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Functional role of CCAN histone fold proteins in mitosis

A thesis presented to the National University of Ireland for the degree of Doctor of
Philosophy

by

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“To know that you don’t know is the beginning of wisdom”

Socrates

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Abbreviations

°C	Degrees Celsius
ACA	Anti-centromere antigen
APC/C	Anaphase Promoting Complex/Cyclosome
BSA	Bovine Serum Albumin
BUB	Budding uninhibited by benzimidazole
CAD	CENP-A distal
CATD	CENP-A Targeting Domain
CCAN	Constitutive centromere associated network
CDK1	Cyclin-dependent kinase 1
cDNA	Complementary DNA
CENP	Centromere Protein
CO ₂	Carbon Dioxide
CPC	Chromosome passenger complex
DAPI	4',6-diamidino-2-phenylindole
dCTP	Deoxycytidine triphosphate
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide-5'-triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene-glycol tetraacetic acid
EtOH	Absolute Ethanol
FACS	Fluorescence-activated cell sorting
FBS	Foetal Bovine Serum
g	Gram(s)
GFP	Green fluorescent protein
H3P	Phospho-serine-10 of histone H3
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	Histone fold domain
HRP	Horseradish peroxidase
ICEN	Interphase centromere complex
IF	Immunofluorescence
kDa	Kilodaltons
LAP	Localisation and affinity purification
LSB	Laemli sample buffer
MAD	Mitotic-arrested deficient
MAP	Microtubule-associated protein
MeOH	Methanol
MgCl ₂	Magnesium chloride
mL	Millilitre
mM	Millimolar

Mnase	Micrococcal Nuclease
MON	Monastrol
mRNA	messenger RNA
MT	Microtubule
MTOC	Microtubule-organizing centre
NAC	Nucleosome associated complex
NaCl	Sodium chloride
NEAA	Non-essential amino acids
NEB	Nuclear envelope breakdown
nm	Nanometre(s)
NOC	Nocodazole
ns	Non-significant
NT	Non-targeting
NuMa	Nuclear protein of the mitotic apparatus
PA	Photoactivatable
PBS	Phosphate Buffered Saline
PBS-T	PBS-Tween
PBS-TX	PBS-Triton-X
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI	Propidium Iodide
PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative Real-time PCR
RNA	Ribonucleic acid
RNAi	RNA interference
RPM/ rpm	Revolutions per minute
RT	Room temperature
S.D	Standard deviation
SAC	Spindle Assembly Checkpoint
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard Error of the Mean
siRNA	Small interfering RNA
STS	Staurosporine
TAP	Tandem affinity purification
TPX2	Targeting protein for Xklp2
Tris	Tris(hydroxymethyl)aminomethane
WT	Wild type
ZM	ZM447439 Aurora kinase inhibitor

Abstract

Centromeres are chromosomal loci that direct segregation of the genome by specifying kinetochore assembly in mitosis and meiosis. Kinetochores are built upon centromeric chromatin, which contains the histone H3 variant CENP-A nucleosomes and a group of over 15 associated proteins comprising the centromere-associated network (CCAN). The CCAN contains two novel histone fold domain complexes, CENP-T/W and CENP-S/X, which are essential for error-free mitotic progression. The CCAN nucleates both kinetochore formation and spindle assembly checkpoint (SAC) activity. Together, these activities both attach chromosomes to the spindle and signal the presence of improperly attached chromosomes. Failure to correct kinetochore-spindle attachments leads to aneuploidy, a characteristic of cancer. Chronic checkpoint activation can result in mitotic slippage and consequent aneuploidy or polyploidy, cell death via apoptosis, after failed mitotic exit, or execution of a death pathway directly from mitosis. All these outcomes from prolonged mitosis are associated with increased caspase-3 activity, suggesting its involvement in a normal mitosis.

Chapter 2 of this thesis, investigates the alleged link between caspase-3 and mitosis. We found no evidence that active caspase-3 is required for mitotic checkpoint activity in a normal mitosis, supported by no observable influence of caspase-3 depletion on mitotic kinetics.

Chapter 3 examines the functional role of the CENP-T/W complex in mitosis. Both proteins were required for a robust mitosis in each cell cycle as well as proper mitotic checkpoint function. These results are consistent with the unique role that the CENP-T/W complex plays in specifying an early step of kinetochore assembly late in the cell cycle.

Chapter 4 examines the mechanism of multipolar spindle formation following CENP-W depletion. Our analysis revealed that multipolar spindle formation occurs during mitosis. Evidence is presented for a physical model in which unbalanced spindle forces lead to pole fragmentation.

These studies provide novel insight into kinetochore assembly and its role in mitotic spindle structure and function and identify the CENP-T/W complex as a potential target for anti-cancer drug development.

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Thesis structure and Declaration of contribution

Thesis Structure

Each of the three research chapters (Chapters 2-4) have been written in the form of standalone manuscripts with a view to submitting them to international, peer-reviewed journals for publication. Parts of Chapter 3 have already been used as the basis of a publication (Prendergast et al., 2011). Chapter 4 has comprised a basis for first-author publication and it is expected that will be submitted shortly. This format has led to some minimal repetition of CENP-W associated phenotype; however, the remainders of each manuscript are unique. The use of this format has also resulted in the less conventional listing of references at the end of each chapter/manuscript rather than together at the end of the thesis document.

Declaration of contributions

I declare I did not obtained a degree in this University, or elsewhere, on the basis of this work. All work presented in this thesis was performed by myself with the following exceptions:

Chapter 2: the caspase-3 like activity assay was performed under supervision of Dr. Howard Fearnhead who also provided the necessary reagents; the collection of the cell cycle fractions following release from double thymidine block was a collaborative work between myself and Nadine Quinn

Chapter 3: the qRT-PCR experiment (Figure S3.6B) was performed by Nadine Quinn. The above contributions have been acknowledged on the title page of each chapter by the inclusion of the contributors as co-authors.

Chapter 1 and 5 comprise of my own study on the literature review as well as my critical conclusions and future directions based on the data I obtained, respectively. Since all the work included in the thesis was carried out in Prof. Kevin Sullivan's laboratory, the mention of the supervisor's name on the title pages of these two chapters is dictated by the article-based style, as it would have been in the publications submitted to the peer-reviewed journals.

CHAPTER 1

General introduction

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Key words: mitosis, centromere, kinetochore, spindle assembly checkpoint (SAC), centrosome, multipolarity, apoptosis, caspase-3, aneuploidy,

1.1. Overview

In order to replicate for normal growth and development, cells must double their chromosomes and transport them into new daughter cells at cell division. The eukaryotic cell cycle involves a series of controlled events which are responsible for this faithful duplication and maintenance of genome stability (Norbury and Nurse 1992) and in general can be divided into two distinct stages: interphase and mitosis. Interphase consists of three phases: gap-1 phase, synthesis phase and gap-2 phase. Cells enter the first gap phase (G1), where they prepare for DNA replication, cells then proceed into the synthesis phase (S phase) where DNA replication occurs. Following S phase, cells enter G2 phase and prepare for mitosis. The mitotic phase consists of mitosis and cytokinesis. Mitosis can be sub-divided into 5 different stages. Prophase is the first stage of mitosis during which the replicated chromosomes condense and centrosomes move to opposite sides of the cell to organise the spindle microtubules. Cells then enter prometaphase during which the nuclear envelope breaks down, and the microtubules elongate and attach kinetochores. Tension is then applied by these spindles and the chromosomes bi-orient during metaphase. The spindles then shorten and sister chromatids begin to move towards opposite poles of the cell during anaphase. Following anaphase, cells enter telophase and the sister chromatids arrive at opposite poles of the cell. During cytokinesis, the cytoplasm and cell membrane are divided between the daughter cells that now enter G1 phase and the cycle starts again (Figure 1.1).

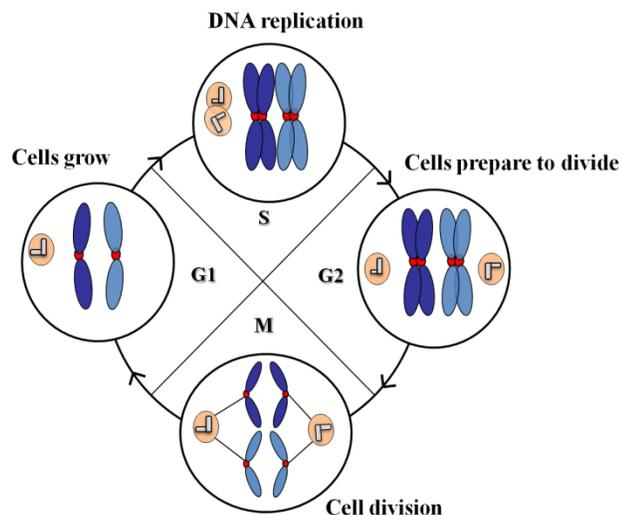


Figure 1.1. Overview of the cell cycle. The eukaryotic cell cycle is composed of two distinct phases: Interphase and Mitosis. During interphase, cells undergo growth, DNA (blue) is replicated and centrosomes (orange) are duplicated. Cells then undergo a second round of growth before entering mitosis. During mitosis, chromosomes are equally distributed to daughter cells. Notice: for simplicity, to visualize DNA replication, chromatin is shown as condensed chromosomes also during interphase, whereas in live cells chromosomes are only present in mitosis.

Among all the mitotic phases, prometaphase is a very eventful time of the eukaryotic cell cycle during which two intricate structures coordinate to accurately segregate duplicated chromosomes. One is called the mitotic spindle and forms a tight attachments with the second one – specialized structures on each of the sister chromatids known as kinetochores. The latter assembles during G2 phase of the cell cycle upon centromere, the unique site of the chromosomal loci, specialized by the appearance of the CENP-A containing nucleosomes, a vertebrate conserved variant of histone H3 that serves as the epigenetic mark for kinetochore assembly (Cleveland *et al.* 2003; Allshire and Karpen 2008). Therefore, kinetochores mediate the microtubule dependent movement of chromosomes during mitosis towards the correct configuration called biorientation (Cheeseman and Desai 2008). Biorientation contributes to chromosome congression to the metaphase plate, and any errors in chromosome attachments activate the spindle assembly checkpoint (SAC), localized at the kinetochore sites, which delays cells at the metaphase-anaphase transition until correct bipolar attachment is achieved (Carroll and Straight 2006). In eukaryotic cells, all of these events are very tightly and timely coordinated with each other to maintain their ploidy, which is critical for cell surveillance (Holland and Cleveland 2009). In this review, we characterize the main features of centromere/kinetochore assembly and how they are coupled with dynamic nature of mitotic spindle formation in order to prevent from chromosome missegregation, which could lead to cancer (Weaver and Cleveland 2006).

1.2. The centromere and kinetochore assembly

Centromeres are specialized chromosomal regions of the mitotic or meiotic cells, which ensure the accurate segregation of chromosomes during cell division by directing the assembly of kinetochores in the late G2 and M phase (Sullivan 2001). By conventional electron microscopy (EM), the vertebrate kinetochore is a trilayered structure (Jokelainen 1967), that can be schematically subdivided into distinct functional modules (Cheeseman and Desai 2008). The first module, comprising the inner core (also called inner plate) is implicated in the interactions of kinetochore with centromeric chromatin (Cleveland *et al.* 2003). It is built on CENP-A nucleosomes and contains the DNA-binding proteins, i.e. CENP-B, structural proteins such as CENP-C, -H and -I and associated binding partners, that are collectively named constitutive centromere associated network (CCAN) (Hori *et al.* 2008), also known as ICEN/NAC/CAD complex (Foltz *et al.* 2006). The inner core is linked by a 40 nm fibrous gap (interzone) to a dense outer plate. That second module is ~ 40 nm thick, and together with extending outward ~ 150 nm fibrous corona containing CENP-F, CENP-E and cytoplasmic dynein (Cooke *et al.*

1997), provides the core of the microtubule-binding interface (Cheeseman and Desai 2008). The latter is comprised of 10 proteins forming the KMN network (Knl-1, Mis12 and Ndc80 complex), which main role is to capture microtubules, but it can also serve as a recruitment pad for additional proteins, such as molecular motors or checkpoint proteins (Martin-Lluesma *et al.* 2002; DeLuca *et al.* 2003). The above two modules of inner and outer domain contribute a structural core of the kinetochore that physically links chromosomes and microtubules (Figure 1.2). The additional modules, associated with both domains are implicated in the control of the state of these kinetochore-microtubule attachments. They include the components of the spindle assembly checkpoint (SAC) proteins, that inhibit anaphase until kinetochores acquire sufficient numbers of microtubules (Cleveland *et al.* 2003; Musacchio and Salmon 2007) and components of the chromosome-passenger complex (CPC), that recognizes and corrects erroneous attachments to result in biorientation (Ruchaud *et al.* 2007).

