30 min at RT, mixed and incubated for 1h at RT. The medium was replaced with 400µL of Schneider's medium without FBS and the previous solution (A+B) was slowly added to each well, incubated for 4h at 25ºC and then replaced with Schneider's medium supplemented with 10% of FBS. After 48h, the antibiotic for selection was added (Blasticidin 25µg/mL) (Fluka). Every 4 days the medium was replaced and the drug was added until the cells reached a density of 6x10^6 cells/mL. Selection was carried out for 1 month.

4.6. Double-Stranded RNA interference (dsRNA), cell culture and drug treatment

The depletion of Mis12 in *Drosophila* S2 cells by RNAi was performed as previously described (Maiato et al., 2003). Double-stranded RNA (dsRNA) was generated from PCR product using specific primers that incorporate a 5' T7 RNA polymerase promoter site (underlined sequence) and used as templates for the transcription reaction using MEGAscript T7 kit (Ambion) according to the manufacturer's instructions. 30 µg of dsRNA was used in all dsRNA experiments. S2 cells were grown in Schneiders's medium (Sigma) supplemented with 10% foetal bovine serum (GIBCO) without antibiotics, at 25ºC. At selected time points, cells were collected and processed for immunofluorescence, time-lapse microscopy and immunoblotting. Treatment with drugs was performed as follows: 20mM MG132 (Calbiochem) for 2 hours to inhibit the proteosome and arrest cells in mitosis in a checkpoint independent manner; 30mM colchicine (Sigma-Aldrich) for 1hour to induce microtubule depolymerisation.

Protein	Sequence
Mis12	**FW** 5' TAATACGACTCACTATAGGGATGGACTTCAATAGCCTAGCC 3'
	RV 5' TAATACGACTCACTATAGGGTTAATCAGTCTCCTTCTTTAT 3'

4.7 Immunofluorescence in S2 cells

Cells were centrifuged onto slides (5 minutes at 1500 rpm) and processed for simultaneous fixation and extraction in 3.7% methanol-free formaldehyde (Sigma-Aldrich), in 1x PBS, 0.5% Triton X-100 (Sigma-Aldrich) for 10 minutes followed by 3x for 5 minutes washes in 1x PBS, 0.05% Tween20 (Sigma-Aldrich). For sequential fixation and extraction protocol (used to visualize spindle morphology) the fixation solution was prepared using 3.7% methanol-free formaldehyde in 1x PEM for 12 minutes and then extraction was performed 3x for 5 minutes using 1x PBS, 0.5% Triton X-100. Primary antibody incubations were prepared in blocking solution (1x PBS, 0.05% Twee20, 10% FBS) for at least 1 hour at (RT) or overnight at 4°C, followed by 3x for 5 minutes washes in 1x PBS, 0.05% Tween20.

Secondary antibody incubations were performed during 45 minutes, followed by 3x 5 minutes washes. Slides were then mounted using Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories, Inc.Burlingame, CA 94010). Z-series optical sections were collected with 0,24 µm using a 63x objective on an AxioImager Z1 (Carl Zeiss, Germany) microscope, using an AxioCam MR ver.3.0. Data stacks were deconvolved using the Huygens Essential version 3.0.2p1 (Scientific Volume Imaging B.V., The Netherlands). Images treated using Fiji and Adobe Photoshop CS (Adobe Microsystems, CA).

4.8 SDS-PAGE and Western Blot

For immunoblotting, cells were collected by centrifugation, washed 1x in PBS and ressuspended in Ripa lyses buffer supplemented with 1x protease (Complete, Roche) and phosphatase (Sigma-Aldrich) inhibitors. Whole cell protein extracts were quantified by Bradford method and 50µg of protein was used. After boiling for 5 minutes at 95ºC, the extracts were loaded on polyacrylamide gel, electrophoresis and transferred to a nitrocellulose membrane (Whatman) using a wet system (BioRad) for 1,5 hour at 100V. To analyse transference efficiency, membrane was incubated for 5 minutes with Ponceau S. Nitrocellulose membrane was blocked 30 minutes at RT with 5% non-fat milk in 1x PBS, 0.05% Tween20. All antibodies were diluted in the same blocking solution and incubated for 1 hour at RT or overnight at 4ºC. After incubation with primary antibody, membrane was washed 3x for 5 min with 1x PBS, 0.05% Tween20 and incubated for 45 min with secondary antibody. After wash 3x for 5 minutes with 1X PBS, 0.05% Tween20 blots were developed by ECL method.

4.9 Antibodies

The primary antibodies used for immunofluorescence were newly generated anti-Mis12 (rabbit and rat) (1:4000), anti-Mad2 (Rb 1223) (1:10), anti-BubR1 (Rb 666) (1:1500) (Logarinho et al., 2004); anti-phospho Histone H3 (Ser10) (Upstate) (1:1000), anti α-tubulin (clone B-5-1-2) (Sigma) (1:5000), anti-Ndc80 rabbit (kindly provided by Byron Williams and Michael Goldberg) (1:1500), anti-Spc105 (sheep)

(Venkei et al., 2011) (1:500) and anti-CID antibody (C.E.S. and Soren Steffensen, IBMC, University of Porto, Portugal, unpublished) (1:2000). Secondary antibodies used for immunofluorescence were Alexa 488, Alexa 568, Alexa 647 from mouse, rabbit, rat (Molecular Probes) (1:2000). Primary antibodies used for immunoblotting were anti-α-tubulin mouse DM1A (Sigma-Aldrich), anti-Mis12, anti-Ndc80 (Byron Williams and Michael Goldberg) (1:5000). Secondary antibodies used for immunoblotting were HRP mouse, rabbit and rat (Vector Laboratories).

4.10 BrdU analysis

S2 cells were plated on glass coverslips previously coated with 0.25mg/mL concanavalin A (Sigma-Aldrich) and incubate during 1h at 25ºC. 10 µL of BrdU (Bioscience Pharmingen) were added and incubated for 30 min. Cells were washed 2x with 1X PBS and fixed with 3,7% Formaldehyde (Sigma-Aldrich) in PBS 0.1%Triton X-100 (MercK) for 15 minutes at RT. Cells were washed 3x in PBS 0.1% Triton X-100 and incubated with DNase I (Ambion) for 1h at 37ºC for DNA denaturation. Blocking was performed in PBS 0.1% Trinton X-100 with 10% of FBS for 45 min at RT. Cells were incubated O.N at 4 ºC in primary antibodies diluted in blocking solution. After washing 3x with PBS 0.1% Trinton-X100, cells were incubated with secondary antibodies for 45 min at RT. Coverslips were washed again three times with PBS 0.1 Triton X-100% for 5 min and mounted in Vectashield medium containing DAPI (Vector). Images were acquired on an AxioImager Z1 (Carl Zeiss, Germany) connected to an Axiocam MR ver.3.0 (Carl Zeiss, Germany) using a 63X objective and deconvolved with Huygens Pro (Scientific Volume Imaging, Hilversum, The Netherlands), projected using Fiji software and processed with PhotoShop CS (AdobeMicrosystems, CA).

4.11 FRAP (Fluorescence Recovery After Photobleaching)

FRAP analysis was performed at 25ºC using a laser scanning confocal microscope (Leica SP2 AOBS SE) using a 63X oil objective with 8X digital zoom with the confocal pinhole set to 3 Airy unit in a 256:256 format. Four baseline scans were acquired using 10% of full laser power and then the photobleaching of

a circular region was performed by repetitively scanning the bleach region 4x using 100% laser power. Images were collected every 0.995s during the recovery phase for a total of 140 s. The analysis was performed using Fiji software. Correction of the fluorescence due to photobleaching was achieved by subtraction of the background to the total fluorescence in the bleached area dividing for the control fluorescence without the background. FRAP curves were analysed using nonlinear regression by fitting the data points to a single exponential curve (one phase association) using Prism software version 4.0 (GraphPad Software Inc., San Diego, CA).

Mobile fraction = $(RFI_{t\infty} - RFI_{t0})/(1- RFI_{t0})$

Mean Residence time = $1/k$

k = constant

Chapter 3

Stability of Kinetochore-Microtubule attachment and the role of different KMN network components in *Drosophila*

1. INTRODUCTION

Proper cell division is essential to generate two genetically identical daughter cells and maintain genomic stability. The kinetochore is a multi-protein structure located at the centromeres that provides the interface between the chromosomes and spindle microtubules, essential for the segregation of sister chromatids during anaphase. Additionally, it also participates in a surveillance mechanism known as the Spindle Assembly Checkpoint (SAC).

The size and complexity of kinetochores vary considerably among different species, however, the network of proteins that are transiently recruited and involved in microtubule capture, spindle checkpoint response and regulation of microtubule dynamics are mostly conserved. The interaction between microtubules and chromosomes involves a conserved super-complex of proteins, known as the KMN network (reviewed in Cheeseman and Desai, 2008). The complete KMN network appears to assemble during prometaphase, however in most species, the Mis12 complex localizes at the kinetochores throughout the cell cycle (Liu et al., 2010; Cheeseman et al., 2008; Kiyomitsu et al., 2007).

The kinetochore assembly pathway has been widely studied in different organisms. The connection of the Ndc80 complex and the KNL1 complex (KNL1 and Zwint-1 in humans) to the inner kinetochore appears to differ between different cell types. The results suggest that the Mis12 complex is necessary for the localization of the Ndc80 complex (Venkei et al., 2012; 2011; Przewloka et al., 2011; 2007; Kline et al., 2006; Cheeseman et al., 2004; Scharfenberger et al., 2003). However, it has been reported that kinetochore localization of Ndc80 in humans, budding and fission yeast does not depend on KNL1 (Liu et al., 2010; Cheeseman et al., 2008; Kiyomitsu et al., 2007). In human cells, CENP-T plays a major role as a link to the Ndc80 complex (Nishino et al., 2013). Interestingly, the Mis12 complex and Spc105 are interdependent for their kinetochore localization in yeast and *Drosophila* (Venkei et al., 2012; Przewloka et al., 2007; Cheeseman et al., 2004). The Mis12 complex is thought to play a central role in kinetochore assembly

(reviewed in Cheeseman and Desai, 2008). In *Drosophila*, it interacts with the centromere protein Cenp–C via the Nnf1 subunit (Przewloka et al., 2011) however in vertebrates it is not clear which subunit of the Mis12 complex is responsible for the interaction (Screpanti et al., 2011). The most distal subunit of the Mis12 complex is Nsl1, that interacts with the Ndc80 complex via Spc25/Spc24 and also Spc105 using different interfaces (Petrovic et al., 2010).

The Ndc80 complex interacts with microtubules through the N-terminal tail and calponin homology domain of Ndc80 (DeLuca and Musacchio, 2012; Tooley and Stukenberg, 2011; Alushin et al., 2010; Joglekar et al., 2010; Guimaraes et al., 2008; Miller et al., 2008; Wilson-Kubalek et al., 2008). Point mutations in the calponin homology domain prevent stable kinetochore-microtubule attachments *in vivo* and *in vitro* and the affinity of the Ndc80 complex for microtubules is compromised (Sundin et al., 2011; Tooley et al., 2011; Ciferri et al., 2008; Alushin et al., 2010; Miller et al., 2008; Guimaraes et al., 2008; Ciferri et al., 2007; DeLuca, 2002). Recently, it has been shown that the Ndc80 complex has a bent in its structure that corresponds with a break in registry of the central coiled-coil region within NDC80 (Wang et al., 2008). This region of Ndc80 complex is called loop domain and it is involved in stabilizing kinetochore-microtubule attachments (Zhang et al., 2012; Varma et al., 2012; Matson and Stukenberg, 2012), thus providing a total of three different kinetochore-microtubule binding domains within the Ndc80 complex (Maure et al., 2011).

Within the KMN network Ndc80 is not the only protein involved in microtubule binding. Despite of Mis12 complex not being able to co-sediment with microtubules alone, its presence significantly increases the microtubule-binding capacity of KNL1/Spc105 (Cheeseman et al., 2006). Furthermore, it has been shown 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). Several studies have been addressing the role of KMN network in kinetochore-microtubule attachment. When the Mis12 complex is absent defects in chromosome alignment and biorientation, unstable kinetochore-microtubule interactions and abnormal chromosome

segregation occur (Venkei et al., 2011; Przewloka et al., 2007; Kline et al., 2006; Obuse et al., 2004b; Goshima et al., 2003). Similarly, depletion of the Ndc80 complex results in impairment of kinetochore-microtubule attachment and chromosome missegregation. When Spc105 is depleted, a stronger phenotype is observed in respect to the stability of kinetochore-microtubule interactions. In *C.elegans* depletion of KNL1 causes a “kinetochore null” phenotype (Desai et al., 2003). A similar phenotype is observed in *Drosophila*, where depletion of Spc105 results in a severely impaired chromosome congression, alignment and segregation, leading to a dramatic decrease in cell viability (Przewloka et al., 2007). In human cultured cells the phenotype is milder. Nevertheless, stable kinetochore-microtubule fibers are significantly reduced and chromosome missegregation is observed. This is most probably due to the KNL1-independent kinetochore localization of Ndc80 (Cheeseman et al., 2008; Kiyomitsu et al., 2007).

It is well established in metazoans that microtubule subunits are continuously added to microtubule plus ends at the kinetochore in mature K-fibers and is counter balanced by their removal at their minus ends at spindle poles. This condition is known as microtubule poleward flux (Mitchison, 1989).

In *Drosophila* S2 cells, kinetochore-microtubule flux requires cytoplasmic linker associated protein (CLASP) mediated incorporation of tubulin subunits at kinetochores (Maiato et al., 2005) balanced by KLP10A-dependent MT depolymerization at the poles. Since, the depletion of KMN network perturbs the kinetochore-microtubule interaction, it is probable that in these conditions the kinetochore-microtubule flux is altered significant biological impact.

The role of KMN network in SAC response is not yet well understood. The first studies in human cultured cells only reported a mitotic delay after hDsn1 depletion but not after hMis12 depletion (Obuse et al., 2004b; Goshima et al., 2003). Subsequently, others reported a mitotic arrest after depletion of the four subunits separately (Kline et al., 2006). More recently, a delay in anaphase onset has been observed in Mis12 and Nsl1 *Drosophila* mutants embryos (Venkei et al., 2011). Studies performed in budding yeast reported an impairment of checkpoint response in Spc24 and Spc25 conditional lethal mutants (Janke et al., 2001), while in *Xenopus* and human cultured cells a mitotic arrest is observed upon depletion of different proteins of the Ndc80

complex (McCleland et al., 2004; Bharadwaj et al., 2004; DeLuca, 2002). However, it has been shown that the extent of depletion of Ndc80 complex has different outcomes in the kinetochore localization of Mad2 and SAC response (Meraldi et al., 2004) that may explain the observed discrepancy in results. More recently, it has been shown that the deletion of the N-terminal 207 amino acid region containing both the tail domain and a calponin homology (CH) domain of Ndc80 protein abolishes the SAC (Guimaraes et al., 2008). Also, recent findings suggest that KNL1 plays a role in SAC activation and silencing. It has been shown that SAC activation at kinetochores is dependent on recruitment of Bub1-Bub3 to the MELT motifs on KNL1 through phosphorylation by Mps1 (Yamagishi et al., 2012; London et al., 2012; Shepperd et al., 2012). The interaction of TPR domains of BubR1 and Bub1 with KI motifs in the N-terminal region of KNL1 are also important for the SAC in human cells (Krenn et al., 2012; Bolanos-Garcia et al., 2011; Kiyomitsu et al., 2011). A second pathway of SAC activation involving ZWINT was described in metazoans. ZWINT is associated both to KNL1 and the RZZ complex and is necessary for the recruitment of MAD1 to kinetochores (Kiyomitsu et al., 2007; Kops et al., 2005). KNL1 also has a binding motif to PP1, which recently has been shown to be involved in SAC silencing (Rosenberg et al., 2011; Pinsky et al., 2009; Vanoosthuyse and Hardwick, 2009). Additionally, a second mechanism of SAC silencing has been identified in C. *elegans* involving the N-terminus of KNL-1 (Espeut et al., 2012).

While depletion of different KMN network components in different model systems leads to apparently similar phenotypes, a directly comparable detailed study of the different KMN components in the same system is lacking. Therefore, in this study we have analysed in detail the interactions between kinetochores and microtubules in *Drosophila* tissue culture cells upon depletion of different KMN components, as well as the outcome in terms of chromosome segregation. Additionally, we also addressed the role of the individual components of KMN network in SAC signalling.

2. RESULTS

2.1 KMN network components depend partially on each other for kinetochore localization

Kinetochores are highly dynamic structures whose composition changes to fulfil different requirements at specific stages of mitosis. The structural core of the kinetochore contains a super-complex of proteins, the KMN network named after its constituents: KNL-1/Spc105/Blinkin, Mis12 and Ndc80 complexes. All together the KMN network is responsible for the proper interaction between chromosomes and microtubules. Therefore, it is of crucial importance to study the function of each individual component of the KMN network in different species in order to understand the evolutionary adaptations that may have occurred within this structure.

To further dissect the role of the KMN network assembly and the role of each component, we performed double-stranded RNA (dsRNA) interference in *Drosophila* S2 tissue culture cells. Mis12 and Spc105 were depleted individually while to deplete the Ndc80 complex we performed simultaneous depletion of two subunits, Ndc80 and Nuf2. The protein levels upon dsRNA treatment were monitored by immunofluorescence and western blot (Fig. 3.1). An efficient depletion of Mis12 was achieved after 120h of RNAi treatment (Fig. 3.1 A and B). The Ndc80 complex (Ndc80 and Nuf2) and Spc105 protein levels were evaluated after 96h post-treatment (Fig. 3.1 A, C and D, B´, C´ and D). More than 90% of depletion of protein levels was observed for all the components tested (Fig. 3.1 A).