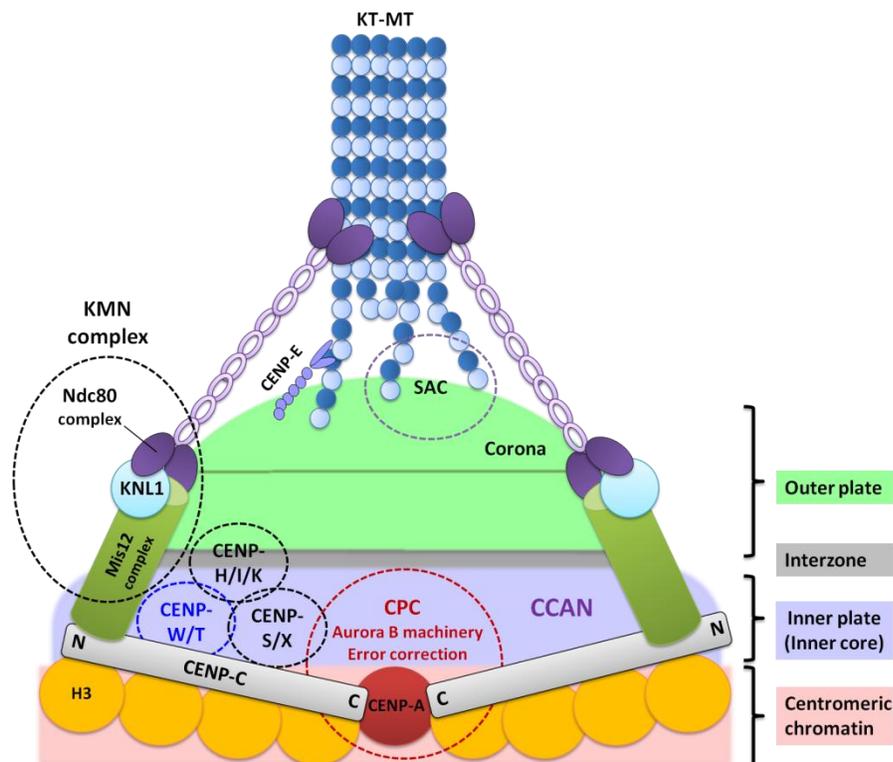


Figure 1.2. Schematic of the proteins that comprise the centromere and kinetochore. The inner centromere in vertebrates is comprised of 16 proteins which are collectively known as the CCAN (purple panel) and assemble upon the CENP-A containing centromeric chromatin (rosy panel). The kinetochore assembles on the CCAN and contains a key microtubule binding complex KMN, consisting of KNL1, the Mis12 complex and the Ndc80 complex, which together with CENP-E facilitates kinetochore-MT attachments. These proteins comprise the outer plate (green panel), which is separated from the inner plate by fibrous gap called interzone (grey panel). Errors in these attachments are sensed and corrected by cooperative work of the Aurora B machinery (Chromosome Passenger Complex, CPC) and the spindle assembly checkpoint (SAC) action (Adapted from Screpanti *et al.* 2011; Musacchio & Salmon, 2007).

1.2.1. Centromere identity

Centromeres generally appear as constricted regions of mitotic chromosomes and in most organisms they occupy distinct position on each chromosome (Przewloka and Glover 2009). With the exception of budding yeast *Saccharomyces cerevisiae*, exhibiting so-called point centromere, built upon a small 125-bp defined DNA sequence (Fitzgerald-Hayes *et al.* 1982), all higher eukaryotic centromeres are regional and assemble on tandem repeats of highly repetitive (satellite) elements (Allshire and Karpen 2008), and the DNA beneath them varies quantitatively and qualitatively among different species (Cleveland *et al.* 2003). This high divergence of centromeric DNA sequence and a stable or temporary acquisition of neocentromeres on unique DNA sequences (Marshall *et al.* 2008; Wade *et al.* 2009) has led to the conclusion that kinetochore establishment is not dictated by the underlying DNA information, but must be epigenetically determined. One common feature of all centromeres is the presence of the histone H3 variant, CENP-A (also known as CenH3). Since CENP-A is the foundation upon which the centromere is assembled it is thought to be the epigenetic mark which specifies centromere identity and function (Palmer *et al.* 1991; Meluh *et al.* 1998; Morris and Moazed 2007; Dalal 2009).

1.2.1.1. CENP-A

CENP-A was originally identified from autoimmune sera derived from patients with limited systemic sclerosis – CREST syndrome (Tan *et al.* 1980). These sera recognized the centromere region with three canonical human centromeric proteins: CENP-A, CENP-B and CENP-C (Earnshaw and Rothfield 1985). CENP-B was shown to associate with alpha satellite DNA (Masumoto *et al.* 1989) while CENP-C was identified as a component of the inner kinetochore (Saitoh *et al.* 1992) that is required for its assembly (Tomkiel *et al.* 1994). CENP-A was isolated from nucleosomes following micrococcal nuclease (MNase) digestion (Palmer and Margolis 1985) and found to co-purify with core nucleosome components (Palmer *et al.* 1987). Analysis of partial amino acid sequence of CENP-A suggested that it shared homology with histone H3 (Palmer *et al.* 1991) and this was confirmed by isolation of full length cDNA for human CENP-A (Sullivan *et al.* 1994). The C-terminal domain of CENP-A contains the histone fold domain (HFD), characteristic of all core histones, and shares approximately 60% homology with histone H3, while the N-terminal domain does not share any homology and its length differs significantly between species (Sullivan *et al.* 1994). This C-terminal HFD is comprised of three alpha helices separated by loops, and it is required for targeting CENP-A to the centromere (Sullivan *et al.* 1994). This is called CENP-A targeting domain (CATD) and was shown to be

sufficient for targeting not only CENP-A to centromeric nucleosomes, but when this was substituted to H3, the resulting H3^{CATD} chimeric protein was targeted to centromeres and capable of maintaining centromere function in the absence of CENP-A (Black *et al.* 2004; Black *et al.* 2007).

In vitro nucleosome assembly studies have shown that CENP-A can replace histone H3 in octamers containing H4, H2A and H2B histones (Yoda *et al.* 2000). However, nucleosomes containing CENP-A seem to be quite unusual and their composition may vary between species (Allshire and Karpen 2008). The (CENP-A: H4)₂ tetramer is more compact and rigid than that of the (H3:H4)₂ tetramer, a property which is conferred by the CATD of CENP-A (Black *et al.* 2007) and generates unique chromatin features which may physically mark centromere locations. Another striking feature was revealed by findings of centromeric nucleosomes inducing positive DNA supercoils as compared with the negative supercoiling of H3 nucleosomes (Furuyama and Henikoff 2009). All functional centromeres contain CENP-A nucleosomes and this chromatin is embedded in highly condensed heterochromatin which is characterised by methylation of histone H3 on lysine 9 (H3K9me), and this heterochromatin is involved in centromere chromatid cohesion (Bernard *et al.* 2001). Finally, centromeric chromatin was also found to be enriched in a histone H2A variant called H2A.Z in vertebrate cells (Foltz *et al.* 2006) and histone modifications in centromeric chromatin differ from those found in both euchromatin and heterochromatin (Sullivan and Karpen 2004). Overall, the above mentioned differences between canonical H3 and CENP-A containing nucleosomes emphasize an epigenetic mechanism for creating a special environment for proper centromere formation.

Additional evidence arises from the assembly studies of centromeric chromatin which does not occur during S phase like canonical histones (Shelby *et al.* 2000). In human cells, CENP-A synthesis is not coupled with DNA replication, with mRNA and protein accumulating during the G2 phase of the cell cycle suggesting a separation of CENP-A and histone H3 assembly (Shelby *et al.* 1997; Shelby *et al.* 2000). Indeed, the newly synthesised CENP-A has been shown to assemble after mitotic exit, in G1 phase (Jansen *et al.* 2007). In general, CENP-A is deposited on centromeric DNA in two steps. The first step involves removal of CENP-A nucleosomes from the template DNA and dilution of the parental CENP-A nucleosomes between the replicated DNA, without substituting new fibers with new CENP-A in S phase. The second step involves loading of newly synthesized CENP-A that can be first detected in late telophase cells and continues into G1 phase but is not seen at any other stage of the cell cycle (Jansen *et al.* 2007). To date, it is still unknown how centromeric nucleosomes become positioned in

chromatin. Three different models have been proposed to explain how the cell compensates for CENP-A dilution following S phase (Probst *et al.* 2009). The placeholder model suggests H3 nucleosomes fill the gaps left by CENP-A nucleosomes (Dunleavy *et al.* 2011). The gap model suggests the gaps are left empty until new CENP-A is deposited during G1. The splitting model proposes that CENP-A nucleosomes are split and mixed with H3 to form full nucleosomes. Regardless of the model, it is clear that this unique timing of assembly of CENP-A indicates that for a long period in the cell cycle centromeres contain only 50% of the maximal amount of CENP-A, which is speculated to contribute to the flexibility of the centromeric heterochromatin during mitosis.

All the findings mentioned above point to CENP-A as the leading epigenetic marker, primarily directing active centromere assembly (Przewloka and Glover 2009). Although in *Drosophila* ectopic expression of CENH3 (homologue of CENP-A) is sufficient for centromere formation (Mendiburo *et al.* 2011), in human cells the overexpression of CENP-A resulting in ectopic misincorporation, does not trigger ectopic kinetochore formation (Van Hooser *et al.* 2001; Gascoigne *et al.* 2011). These observations suggest, that CENP-A itself is required, but not sufficient for the establishment of a functional centromere and kinetochore in human cells. Indeed, in the years following CENP-A, CENP-B and CENP-C discovery additional centromere components were added to the list as proteins that localize constitutively to kinetochores throughout the cell cycle.

1.2.1.2. Constitutive Centromere Associated Network (CCAN)

Proteomic studies yielded a large increase in the number of proteins known to reside at centromeres. As previously discussed, CENP-A, -B and -C were the first centromere proteins to be identified and were originally identified as autoantigens recognised by anti-centromere antibodies (Earnshaw and Rothfield 1985). A large group of proteins have been identified in addition to these 3 constitutive centromere proteins, which assemble onto centromeric chromatin in a CENP-A dependent manner but which also influence assembly of CENP-A (Obuse *et al.* 2004; Foltz *et al.* 2006). Obuse *et al.* (2004) isolated CENP-A chromatin from HeLa interphase nuclei by chromatin immunoprecipitation and identified the interphase centromere complex (ICEN complex), which was composed of previously known centromere proteins CENP-A, -B, -C, -H and human Mis-12 as well and approximately 40 proteins of unknown function. Following that discovery, three almost simultaneous studies that used different approaches in human and

chicken cells considerably increase the number of centromeric proteins to 16 by identification of 11 new ones (Foltz *et al.* 2006; Izuta *et al.* 2006; Okada *et al.* 2006).

Foltz *et al.* (2006) demonstrated using tandem affinity purification (TAP) in conjunction with CENP-A in HeLa cells, the existence of a set of tightly bound proteins which they termed the CENP-A nucleosome-associated complex (NAC). The CENP-A NAC was found to be comprised of the previously identified CENP-C, -H and -U as well as the previously unidentified centromere proteins CENP-M, -N and -T (Foltz *et al.* 2006). Iterative TAP tag purifications with CENP-M, -N and -U identified a second set of proteins termed the CENP-A distal complex (CAD) (Foltz *et al.* 2006). The CAD was found to contain CENP-I, -K, -L, -O, -P, -Q, -R, and -S.

Okada *et al.* (2006) took a complementary approach, focusing on the proteins which were associated with the inner kinetochore proteins, CENP-H and CENP-I and identified a multi-subunit complex, which they termed the CENP-H-I complex. FLAG/GFP tagged CENP-H and CENP-I cell lines were generated in DT40 cells, in which the endogenous protein was completely replaced (Okada *et al.* 2006). Affinity purifications with these tagged proteins from chromatin fractions identified 5 previously unidentified centromere proteins (CENP-K, -L, -M, -O and -P). Simultaneous experiments in HeLa cells with LAP tagged CENP-H, -O and -P, identified four additional proteins to those identified in DT40 cells; CENP-N, -Q, -R and -U (Okada *et al.* 2006). In total the purifications from chicken and human cells identified a multi-subunit complex which consists of 11 interacting proteins (CENP-H, -I, -K to -R and CENP-U) (Okada *et al.* 2006). Okada *et al.* (2006) divided this complex into 3 functional and physical groups, based on phenotypic analysis and localisation dependencies: CENP-H, CENP-M and CENP-O class proteins.

Later, all newly found CENPs, together with CENP-W that was identified as an interacting partner of CENP-T (Hori *et al.* 2008), and CENP-X which was found in complex with CENP-S (Amano *et al.* 2009) were collectively named the constitutive centromere associated network (CCAN) (Hori *et al.* 2008). More recent studies which have integrated quantitative proteomics and bioinformatics analysis have predicted the existence of nearly 100 additional centromere and kinetochore associated proteins (Ohta *et al.* 2010).

1.2.1.3. HFD complexes of the CCAN

Within the CCAN components, there are four additional proteins that contain histone fold domains (HFD), similar to CENP-A. They form two distinct complexes: one is CENP-W/T and the other CENP-S/X complex (Hori *et al.* 2008; Amano *et al.* 2009).

CENP-W was originally identified as a novel HFD containing, CENP-T interacting factor. Hori *et al.* (2008) demonstrated, under stringent condition, that CENP-T can be separated from the rest of the CCAN and is distinct from the CENP-H containing complex proteins. However, in addition to two existing HFD CENPs (CENP-T and CENP-S), a novel 11 kDa protein was identified and re-named CENP-W (previously named CUG2) due to its co-purification with CENP-T and its constitutive localization to centromeres (Hori *et al.* 2008). These two proteins were found to form a complex through their histone fold domains and associate with centromeric DNA (Hori *et al.* 2008). The HFD of CENP-T is necessary to target the protein to centromeres. Neither CENP-T nor CENP-W associate with CENP-A nucleosomes, rather the complex associates with the canonical H3 nucleosomes which are interspersed with CENP-A nucleosomes at centromeric chromatin, similar to CENP-C (Hori *et al.* 2008). CENP-W/T complex has been suggested to be the connection between the centromere and the outer kinetochore in vertebrate cells (Hori *et al.* 2008). While CENP-A nucleosomes have been demonstrated to be essential for specifying centromere identity as previously discussed, over-expression of CENP-A alone is not sufficient to direct kinetochore assembly in humans (Van Hooser *et al.* 2001). In *Drosophila*, ectopic expression of CENP-A^{CID} results in kinetochore assembly (Heun *et al.* 2006; Mendiburo *et al.* 2011), while in humans ectopic expression of CENP-A only results in the mis-localisation of three proteins, CENP-C, CENP-N and Mis18 (Gascoigne *et al.* 2011). Gascoigne *et al.* (2011) demonstrated that CENP-T along with CENP-C can direct kinetochore assembly in the absence of CENP-A nucleosomes. Ectopic co-localisation of CENP-C and CENP-T resulted in formation of significantly bigger yet functional kinetochores which were capable of interacting with microtubules directing chromosome assembly (Gascoigne *et al.* 2011). Therefore, CENP-W/T along with CENP-C have been suggested to provide the platform for outer kinetochore assembly in vertebrate cells (Gascoigne *et al.* 2011; Prendergast *et al.* 2011).

CENP-S was originally identified as a member of the CENP-A distal (CAD) complex, found in affinity purifications with CENP-M and -U (Foltz *et al.* 2006). Like CENP-T, CENP-S was found to be a distinct member of the CCAN, as purifications with CENP-S did not contain

members of the CENP-H or -O class proteins, but did reveal small amounts of CENP-T as well as a novel centromere protein, CENP-X (Amano *et al.* 2009). Sequence analysis of CENP-S and CENP-X revealed that both proteins contain putative HFDs. Both proteins are interdependent on each other for their centromere localisation but are recruited downstream of CENP-H class proteins. Depletion of either protein resulted in mitotic abnormalities in both human and chicken cells, smaller outer kinetochore plates and they were found to be required for the assembly of outer kinetochore proteins including; KNL1, Ndc80 and Hec1 (Amano *et al.* 2009).

Apart from their role at centromeres, CENP-S/X complex have been suggested to function in a DNA damage pathway, since it is identical to MHF1/MHF2. The latter proteins are the binding partners of FANCM, a member of Fanconi anemia nuclear core complex (Singh *et al.* 2010; Yan *et al.* 2010). Fanconi anemia is a disease of cancer predisposition which is characterised by bone marrow failure, developmental defects, and an increased sensitivity to DNA interstrand crosslinking (ICLs) agents (Thompson and Jones 2010). FANCM is an ATP dependent branch-point translocase which acts on Holliday junctions and function at stalled and broken replication forks produced by ICLs (Singh *et al.* 2010; Yan *et al.* 2010). Although centromeric localization of FANCM has not been reported, CENP-S and CENP-X were found to exist as a heterodimer in association with the FANCM protein and depletion of CENP-S/X complex in HeLa cells caused an increase in chromosomal aberrations and an increased sensitivity to ICLs (Singh *et al.* 2010; Yan *et al.* 2010). Moreover, FANCM depleted cells exhibited supernumerary centrosomes, therefore it is speculated that the CENP-S/X-FANCM association could play a role in chromosome segregation, possibly by ensuring the faithful replication of structures associated with centromeric DNA (Thompson and Jones 2010).

In addition, a recent study demonstrated that the two centromeric HFD complexes discussed above co-assemble to form a stable CENP-T-W-S-X heterotetramer that shares structural and functional properties with canonical histones (Nishino *et al.* 2012). These crystal structure-based findings further reveal a new level of complexity for centromeric chromatin assembly.

1.2.1.4. The function of the CCAN in kinetochore assembly

The role that CCAN proteins play in the establishment of centromeric chromatin and therefore kinetochore assembly is an active area of investigation. Although all CCAN members assemble downstream of CENP-A, several have been implicated in the establishment of CENP-A chromatin as well as in kinetochore function and microtubule modulation. The CCAN can be divided into subgroups based principally on the nomenclature used by Okada *et al.* (2006). The

inner kinetochore consists of five groups of CENPs: CENP-C, CENP-W/T/S/X, CENP-H/I/K, CENP-N/L/M and CENP-O/P/Q/R/U (Perpelescu and Fukagawa 2011). The classification of groups is based on targeting/recruiting interdependencies, knockout phenotypes, and some biochemical data (Cheeseman and Desai 2008).

All components of the CCAN with the exception of CENP-B protein and CENP-O class proteins are essential and necessary for the proper chromosome congression and segregation (Przewlaka and Glover 2009). CENP-B is a DNA-binding protein similarly to CENP-C, however, contrary to the latter, CENP-B appears not to be required for CCAN formation, since it is absent from fully functional neocentromeres (Tyler-Smith *et al.* 1999) and CENP-B knockout mice survive (Perez-Castro *et al.* 1998). While the function of CENP-B remains obscure, much progress has been made towards elucidating the functions of CENP-C. Its depletion leads to severe defects, such as chromosome missegregation, loss of mitotic checkpoint function, consistent with CENP-C along with CENP-T to be capable of directing the assembly of a functional kinetochore in the absence of CENP-A nucleosomes (Gascoigne *et al.* 2011). Additional studies have demonstrated that CENP-C directly binds to the Mis12 complex thus providing a link between centromeric chromatin and the outer kinetochore (Przewlaka *et al.* 2011; Screpanti *et al.* 2011).

Functional studies performed in vertebrate cells suggested the existence of differential link made by CENP-T and CENP-W between centromere and kinetochore than the one provided by CENP-C. Knockout experiments in DT40 cells revealed that CENP-T is recruited upstream of CENP-H and CENP-O class CCAN members but parallel to CENP-C in centromere assembly (Hori *et al.* 2008). The localization of CENP-W and CENP-T at centromere has been shown to be interdependent (Hori *et al.* 2008). Consistent with a discussed above function of CENP-W/T complex in kinetochore formation (Gascoigne *et al.* 2011), depletion of these two proteins in both chicken and human cells leads to severe mitotic defects, with a loss of kinetochore association of most of the CCAN complexes (Hori *et al.* 2008), as well as the outer kinetochore proteins such as Hec1 (Gascoigne *et al.* 2011) and profound mitotic delay combined with a spindle rolling phenotype following siRNA depletion of CENP-W (Prendergast *et al.* 2011).

CENP-H and -I were demonstrated to be necessary for the deposition of newly synthesised CENP-A at centromeres, as well as recruitment of Hec1 protein to the outer kinetochore. Incorporation rates of CENP-A GFP were drastically reduced in CENP-H/-I mutant cells (rates of between 15-30%) when compared to incorporation in wild-type cells (rates of approximately 80%), indicating CENP-H/-I play a role in the incorporation of nascent CENP-A (Okada *et al.*

2006). This was surprising as CENP-H/-I depend on CENP-A for their centromere localisation and lead to the hypothesis that incorporation of nascent CENP-A requires the presence of pre-existing CENP-A nucleosomes with CENP-H/-I acting as an intermediary (Okada *et al.* 2006). CENP-K in association with KNL-1 is involved in the recruitment of the Ndc80 complex to kinetochores during mitosis (Cheeseman *et al.* 2008). Simultaneous depletion of both these proteins severely disrupts kinetochore assembly and results in chromosome segregation defects (Cheeseman *et al.* 2008). Another study demonstrated that in human cells CENP-I protein is required for further recruitment and kinetochore localization of CENP-F and one of the major mitotic checkpoint protein – Mad2 (Liu *et al.* 2003). All three proteins, CENP-H/I/K are interdependent for their centromere localisation, and depletion of either also results in the loss of CENP-L, -M, -O, -P and -Q (Okada *et al.* 2006). However, with the exception of CENP-L, which siRNA depletion was demonstrated to induce monopolar spindle formation (McClelland *et al.* 2007), the group of latter proteins have been shown to be non-essential, but their depletion results in a G2/M accumulation when compared to wild type DT40 cells (Hori *et al.* 2008b). This CENP-O class plays an important role in the recovery of cells from spindle damage and have been proposed to protect cells from premature sister chromatid separation until all microtubules have correctly attached to kinetochores (Hori *et al.* 2008b). In support of this hypothesis CENP-Q was found to bind to microtubules *in vitro* (Amaro *et al.* 2010).

The above mentioned functional studies on knockout chicken cell lines or knockdown analyses from different models demonstrated it clearly, that single centromeric proteins of the CCAN or centromeric subcomplexes differentially influence kinetochore assembly and function.

1.2.2. Kinetochore identity

The function of the centromere is to nucleate the assembly of the mitotic kinetochore to provide the cell a means of connecting to spindle microtubules and regulating this association via mitotic checkpoint signaling (Glynn *et al.* 2010). As discussed above, CENP-A chromatin forms the foundation for kinetochore assembly from yeast to humans (Cheeseman and Desai 2008). Following CENP-A, centromeric proteins are sequentially recruited throughout the cell cycle, to mark the location on chromosomes to which outer kinetochore proteins start to localize in late G2 phase, through all stages of mitosis until anaphase onset. However, at the outer kinetochore and fibrous corona, a less linear hierarchy occurs, reflecting changes in the composition influenced by the microtubule attachment state and local signaling pathway (DeLuca *et al.* 2006; Cheeseman and Desai 2008). Despite this dynamism, centromere and kinetochore proteins form biochemically stable complexes that comprise the structural core of the kinetochore.

The KMN network

In higher eukaryotes, kinetochore proteins with MT-binding capacity can be subdivided into two main groups: proteins that form the core attachment site (Ndc80/Hec1) and are responsible for stable end-on attachment of the chromosomes to the mitotic spindle and proteins that promote kinetochore motility (e.g. CENP-E or dynein) or control microtubule dynamics, either by promoting microtubule polymerization (e.g. CLASP, MAP125, EB1) or depolymerization (MCAK). Here, we will focus on the first group of proteins.