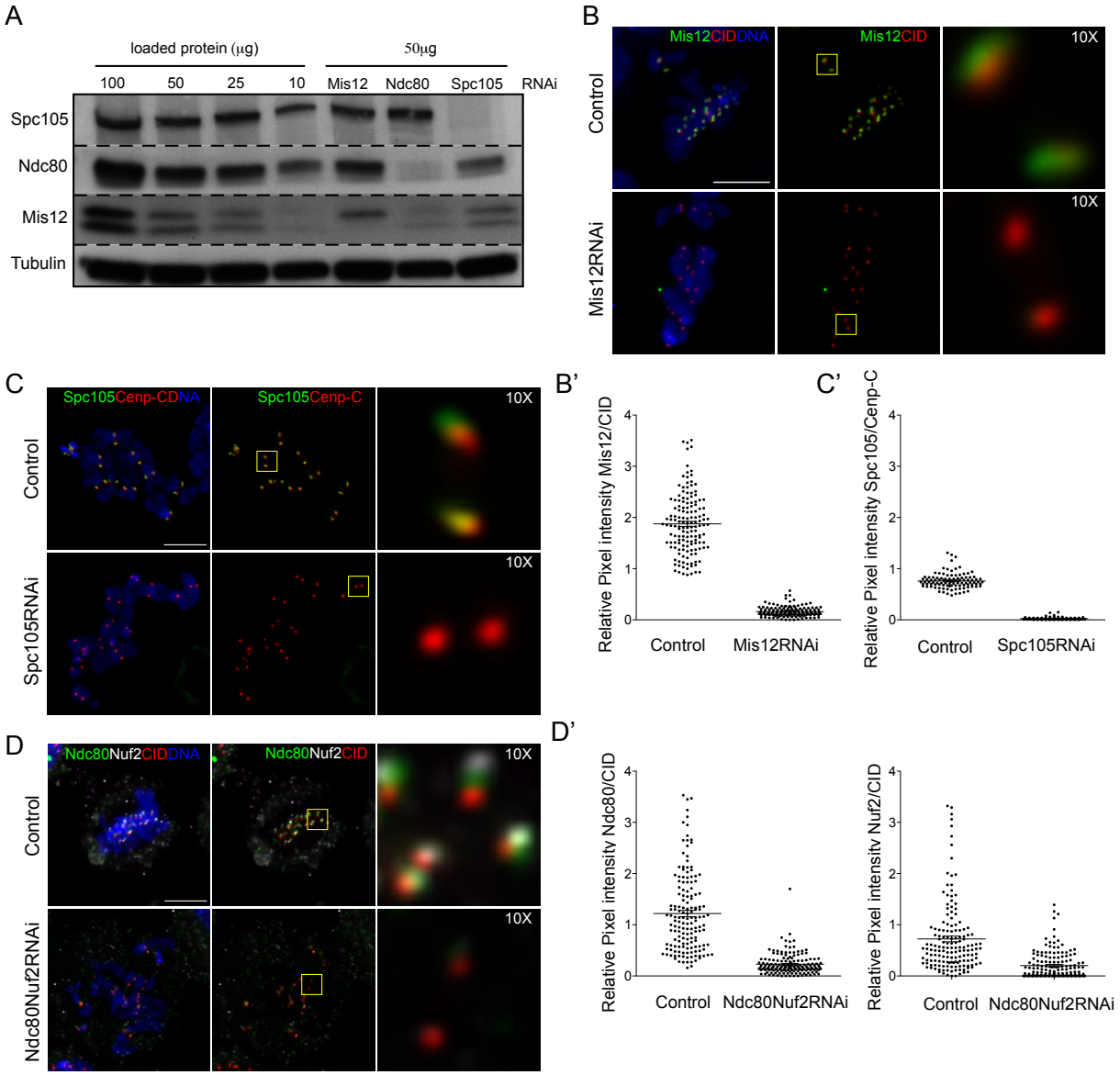


Figure 3.1 KMN network depletion by dsRNA. (A) Antibody titration of KMN components. 100, 50, 25, 10µg of S2 cells total protein extracts were separated by SDS-PAGE and the presence of Spc105, Ndc80 and Mis12 proteins was detected by western blot. The level of the same proteins upon dsRNA treatment was evaluated in protein-depleted extracts where 50µg of protein was loaded. (B, C, D) Immunofluorescence analysis of control or depleted cells for Mis12, Spc105 and Ndc80/Nuf2 showing CID or CENP-C (red), Mis12, Ndc80, Spc105 (green), Nuf2 (white) and DNA (blue). CID and CENP-C were used as centromere markers. Magnifications of selected regions are shown on the right panel. Scale bar is 5µm. (B', C', D') Quantification of the mean pixel intensity of Mis12, Spc105, Ndc80 and Nuf2 at kinetochores relative to the centromeric signals (CID and CENP-C) using immunofluorescence images shown in (B, C, D) where each dot represents an individual kinetochore, (n>100). Error bars represent SEM.

Next we investigated the dependencies of kinetochore recruitment between the different KMN components during mitosis. Surprisingly, a reduction of Mis12 signal at kinetochores was observed upon depletion of Ndc80/Nuf2 and Spc105 (Fig. 3.2 A and D). Ndc80 levels were highly reduced when we depleted either Mis12 or Spc105 (Fig. 3.2 B and D). A reduction in Spc105 at kinetochores is observed when the Mis12 protein is depleted (Fig. 3.2 C and D). These results indicate that in *Drosophila* there is a partial dependency of Mis12 on Ndc80/Nuf2 and Spc105 for its kinetochore localization. Additionally, the Ndc80 complex depends on both Mis12 and Spc105 to localize to kinetochores and Spc105 to a lesser extend depends on Mis12.

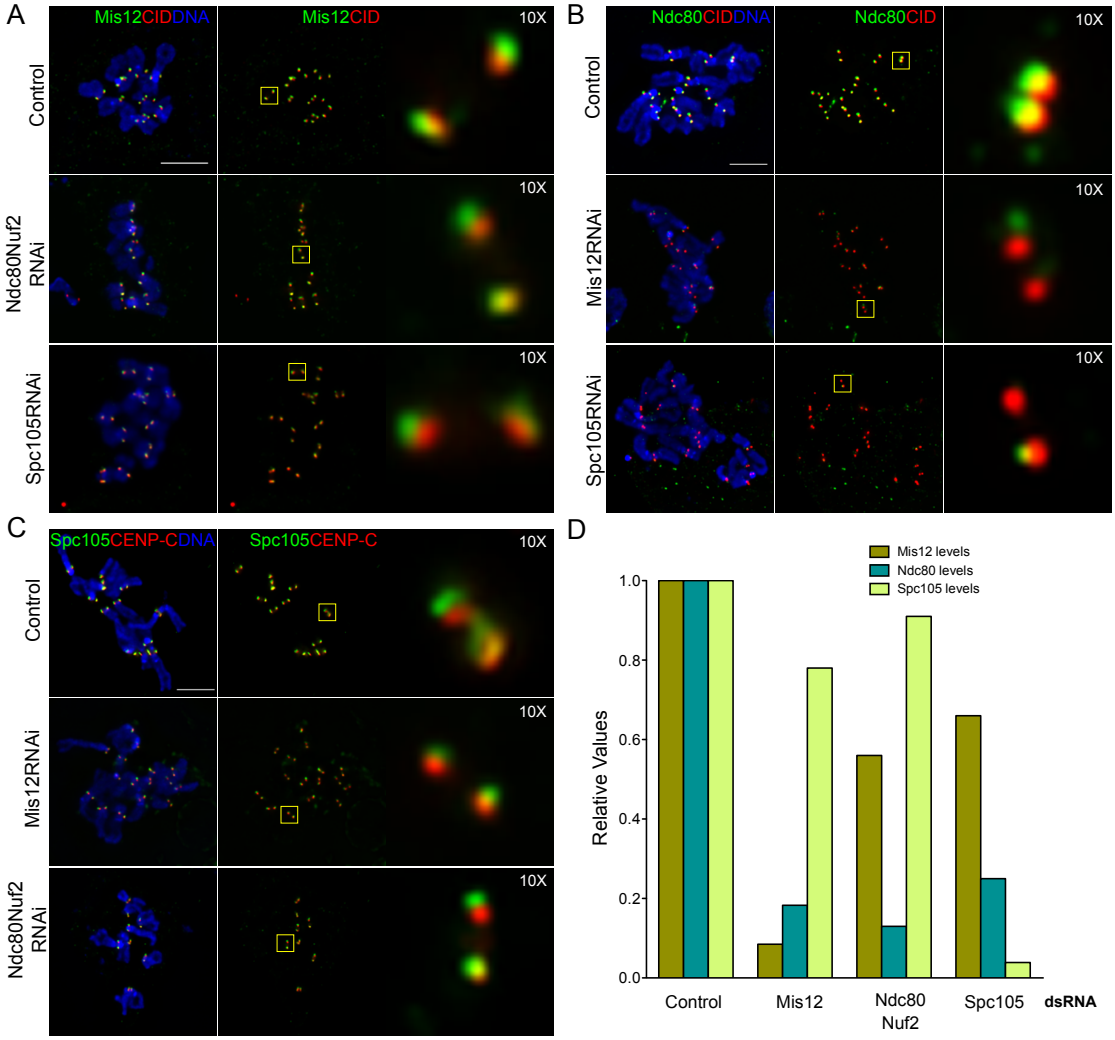


Figure 3.2 KMN network proteins are interdependent for localization at kinetochores. (A, B, C) Immunofluorescence of KMN network proteins. CID and CENP-C (red) were used as centromeric markers and the KMN network components were labelled in green. Magnifications of selected regions are

shown on the right panel. Scale bar is 5µm. (D) Quantification of the mean pixel intensity of Mis12, Ndc80, and Spc105 at kinetochores relative to the centromeric signals (CID and CENP-C) using immunofluorescence images shown in (A, B, C), n=150 kinetochores per condition from two independent RNAi experiments.

To determine whether depletion of Mis12 also affected other kinetochore proteins we also monitored the localization of ZW10 and CENP-meta (CENP-E *Drosophila* homologue). A decreased in protein localization was observed for both proteins, showing that Mis12 also affects other components of the outer kinetochore (Fig. 3.3).

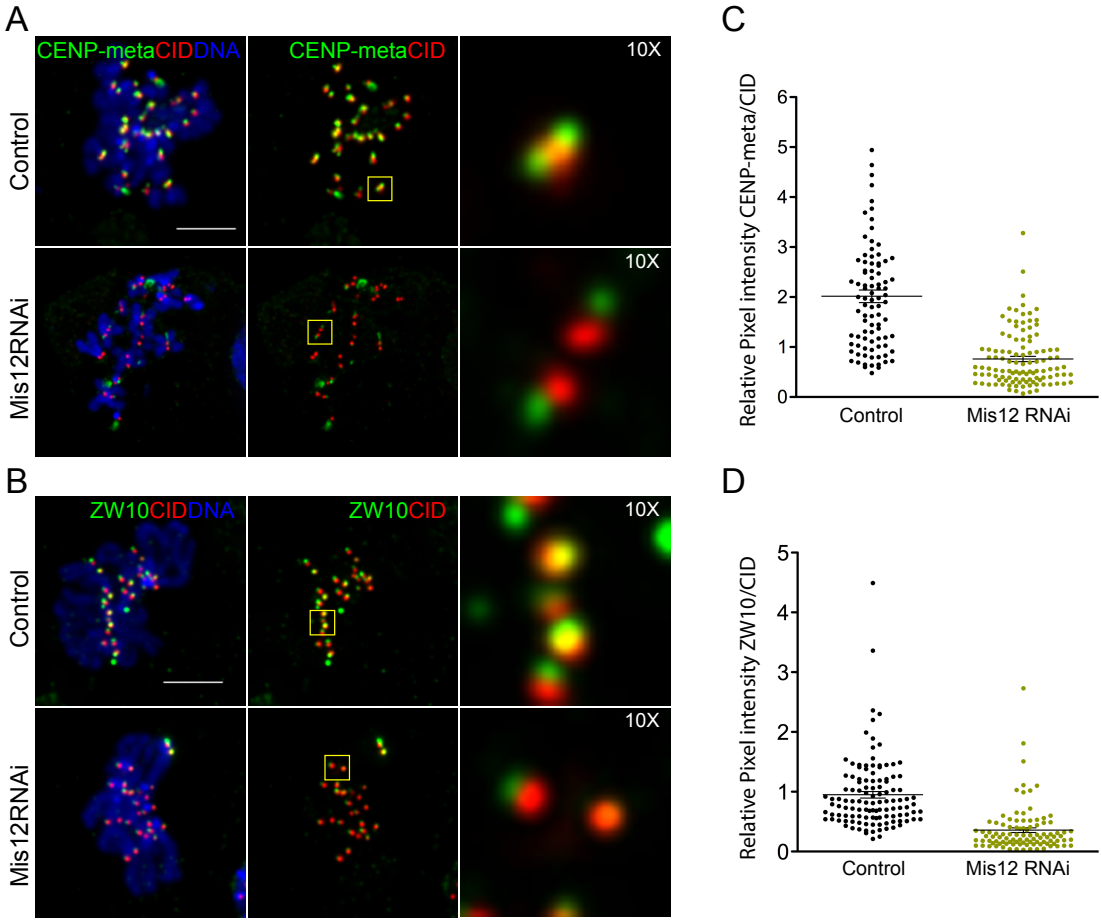


Figure 3.3 Kinetochore localization of CENP-meta and ZW10 after depletion of Mis12 protein. (A and B) Immunofluorescence in S2 cells showing CENP-meta and ZW10 (green), CID (red) and DNA (blue). Magnifications of selected regions are shown on the right panel. (C and D) Quantifications of the mean pixel intensity of CENP-meta and ZW10 at kinetochores relative to the centromeric signals CID using immunofluorescence images shown in (A and B), n>100 kinetochores per condition from two independent RNAi experiments.

2.2 Kinetochore-microtubule attachment after depletion of KMN network components

Previously, it has been shown that the KMN network plays a crucial role in kinetochore-microtubule attachment. However, a detailed analysis of the contribution of each component in the same cell type was lacking. Accordingly, we analysed the kinetochore-microtubule attachment after depletion of the different components (Fig. 3.4). No metaphase cells were found upon depletion of Mis12, Ndc80/Nuf2 or Spc105, and most mitotic cells were in prometaphase with chromosomes dispersed along the mitotic spindle. However, closer analysis of these cells revealed that almost all the kinetochores were still able to establish lateral interactions with microtubules (Fig. 3.4 A). To further evaluate the nature of these kinetochore-microtubule attachments and to exclude the possibility that this was due to an interaction with immature kinetochore fibers, cells were incubated with MG132 prior to fixation to prevent them from exiting mitosis allowing to extend the time chromosomes have for alignment (Fig. 3.4 B). In this condition, chromosomes in control cells reach biorientation and align to the metaphase plate (Fig. 3.4 B). However, after depletion of Mis12, Ndc80/Nuf2 or Spc105, and MG132 incubation for 2 hours chromosomes are not able to congress and the kinetochore-microtubule interactions remained lateral (Fig. 3.4 B and D). This indicates that the lateral kinetochore-microtubule attachments we observed do not depend on the time cells spend in mitosis but it is intrinsic to the modified kinetochores after specific KMN network components depletion. Since in the absence of Ndc80 complex the motor proteins dynein and CENP-E are still able to localize to kinetochores (DeLuca et al., 2005), we investigated if these proteins were involved in the establishment of the lateral interactions we observed upon depletion of the Ndc80 complex. Accordingly, we co-depleted with Ndc80/Nuf2 and ZW10 a component of the RZZ complex, known to be responsible for dynein localization to the kinetochore (Starr et al., 1998), or the plus-end directed motor protein CENP-meta (Fig. 3.4 E). The results show that kinetochores still bind microtubules laterally (Fig. 3.4 C and D) indicating that these motor proteins do not provide the molecular mechanism

responsible for these lateral attachments and suggesting that Spc105 is likely to play a significant role in this process.