The structural core of the microtubule-attachment site is compromised by the components of KNL-1 – Mis12 – Ndc80 supercomplex, collectively named the KMN network (Cheeseman *et al.* 2006). The Mis12 complex is the first to be recruited to centromeres by associating directly with the proteins of the CCAN, presumably via CENP-C dependent pathway (Liu *et al.* 2006; Milks *et al.* 2009). It is composed of four proteins: Mis12, Nnf1, Nsl1 and Dsn1, all of which are required and their localization is interdependent, meaning that loss of any single component results in a similar phenotype, such as defects in chromosome alignment, orientation and segregation (Kline *et al.* 2006). However, chromosomes still attach to spindles in Mis12 depleted cells, but tension between sister kinetochores is decreased and spindles become very long, with a frequent lagging chromosomes appearance (Kline *et al.* 2006). The only exception from the above interdependence was reported in *C.elegans* studies where depletion of only Dsn1 alone led to a more severe, “kinetochore null” phenotype than depletion of the other subunits (Desai *et al.* 2003; Cheeseman *et al.* 2004). Loss of Mis12 complex compromises not only binding of the outer kinetochore proteins, such as Ndc80, BubR1 or CENP-E, but also results in lower levels of centromeric CENP-H and even CENP-A (Kline *et al.* 2006), which further emphasize the notion of interdependency between the CCAN and outer kinetochore proteins. In fact, it has been hypothesized that CCAN may directly influence the formation of KMN network, by inducing local stabilization of Dsn1, which is otherwise unstable, by the assembly of Mis12 complex at the beginning of mitosis (Cheeseman and Desai 2008).

The Ndc80 complex is the main microtubule-binding module which together with the above discussed Mis12 complex collaborates to form and maintain proper end-on attachments at the kinetochore site (DeLuca *et al.* 2006; Ciferri *et al.* 2007). It is comprised of the four proteins, that form stable dimers: Spc24/Spc25 and Nuf2/Ndc80 (alternatively called Hec1) (Ciferri *et al.* 2005). In contrast to Mis12 complex, the localization of the four subunits is not completely interdependent and depletion of the Spc24/Spc25 dimer affects Nuf2/Ndc80 localization, but not

vice versa, consistent with the physical features of Spc24/Spc25 dimer (Ciferri *et al.* 2007; Przewloka and Glover 2009). Its C-terminal globular domains face the centromeric site, while long N-terminal coiled-coils directly bind to rod-like structure with globular domains at both ends of Nuf2/Ndc80 dimer. However, it is the latter Nuf2/Ndc80 dimer that seems to be critical for microtubule binding, due to the N-terminal regions of both proteins (Ndc80 and Nuf2) containing Calponin-homology (CH) domains that make a cooperative, predominantly electrostatic interactions with microtubules (Ciferri *et al.* 2008). Moreover, the latter interactions are facilitated by the unique feature of the Ndc80/Hec1 protein, which contains on the N-terminus, closer than the CH domain, an unstructured tail essential for the above microtubule contacts. Therefore, consistently with these features for microtubule binding, the loss of Ndc80 complex results in severe spindle attachment defects, disorganized metaphase plates and extensive chromosome missegregation in all systems (Ciferri *et al.* 2007; Przewloka *et al.* 2007).

In human cells, the KNL-1 (also called Blinkin or Spc105) serves more complementary function and behaves as a potential center for kinetochore checkpoint signaling, since it has been shown to bind directly the mitotic checkpoint proteins Bub1 and BubR1 via its N-terminal region (Kiyomitsu *et al.* 2007). Therefore, RNAi-based KNL-1 depletion resembles the consequences of these two checkpoint protein depletion. However, KNL-1 exhibits the redundant concentration-dependent microtubule binding capacity (Cheeseman *et al.* 2006), particularly strong when combined with the Mis12 and Ndc80 complexes. The link made by such supercomplex to connect the centromere to the microtubule fiber of the mitotic spindle must be sufficiently dynamic, since the structure requires a mutual exchange of signals between inward and outward components; and on the other hand must be strong enough to sustain the pulling forces during anaphase (Przewloka and Glover 2009). The relation of the CCAN attachment to the KMN network and regulation of this binding during kinetochore assembly remains a matter of debate. The recent EM study of the kinetochores revealed that the inner kinetochore component CENP-T, which directly interacts with Ndc80 (Gascoigne *et al.* 2011) is flexible and responsible for the kinetochore stretching when tension is applied from mitotic spindles (Suzuki *et al.* 2011). Moreover, it has been shown that the amount of CCAN and KMN components at kinetochores does not change significantly from late prophase until late anaphase, irrespectively of the microtubule-attachment status (Cheeseman and Desai 2008), in contrast to SAC components.

1.2.3. The SAC pathway

Ultimately, the function of the kinetochore protein complex in mitosis, besides the formation of the above discussed microtubule binding sites, is to monitor these attachments and respond immediately to any changes or lack of the kinetochore-microtubule structure (Glynn *et al.* 2010). Therefore, unattached kinetochore recruits additional proteins to facilitate microtubule interactions and signal cell cycle arrest, particularly at metaphase-anaphase transition. This surveillance strategy is mediated via the spindle assembly checkpoint (SAC), also known as mitotic checkpoint (Musacchio and Salmon 2007), which is required to block sister chromatid separation until all chromosomes achieve proper bioriented attachments on the mitotic spindle (Cheeseman and Desai 2008). The main target of the SAC is a large E3 ubiquitin ligase complex called the Anaphase Promoting Complex/Cyclosome (APC/C), however how unattached kinetochores generate the inhibition signal is still not completely understood.

Components of the spindle assembly checkpoint were first identified and characterized in yeast (Hoyt *et al.* 1991; Li and Murray 1991) and subsequently their orthologs were found in mammals. The core of the SAC proteins involve evolutionary conserved MAD (mitotic-arrested deficient) Mad1, Mad2 and Mad3 (BubR1 in humans) proteins and BUB (budding uninhibited by benzimidazole) Bub1 and Bub3 proteins (Musacchio and Salmon 2007; Logarinho and Bousbaa 2008). Additional proteins involved in this signaling pathway include protein kinases, such as multipolar spindle-1 (Mps1) or Aurora B; proteins that regulate SAC activity in higher eukaryotes, like CENP-E, dynein/dynactin, together with Lis1 and CLIP170; and so-called RZZ (ROD-ZW10-Zwilch) complex (Musacchio and Hardwick 2002; Logarinho and Bousbaa 2008). The RZZ functions both in assembling other checkpoint proteins at the kinetochore and in facilitating their removal once the checkpoint is satisfied (Karess 2005).

The main SAC effector is the mitotic checkpoint complex (MCC) composed of the three checkpoint proteins Mad2, BubR1 and Bub3, which during prometaphase associate with the APC/C activating molecule Cdc20 (Figure 1.3A) (Hwang *et al.* 1998). The formation of the MCC acts as the diffusible effector of APC/C inhibition. The APC/C tightly controls the metaphase-anaphase transition by the ubiquitination and subsequent targeting for degradation of its two main substrates, cell cycle regulatory proteins – securin and cyclin B1. Securin binds and inhibits protease Separase, which activity is required to degrade centromere associated cohesin to allow chromosome segregation during anaphase (Peters *et al.* 2008). Cyclin B is the activating subunit of the cyclin-dependent kinase-1 (CDK1), which activity is necessary for maintaining the cell in mitosis. CDK1/cyclin B1 is formed as an inactive complex as cyclin B1

accumulates during S and G2 phase and is activated by dephosphorylation of CDK1 at the entry into mitosis (Clarke and Karsenti 1991). When all kinetochores are correctly bioriented on the mitotic spindle upon metaphase chromosome congression, the SAC is silenced, Cdc20 is released and APC/C complex is activated (Figure 1.3B). Subsequently the ligase tags securin and cyclin B1 with polyubiquitin chains, causing their proteolytic destruction by the 26S proteasome. This, in turns, activates Separase that cleaves cohesin, driving sister chromatid separation; and inactivates CDK1 kinase, initiating mitotic exit followed by cytokinesis (Figure 1.3C) (Peters 2006).

The crucial step in the creation of the MCC complex is the activation of Mad2 at unattached kinetochore, catalyzed by Mad1-Mad2 complex, which triggers Mad2 conformational change from open to close form (Figure 1.3A) (Luo *et al.* 2002; Sironi *et al.* 2002). Mad2 conversion is further amplified in the cytosol by a positive feedback loop, explaining how even a single unattached kinetochore is sufficient to sustain the checkpoint signal. However, a recent evidence suggests that the MCC is able to assemble and prevent mitotic exit even in the absence of kinetochores – it just happens more slowly (Kulukian *et al.* 2009). During G2 phase, BubR1 can function as a pseudo-substrate inhibitor of the APC/C, forming independently of unattached kinetochores, since the latter have not yet assembled (Kulukian *et al.* 2009). During prometaphase, Mad1 is recruited to the kinetochores and, unlike other MCC components that undergo dynamic exchange at the kinetochore, remains stably bound until kinetochores attach to the spindle (Emre *et al.* 2011). Kinetochore bound Mad1 recruits Mad2 which, in turns, promotes formation of a Mad2-Cdc20 intermediate that can transfer Cdc20 into a complex with BubR1 and therefore block APC/C activity, much more efficiently than in G2 phase (Kulukian *et al.* 2009). Therefore, kinetochore platform is thought to be required for a robust checkpoint signal and MCC complex formation.

In addition, recent studies of the KMN complex reveal its potential role in coordinating the activity of the protein kinases in generating a signal through a phosphorylation cascade (Burke and Stukenberg 2008). As previously mentioned, the KMN in fact serves as a binding site for many of the checkpoint proteins (Przewloka and Glover 2009). Several members of the KMN network directly associate with SAC proteins, for example KNL-1 with Bub1 and BubR1 (Kiyomitsu *et al.* 2007) and also Ndc80 component, Hec1 has been shown to be required for kinetochore localization of Mad1-Mad2 complex and Mps1 kinase recruitment (Martin-Lluesma *et al.* 2002).

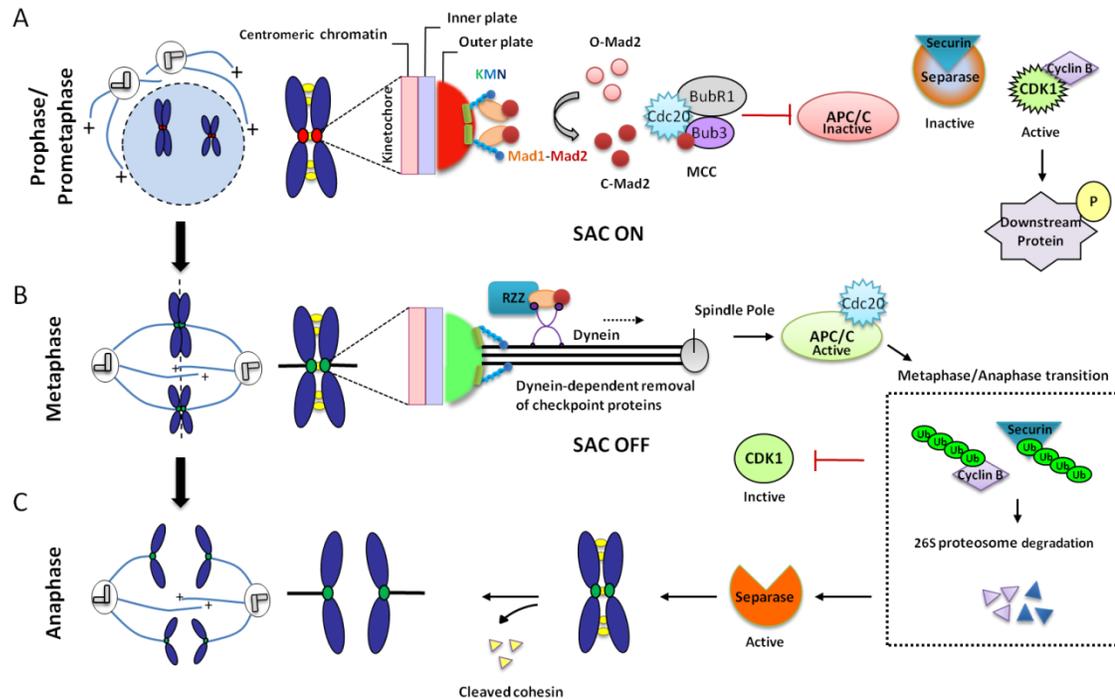


Figure 1.3. Relationship of the SAC with the cell cycle machinery. Spindle assembly checkpoint (SAC) regulates metaphase-anaphase transition, blocking mitosis progression in the presence of unattached kinetochores. To enter mitosis, cells require the activation of the Ser/Thr kinase CDK1. **(A)** In early mitosis (prophase and prometaphase) SAC is active. Mad1-Mad2 complexes assemble on unattached kinetochores, where they facilitate Mad2 conformational change (from open to close) and binding to Cdc20. Mad2-Cdc20 together with BubR1 and Bub3 forms the mitotic checkpoint complex (MCC), which inhibits the activity of APC/C by binding to its co-factor Cdc20. **(B)** At metaphase, all kinetochores are bi-oriented on the spindle, the SAC is switched off and some of its components (e.g. Mad1-Mad2 and RZZ complex) are removed from the kinetochores by the action of the motor protein dynein. In the absence of MCC, Cdc20 is released and can activate the ubiquitin ligase APC/C which ubiquitinates cyclin B1 and securin, which triggers their proteasome dependent degradation and initiates the mitotic exit program. **(C)** Degradation of securin causes activation of separase, which cleaves cohesin allowing chromosome segregation during anaphase (Adapted from Musacchio & Salmon, 2007; Tanaka & Hirota, 2009).