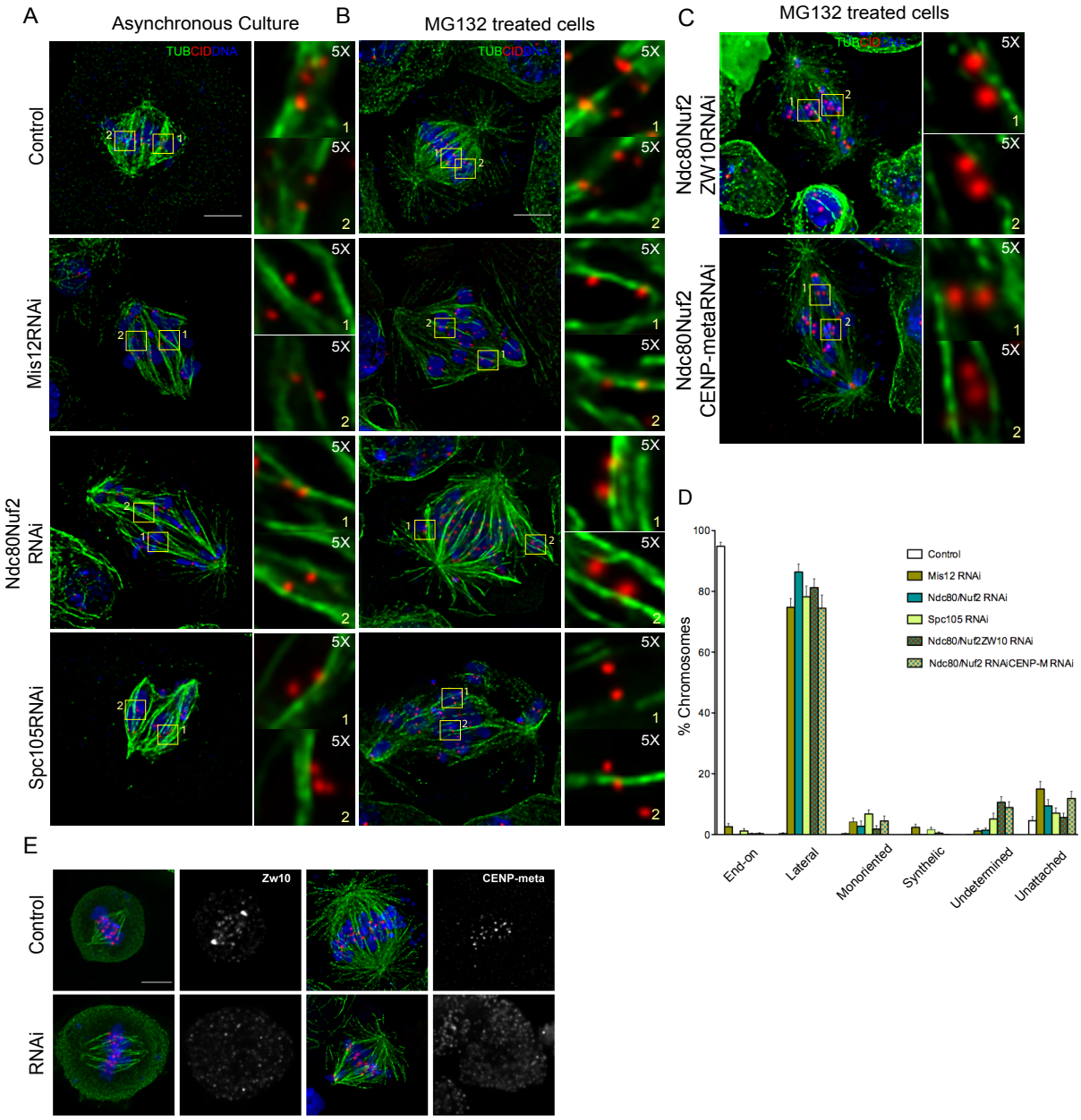


Figure 3.4 Kinetochore–microtubule interactions after depletion of KMN network components. (A-C) Kinetochore-microtubule interactions accessed either in asynchronous culture or after MG132 incubation. CID (red) was used as centromeric marker, α-tubulin (green) and DNA (blue). Magnifications of insert regions are shown on the right panel corresponding to 3 z-stacks of the focal plane of CID signals. (D) Overall quantification of kinetochore-microtubule interactions in the presence of MG132 after depletion of the different KMN network components alone or the Ndc80 complex together with ZW10 or CENP-meta. Error bars represent SEM. Note that none of the depletions completely abolished kinetochore-microtubule interactions. (E) Immunofluorescence analysis of control or depleted cells for ZW10 (depletion

level determined by ZW10 streaming on the spindle, 11/12 control cells and 0/22 depleted cells exhibited streaming) or CENP-meta (93% reduction on protein levels was observed in depleted cells by immunofluorescence, n=150 kinetochores), showing DNA (blue), CID (red) and α-tubulin (green) in the merged image and the ZW10 or CENP-meta in single channel. Scale bar is 5μm.

2. 3 Mis12, Ndc80/Nuf2 and Spc105 proteins have different contributions in kinetochore-microtubule attachment

It has been reported that kinetochores that bind laterally to microtubules do not establish stable interactions (Inoué, 1964). To determine the level of stability of kinetochore-microtubule interactions after depletion of the different KMN network components, cells were exposed to cold treatment (0º C) for 10 minutes prior to fixation and antibody staining. Under these conditions only microtubules stably bound to kinetochores should resist to cold-induced depolymerisation (Rieder, 1981). As a control for efficient depolymerisation, we depleted Cenp-C (Fig. 3.5 A), a protein that forms the interface between the centromere and the kinetochore and that after depletion results in a kinetochore null phenotype in S2 cells (Orr and Sunkel, 2010). The results show that after depletion of Mis12 or Ndc80/Nuf2 most cells contain stable, though fewer, microtubule bundles when compared to control cells (Fig. 3.5 A and B) and a significant proportion of chromosomes remain attached (Fig. 3.5 A and B). Even after depletion of Spc105, microtubule bundles are still visible and over 40% of chromosomes remain attached to microtubules. However, when we co-depleted ZW10 or CENP-meta with the Ndc80 complex we observed a significant increase in unattached kinetochores (50% and 60% respectively) similar to the values observed for Spc105 dsRNA (Fig. 3.5 A and B). These results suggests that while dynein and/or CENP-meta are not required for the latter kinetochore microtubule interaction, these motor proteins might be playing an essential role in promoting the stability of the lateral attachments.

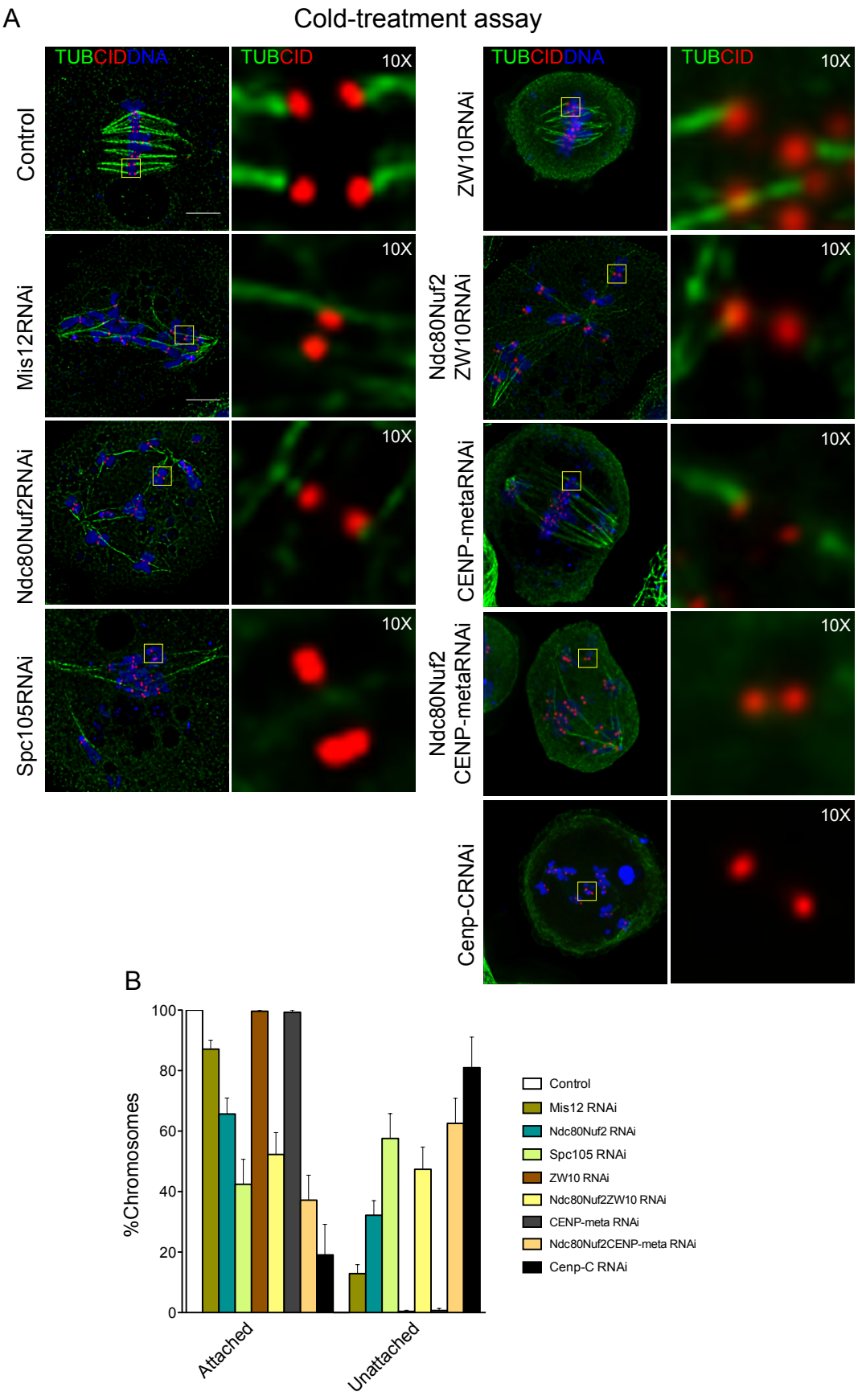


Figure 3.5 Stability of lateral attachments. Immunofluorescence analysis of control or depleted S2 cells for different KMN components after cold-treatment.

(A) Immunofluorescence images show DNA (blue), CID (red) and α-tubulin (green). Magnifications of selected regions are shown on the right panel. Scale bar is 5μm. (B) Quantification of kinetochore-microtubule interactions from (A). Control cells (n=19), Mis12RNAi (n=19), Ndc80Nuf2RNAi (n=21), Spc105 (n=12), Ndc80Nuf2ZW10RNAi (n=20), Ndc80Nuf2CENP-metaRNAi (n=17) from two independent RNAi experiments. The graph shows the percentage of chromosomes attached per cell including those that are bioriented, monoriented and laterally attached. Error bars represent SEM.

Previous studies in different model systems suggest that depletion of KMN network proteins prevented all types of kinetochore attachment (review in Varma and Salmon, 2012). To further explore the nature of the interaction between kinetochores and chromosomes that was observed after depletion of KMN network components, we subjected these cells to the MG132-Taxol assay, previously developed to analyse strength and stability of kinetochore-microtubule interactions (Maia et al., 2007). In this assay, cells depleted of a KMN network protein were incubated with MG132 to prevent mitotic exit followed by a high dose of taxol for a short period to cause collapse of the spindle prior to fixation (Fig. 3.6 A and B). Depletion of Mis12, Ndc80/Nuf2 or both complexes at the same time, confirms that chromosomes are able to maintain a strong interaction with microtubules that is sufficiently stable to withstand the forces exerted by the collapse of the spindle after taxol incubation.

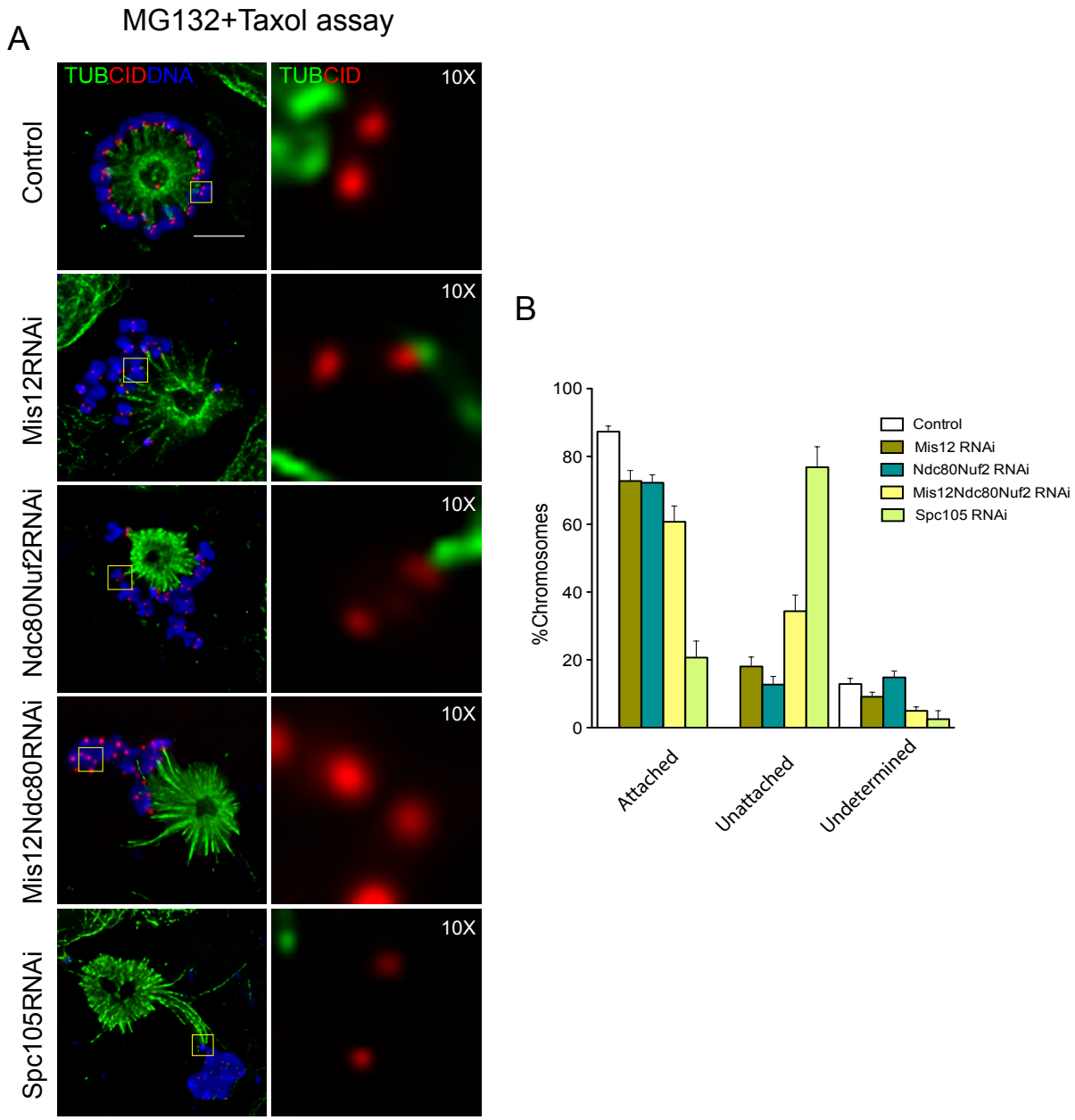


Figure 16 Stability of lateral attachments. Immunofluorescence analysis of control or depleted S2 cells for different KMN components after MG132+Taxol assay. (A) Immunofluorescence images show DNA (blue), CID (red) and α-tubulin (green). Magnifications of selected regions are shown on the right panel. Scale bar is 5μm. (B) Quantification of kinetochore-microtubule interactions from (A). Control cells (n=35), Mis12 RNAi treated cells (n=39), Ndc80Nuf2 RNAi treated cells (n=49), Mis12Ndc80Nuf2 RNAi treated cells (n=28), Spc105 RNAi treated cells (n=10) from two independent RNAi experiments. The graph shows the percentage of chromosomes attached per cell including those that are bioriented, monooriented and laterally attached. Error bars represent SEM.

To address whether these stable kinetochore-microtubule interactions were able to promote force across sister kinetochores, we measured the interkinetochore distance in control and RNAi treated cells after a short incubation with MG132 to prevent mitotic exit, using CID as the centromere marker. We found that the mean interkinetochore distance in control cells was almost double that observed after depletion of Mis12, Ndc80/Nuf2 and Spc105 (Table 1). As a control for loss of tension across sister kinetochores, control and dsRNA treated cells were subjected to colchicine incubation to depolymerize all microtubules before fixation. In all conditions we observed a decrease of the interkinetochore distance (Table 1). All together, these results suggest that the interaction established between kinetochores and microtubules after depletion of KMN network components although partially stable, is not able to promote sufficient forces between sister kinetochores. Nevertheless, these observations suggest for the first time that the lateral interactions established between kinetochores and the microtubule lattices are significantly stronger than previously thought and that motor proteins (dynein and CENP-meta) play a role in the stability of the lateral attachment.

Table 1. Interkinetochore distance in *Drosophila* S2 cells treated with MG132 or colchicine.

	MG132	**Colchicine**
Control	1,25±0,18 (n=182)	0,77±0,19 (n=118)
Mis12 RNAi	0,74±0,16 (n=101)***	0,78±0,19 (n=100) ns
Ndc80Nuf2 RNAi	0,78±0,21 (n=100)***	0,68±0,24 (n=90)**
Spc105 RNAi	0,72±0,17 (n=135)***	0,80±0,18 (n=61) ns

n = pairs of kinetochores, *** p<0,0001**,** **p<0,001**,** ns (not statistically significant).

2.4 *Drosophila* Mis12 does not bind directly to microtubules

It has already been described that Ndc80 and Spc105 proteins exhibit microtubule-binding and bundling activity (Tooley and Stukenberg, 2011; Joglekar et al., 2010; Pagliuca et al., 2009; Cheeseman et al., 2006). Moreover, Aurora B regulated kinetochore-microtubule binding by

phosphorylating different components of KMN network (Welburn et al., 2010). In C. *elegans* it has been shown that Mis12 does not co-sediment with microtubules. However across species there are always differences in function of KMN network proteins. Therefore, we investigated whether *Drosophila* Mis12 protein could bind microtubule directly. Accordingly, we performed an overlay assay with purified recombinant Mis12 recombinant protein, a commercial available microtubule associated proteins (MAPS) (Cytoskeleton) as a positive control and BSA as a negative control. All the samples were incubated with GDP or GTP and further on incubated with polymerized tubulin. The results were analysed by SDS-page and wester-blot where only MAPs showed a specific band for α–tubulin. In order to test specificity of the overlay assay, the experiment was repeated without polymerized bovine tubulin. Total cell extracts and MAPs were used and incubated with and without GTP, no reactivity was found for the MAPs and a specific band for α–tubulin was obtained for the total cell extracts meaning that the results obtained in the overly assay where due to the previous incubation with polymerized microtubules (Fig. 3.7 B).