1.2.4. Microtubule attachments, troubleshooting and aneuploidy

To achieve faithful chromosome segregation, kinetochores of duplicated chromosomes interact with microtubules coming from opposite poles of the mitotic cell, a process called bi-orientation (Rieder and Salmon 1994). Errors in this process can lead to aberrant distribution of chromosomes between daughter cells and therefore contribute to aneuploidy (Kops *et al.* 2005). Aneuploidy is defined as the state of having an abnormal chromosome content which has been described as the most common characteristic of cancer cells (Weaver and Cleveland 2006). Some cancer cells are stably aneuploid, following chromosomal redistribution earlier during tumorigenesis. However, more often cancer cells derive from an increase in the rate of gain or loss of whole chromosome, a condition known as chromosomal instability (CIN) (Lengauer *et al.* 1998; Kops *et al.* 2005). Aneuploidy or CIN can often arise from defects in chromosome segregation during mitosis as a result of microtubule misattachments or defects in the mitotic checkpoint (Cahill *et al.* 1998; Weaver and Cleveland 2005). The previously discussed SAC signaling which monitors lack of kinetochore-microtubule attachment is the main surveillance pathway of the mitotic cell to prevent from chromosome missegregation. The other one is fulfilled by Aurora B kinase-dependent correction mechanism of the erroneous attachments. These two main pathways are interdependent and cooperate to maintain ploidy of the mitotic cells.

Error correction and SAC silencing

Bioriented kinetochores are under tension arising because of the pulling forces exerted by microtubules and associated motors and the resistance of the physically attached sister centromeres, bound together through combined action of the cohesin complex and topoisomerase II (Przewlaka and Glover 2009). Because of these resistant forces cohesin complex is essential for the correct chromosome alignment and SAC silencing (Peters *et al.* 2008). Lack of tension, indicating incorrect or absent microtubule binding, or triggered by cohesin depletion, activates the SAC and delays onset of anaphase (Toyoda and Yanagida 2006; Maresca and Salmon 2009). Therefore, applying the tension by microtubule attachment is thought to be the main cue for mitotic checkpoint silencing and subsequent mitotic exit rather than microtubule attachment per se (Maresca and Salmon 2009; Uchida *et al.* 2009).

On their route to biorientation, kinetochores go through two distinct types of microtubule attachment (Tanaka and Desai 2008). Initially, kinetochores make only lateral contact with microtubules and this is followed by the rapid poleward movement of the attached

chromosomes, mediated by minus-end directed dynein together with the RZZ complex (Vallee 1990; Savoian *et al.* 2000). The RZZ complex associates with the KMN network via another checkpoint protein called Zwint-1, that engages in a dimeric complex with KNL-1. The RZZ is required for kinetochore localization of Mad1-Mad2 complex (Kops *et al.* 2005), and yet another protein called the spindly, which in turns, is required for dynein recruitment and together with the latter is involved in regulation and conversion of these lateral attachments, at later stages of mitosis, into stable end-on attachments (Griffis *et al.* 2007; Civril and Musacchio 2008; Gassmann *et al.* 2008). The bundle of microtubules attached to kinetochores in end-on orientation is called a k-fiber. In vertebrates, stable end-on kinetochore-MT attachments seem to be dependent as well on the recently discovered complex of three proteins, Ska1, Ska2 and Ska3 (Gaitanos *et al.* 2009; Guimaraes and DeLuca 2009). The Ska complex together with core microtubule lattice-binding site (KMN network) tethers the kinetochores to microtubule plus-end through a “sliding collar” whose motility is coupled to the polymerization and depolymerization of kinetochore microtubules (Civril and Musacchio 2008; Amaro *et al.* 2010). Therefore, the eukaryotic Ska complex has been suggested to represent functional similarities of a large Dam1 complex present only in yeast (Welburn *et al.* 2009). The formation of the end-on attachments is critical for biorientation (also called amphitelic orientation) and generation of load bearing attachments. It is also necessary for SAC signal silencing, since establishment of end-on attachments suppresses the kinetochore accumulation of the RZZ complex and of other proteins whose localization depends on the RZZ, which is followed by dynein-dependent stripping of the complex and associated proteins (Mad1-Mad2) and their poleward transport (see Figure 1.3B, SAC OFF) (Howell *et al.* 2001; Gassmann *et al.* 2010).

However, since the interaction between kinetochores and microtubules is a stochastic process, besides the lateral microtubule attachments, in each mitosis, there is a risk of erroneous attachment formation, that in most cases can be sensed by the SAC. Such incorrect intermediate orientations include monotelic – when the kinetochore of only one sister chromatid is connected to k-fibers, syntelic – when sister kinetochores are attached to the same pole; and merotelic attachments – when one kinetochore interacts with the microtubules coming from opposite spindle poles (Figure 1.4A). All of these erroneous attachment are immediately corrected via combined action of the two signaling pathways. The monotelic and syntelic attachments can be sense by the SAC, which delays the exit from mitosis until the correct attachment is achieved (Cimini 2008). However, the SAC is not able to detect merotelic attachments, since in this case there are no unattached kinetochores, but a single kinetochore is attached to microtubules from

both spindle poles. Hence, cells with merotelic attachments can exit mitosis without any significant delay (Cimini *et al.* 2004) leading to chromosome missegregation due to generation of the lagging chromosomes, which by being attached to both poles cannot move during anaphase onset and eventually are pushed into one of the daughter cells. Following cytokinesis and nuclear envelope reassembly, these lagging chromosomes often form micronuclei, that are highly frequent in cancer cells and are believed to be a biomarker for cancerogenesis (Majer *et al.* 2001; Cimini *et al.* 2002). Therefore, most merotelic attachments are corrected before anaphase onset (Cimini *et al.* 2004) by activating Aurora B dependent specific pathway of the checkpoint signaling that is able to sense the lack of tension between sister kinetochores (Biggins and Murray 2001). The Aurora B kinase stands a little apart from the other SAC checkpoint kinases in that it has a specific ability to discriminate between correct and incorrect kinetochore-microtubule attachments by selectively destabilizing the latter. The Aurora B kinase resides near the centromeric chromatin, where together with INCENP (inner centromere protein), borealin and survivin comprise the chromosome passenger complex (CPC), which relocates from the inner centromere to the central spindle at the metaphase-anaphase transition (Ruchaud *et al.* 2007). Aurora B corrects the misattachments by decreasing the kinetochore affinity for microtubules interaction, by phosphorylating key components of the kinetochore responsible for microtubule binding (such as Ndc80/Hec1 complex and microtubule depolymerizing kinase MCAK) (Andrews *et al.* 2004; Maresca and Salmon 2010). The flexibility in centromere and kinetochore structure helps to perform Aurora B action – the kinase located at the inner centromere that influences kinetochore-microtubule attachments situated at least 200 nm away. In fact, the flexible and expandable properties of the centromeric region are thought to resemble springs that, as mentioned above, can respond to the pulling forces applied by the spindle, resulting in the extension of kinetochore and centromeric springs (Ribeiro *et al.* 2009). However, regardless of the status of the centromeric spring, only proper tension applied to kinetochore springs satisfies the spindle assembly checkpoint (Figure 1.4B) (McEwen and Dong 2009). When misattachments occur, the unbalanced tension resulting from merotelic orientation is thought to position one of the two kinetochores more towards Aurora B phosphorylation gradient and consequently microtubules are released, mainly due to the phosphorylation of the Ndc80 protein (Liu *et al.* 2009). The unattached kinetochores acquire SAC proteins which in turns respond to the lack of tension and facilitate the establishment of a proper bipolar attachments (Santaguida and Musacchio 2009).

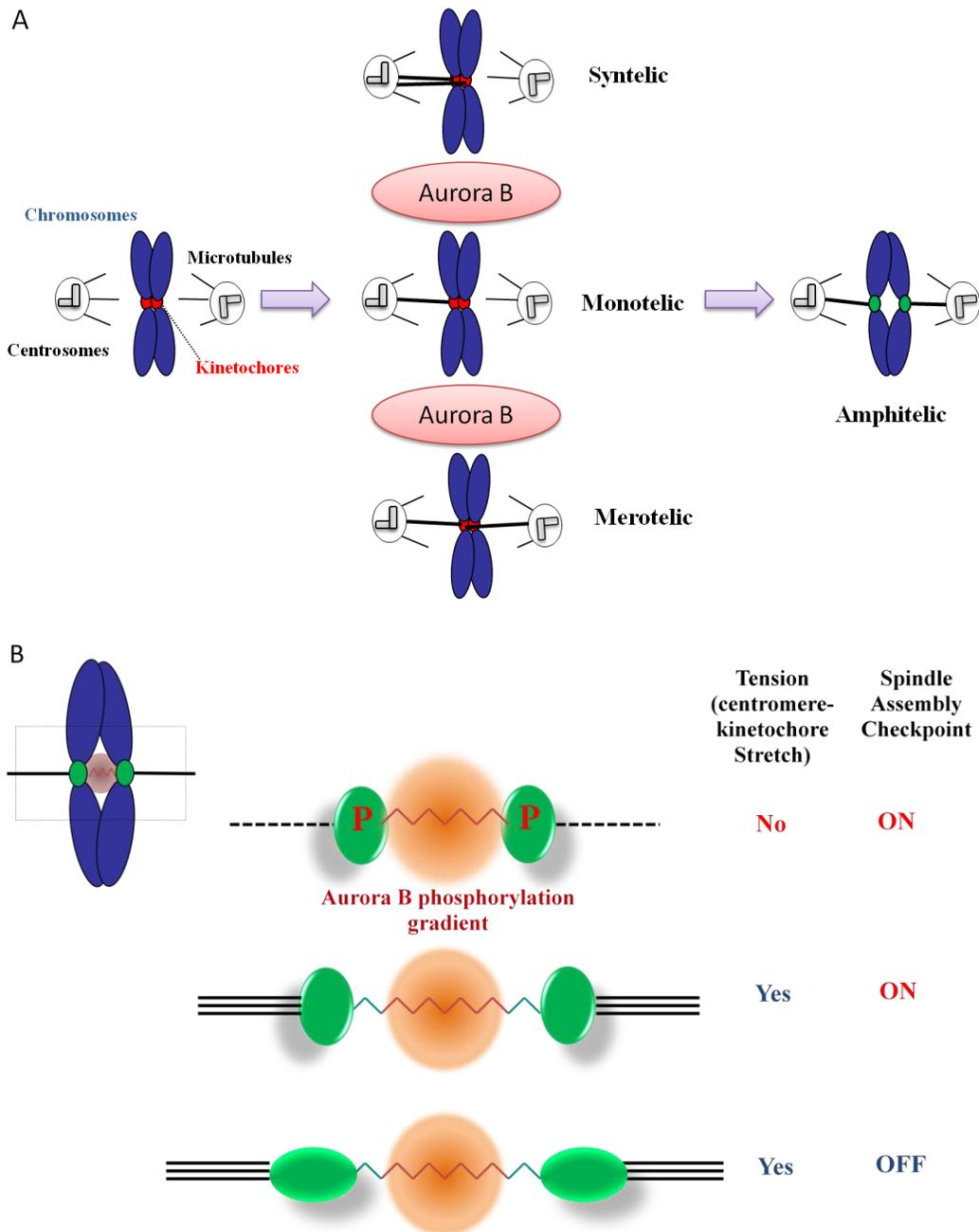


Figure 1.4. Aurora B kinase involvement in improper kinetochore-MT attachment correction and silencing of the SAC. (A) Schematic representation of the erroneous kinetochore-MT attachments are corrected by Aurora B kinase activity. As the capture of the kinetochores by microtubules is a stochastic process, wrong orientations, termed syntelic or merotelic (red labeled kinetochores), can occur. The Aurora B kinase activity is responsible for establishment of a proper amphitelic kinetochore-MT attachments (green labeled kinetochores). (B) Kinetochore stretching inactivates the SAC. When tension is low, the microtubule attachment sites position within the Aurora B phosphorylation gradient (orange) where microtubule-releasing activity is thought to be high. Therefore SAC silencing depends on kinetochore stretching rather than centromere stretching between sister kinetochores (Adapted from Tanaka and Hirota, 2009).