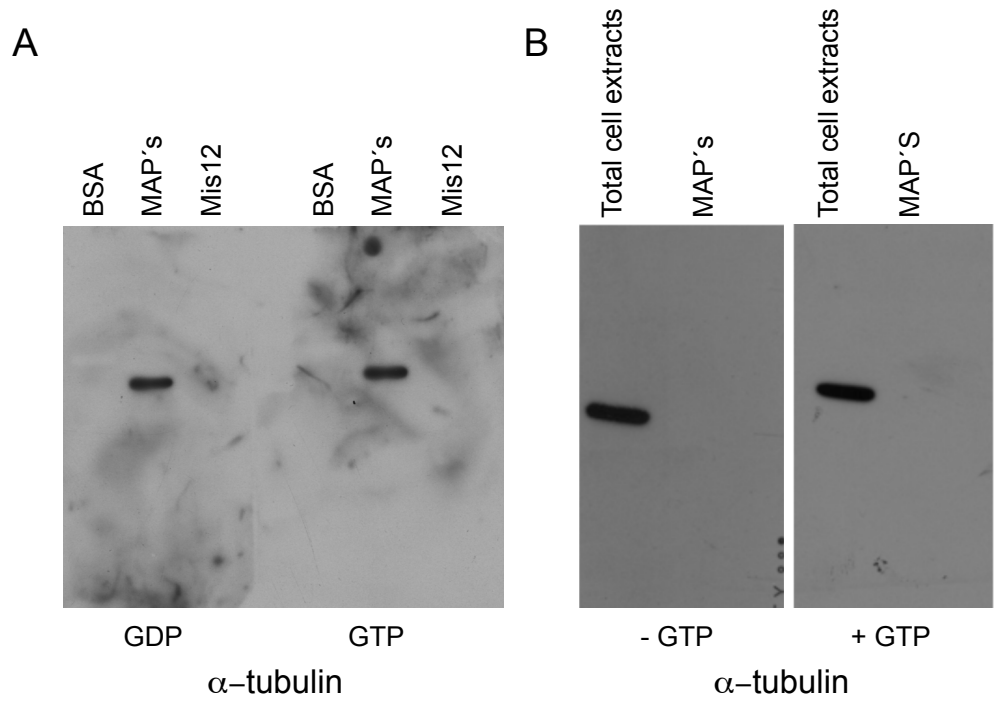


Figure 3.7 Overlay assay of recombinant Mis12 protein. (A) 12% SDS-page was performed to run the proteins samples. BSA used as negative control, MAP´s

as positive control and Mis12 recombinant as the protein of interest. Western-blot was made and the proteins were incubated with polymerized tubulin in presence of GDP or GTP. Hybridization against α–tubulin was performed. (B) SDS-page and western-blot were done with total cell extracts and MAP´s. Hybridization with anti-tubulin antibody was made but without previous incubation of polymerized tubulin and in presence or absence of GTP.

2.5 Chromosome segregation upon individual KMN network depletions.

So far, our results are consistent with a very significant role for Spc105 in kinetochore-microtubule interactions while Ndc80 could play a role in facilitating end–on microtubule binding (Maure et al., 2011). These observations led us to explore the outcome of chromosome segregation *in vivo* after depletion of individual KMN components. Cells stably expressing CID-mCherry (*Drosophila* CENP-A homologue) and GFP-Tubulin were treated with dsRNA against different KMN components and followed during mitosis (Fig. 3.8 A-D and supplementary movies S1-S4). In order to study kinetochore behavior, we manually tracked kinetochore pairs from prometaphase until late anaphase in all conditions (Fig. 3.8 A'-D'). The results show that chromosomes in control cells display minor oscillations and reach biorientation within approximately 10 minutes prior to anaphase onset (Fig. 3.8 A and A'). Mis12 and Ndc80 depleted cells behave differently from control. Cells depleted either of Mis12 or Ndc80/Nuf2 exhibit significant chromosome movements towards and away from metaphase plate and at anaphase onset a high percentage of chromatids do not separate so that sister chromatids segregate together (Fig. 3.8 B, B', C and C'). Chromosomes in cells depleted of Spc105 do not oscillate during prometaphase and at anaphase onset sister chromatids do not separate and some chromosomes completely fail to segregate remaining at the equatorial plane of the cell (Fig. 3.8 D and D').

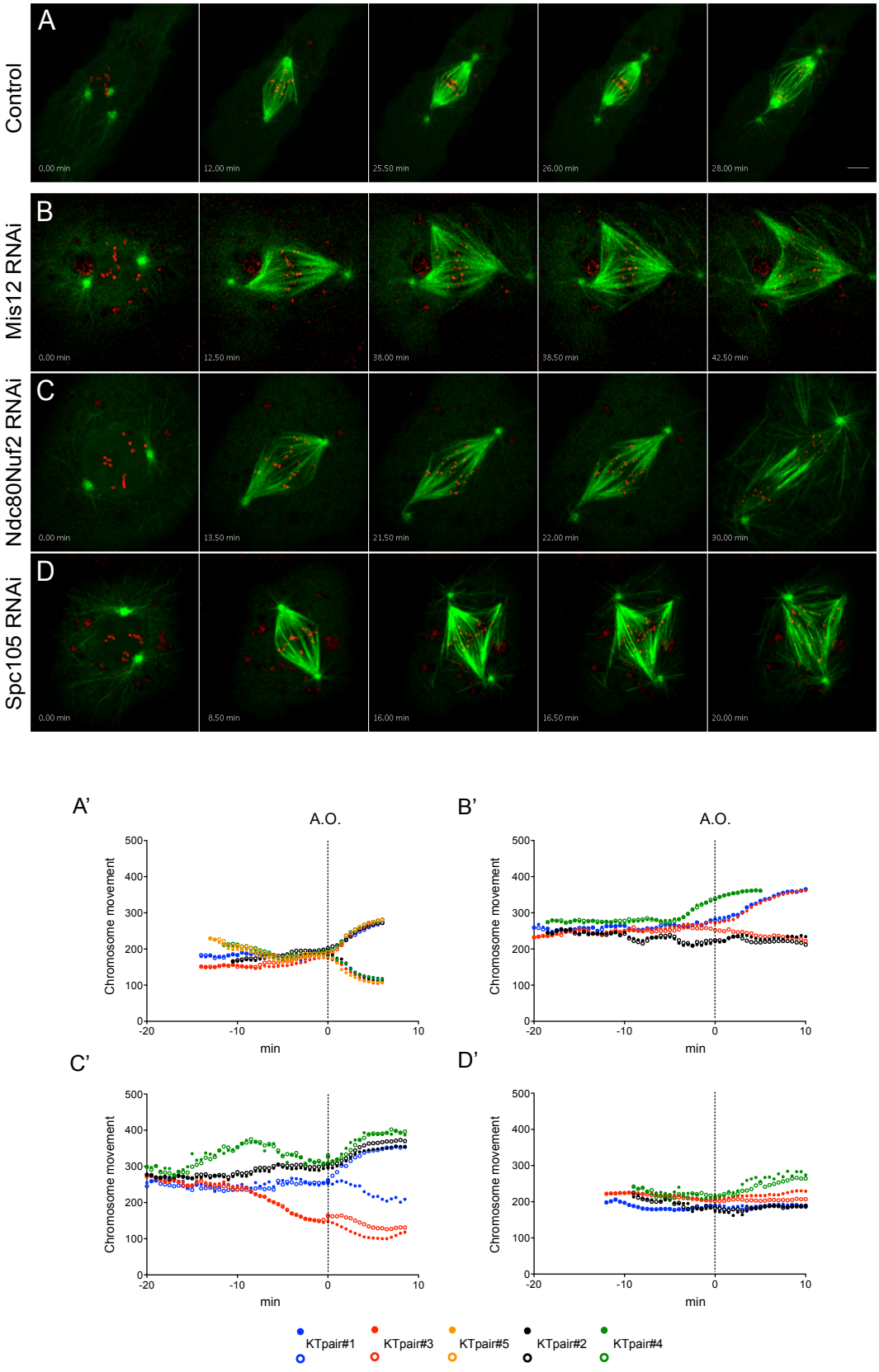


Figure 3.8 Figure 3.8 Time-lapse microscopy of S2 cells stably expressing GFP–Tubulin and CID–mCherry recorded every 30s to evaluate chromosome segregation. (A-D) Stills were taken from representative movies and (A'-D') quantitative analysis of kinetochore tracking of the corresponding condition.

Several pairs of kinetochores were manually tracked from prometaphase until late anaphase after depletion of different KMN network components.

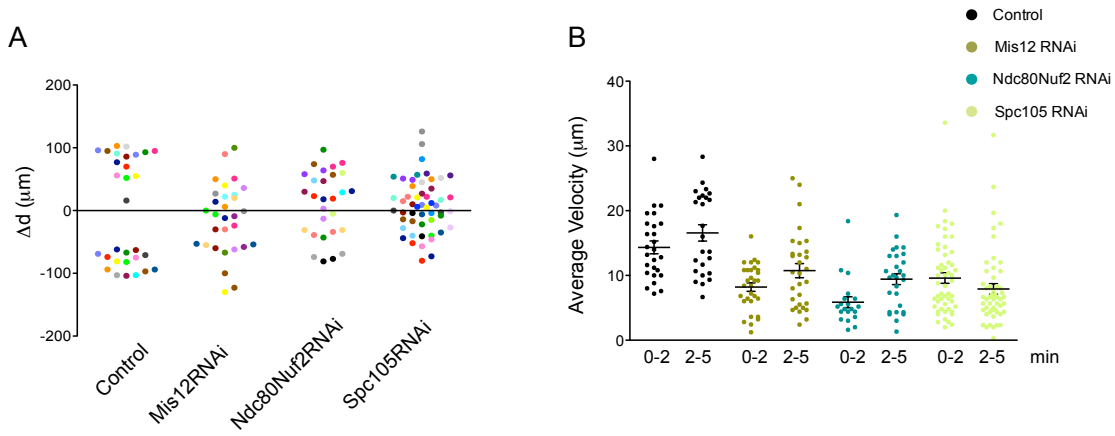


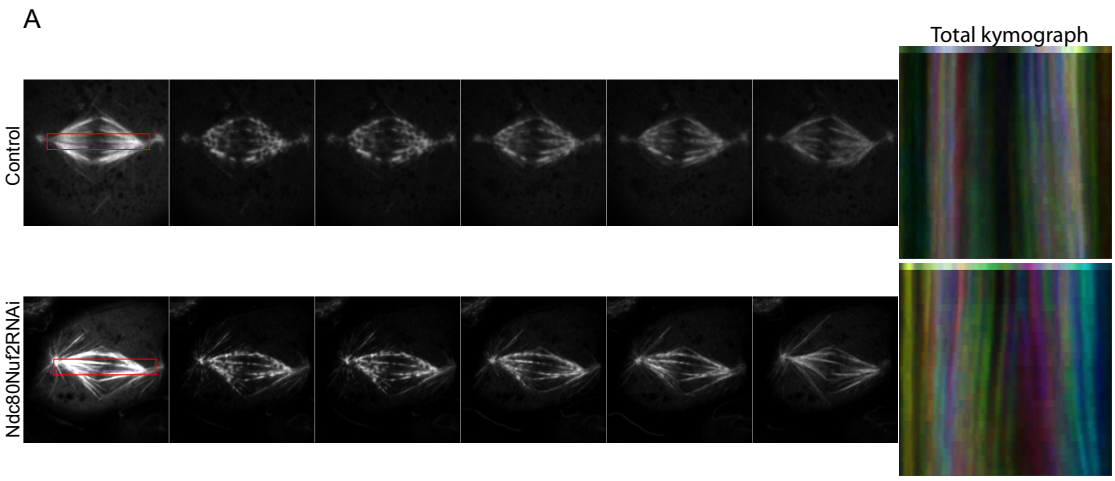
Figure 3.9 (A) Outcome of chromosome segregation in different conditions. The graph reflects the final position of pairs of kinetochores relative to initial position at time of anaphase onset from the time-lapse movies of S2 cells stably expressing GFP–Tubulin and CID–mCherry recorded every 30s to evaluate chromosome segregation. Each kinetochore pair is represented by two dots of the same colour (B) Average velocity of individual kinetochores during anaphase. Anaphase was divided in two phases, 0-2 minutes and 2-5 minutes. Error bars represent SEM.

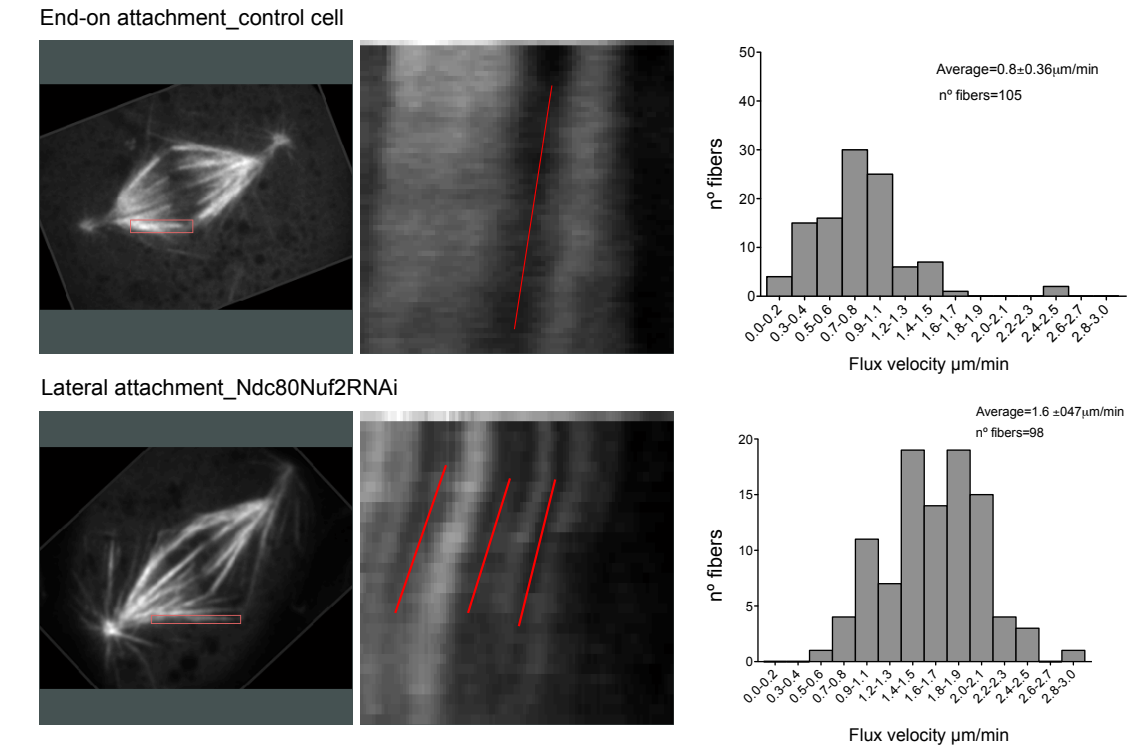
Manual kinetochore tracking, allowed us also to calculate the initial and final position of kinetochores within the cell. This analysis showed that chromosome segregation after depletion of Spc105 is significantly more compromised than depletion of any other KMN component (Fig. 3.9 A). We then evaluated the ability of chromosomes to move along spindle microtubules by determining their velocity during anaphase. We find, as expected, that the average velocity of kinetochores during anaphase for cells depleted of any KMN component is much slower than in controls. However, it is surprising that even after depletion of Spc105 kinetochores are still able to migrate polewards, although with a velocity that is about half of control kinetochores. Further analysis revealed that kinetochores depleted of either Mis12 or Ndc80 although showing a slower poleward movement when compared to control cells, display first a slow phase followed by a faster phase, while after depletion of Spc105 no differences are observed between these two time intervals (Fig. 3.9 B). These results show that depletion of Mis12 or Ndc80 do not prevent kinetochores from showing significant

poleward movement while depletion of Spc105 severely compromise the ability of kinetochores to move along microtubules.

2.6 Lateral interactions display an increased poleward “flux”

Proper interactions between kinetochores and microtubules are responsible for the correct biorientation and segregation of chromosomes. Poleward flux is present in both ipMTs and kMTs being slower in the last ones (Maddox et al., 2003). We investigated whether lateral attachments resulting from KMN protein depletion have any impact in flux velocity. To address this question we simultaneously depleted Ndc80 and Nuf2. We choose to deplete these two KMN network components since depletion of the Ndc80 complex do not seem to affect other proteins of outer kinetochore as CENP-E that have been already reported as affecting flux (Maffini et al., 2009). To test this possibility an improved methodology for speckle microscopy (Inducible Speckle Imaging or ISI) was used in S2 cells stably expressing GFP-tubulin and CID-mCherry. Kymographs were aligned placing the mitotic spindle parallel to an imaginary X-axis. CID-mCherry signal at kinetochores was used as a reference for the lateral attachments of kinetochores to microtubules, allowing us to determine the rate of plus-end microtubule polymerization. The frames were taken every 2 sec for a period of 2 minutes (Fig. 3.10 A and B). In control cells kMTs flux is 0.8±0.36 μm/min (supplementary movie S5) where microtubules laterally attached to kinetochores in Ndc80Nuf2 RNAi exhibited a flux of 1.6±0.17 μm/min (supplementary movie S6) (Fig. 3.10 A and B).


A
Total kymograph
Control
Ndc80Nuf2RNAi


B
End-on attachment_control cell
Average=0.8±0.36µm/min
nº fibers=105
nº fibers
Flux velocity µm/min
Lateral attachment_Ndc80Nuf2RNAi
Average=1.6 ±047µm/min
nº fibers=98
nº fibers
Flux velocity µm/min

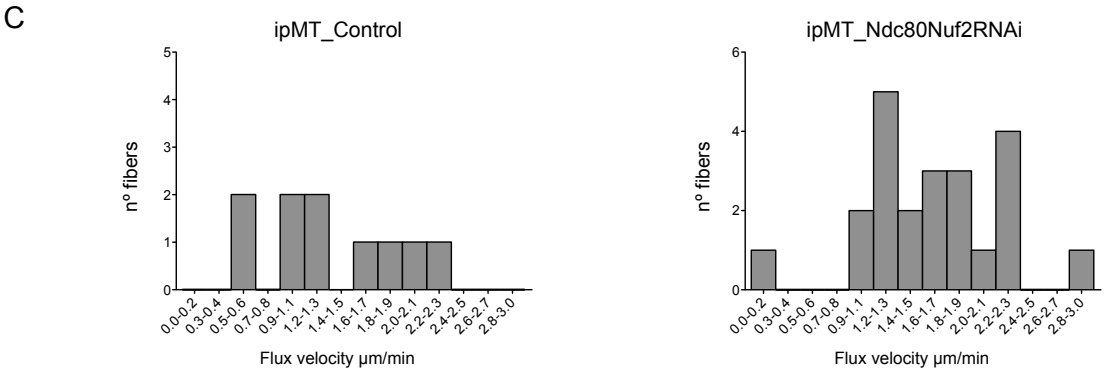

C
ipMT_Control
nº fibers
Flux velocity µm/min
ipMT_Ndc80Nuf2RNAi
nº fibers
Flux velocity µm/min

Figure 3.10 Microtubule flux of lateral interactions. To carry on the experiment a S2 cell line stable expressing GFP-tubulin and CID-mcherry was used. (A) lSI images of control cells and Ndc80Nuf2 depleted cells. First frame before bleaching and consecutive frames reveal the dynamicity of microtubules. the Total cell kymographs are also represented. (B) Detailed lSI experiment and quantifications of end-on attachment in control cells and lateral attachment after depletion of Ndc80/Nuf2 with the respective kymograph of the fiber highlight in red. Average (± s.d.) of three independent experiments (n=105, control end-on attached fibers; n=98, Ndc80Nuf2RNAi lateral attached fibers). (C) Quantification of ipMT in control and Ndc80/Nuf2 depleted cells.