1.3. Spindle microtubule assembly, centrosomes and multipolarity

In addition to kinetochore assembly, facilitating the proper bipolar attachments of the sister chromatids in mitosis, the mitotic spindle is essential to fulfill the accurate chromosome segregation into two daughter cells. During mitosis, in most of the organism, the mitotic spindle is assembling into bipolar organization from spindle poles derived from duplicated centrosomes in the preceding interphase. Because of the bi-directionality of the cell division it is extremely important to keep the number of centrosomes constant in a cell and limit its duplication to exactly once per cell cycle. The presence of more than two centrosomes (centrosome amplification) severely disturbs mitotic process and cytokinesis via formation of more than two spindle poles, leading to increased frequency of chromosome segregation error (chromosome instability). This feature, very characteristic of cancer cells, was observed for the first time a century ago by the German zoologist Theodor Boveri, who recognized a crucial link between centrosome defects, aneuploidy and the development of cancer (Boveri 2008). Boveri found that in sea urchin eggs following dispermic fertilization, multipolar mitotic spindles formed due to double the number of sperm-derived centrosomes, and the consequence of that multipolar spindle division in these cells led to unequal chromosome segregation into daughter cells. He proposed the revolutionary theory that imbalance of chromosomes (aneuploidy) may promote tumorigenesis and play a causative role in the origin of malignant tumors (Boveri 2008; Holland and Cleveland 2009). He also suggested that chromosome aberrations observed in cancer cells were a result of multipolar mitosis in cells with supernumerary centrosomes. A hundred years later, Boveri's theory is still current and the object of extensive further investigation and development.

1.3.1. Centrosome structure, function and cell cycle

Centrosomes are major microtubule-organizing centers of animal cells (Luders and Stearns 2007), usually positioned near the nucleus of the cell. They possess two defining functional features: one is the ability to nucleate microtubules, and the other is to duplicate once in each cell cycle, to yield two centrosomes that act as spindle poles in mitosis (Nigg 2006).

Centrosomes and associated components influence the cell shape, polarity and motility, as well as spindle formation, chromosome movement and completion of cytokinesis (Sluder and Hinchcliffe 1999; Khodjakov and Rieder 2001; Piel *et al.* 2001). Therefore, this small non-membranous organelle provides an important structural cortex for coordinating cell cycle regulation.

Structurally, the centrosome of animal cells consists of a pair of centrioles joined by fibers connecting their proximal ends, that serve as a centrosomal organizer, together with a surrounding protein matrix called pericentriolar material (PCM), which serves as a framework to anchor microtubule nucleation sites (Paintrand *et al.* 1992; Ou *et al.* 2004). In addition, other principal components of centrosomes represent γ -tubulin complexes that are responsible for nucleation of microtubules (Schiebel 2000); and fibers composed of centriin that act as calcium-sensitive connections between microtubule arrays, mediating dynamic changes in the structure, orientation and position of the distal region of centrioles (Salisbury 2007). The two centrioles structurally differ from each other: one possesses a set of appendages at the distal end (mother centriole) which are required for microtubule anchoring, and the other is without appendages (daughter centriole) (Bornens 2002). The daughter centriole, however, acquires appendages during the maturation process that ends in the following cell cycle, when the centriole is ready to act as mother. The main function of centrioles in animal cells is the organization of the PCM matrix, which confers long-term stability upon the centrosome. This stability is provided by post-translational modifications, such as polyglutamylolation of centriolar tubulin, which loss causes in HeLa cells centrioles disappearance and defective recruitment of PCM to the spindle poles (Bobinnec *et al.* 1998).

During interphase, centrosomes organize the cytoplasmic microtubule network, which is involved in transport of proteins and small organelles and establishment of cellular shape and polarity. During mitosis, the centrosomes become the core structures of spindle poles and direct the formation and positioning of the mitotic spindles, guaranteeing normal and asymmetric cell division and defining the cytokinetic plane (Doxsey 2001). Although, cells that lack centrosomes, such as higher plants, can still organize a normal bipolar spindle, suggesting a presence of the secondary MTOCs in these cells, if centrosomes are present they dominate in mitotic spindle organization (Luders and Stearns 2007). Therefore, the maintenance of the correct centrosome number during each cell cycle is essential for equal transmission of a genome to daughter cells (Fukasawa 2005). For this reason, the cell must ensure that centrosome activities are tightly coordinated with cell cycle progression. Since each daughter cell receives only one centrosome upon cytokinesis, the centrosome, like DNA, must duplicate only once prior mitosis. Hence, animal cells are equipped with mechanisms that coordinate these two cycles. The key cell cycle regulators exhibiting local activities and residence at the centrosome include: p53, the CDKs/cyclins, protein kinase A (PKA), Aurora-A, Polo-like kinase-1 (Plk1), BRAC1 and BRAC2 and APC/C (Lingle *et al.* 2005).

The centrosome cell cycle can be divided in four main stages: centriole disengagement, centriole duplication, centrosome maturation and centrosome separation (Figure 1.5). Cells begin the cell cycle with exactly one centrosome. In late G1/early S phase the activation of CDK2/cyclin E complex triggers initiation of both DNA and centrosome duplication. The latter begins with the physical separation of the paired centrioles, without removal of the proteinaceous linker between them (G1-G2 tether). This step is followed by formation of procentrioles near the proximal ends of pre-existing centriole, and each of the new ones are joined to the parental centrioles with another protein linker (S-M linker). This semiconservative duplication mechanism provides the means for the two types of centriole number control: firstly, each centriole must duplicate once in every cell cycle; and secondly, exactly one new centriole must form adjacent to each pre-existing parental centrioles. This is under the control of two protein kinases – Plk4 and SAS-6 (Strnad *et al.* 2007; Loncarek *et al.* 2008). During S and G2 phases, procentrioles elongate and two centrosomes progressively recruit more PCM. In late G2, the daughter centriole of the parental pair acquires appendages and two identical centrosomes are generated. At late G2 and M transition, the loose fibrous G1-G2 link between centrosomes is severed following phosphorylation of its components by the protein kinase NEK2, enabling centrosome separation, mediated mainly by Eg5 kinesin activity. The recent data provides evidence that NEK2 pathway becomes essential when Eg5 is impaired, and both pathways are controlled by Plk1 kinase (Mardin *et al.* 2010; Bertran *et al.* 2011). During mitosis, the untethered centrosomes form two spindle poles and direct the establishment of the bipolar mitotic spindles. Notably, the complete formation and maturation of centrioles is achieved in more than one cell cycle (Nigg 2006). Following mitotic exit, at early G1 phase, the tight S-M link between centrioles is lost in a Plk1/Separase-dependent manner and the centrioles disengage from each other. The loss of this link at the beginning of G1 licenses centrioles for a new round of duplication during the following cell cycle. In addition, that special organization of centrioles relative to each other, generated by two different types of linkers plays a key role in the regulation of both centriole duplication and microtubule organization. Although the S-M linker is important to limit centriole duplication to one per cell cycle, the G1-G2 linker allows the duplicated centrosomes to function as a single microtubule-organizing centre during interphase (Nigg and Stearns 2011).

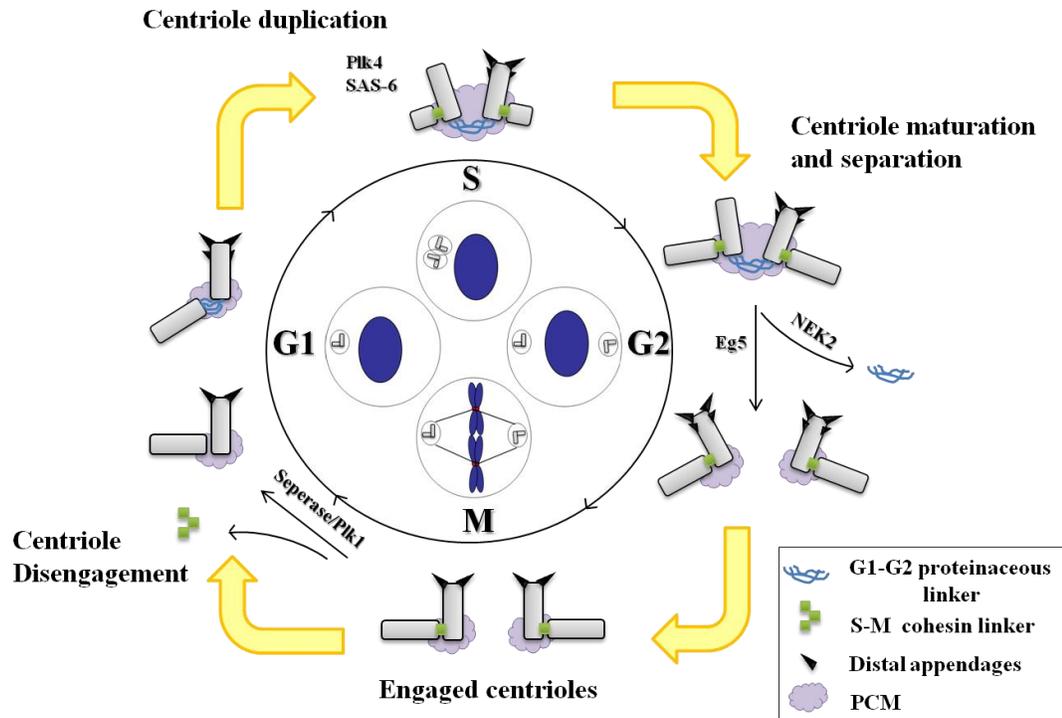


Figure 1.5. The chromosome and centrosome cell cycle. Diagram shows the cell cycle and the centrosome cycle. In the centre the 4 phases of the cell cycle (G1, S, G2, M) are schematized, with particular attention to the centrosome duplication-segregation cycle. Centrosome consists of two centrioles (grey), mother and daughter centriole, that are surrounded by pericentriolar material (PCM, purple) and bound by two distinct linker structures, depending on the cell cycle stage. The G1-G2 tether (blue) connects the proximal ends of the two parental centrioles from G1 to late G2. In G2 and M transition that linker is removed and centrosomes are separated by the co-operative work of NEK2 kinase and Eg5 kinesin. The S-M linker (green) forms during S phase and connects the proximal end of the nascent procentriole to the lateral surface of the mother centriole. This process is under the control of Plk4 and SAS-6 protein kinases. The removal of S-M tight linker in late M phase (disengagement) is an important element of cell cycle control of centriole duplication (Adapted from Nigg, 2006).

1.3.2. Centrosome amplification and cancer

As discussed above, centrosome doubling is strictly coordinated with DNA replication, mitosis and cell division machinery, since the presence of two centrosomes in mitosis is critical for the formation of bipolar mitotic spindles. Loss of that control results in abnormal amplification of centrosomes (presence of more than two centrosomes) which has been found to be a common event in the development of many cancers (Lingle *et al.* 2005). Since Boveri's first observation, several studies have shown that supernumerary centrosomes are common to almost all types of solid and hematological malignancies, including breast, brain, lung, colon, ovary, liver, prostate, bone, gall bladder, neck cancer as well as lymphoma and leukemia (Lingle *et al.* 1998; Pihan *et al.* 1998; Gustafson *et al.* 2000; Kuo *et al.* 2000; Kramer *et al.* 2003; Nitta *et al.* 2006). In cancer cells, aberrations in centrosome number are often associated with structural irregularities like

increased centrosome size, formation of acentriolar bodies and alterations in the expression and phosphorylation status of PCM components (Lingle *et al.* 2002). Furthermore, it has been shown that centrosome abnormalities correlate with increased levels of multipolar mitosis and aneuploidy in cancer cells. All these observations support the idea, initially suggested by Boveri, that the presence of extra centrosomes in cancer cells leads to multipolar mitotic division and consequently to aneuploidy (Lingle *et al.* 2005).