The flux of microtubules in lateral attachments is double when compared to normal end-on attachments. It was not possible to quantify the lateral attachments in control cells since lateral attachments are converted to end-on very rapidly during early prometaphase. However when we analysed the interpolar microtubules in both conditions the poleward flux is very similar, suggesting that the differences observed between control and Ndc80Nuf2 depleted cells are due to the nature of the attachments (Fig. 3.10 C).

2.7 Spindle Assembly Checkpoint after depletion of KMN network proteins

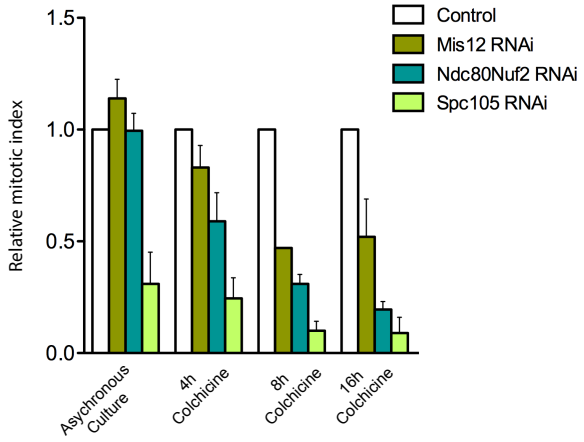
Recent studies indicate that SAC proteins bind to specific receptors within the KMN network (Kiyomitsu et al., 2007; Kline et al., 2006; Martin-Lluesma et al., 2002; Janke et al., 2001). It has been shown that Bub1 and BubR1 recognize the KI motifs within Spc105 in human cultured cells and an impairment of SAC is observed when Spc105 is depleted (Krenn et al., 2012; Bolanos-Garcia et al., 2011; Kiyomitsu et al., 2011). This suggests that kinetochore-microtubule interaction is directly monitored by the SAC (review in Foley and Kapoor, 2013). However, depletion of either Mis12 or Nsl1 has been associated with a delay in mitotic exit in *Drosophila* mutants (Venkei et al., 2011). 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 yet been confirmed (Schittenhelm et al., 2009). In order to further understand the role of KMN network in SAC response we quantified the mitotic index in control versus Mis12, Ndc80 or Spc105 depleted cells

either in asynchronous culture or colchicine treated cells. Despite the chromosomes fail to align upon depletion of each of the KMN network components, cells were not arrested in mitosis in asynchronous culture, since the mitotic index did not differ between control cells and the KMN network depleted cells. The only difference observed was a reduced mitotic index in asynchronous culture for Spc105 depleted cells. When SAC was challenged by treating cells with microtubule poisons (colchicine), an effect on mitotic index was already present for Ndc80 depleted cells after 4h of treatment where only after 8h a decreased in the mitotic index was observed in Mis12 depleted cells. In Spc105 depleted cells the mitotic index is dramatic decreased for all time point treatments after colchicine treatment. These results show that for Ndc80 or Spc105 depleted cells the SAC is severely compromised and a weakened checkpoint phenotype was observed in Mis12 depleted cells (Fig. 3.11 A).

To address further the relationship between SAC proteins and the KMN network, we determined whether depletion of KMN components leads to premature exit from mitosis by *in vivo* time-lapse microscopy using S2 cells stably expressing GFP-Tubulin and the centromere marker CID-mCherry. Control and RNAi treated cells were filmed from mitotic entry until telophase and the time from NEBD to anaphase onset was determined (Fig. 3.11 B). Our results show that Ndc80/Nuf2 or Spc105 depleted cells exhibit premature mitotic exit (18.12 ±5.5 min and 17.04±3.5 min, respectively) when compared to (26.44±8.25 min) in control cells (Fig. 3.11 B and C). However, cells depleted of Mis12 take on average more time to exit mitosis (35.68±9.88 min) than either control cells or cells depleted of Ndc80/Nuf2 or Spc105 (Fig. 3.11 B and C). These observations suggest that after Mis12 depletion sufficient levels of SAC proteins can still localize at kinetochores to generate a wait anaphase signal. To test this hypothesis we co-depleted Mis12 and different SAC proteins. The depletion of ZW10 was used to prevent Mad2 from localizing to kinetochores (Buffin et al., 2005). The results clearly show that the delay in mitotic exit after depletion of Mis12 is SAC dependent, since delocalization of Mad2 (depletion of ZW10) or co-depletion with BubR1 or Mps1 abrogates the delay (Fig. 3.11 B and C). Additionally, we also analysed the behavior of S2 cells when we co-depleted Mis12, Ndc80 and Nuf2. This

resulted in cells not being able to sustain SAC activity exiting mitosis 14 minutes after NEDB (Fig. 3.11 B and C). Interestingly, the time from NEBD to anaphase onset for Ndc80/Nuf2 and Spc105 RNAi are very similar.

A



B

	Mitotic Time (min)		StDv (min)
Control	26.44		8.25
Mis12RNAi	35.68	**	9.88
Ndc80Nuf2RNAi	18.12	*	5.5
Spc105RNAi	17.04	**	3.5
Mis12ZW10RNAi	16.15	**	4.17
Mis12BubR1RNAi	16.22	**	4.94
Mis12Mps1RNAi	12.16	***	3.08
Mis12Ndc80Nuf2RNAi	14.25	**	4.48

Statistically sgnificant: *** p<0,0001, **p<0,001, *p<0,01

C

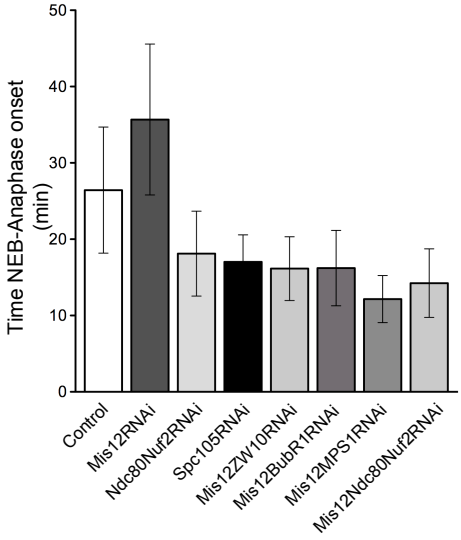
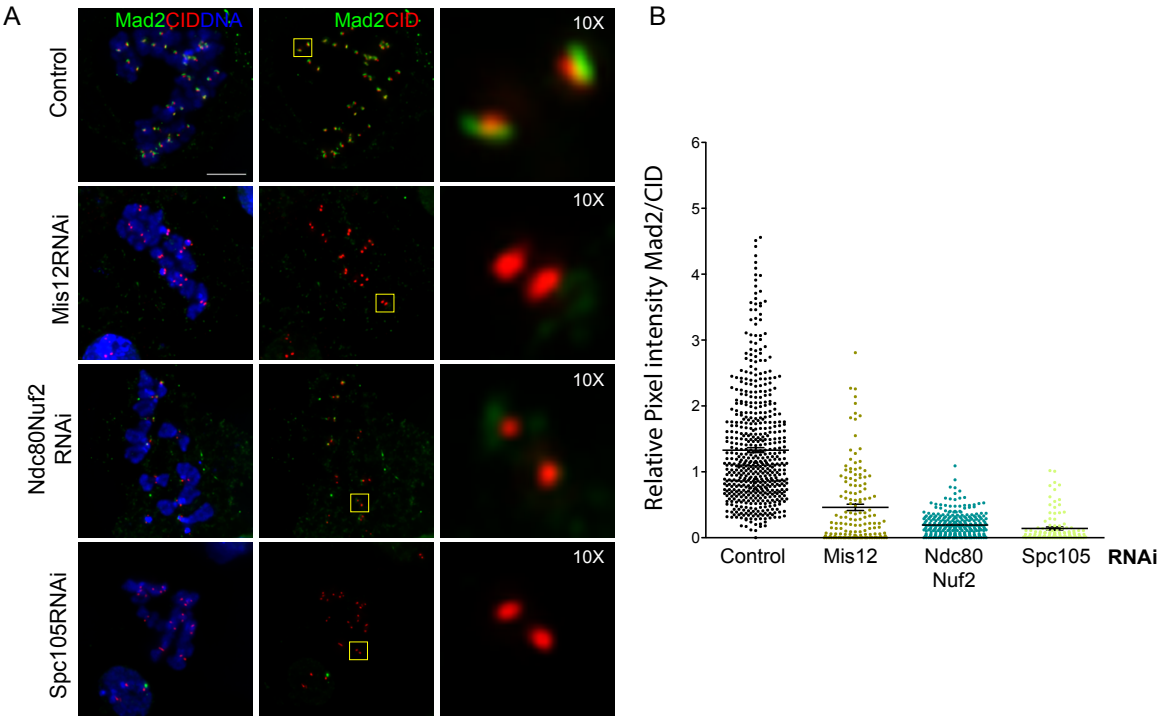
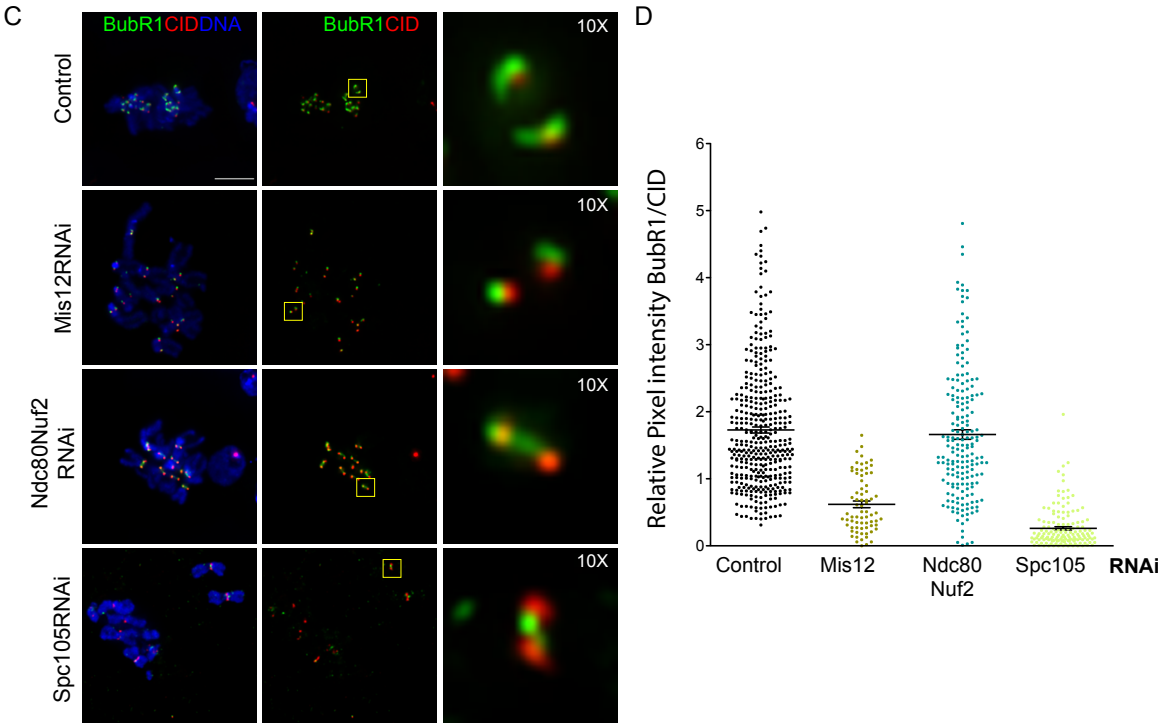


Figure 3.11 SAC response after depletion of different KMN network components. (A) Mitotic index in asynchronous culture and after different incubation time with colchicine, quantifications were made using α-tubulin/PH3 double staining (B) Table and (C) graphical representation of the quantification of mitotic time in control and after depletion of different KMN network components on their own or after co-depletion of different SAC proteins. Mitotic timing was determined from NEBD to anaphase onset in cells stably expressing GFP–Tubulin and CID–mCherry. Values in the graphs represent mean ± SD from at least 10 different cells for each experimental condition.


A
Mad2CIDDNA
Mad2CID
10X
Control
Mis12RNAi
Ndc80Nuf2 RNAi
Spc105RNAi
B
Relative Pixel intensity Mad2/CID
Control
Mis12
Ndc80 Nuf2
Spc105
RNAi


C
BubR1CIDDNA
BubR1CID
10X
Control
Mis12RNAi
Ndc80Nuf2 RNAi
Spc105RNAi
D
Relative Pixel intensity BubR1/CID
Control
Mis12
Ndc80 Nuf2
Spc105
RNAi

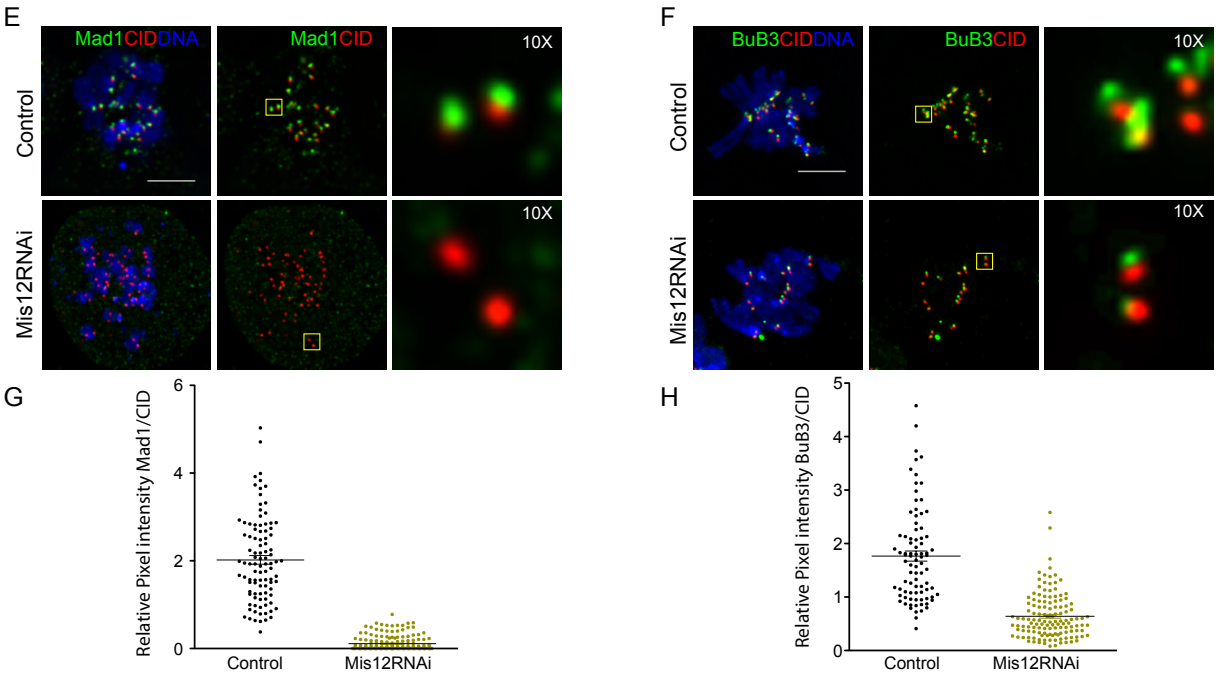


Figure 3.11 SAC proteins localization after KMN network depletion. Levels of SAC proteins by immunofluorescence of SAC proteins (A) Mad2 (C) BubR1 (E) Mad1 (F) BUB3 at kinetochores in S2 cells after depletion of KMN network components. . Mad2 and BubR1, Mad1 and BUB3 (green), CID (red) and DNA (blue). Magnifications of selected regions are shown on the right panel. Scale bar is 5μm. (B, D, G and H) Quantification of the mean pixel intensity of Mad1, Mad2, BubR1 and BUB3 at kinetochores relative to the centromeric signal CID using immunofluorescence images shown in (A, C, E and F) where each dot represents an individual kinetochore, n>100 from two independent RNAi experiments. Error bars represent SEM.

In order to confirm these results and to study the differences observed in the mitotic timing, we analysed the localization of the SAC proteins Mad2 and BubR1 in the absence of various KMN network components. Given the variability of labelling in asynchronous culture, quantification of Mad2 levels was only possible after cells were treated with MG132 and Colchicine (Fig. 3.11 A). Additionally the levels of Mad1 and BUB3 were also tested for Mis12 depleted cells (Fig. 3.11 E-H). After depletion of Mis12, Ndc80/Nuf2 or Spc105 we observed a strong reduction of Mad2 levels (Fig. 3.11 A and B). The levels of BubR1 were only affected upon depletion of Mis12 and Spc105 (Fig. 3.11 C and D) but remained unaffected after Ndc80/Nuf2 depletion, as previously reported by our group. Interestingly, the same study described that Mps1 depends on Ndc80/Nuf2 for kinetochore localization (Conde et al., 2013). Moreover the levels of Mad1 and BUB3 are reduced in Mis12 depleted

cells (Fig. 3.11 E-H). Together our results suggest that the impaired SAC upon Ndc80 complex and Spc105 depletion is due to an inability of the checkpoint proteins Mad2 and BubR1 to localize to kinetochores and the delay in mitotic exit after Mis12 depletion could depend on a more heterogeneous levels of Mad2, BubR1, BUB3 or Mps1 at the kinetochores.

3. DISCUSSION

Here we show that KMN network components play different roles in kinetochore-microtubule interaction and checkpoint signalling during progression through mitosis. Spc105 plays a major role in microtubule attachment probably by providing a platform for microtubule binding, while Ndc80 appears to be involved in transforming a lateral interaction into an end-on kinetochore-microtubule attachment. Furthermore, dynein and CENP-meta appear to confer stability to the lateral kinetochore-microtubule interactions. The role of Mis12 is more complex since it appears to affect the overall stability of the KMN network both with respect to microtubule binding and checkpoint signalling.

The assembly of the KMN network has been previously studied including in *Drosophila* (Venkei et al., 2012; Schittenhelm et al., 2009; Przewloka et al., 2007) and although the kinetochore localization dependencies within the KMN network are conserved among species, there are some exceptions, such as Ndc80, which does not depend on Spc105 for its kinetochore localization (Liu et al., 2010; Cheeseman et al., 2008; Kiyomitsu et al., 2007). In fact, the phenotype caused by the absence of Spc105 in human cultured cells does not display the kinetochore null phenotype observed in other organisms such as *C. elegans* and *Drosophila* (Przewloka et al., 2007; Cheeseman et al., 2004). We have also analysed the dependencies of recruitment between KMN network components in mitosis and although our results are mostly in accordance with the previous studies, some important differences were identified. We find that Mis12 localization is partially affected by the Ndc80 complex. Implying that Mis12 is not only

involved in anchoring the KMN network within the centromere (reviewed in Cheeseman and Desai, 2008) but it is itself also stabilized by the outer kinetochore domain.

Previous studies have suggested that loss of any KMN network component leads to loss of stability of kinetochore fibers and microtubule attachment (Guimaraes et al., 2008; Miller et al., 2008; Kiyomitsu et al., 2007; Kline et al., 2006; DeLuca et al., 2005; DeLuca, 2002). Our study shows that when different KMN network components are absent, at least within the limitations of a knockdown experiment, kinetochores can still interact with microtubules but they do so through lateral attachments. In order to test whether these lateral attachments were due to well-known kinetochore motor proteins we co-depleted dynein and CENP-E, two proteins that still localize to kinetochores upon depletion of the Ndc80 complex (DeLuca et al., 2005). Our results show that these proteins are not involved in the establishment of the lateral interactions. This observation led us to investigate to what extent these lateral interactions were functional. Our data shows that depleted cells were still able to exhibit cold stable kinetochore fibers. Interestingly, it has been already observed that human cultured cells, where only Spc25 has been depleted, exhibit stable kinetochore fibers after treatment with calcium and cold (Bharadwaj et al., 2004). In *Drosophila*, we find that depletion of Spc105 has a much more severe effect on microtubule attachment than depletion of Mis12 or Ndc80. Furthermore, when we co-depleted the proteins ZW10 and CENP-meta together with Ndc80 complex. Although lateral attachments can be formed they are not stable suggesting a synergistic effect upon kinetochore-microtubule interactions between KMN components and these motor proteins. The nature of the lateral interactions were studied by subjecting the cells to the MG132-Taxol assay and the results show complete loss of attachments after depletion of Spc105, in accordance with previous reports (Cheeseman et al., 2008; Kiyomitsu et al., 2007). However, neither the depletion of Mis12 nor the Ndc80 complex resulted in a complete loss of lateral attachments. Presumably, in the absence of Mis12 or Ndc80, microtubules are able to bind the kinetochore through direct interactions with Spc105, which has been previously shown to bind microtubules *in vitro* (Cheeseman et al., 2006) or with proteins that require Spc105 for their kinetochore localization.

Interestingly, *in vivo* functional analysis showed that, at least in cells depleted of Mis12 or the Ndc80 complex, kinetochores that bind laterally move poleward along microtubules during anaphase. Nevertheless, as previously reported, depletion of any KMN network proteins led to severe problems in chromosome alignment, biorientation and segregation (Venkei et al., 2011; Przewloka et al., 2007; Kline et al., 2006; Cheeseman et al., 2004; Goshima et al., 2003; Obuse et al., 2004b). Additionally, we observe that in *Drosophila*, depletion of Spc105 causes a severe loss in cell viability, which is not observed after depletion of either Mis12 or Ndc80 (data not shown). Interestingly, our flux analysis revealed a 2-fold increase in Ndc80Nuf2 depleted cells (1.6±0.17 µm/min) when compared to control cells (0.8±0.36 µm/min). This is the first time that has been described an increase in flux upon depletion of kinetochore proteins. Previously, it has been reported that the depletion of CLASP lead to impairment of microtubule subunit incorporation into K-fibers at the kinetochore, whereas proteins such as KLP10A/Kif2a regulate microtubule depolymerisation at the poles (Matos et al., 2009; Maiato et al., 2005). Our study also reveals that besides the increased flux in lateral interaction the poleward velocity of chromosomes during anaphase is decreased when compared to end-on attachments (Fig 3.9 B). These observations suggests that when microtubules are not end-on attached to kinetochores, the flux is identical to interpolar microtubules since there are no kinetochores offering resistance to the entry of tubulin subunits.

During progression through mitosis, cells monitor the interaction between kinetochores and microtubules through the activation of the SAC, preventing mitotic exit if chromosomes are not properly attached (reviewed in Foley and Kapoor, 2013). SAC proteins monitor kinetochore attachments through transient accumulation at unattached kinetochores resulting in the production of an inhibitor of mitotic exit. It has been shown that KNL1/Spc105 provides the surface for the interaction of Bub1 and possible BubR1 with kinetochores (Krenn et al., 2012; Kiyomitsu et al., 2011; Bolanos-Garcia et al., 2011). It has also been suggested that localization of Mps1 requires the Ndc80 complex. The localization of Mad1/Mad2 complex appears to involve the RZZ complex (Buffin et al., 2005). However, our analysis indicates that the levels of SAC proteins after depletion of KMN components do not fully explain the

differences observed. Mad2 levels are reduced in Mis12 and Ndc80/Nuf2 RNAi and almost absent in Spc105 RNAi, whereas BubR1 levels are decreased in Mis12, almost absent in Spc105 depleted cells and normal for Ndc80/Nuf2 RNAi. Indeed, these results differ from those previously published (Schittenhelm et al., 2009) where Spc105 mutant embryos analysed during mitosis do not seem to abolish SAC function and levels of BubR1 are still detected at kinetochores. It is however possible that the mutant used was not a null allowing a low but significant level of proteins that could contribute towards a partial functional SAC.

Previously, it has been reported that impairment of KNL-1 at kinetochores, either in *C.elegans* or budding yeast, inhibits the formation of load-bearing attachments (Pagliuca et al., 2009). However, recently it has been shown in *C.elegans*, that the extreme N-terminus responsible for the microtubule binding activity of KNL1 is not necessary either for load-bearing attachment formation or checkpoint activation but instead plays a role in checkpoint silencing at the kinetochore (Espeut et al., 2012). Our results also show that depletion of Spc105 has a more dramatic effect in chromosome segregation and cell viability when compared to depletion of other KMN network components. This phenotype cannot be explained purely based on the dependencies of recruitment of KMN network components. However, the impact of Spc105 in the organization of the outer kinetochore could to some extent explain the severity of the phenotype.

We conclude that Spc105 plays a fundamental role for the stability of the kinetochore-microtubule interaction while other KMN network components appear to contribute to orient microtubules properly and to allow segregation to opposite spindle poles. Furthermore, at least in *Drosophila*, dynein and CENP-meta contribute for the stabilization of lateral attachments. The role of different KMN components in the SAC is becoming clearer, however, further work is necessary to determine why depletion of Mis12 still allows a partially functional checkpoint.

4. MATERIAL AND METHODS

4.1. Double-Stranded RNA interference in *Drosophila* S2 cells

The depletion of the proteins in *Drosophila* S2 cells by dsRNA was performed as previously described in Material and Methods Chapter 2. 30μg of dsRNA was used to deplete Mis12, Ndc80, Nuf2 and Spc105 proteins. At selected time points, cells were collected and processed for immunofluorescence, time-lapse microscopy and immunoblotting.

Protein	Sequence
Mis12	**FW** 5' TAATACGACTCACTATAGGGATGGACTTCAATAGCCTAGCC 3'
	RV 5' TAATACGACTCACTATAGGGTTAATCAGTCTCCTTCTTTAT 3'
Ndc80	**FW** 5' TAATACGACTCACTATAGGGCTGGAGAATAAGTTGCATGATCC 3'
	RV 5' TAATACGACTCACTATAGGGCTCTTTATCAGGVACAAATCAC 3'
Nuf2	**FW** 5' TAATACGACTCACTATAGGGGCAAGGTGGATGATTACAAAGAG 3'
	RV 5' TAATACGACTCACTATAGGGTTAAGTGGAATTCATCTGCCAGT 3'
Spc105	**FW** 5' TAATACGACTCACTATAGGGAACCTATGGAAGAGGAAATGAGC 3'
	RV 5' TAATACGACTCACTATAGGGTAATAATAGCGTGTGCCTCGATT 3'
Zw10	**FW** 5' TAATACGACTCACTATAGGGTGGCACCTACGTTCGATT 3'
	RV 5' TAATACGACTCACTATAGGGATCATGCAGCGTGGGAAG 3'
CENP-meta	**FW** 5' TAATACGACTCACTATAGGGTGTTCCCGTCTTTCAACTGG 3'
	RV 5' TAATACGACTCACTATAGGGTCGCCTCTTTAGAGCCAACC 3'
BubR1	**FW** 5' - TAATACGACTCACTATAGGGAGCTTTTTAAATCGACACAGGG - 3'
	RV 5' - TAATACGACTCACTATAGGGAGTAGATGCTTAGTTCCGACGC - 3'
Mps1	**FW** 5' - TAATACGACTCACTATAGGGTCTTCCAAACACCTATGACCG - 3'
	RV 5' - TAATACGACTCACTATAGGGCGTTTAGATATCCCTGCACCA - 3'

4.2 Cell culture, RNAi and drug treatment

S2 cells were grown in Schneiders's medium (Sigma) supplemented with 10% foetal bovine serum (GIBCO) without antibiotics, at 25ºC. RNAi was performed in *Drosophila* S2 tissue culture cells as previously described

(Maiato et al., 2003). 30μg of dsRNA was used to deplete Mis12, Ndc80, Nuf2 and Spc105 proteins. At selected time points, cells were collected and processed for immunofluorescence, time-lapse microscopy and immunoblotting. Treatment with drugs was performed as follows: 20mM MG132 (Calbiochem) for 2 hours to inhibit the proteosome and arrest cells in mitosis in a checkpoint independent manner; 30mM colchicine (Sigma-Aldrich) for 1hour to induce microtubule depolymerization and 100nM taxol (Sigma-Aldrich) for 3 hours to promote microtubule stabilization, before cells were collected for immunofluorescence analysis.

MG132-Taxol assay.

Cells were incubated with 20mM of MG132 (Calbiochem) during 1 hour and then with 100nM of taxol (Sigma-Aldrich) for 3 hours, which induces the collapse of the mitotic spindle into a monopolar structure with all the chromosomes localized around the aster allowing an easy read-out of the microtubule-kinetochore attachments; as described in (Maia et al., 2007). Cells were then processed for immunofluorescence as described below.

Cold-Treatment assay.

$1.0x10^5$ S2 cells were plated in coverslips coated with 100mg/ml concanavalin A (Sigma-Aldrich) in 6 wells plate and incubated with 20mM of MG132 (Calbiochem) for 2 hours. The 6 wells plate was placed on ice for 10 minutes to depolymerize all microtubules except the fibers stably attached to chromosomes. Cells were then processed for immunofluorescence as described below.

4.3 Immunofluorescence in S2 cells

The approach was the same used in Material and Methods of Section 2.

4.4 SDS-PAGE and Western Blot

The approach was the same used in Material and Methods of Section 2.

4.5 Antibodies

The primary antibodies used for immunofluorescence were newly generated anti-Mis12 rabbit and rat (1:4000); anti-Mad2 (Rb 1223) (1:10), anti-BubR1 (Rb 666) (1:1500) (Logarinho et al., 2004); anti-phospho Histone H3 (Ser10) (Upstate) (1:1000), anti α-tubulin (clone B-5-1-2) (Sigma) (1:5000), anti-CENP-C (Rb1) (Heeger et al., 2005) (1:10000), anti-Ndc80 and anti-Nuf2 (kindly provided by Byron Williams and Michael Goldberg) (1:1500); anti-Spc105 (sheep) (Venkei et al., 2011) (1:500), anti-Spc105 (rat) (Conde et al., 2013) (1:2000) and anti-CID antibody (C.E.S. and Sore Steffensen, IBMC, University of Porto, Portugal, unpublished) (1:2000). Secondary antibodies used for immunofluorescence were Alexa 488, Alexa 568, Alexa 647 from mouse, rabbit, rat, guinea pig and sheep (Molecular Probes) (1:2000). Primary antibodies used for immunoblotting were anti-α-tubulin mouse DM1A (Sigma-Aldrich), anti-Mis12, anti-Ndc80, anti-Nuf2 (Byron Williams and Michael Goldberg) (1:5000) and anti-Spc105 sheep (Venkei et al., 2011) (1:8000). Secondary antibodies used for immunoblotting were HRP mouse, rabbit, rat, guinea pig and sheep (Vector Laboratories).

4.6 Time-lapse fluorescence imaging of S2 cells and Chromosome tracking

Depletion of Mis12, Ndc80, Nuf2, Spc105, Zw10, Mps1 was performed in S2 cells stably expressing GFP-Tubulin and CID-mcherry (Coelho et al., 2008) using dsRNA. Cells were then plated at least 15 minutes on 35mm petri dishes (MatTek corporation) previously coated with 0.25 µg/µl concanavalin A (Sigma). Images were collected using a spinning disk confocal (Andor Revolution XD) with an electron multiplying charge-coupled device camera (iXonEM+; Andor) and a CSU-22 unit (Yokogawa) based on an inverted microscope (IX81; Olympus), and a 100X objective was used. Z stacks were acquired at 0.5µm steps every 30s. Acquisition parameters, as exposure time or steps, were controlled by IQ2.1.2 (ANDOR Technology, UK) software.

Image processing and movie assembly was processed using IQ 2.1.2 (ANDOR Technology, UK) software.

4.7 Overlay Assay

Microtubule overlay assays were performed as previously described (Saunders et al., 1997). 500 ng per lane of recombinant Mis12, recombinant MAP's and BSA (Sigma) were fractionated by 12% SDS-PAGE and blotted onto Nitrocellulose membranes (WatProtman). The membranes were pre-incubated in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% low fat powdered milk for 1 h and then washed three times for 15 min in lysis buffer. The filters were then incubated for 30 min in lysis buffer containing 1 mM GDP, 1 mM GTP, or 1 mM GTP-S. MAP-free bovine brain tubulin (Cytoskeleton) was polymerized at a concentration of 2g/ml in lysis buffer by addition of GTP to a final concentration of 1 mM and incubated at 37ºC for 30 min. The nucleotide solutions were removed and the buffer containing polymerized microtubules added to the membranes for incubation for 1 h at 37ºC with addition of taxol at a final concentration of 10 M for the final 30 min. The blots were then washed three times with TBST and the bound tubulin detected using standard Western blot procedures using anti–tubulin antibodies (Sigma) at 2.5 g/ml and the ECL detection system.

4.8 Quantification of microtubule flux

The flux rate was measured by Inducible Speckle Imaging (ISI), which produces, at a specific moment a high-contrast speckle pattern (António J. Pereira PhD thesis). This new technique does not require low fluorescence samples and S2 cells stably expressing GFP-tubulin and CID-mCherry were used to perform the analysis (Coelho et al., 2008). By collecting images every 2 seconds at 25 ºC, using a spinning disk confocal system (ANDOR Technology) we could follow the movement of the fluorescent speckles within the spindle. This movement was represented on a kymograph, keeping the labelled kinetochores as the reference point. The slope of the lines obtained

from individual speckles was used to calculate flux rates on kinetochore microtubules. All kymographs were analysed using a program from a custom routine written in Matlab (Natick, MA) (Matos et al., 2009).

PART III

GENERAL DISCUSSION

Cell division is the key process that allows development and function of all living organisms. During this process cells undergo dramatic changes that during mitosis result in the equal segregation of the chromosomes so as to give rise to two genetically identical daughter cells. Errors in chromosomes segregation occurring during mitosis can result either in loss of cell viability or tumourigenisis. Thus mitosis must be a highly regulated process in order to guarantee faithful chromosome segregation.

The work presented in this thesis aimed to further our understanding on the individual contributions of the three complexes of KMN network in kinetochore-microtubule attachment and SAC signalling, as well as the role played by the kinetochore as a vehicle through which the chromosomes move within the mitotic spindle during early stages of congression and what are the contribution of these events for a correct chromosome segregation.