Mechanisms of centrosome amplification

Supernumerary centrosomes can originate through different mechanisms. Normal centrosome duplication is a tightly regulated process, which allows only one replication in a single cell cycle. However, overduplication may occur when the centrosome cycle becomes uncoupled from chromosome replication (Lingle *et al.* 2005). There are four main possible mechanisms that account for generation of amplified centrosomes (Fukasawa 2005). First, when cells experience multiple rounds of centrosome duplication in a single cell cycle. Formation of complete centrioles usually requires several hours, so centrosome duplication necessitates a prolonged arrest of the cell cycle, a condition common in the presence of DNA damage induced checkpoint delay or overexpression of centrosome number regulators (e.g. Plk4) (Inanc *et al.* 2010). Second, when cells fail to undergo cytokinesis, resulting in doubling of the genome and the number of centrosomes (a single 4N daughter cell inherits both spindle poles, instead of just one), a scenario very characteristic for mitotic slippage caused by impaired SAC or overexpression of oncogenes or tumor suppressors (e.g. p53, Aurora A, BRAC2). Third, when the paired centrioles untimely split and form individual centrosomes (Hut *et al.* 2003; Mattiuzzo *et al.* 2011). Finally, centrosome amplification can arise from de novo formation of acentriolar centrosomes, a phenomenon associated with centriole ablation (Khodjakov *et al.* 2002). Nevertheless, no matter how amplified centrosomes are generated, the presence of more than two centrosomes disturbs the proper mitotic process and destabilizes chromosomes in the subsequent cell cycle, mainly due to multipolar spindle formation (Fukasawa 2005).

Cells can differentially respond to multipolar spindle division following centrosome amplification. In fact, cancer cells possess several mechanisms by which they can successfully divide in the presence of supernumerary centrosomes. Cells can divide in a bipolar manner by inactivating, excluding from the cytosol or clustering extra centrosomes resulting in a pseudo-bipolar spindle formation and a normal mitotic process takes place (Kwon *et al.* 2008). Cells can also divide by multipolar division giving rise to aneuploid mononucleated cells or more

commonly, to polynucleated cells following cytokinesis failure. However, all of the above mentioned multipolar spindle intermediates promote merotelic attachments, by altering spindle geometry and therefore may result in aneuploidy and chromosome instability (CIN) (Ganem *et al.* 2009). In addition, syntelic attachment also can accumulate upon centrosome clustering and may promote further enhancement of merotely, which unresolved will give rise to lagging chromosomes at anaphase, as discussed previously.

1.3.3. The role of motor proteins in mitotic spindle assembly and chromosome segregation

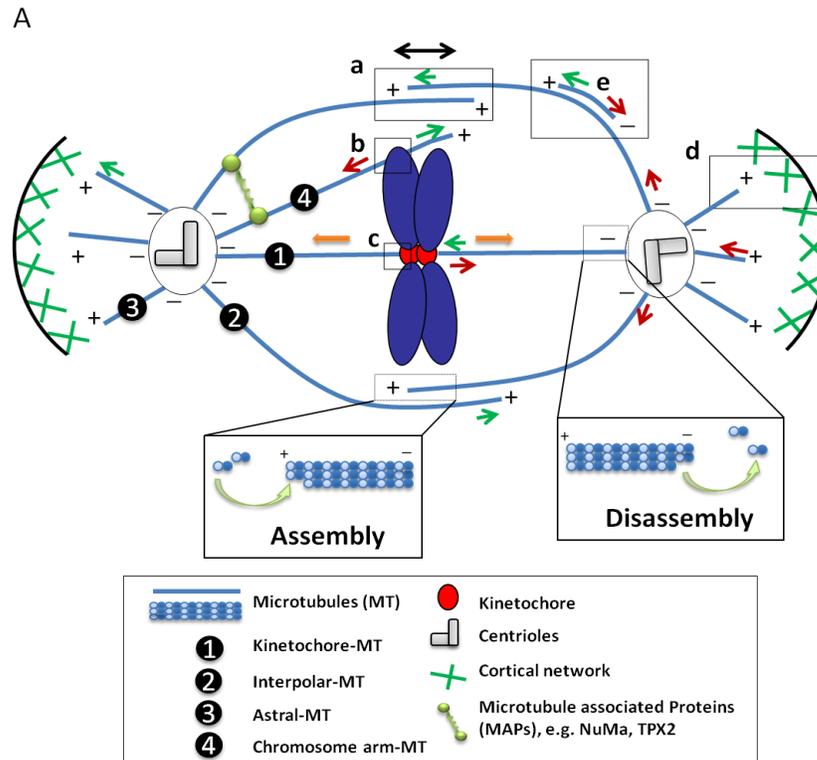
The mitotic spindle is a dynamic, self-organizing into bipolar orientation, microtubule-based mechanochemical structure, that uses energy from nucleotide hydrolysis to segregate sister chromatids accurately into daughter cells during cell division (Sharp *et al.* 2000). In general, the activities of microtubules and their associated motors are responsible for the carefully orchestrated sequences of movements that underlie mitosis. Chromosomes themselves are active participants in generating spindle structure and dynamics. Previously discussed, centromere and kinetochore assembly provides a localized, high-affinity site for the spindle attachment and therefore is a major site for chromosome motility that can move bi-directionally along spindle microtubules. The abundance of regulatory proteins, which are located either at kinetochores or microtubule plus-ends, reflects the dynamism of the mitotic spindle and the complexity of its control (Przewloka and Glover 2009; Amaro *et al.* 2010).

The primary structural element of the spindle is an antiparallel array of microtubules composed of $\alpha\beta$ -tubulin dimers, undergoing transitions between polymerization and depolymerization, coupled to GTP hydrolysis – two events that underlie the dynamic instability of the mitotic spindle (Mitchison and Kirschner 1984; McIntosh and McDonald 1989). A slow-growing minus-end of the mitotic spindle is anchored at centrosomes and fast-growing plus-end is searching the prometaphase cytoplasm in all directions until being captured by kinetochores (O'Connell and Khodjakov 2007). Therefore, at least four main microtubule sub-populations can be recognized within the mitotic spindle (Wittmann *et al.* 2001). First, kinetochore-microtubules (or *k*-fibers) that determine the end-on chromosome attachments to the spindle; second, interpolar microtubules, that derive from opposite poles and interact in an antiparallel fashion to stabilize bipolarity of the spindle; and third, astral microtubules that link spindle poles to the cell cortex and contribute to the separation of spindle poles and positioning of the latter relative to each other and to the cell cortex. The fourth set of MTs link centrosomes to chromosome arms (Figure 1.6A). It has been established near a complete list of spindle molecules (Hutchins *et al.* 2010). Interestingly, a vast quantity of them represent mitotic motors that are located at distinct

sites of mitotic spindle and use ATP hydrolysis to generate forces relative to all four sets of spindle MTs described above (Figure 1.6B).

Microtubule-based motor proteins are comprised of two main families of ATP-dependent force-generating enzymes – kinesins and dyneins (Holzbaur and Vallee 1994; Vale and Fletterick 1997). They perform their motor action using at least three different mechanisms: 1) cross-bridging and sliding MTs relative to adjacent MTs, 2) transporting specific mitotic cargos along the surface of MTs, and 3) regulating MT assembly dynamics and coupling movement to MT polymerization and depolymerization (Sharp *et al.* 2000). In addition, chromosome specific mitotic movements are not driven by individual motors, but rather result from shifts in a dynamic balance of antagonistic and complementary forces exerted by multiple motors within the spindle, e.g. plus-end directed motors can drive chromosomes toward the metaphase plate while minus-end directed motors could drive them toward the spindle poles. This balance of forces is observed at distinct essential points in the spindle, where motors are likely to act: kinetochores, midzone, cortex and chromosome arms (Sharp *et al.* 2000).

In the midzone, motors can crosslink and slide antiparallel MTs by forming bipolar structures with motor domains position at both ends (bipolar kinesins) or by forming asymmetric structures that bind one MT as cargo, causing respectively outward or inward force generation (Sharp *et al.* 2000). Owing that MT-polarity patterns in the spindle, antiparallel MT sliding allows plus-end directed motor Eg5 to push the poles apart (centrosome separation) and minus-end directed dynein to pull them together. This compensating balance of forces at the early stages of mitosis facilitates spindle assembly towards establishment of a bipolar array (Tanenbaum *et al.* 2008). Cytoplasmic dynein/dynactin can also crosslink and slide astral MTs in relation to an actin matrix at the cell cortex. This allows dynein to pull the spindle pole apart and position the entire spindle within the cell (Holzbaur and Vallee 1994). Motors that can bind chromosome arms transport them as cargo along adjacent spindle MTs. Plus-end directed chromokinesin drives chromosomes towards the metaphase plate (plateward) and at the same time positions the spindle poles away from chromosome mass (Rieder and Salmon 1994; Wang and Adler 1995).

**B**

Position within the spindle	Motor family	Functional directionality	Role
Midzone/interpolar MTs (Box a and e)	Bipolar kinesins (Eg5) C-terminal kinesin	Pole-pole: outward Pole-pole: inward	MT-MT sliding MT transport
Chromosome (Box b)	Chromokinesins	Chromosome: poleward Pole-pole: outward	Chromosome transport
Kinetochore (Box c)	CENP-E	Chromosome: poleward/plateward Pole-pole: inward/outward	Kinetochore transport/ MT plus-end anchor
	MCAK/XKCM1	Chromosome: poleward Pole-pole: inward	MT disassembly
	Dynein/dynactin	Pole-pole: inward Chromosome: poleward	Kinetochore transport
Cortical (box d)	Dynein/dynactin	Pole-pole: outward	Actin-MT sliding

Figure 1.6. The dynamic nature of the spindle. (A) The spindle contains different subpopulations of microtubules (MTs) – kinetochore and non-kinetochores (astral and interpolar) microtubules. They provide a mean for variety of dynamic processes occurring in the spindle, including microtubules undergoing dynamic instability (green and red arrows represent growing and shrinking microtubules, respectively), poleward microtubule flux (orange arrows), chromosome movements (black arrow), motor-driven antiparallel sliding and microtubule transport mediated by motor proteins with distinct localization within the mitotic spindle (boxes a-e). Further stabilization of this dynamic structure is achieved by cross-linking focused microtubule ends at spindle poles (MAPs proteins). (B) Table represents cumulative knowledge of sites of action (depicted in A) and functional directionality of motor proteins (Adapted from Sharp et al. 2000 and Wittmann et al. 2001).

At kinetochores, the balance of forces toward spindle poles and spindle equator is accomplished by the complementary activities of two motor proteins – plus-end directed CENP-E and minus-end directed dynein (Schaar *et al.* 1997). During early stages of mitosis, minus-end directed dynein located on the fibrous corona can transport laterally associated kinetochores along the MT surface towards the spindle poles (poleward). Simultaneously, plus-end directed CENP-E translocates along the kinetochore fibers of an already bi-oriented chromosome to transport mono-oriented kinetochores plateward (towards the spindle equator) until the plus-end of the MT is encountered (Glynn *et al.* 2010). It can also anchor the kinetochore to the shortening plus-end of the MT to couple poleward kinetochore motility with MT shrinkage. The relevance in maintaining the balance of forces generation between these two functionally distinct motor proteins is reflected in a numerous studies documenting defects in chromosome congression following inhibition or depletion of CENP-E or dynein components (Wood *et al.* 1997; Starr *et al.* 1998; Tanenbaum *et al.* 2008). In addition to transporting properties, microtubule motors couple MT assembly dynamics to kinetochore motility. This complementary class of motors is comprised of microtubule destabilizing proteins, such as kinesin-13 (MCAK/XKCM1) and kinesin-8 family members that function in depolymerizing microtubules proximal to the kinetochores as the chromosomes move towards the pole during anaphase (Maney *et al.* 1998; Cheeseman and Desai 2008). Counteracting the depolymerizing effect are microtubule-associated proteins (MAPs) that bind to and stabilize microtubules (Wittmann *et al.* 2001) at kinetochores along with cytoplasmic linker protein (CLIP)-associated protein (CLASP1 and CLASP2 family), MAP215 and CLIP170 (Glynn *et al.* 2010).