The work in the first chapter of this thesis came in the sequence of previous findings. In *Drosophila* S2 cells, CENP-meta/CENP-E seems to promote destabilization of kinetochore-microtubule attachment (Maia et al., 2007). Interestingly, previously it has been suggested that CENP-E could contribute for the stability of kinetochore microtubule attachment (Putkey et al., 2002), nevertheless, it was demonstrated by fluorescence decay after photoactivation (FDAP) that kinetochores microtubules have a higher half-time turnover, in CENP-E depleted cells (Maffini et al., 2009). These data, in mammalian cells is in agreement with the results observed in *Drosophila* S2 cells where CENP-meta/CENP-E appears to promote kinetochore-microtubule instability (Maia et al., 2007). Surprisingly, the same work also demonstrated that upon depletion of BubR1, impairment on kinetochore-microtubule interactions was observed and that this destabilization was reverted after co-depletion of BubR1 and CENP-E/CENP-meta. Previously, it was already reported that PLK1 phosphorylates BubR1, which recruits PP2A-B56 phosphatase to kinetochores during prometaphase (Suijkerbuijk et al., 2012), resulting in the negatively regulation of Aurora B activity. However, how CENP-E participates in this pathway is still elusive. So, we hypothesized that the correction of defective microtubule attachment could be mediated by

CENP-E through a direct or indirect regulation of Aurora B. The results in chapter one of this thesis do not support this model. The chromosomes that fail to align and stay at spindle poles after CENP-E depletion exhibit high levels of Aurora B activity resulting in a continuous destabilization of kinetochore attachment. Instead, the high levels of active Aurora B on polar chromosomes is indicative that Aurora B might support CENP-E in its function of helping the sliding of the kinetochores along microtubule bundles during chromosome congression as was already proposed by others (Cai et al., 2009; Kapoor, 2006). We have shown that Aurora B kinase activity destabilizes the attachments of polar chromosomes present in CENP-E-depleted cells. Apart from activating the SAC, the destabilization of kinetochore-microtubule attachment seems to free monoriented or synthelic chromosomes so they can slide towards the metaphase plate and achieving amphithelic attachment. However in our and other studies, in CENP-E depleted cells, half of the cells are still able to congress their chromosomes to the metaphase plate and exit mitosis (Maia et al., 2010; Johnson et al., 2004; McEwen et al., 2001). The reason why this happens is most probably due to independent mechanisms for chromosome congression providing strong support for the existence of redundant mechanisms for such an important cellular function (Putkey et al., 2002; McEwen et al., 2001; Yucel et al., 2000). However, this model fails to explain how polar chromosomes with high levels o Aurora B are still able stabilize microtubule attachments and silence SAC. The role of CENP-E in microtubule destabilization has already been proposed (Maffini et al., 2009; Maia et al., 2007), so it could be that when CENP-E is not present, microtubules themselves became more stable (Yang et al., 2008). Alternatively, the kinetochore-microtubule interactions are less prone to be destabilized and the probability of 1-2 polar chromosomes to congress to the metaphase plate increases even in the presence of high levels of Aurora B. This model is supported by the recent data that demonstrated that in early prometaphase the levels of phosphorylated KMN network components by Aurora B are increased, however, these levels are much lower when compared to situations with no tension (Welburn et al., 2010). Moreover, the initial interactions between kinetochores and microtubules are lateral (Magidson et al., 2011) and the KMN network does not seem to participate in

these initial interactions (Magidson et al., 2011; Cai et al., 2009). Thus, Aurora B and B56-PP2A cannot disturb the initial attachment, giving time the cell to congress and switch from lateral to end-on attachment. Our results in the third part of this thesis also point in the same direction. We have demonstrated that upon depletion of KMN network components the interactions established between kinetochores and microtubules are lateral, even though, when subjected to cold or MG+Taxol treatment the attachments in Mis12 and Ndc80 depleted cells were surprisingly more stable that what initially thought (Feijão et al., 2013). Moreover, we have also shown that at least in *Drosophila* S2 cells, kinetochore dynein and CENP-meta/CENP-E are not involved in the initial lateral attachments in the absence of KMN network, contributing instead to their stability.

When we depleted Spc105 a more severe phenotype was observed, this phenotype can be explained taking in consideration two different results. First, in *Drosophila* the centromere-kinetochore structure evolved differently when compared to other species. The CCAN is not a conserved structure, being CENP-C the only conserved protein of the above-mentioned structure (Meraldi et al., 2006). In organisms where CCAN is conserved, the Ndc80 complex does not depend totally on the rest of KMN network to localize at kinetochores since it binds directly to the inner kinetochore through interaction with CENP-T (Nishino et al., 2013; Suzuki et al., 2011). Therefore in *Drosophila* S2 cells upon Spc105 depletion, the two known microtubule-binding activities are abolished. Second, the impairment of recruitment to kinetochores of checkpoint proteins, as well as proteins that are directly involved in kinetochore-microtubule attachment by depletion of Spc105 have been reported in different studies (Feijão et al., 2013; Chan et al., 2012; Krenn et al., 2012; Kiyomitsu et al., 2011; Gassmann et al., 2008). These two sets of results together can explain why in *Drosophila* S2 cells the depletion of Spc105 results in kinetochore null phenotype. A similar phenotype was also observed in S2 cells after depletion of CENP-C (Orr and Sunkel, 2010) or in C.*elegans* after depletion of KNL-1 (Desai et al., 2003). In fact, Spc105 has been described to be the platform for recruitment of different SAC proteins (Krenn et al., 2012; Kiyomitsu et al., 2011; Bolanos-Garcia et al., 2011). Ndc80, which has been described as necessary for the localization of Mps1 at

kinetochores (Conde et al., 2013; Martin-Lluesma et al., 2002), in our study also revealed to be SAC deficient. Despite of having the same phenotype in terms of SAC response, and apparently similar problems is chromosome alignment, biorientation and segregation, Spc105 depleted cells also display a severe loss in cell viability.

Supporting these findings our *in vivo* functional analyses demonstrated that either in Mis12 or Ndc80 depleted S2 cells kinetochores that bind laterally to microtubules still exhibit poleward movement during anaphase, where in Spc105 depleted cells this is severely impaired. These results cannot be explained taking in consideration the KMN network dependencies, since our results show that when Spc105 is removed, Mis12 is still able to localize at kinetochores and the reverse is also true. What is consensual between our and other studies in *Drosophila*, (Feijão et al., 2013; Venkei et al., 2012; Schittenhelm et al., 2009; Przewloka et al., 2007) is that Ndc80 needs both proteins to localize to kinetochores. Finally, in Mis12 S2 depleted cells we observed a slight mitotic delay that is somewhat suprising given that upon depletion of Mis12, the levels of Mad2, Mad1, BubR1 and Bub3 were significantly decreased. However, we were also able to demonstrate that when Mis12 was co-depleted with different checkpoint proteins, the delay was abolished, showing that perhaps this phenotype might be due to heterogeneous levels of SAC proteins at kinetochores.

The results of *Drosophila* Mis12 characterization reveal additional differences to previous studies. We demonstrated that Mis12 protein is not a constitutive protein at kinetochore as previously reported (Venkei et al., 2012; Kline et al., 2006; Goshima et al., 2003; 1999). We found that Mis12 delocalizes from the centromeric region as labeled by CID during a small interval in interphase during the G1/S or the S/G2 transition. Our analyses of Mis12 dynamics also showed a minor increase in the turnover of the protein during mitosis, however the mobile fraction was identical during interphase and mitosis. These results are in conflict with previous ones (Hemmerich et al., 2008; Joglekar et al., 2006) but further work is needed to resolve this issue.

The work presented in this thesis, suggests evolutionary adaptations in *Drosophila* kinetochore. Spc105 seems to share with the Ndc80 complex a

more active role in kinetochore-microtubule attachment, as well as maintaining the outer kinetochore identity. Additionally, Ndc80 together with Mis12 helps to reorient the kinetochore promoting conversion from lateral to end-on attachment also known to be essential to faithful chromosome segregation.

In conclusion, our studies have provided new insights on how chromosomes, through kinetochores make their way towards the metaphase plate and how can kinetochores delay mitotic exit when errors occur thus avoiding aneuploidy. Furthermore, how different kinetochore protein complexes can contribute to chromosomes attachment to the mitotic spindle and the final outcome on the process of cell division.

Part IV

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Part V

APPENDIXES

APPENDIX 1

Abbreviations

a.a.: aminoacids

Ab: Antibody

APC: Adenomatous Polyposis Coli

APC/C: Anaphase-Promoting Complex/Cyclosome

ATM: Ataxia telangiectasia mutated

BDGP: Berkeley *Drosophila* Genome Project

bp: base pairs

BSA: Bovine Serum Albumin

Bub: budding uninhibited by benzimidazole

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

CAK: Cdk-activating kinase

CCAN: Constitutive Centromere-Associated Network

cDNA: complementary DNA

Cdk: Cyclin-dependent kinase

CENP: Centromere-associated Protein

CID: Centromere identifier

CLASP: CLIP-Associated Proteins

CPC: Chromosomal Passenger Complex

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

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

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DPBS: Dulbecco's Phosphate Buffered Saline

DNA: deoxyribonucleic acid

dsRNA: double stranded RNA

E. coli: Escherichia coli

ECL: Enhanced ChemiLuminescence

EDTA: Ethylenediaminetetracetic acid

EGFP: Enhanced Green Fluorescent Protein

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

EM: Electron Microscopy

FBS: Fetal Bovine Serum

FDAP: Fluorescence Decay After Photoactivation,
FRAP: Fluorescence Recovery After Photobleaching

g: gram

G1: Gap phase 1

G2: Gap phase 2

GFP: Green Fluorescent Protein

HeLa: Human immortal cell line

HJURP: Holiday JUnction Recognition Protein

hr: hours

HRP: Horse redish peroxidase

Hsp: Heat-shock protein

IB: Immunoblotting

IF: immunofluorescence

ipMTs: interpolar microtubules

KBD: Kinetochore-Binding Domain

kDa: kiloDalton(s)

K-fiber: kinetochore fiber

Klp: Kinesin-like protein

kMTs: Kinetochore Microtubules

KNL1: kinetochore null

Kt: Kinetochore

L: Liter

LB: Luria-Bertani culture medium

M phase: Mitosis

M: Molar

Mad: Mitotic-arrest deficient

MAP: Microtubule Associated Proteins

MBD: Microtubule-Binding Domain

MCAK: Mitotic Centromere Associated Kinesin

MCC: Mitotic Checkpoint Complex

min: minutes

MIPs: Microtubule Interacting Proteins

Mis12

ml: mililiter

mM: milimolar

MPF: Maturation/Mitosis-Promoting Factor

mRFP: monomeric Red Fluorescence Protein

mRNA: messenger RNA

MT(s): Microtubule(s)

MTOC: Microtubule-Organizing Center

n: number of samples in the study

NEBD: Nuclear Envelope Breakdown

nm: nanometer

nM: nanoMolar

OD: Optical density

ORF: Open Reading Frame

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

PAGE: Polyacrilamide Gel Electrophoresis

PCR: Polimerase Chain Reaction

PEM: PIPES-EGTA-Magnesium Chloride buffer

PBS: Phosphate-buffered saline

RNA: ribonucleic acid

RNAi: RNA interference

rpm: Rotations per minute

ROD: rough deal

RT: room temperature

RZZ: Rod-ZW10-Zwilch

SAC: Spindle Assembly Checkpoint

SAFs: Spindle assembly factors

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

S phase: DNA synthesis phase

***S. pombe*:** *Schizosaccharomyces pombe*

S2: *Drosophila* Schneider 2 cell line

SCF: (Skp1/Cullin/F-box protein)

SD: standard deviation

SDS: Sodium dodecyl sulphate

SDS-PAGE: Sodium dodecyl sulfate-Polyacrylamide Gel Electrophoresis

sec: seconds

SEM: Standard Error of the Mean

TIM: Time In Mitosis

TPR: Tetracopeptide domain

Tris: Tris(hidroximethyl)aminomethane

t-test: Student's *t* test

UV: ultraviolet

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

YFP: Yellow Fluorescent Protein

µg: microgram

µl: microliter

µm: micrometer

µ**M:** microMolar

ZM447439: Aurora B kinase small-molecule inhibitor

ZW10: zeste white 10

+TIPs: Microtubule Plus-end Tracking Proteins

APPENDIX 2

Recipes

Buffer A

6M Guanidine Hydrochloride

20mM Tris-HCl

0,5M NaCl

5mM imidazole

1mM 2-mercaptoethanol, pH 8

Buffer B

8M urea

20mM Tris-HCl

0,5M NaCl

1mM 2-mercaptoethanol, pH 8

Isolation buffer:

2M urea

20mM Tris-HCl, pH 8

1mg/mL of lysozyme)

LB Medium:

1% Tryptone

0.5% Yeast extract

1% NaCl

LB Agar:

1.5% (w/v) Agar in LB medium

Ampicillin plates:

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

Tetracycline plates:

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

PBS (Phosphate-Buffered Saline) pH 7.4:

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

PEM:

100 mM Pipes
10 mM EGTA
1 mM MgCl2

2x SDS-PAGE sample buffer:

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

Protein Electrophoresis:

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

Ponceau S:

0.1% Ponceau
5% acetic acid

TBS:

50 mM Tris-HCl, pH7.5
150 mM NaCl

Transfer Buffer:

25 mM Glycine

192 mM Tris pH 8.3

Enhanced Chemiluminescent (ECL):

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

Solution B: 10 ml Tris 100 mM pH 8.5 and 6 µl H_2O_2 30% (Merck)

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

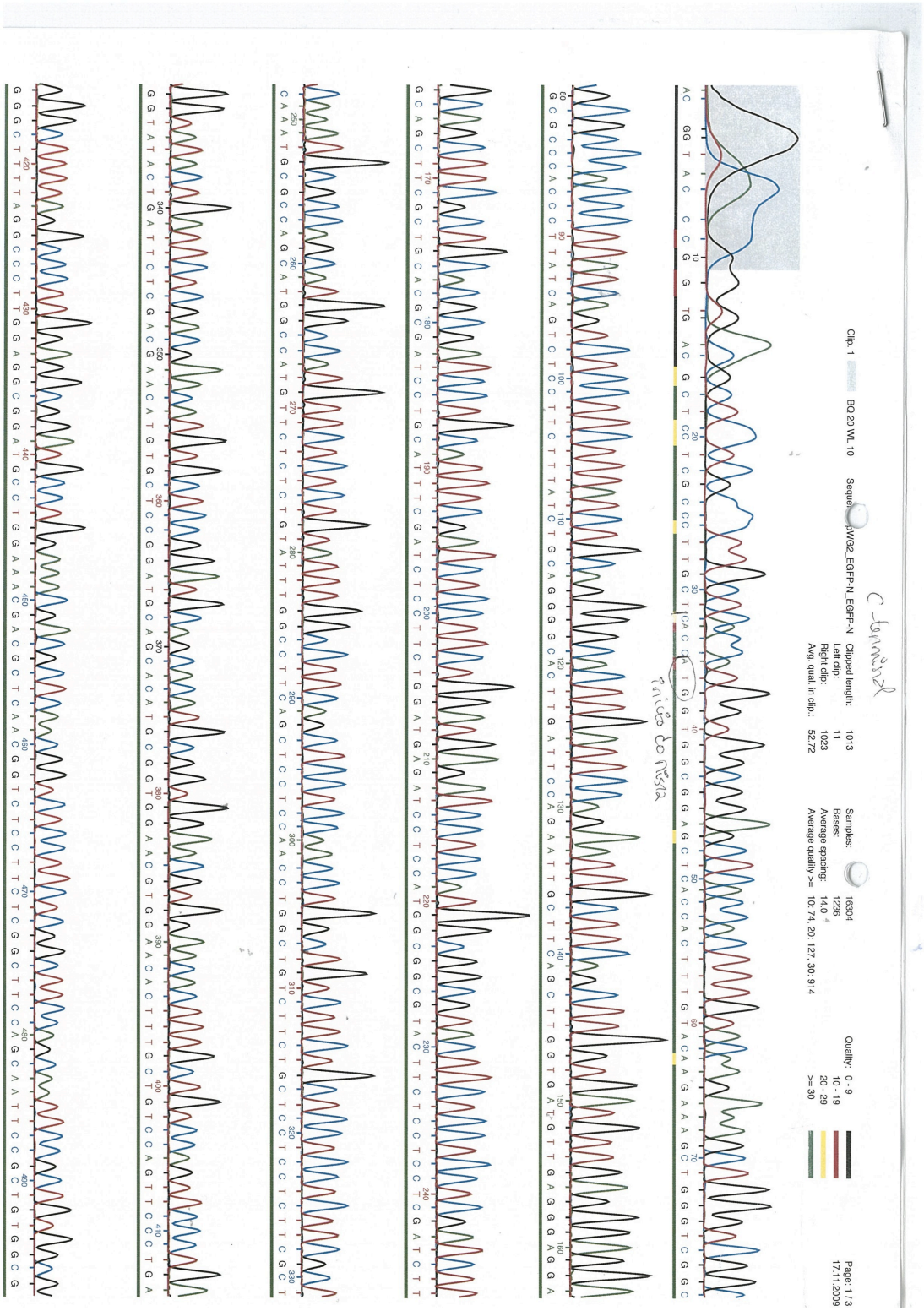
Schneider's Insect Medium:

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

APPENDIX 3

Plasmids

pGW-Mis12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BQ 20 Wi. 10
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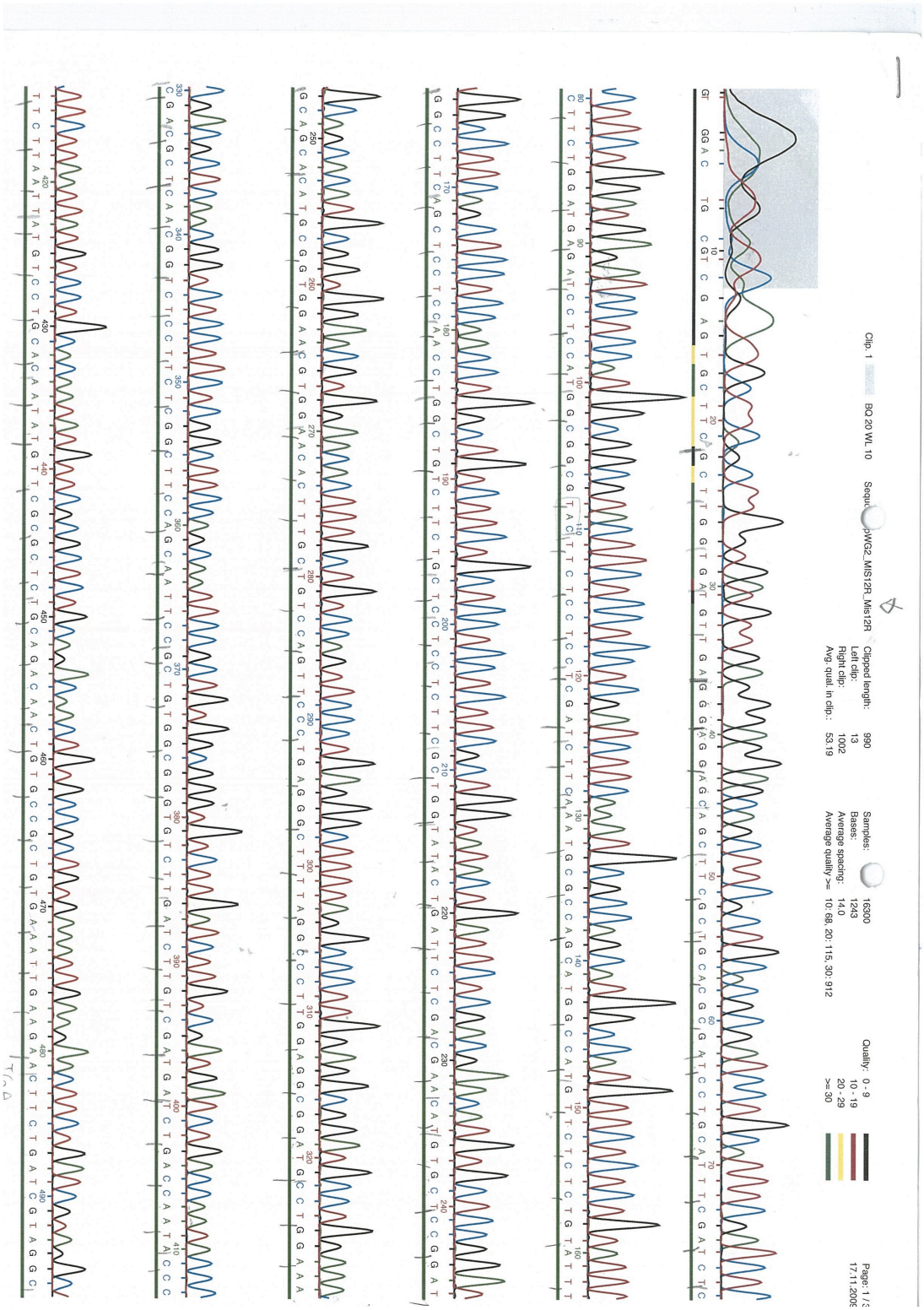
Right clip: 1023
Avg. qual. in clip.: 52.72
Samples: 16304
Bases: 1236
Average spacing: 14.0
Average quality >= 10: 74, 20: 127, 30: 914
Quality: 0 - 9
10 - 19
20 - 29
>= 30
Page: 1 / 3
17.11.2009

pWG-Mis12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Avg. qual. in clip.: 53 19
Samples: 16300
Bases: 1243
Average spacing: 14.0
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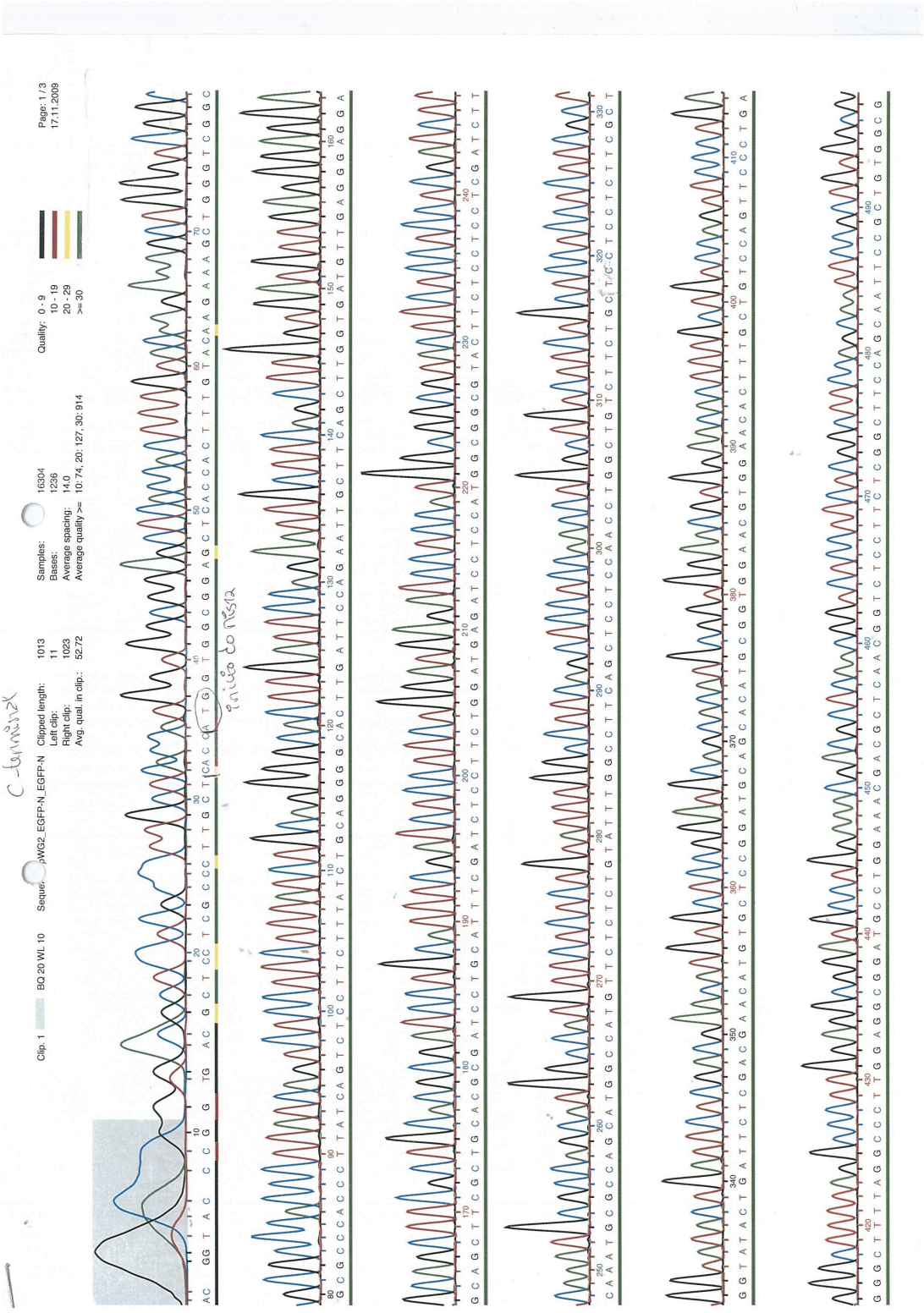
Quality: 0 - 9
10 - 19
20 - 29
>= 30
Page: 1 /
17.11.200

pWG-Mis12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Clipped length: 1013
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Right clip: 1023
Avg. qual. in clip.: 52.72
Samples: 16304
Bases: 1236
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Average quality >=: 10: 74, 20: 127, 30: 914
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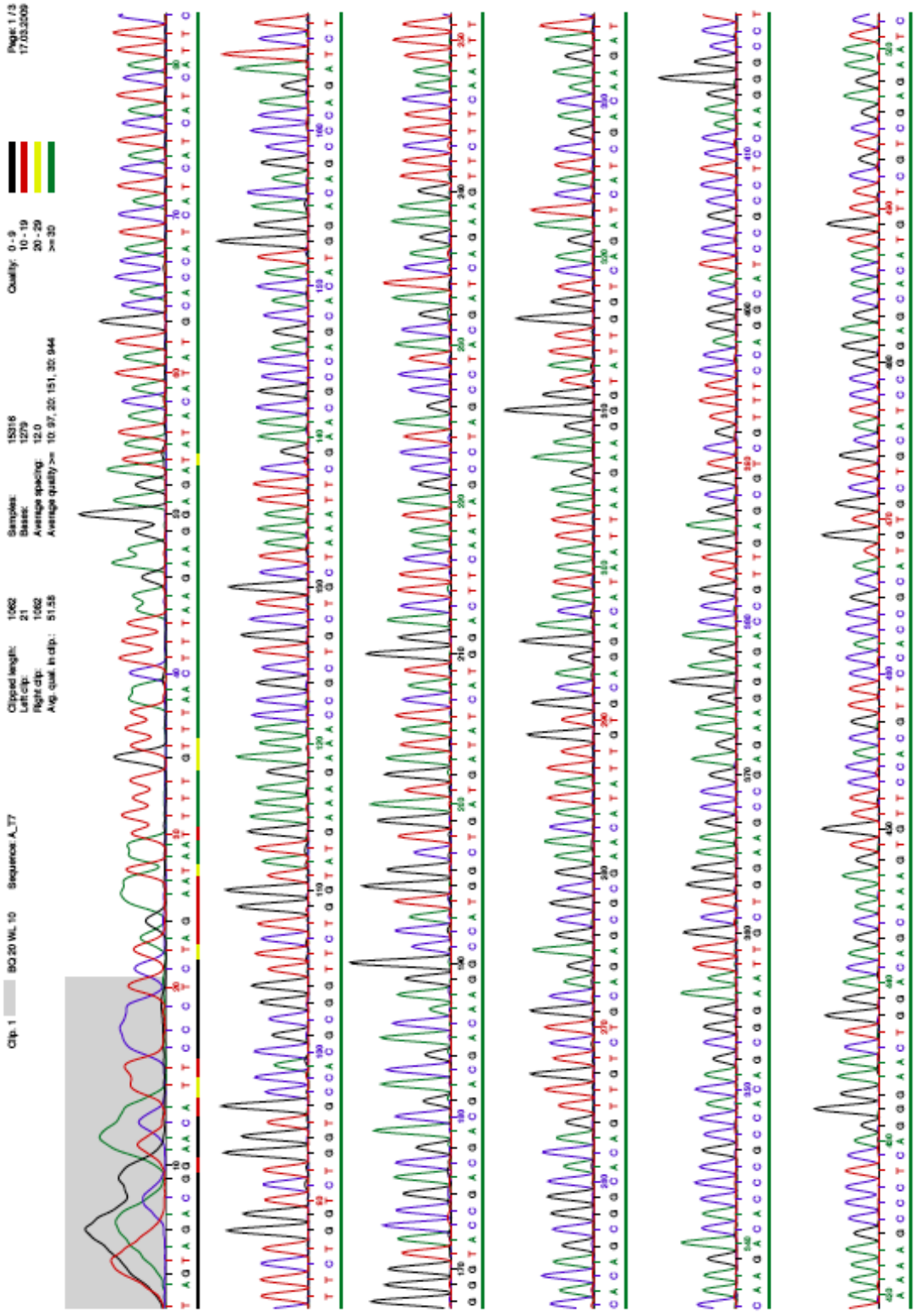
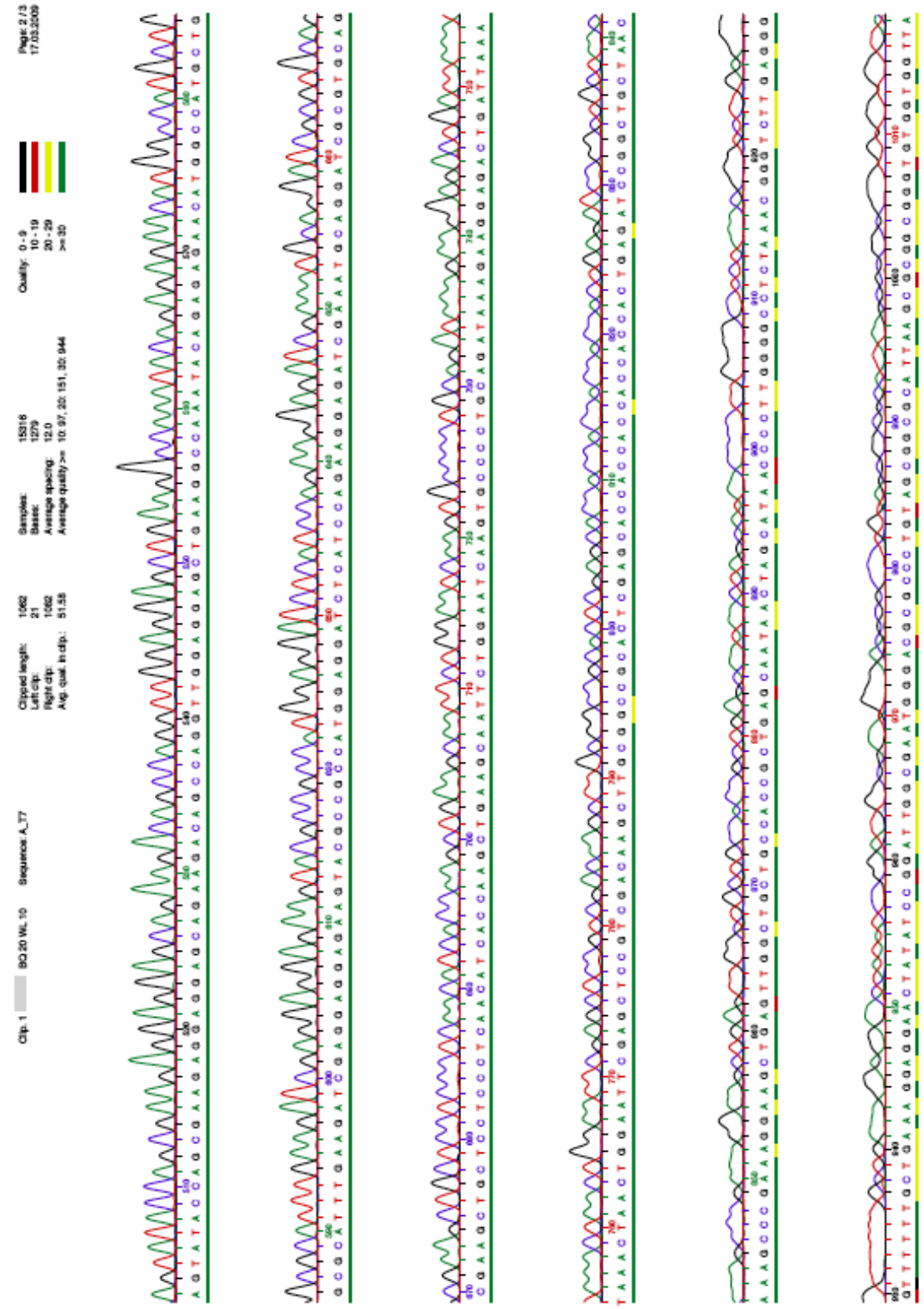
10 - 19
20 - 29
>= 30
Page: 1 / 3
17.11.2009

pET30 (A)_Mis12

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20 - 29
>= 30
Page: 2 / 3
17.03.2009

APPENDIX 4

Supplementary Movie Legends

Supplementary Movies Legends

Movie S1 – Time-lapse microscopy of untreated S2 cells stably expressing GFP-Tubulin and Cid-mCherry. Chromosomes display minor oscillations and reach biorientation within approximately 10 minutes prior to anaphase onset. Images were collected using a spinning disk confocal system (see “Materials and methods” section) at intervals of 30s. NEBD is indicated by the rapid entry of GFP-Tubulin into the nuclear space and anaphase onset takes place when centromere identifier (Cid) separation is observed.

Movie S2 - Time-lapse microscopy of Mis12 depleted S2 cells stably expressing GFP-Tubulin and Cid-mCherry. Chromosomes exhibit significant movements towards and away from metaphase plate, and by the time of anaphase onset a high percentage of chromatids do not separate so that sister chromatids segregate together. Images were collected using a spinning disk confocal system (see “Materials and methods” section) at intervals of 30 s. NEBD is indicated by the rapid entry of GFP-tubulin into the nuclear space and anaphase onset takes place when centromere identifier (Cid) separation is observed.

Movie S3 - Time-lapse microscopy of Ndc80/Nuf2 depleted S2 cells stably expressing GFP-Tubulin and Cid-mCherry. Chromosomes exhibit significant movements towards and away from metaphase plate, and by the time of anaphase onset a high percentage of chromatids do not separate so that sister chromatids segregate together. Images were collected using a spinning disk confocal system (see “Materials and methods” section) at intervals of 30 s. NEBD is indicated by the rapid entry of GFP-Tubulin into the nuclear space and anaphase onset takes place when centromere identifier (Cid) separation is observed.

Movie S4 - Time-lapse microscopy of Spc105 depleted S2 cells stably expressing GFP-Tubulin and Cid-mCherry. Chromosomes do not oscillate as

much as in movies S2 and S3 during prometaphase and at anaphase onset sister chromatids do not separate and some chromosomes fail to segregate. Images were collected using a spinning disk confocal system (see “Materials and methods” section in chapter 3) at intervals of 30 s. NEBD is indicated by the rapid entry of GFP-Tubulin into the nuclear space and anaphase onset takes place when centromere identifier (Cid) separation is observed.

Movie S5 - Time-lapse microscopy using inducible speckle imaging (ISI) in control S2 cells stably expressing GFP-Tubulin and Cid-mCherry (see “Materials and methods” section in chapter 3) collecting images every 2 seconds for 2 minutes.

Movie S6 - Time-lapse microscopy using inducible speckle imaging (ISI) in Ndc80/Nuf2 depleted S2 cells stably expressing GFP-Tubulin and Cid-mCherry (see “Materials and methods” section in chapter 3) collecting images every 2 seconds for 2 minutes.