Further stabilization of the bipolar array also requires focusing of the minus ends of the sorted microtubules to form spindle poles. This is regulated by another types of MAPs, such as NuMa (nuclear protein of the mitotic apparatus) and TPX2 (targeting protein for Xklp2) (Dionne *et al.* 1999; Gruss *et al.* 2002). NuMa is thought to be a principal crosslinker of microtubule minus ends at spindle poles. It forms a complex with cytoplasmic dynein/dynactin and probably other spindle pole proteins, that coalesce disperse microtubules into a focused pole of mitotic spindle. Complementary function in this process is accomplished by TPX2 protein, which has been shown to translocate within the spindle pole and mitotic spindles in a dynein-dependent manner (Wittmann *et al.* 2000; Garrett *et al.* 2002). However, the role of this RanGTP-regulated protein extends beyond the spindle pole focusing. Several studies have shown that TPX2 exhibits many different activities such as MT nucleation and stabilization by crosslinking, spindle pole organization and finally targeting and activation of Aurora A (Wittmann *et al.* 2000; Garrett *et*

al. 2002; Gruss *et al.* 2002; Kufer *et al.* 2002). Depletion of TPX2 results in spindles with a reduced density of microtubules and multipolar spindle formation (Garrett *et al.* 2002). Spindle pole abnormalities were also observed following overexpression of TPX2. In this case the assembly of numerous asters not associated with chromosomes or of extremely dense monopolar spindles were reported (Gruss *et al.* 2002; Ma *et al.* 2010), indicating an important role of this MAP protein in nucleation and organization of a bipolar spindle.

In conclusion, given the structural organization of the mitotic spindle and its polarity, kinetochores and centrosomes derived regulatory machinery, together with multiple motors with distinct localizations, transport properties and mechanisms provide the cell with a precisely controlled, coordinated means for driving coherent mitotic movements at cell division. Spatially, forces exerted within the spindle by the microtubule associated molecules may be highly localized, such as at the kinetochore, or distributed, such as those generated along the length of spindle microtubules or chromosome arms.

1.4. Mitotic checkpoint as a target for chemotherapy

Defects in all of the above mentioned mechanisms ensuring and controlling proper chromosome segregation can cause cell death as a result of chromosome missegregation, but might also be a trigger for oncogenic transformations, mediated through an aneuploidy (Weaver and Cleveland 2006). Therefore, mitotic regulation is important not only for its relevance to tumorigenesis, but can also serve as a successful target for cancer therapy (Weaver and Cleveland 2005; Tanaka and Hirota 2009). In fact, drugs that cause cells to arrest in mitosis (antimitotic drugs), such as vinca alkaloids or taxanes (microtubule poisons) are common treatments for a variety of human tumors, including breast, ovarian and non-small-cell lung cancer (Masuda *et al.* 2003; Lee *et al.* 2004; Yamada and Gorbsky 2006). Extensive research of antimitotic drug treatment, derived from culture cell studies revealed that long-term action of spindle poisons can have several outcomes (Weaver and Cleveland 2005). The most obvious is chronic mitotic arrest until the drug is cleared. In most cases, this effect is cytostatic with renewed cell cycling upon drug removal. The second outcome is known as adaptation or mitotic slippage, in which cells exit long-term arrest in the presence of drug, fail cytokinesis and enter G1, leading to tetraploidy. Adapted cells could then continue cycling, senescence or undergo apoptosis from G1. A final possibility is execution of a death pathway directly from mitosis known as mitotic catastrophe. Whether or not mitotic checkpoint silencing is a prerequisite of adaptation is currently unknown and a subject for a heated discussion (Rieder and Maiato 2004; Yamada and Gorbsky 2006; Lee

et al. 2011). Similarly, the length of time for which intracellular drug concentrations persist at levels sufficient to sustain a checkpoint response or the mechanism linking mitotic delay to subsequent death remains still unknown.

1.4.1. Apoptotic machinery in mitotic events

From a clinical standpoint, cell death is the most desired outcome of antimetabolic drug-based cancer therapy and in mammalian cells has been associated with activation of the apoptotic machinery (Park *et al.* 2004; Baek *et al.* 2005; Yamada and Gorbsky 2006). Apoptosis is a highly-regulated, energy-dependent form of programmed cell death, which has been observed under physiological and pathological conditions (Thompson 1995; Lockshin and Zakeri 2007). Various stress stimuli such as DNA damage, viral infection, nutrient withdrawal, protein aggregation or oxidative stress are involved in initiation of apoptosis (Danial and Korsmeyer 2004). There is a common, evolutionarily conserved mechanism underlying this type of cell death, which involves proteolytic activity of caspases to manifest morphological characteristics of apoptosis (Steller 1995; Riedl and Shi 2004). The latter represent mainly: cleavage of structural and functional components resulting in cell shrinkage, their detachment from neighbouring cells, membrane blebbing, chromatin condensation and DNA cleavage into (oligo)nucleosomal fragments (Kerr *et al.* 1972; Janicke *et al.* 1998). Finally, cells break up to form apoptotic bodies, and the phosphatidylserine is externalized on the plasma membrane, triggering the signal for phagocytosis, which completes apoptosis without an inflammatory response (Fadok 2003; Fadok and Henson 2003).

Caspases are cysteine-dependent aspartate-specific proteases. This denotes two key characteristics of these proteases (Alnemri *et al.* 1996). Like other cysteine proteases, caspases use cysteine side chain as a nucleophile for substrate cleavage. The cleavage is highly specific for substrates, which contain XEXD amino acid sequence (Thornberry *et al.* 1997). With respect to function, two biologically different subfamilies of mammalian caspases can be distinguished: inflammatory caspases (caspase-1, -4, -5, -11, -12, -14), and apoptosis-related caspases, which can initiate (caspase-2, -8, -9, -10) or execute death program (caspase-3, -6 and -7) (Thornberry *et al.* 1997). Different caspases are also involved and mediate two independent pathways of programmed cell death. The first extrinsic pathway is triggered by death receptors that activate initiator caspase-8 to start a cascade involving executioner caspase-3 and -7. The second intrinsic pathway is triggered by different non-receptor stimuli, such as DNA damage and cytotoxic stress, which activates initiator caspase-9, while the main executioners are caspase-3 and -7 (Pop and Salvesen 2009). Regulation of these apoptotic pathways is important for the

survival of cells and the response to damage signals, including microtubule poisons that arrest proliferating cells in mitosis. Indeed, findings from a great amount of studies following treatment with antimetabolic drugs have revealed that apoptosis during mitosis occurs primarily via the intrinsic pathway involving caspase-9 and -3 (Allan and Clarke 2007; Shi *et al.* 2008).

There are evidence that indicate the involvement of apoptosis in antimetabolic drug-mediated cell death. Prolonged mitotic arrest has been associated with several apoptotic markers including activation of caspase-3 (Shin *et al.* 2003; Hsu *et al.* 2006), Fas-associated death domain protein and caspase-10 (Park *et al.* 2004), Bax (Tao *et al.* 2005) and accumulation of p53 (Cahill *et al.* 1998; Vogel *et al.* 2004). Cell death following abrogation of the spindle assembly checkpoint (BubR1 and Mad2) was reported to induce apoptotic markers, such as poly-ADP-ribose polymerase (PARP) and appearance of Annexin V-positive cells (Bharadwaj *et al.* 2004) or caspase-3 activation (Kops *et al.* 2004). The latter, caspase-3 up-regulation at G2/M transition (Hsu *et al.* 2006) and increased activity of this executioner protein during prolonged mitosis is thought to provide a favorable environment for the initiation of apoptosis already in mitosis or following the slippage, especially that Bub1 and BubR1 checkpoint proteins possess caspase cleavage sites, which in mitotic arrested cells might facilitate the slippage (Baek *et al.* 2005; Kim *et al.* 2005). However, the recent live-cell based study suggested that caspase activity is not required for the mitotic checkpoint or mitotic slippage (Lee *et al.* 2011), and whether a cell dies before slipping appears to be dictated by pro- and antiapoptotic signals (Gascoigne and Taylor 2008). Consistently, some of these signals have been found to be regulated by CDK1/cyclin B activity, which apparently can alter the balance between the pro- and antiapoptotic signals over the prolonged mitotic arrest (Allan and Clarke 2007; Harley *et al.* 2010). In nocodazole-treated HeLa or U2OS cells CDK1 kinase phosphorylates the anti-apoptotic protein Mcl-1, triggering its progressive destruction by APC/C pathway (Harley *et al.* 2010). Contrary, the same CDK1/cyclin B activity was also reported to restrain apoptotic routes in spindle poison treated HeLa cells by directly inhibiting caspase-2 (Andersen *et al.* 2009) and caspase-8 (Matthess *et al.* 2010). Bearing that in mind, it is clear that death following spindle poison treatment is governed by a temporal mechanism that modulate the balance between pro- and antiapoptotic factors. However, what regulates these factors in respect to different antimetabolic drugs or different cancer cell lines and, more importantly, how these factors can be altered towards more desired cell death outcome, remains to be discovered.

1.4.2. Mitotic checkpoint defects and antimitotic drug resistance

Several lines of evidence suggest the relationship between chromosome segregation defects, aneuploidy and cancer (Holland and Cleveland 2009). One candidate involved in this relationship is the SAC. Although knockout of SAC genes are lethal to cells (Baker *et al.* 2004; Ricke *et al.* 2008), a partially defective SAC may lead to aneuploidy, allowing missegregation of small number of chromosomes. To date, all of the evidence linking tumorigenesis with the mitotic checkpoint implicate a weakened mitotic checkpoint in the transformation process, which has been defined as a reduced ability to sustain mitotic arrest in the presence of an antimitotic drug (Cahill *et al.* 1998; Yamada and Gorbsky 2006). Mutations in mitotic checkpoint genes have been also associated with antimitotic drug resistance in cancer cells (Kops *et al.* 2005), such as breast, colorectal, ovarian and lung cancer as well as certain types of leukemia (Weaver and Cleveland 2006).

However, the exact role of the SAC in controlling tumorigenesis is not completely understood. Mice heterozygous (*Mad2*) or hypomorphic (*BubR1*) for SAC genes showed an increased frequency of aneuploidy or enhanced tumor formation (Baker *et al.* 2004; Holland and Cleveland 2009). Similarly, mice heterozygous for *Bub3* (Babu *et al.* 2003) or *BubR1* (Dai *et al.* 2004) exhibited elevated rates of tumorigenesis when treated with carcinogen. On the other hand, *Mad2* overexpression triggers tumorigenesis, and leads to a wide variety of neoplasias, tetraploidy and chromosome instability (Sotillo *et al.* 2007). Moreover, defects in components influencing SAC function, like formation of proper kinetochore-microtubule attachment, may also be responsible for aneuploidy and tumor formation (Ricke *et al.* 2008). In mouse fibroblasts and hepatocytes, the depletion of CENP-E, which works together with *BubR1*, increased frequency of chromosome missegregation (Weaver *et al.* 2003). *BubR1* depletion alone has also been observed in a subset of colon cancer cell lines (Cahill *et al.* 1998). Furthermore, germline mutations in *BubR1* have been associated with the rare recessive disease called Mosaic Variegated Aneuploidy (MVA) syndrome, which causes childhood cancer (Hanks *et al.* 2004).

Overall, it is clear from studies in cultured cells treated with antimitotic drugs that there is a link between spindle checkpoint-mediated mitotic arrest and antimitotic drug-mediated cell death, and that partial loss of the mitotic checkpoint function may render cells somehow more resistant to antimitotic drugs. Weakened spindle checkpoint is commonly observed among cancer cells, and thus the molecular linkages between this cancer-related change in cellular physiology and responses to different drug treatments should be more explored.

1.5. Aims

The complexity of the combined action of the several multiprotein complexes, discussed earlier, that assemble with the high precision exactly once per cell cycle in order to segregate chromosomes in a very accurate manner, still remains an open window for investigation. In fact, the centromere assembly, that underlies most of the processes involved in equal chromosome segregation (e.g. kinetochore-microtubule interface, microtubule capturing and error correction as well as signaling from unattached kinetochores) is very complicated and still not entirely understood. Particularly, the role of CENP-W and CENP-T proteins, which were proposed to play the main function in switching the centromeric chromatin into mitotic state owing to their histone fold domains, remained elusive prior to our published work and required to be tested in human cells for a better understanding of the whole centromere assembly process. In a broader aspect, the knowledge of the chromosome segregation machinery is extensively developed and utilized in the battle against cancer, that uses the relationship between the function of the mitotic checkpoint proteins, related to centromere/kinetochore assembly, and sensitivity to antimitotic drugs. Finding out the components of the apoptotic pathway involved in these mitotic processes, which can directly or indirectly change the balance between the fate following extended mitotic arrest would be of a great benefit for the improved anti-cancer therapies.

The specific aims we set out to achieve in this doctoral work were:

- 1) investigation of the histone fold domain (HFD) centromere proteins, CENP-W/T complex of the CCAN for its requirement in mitosis and kinetochore assembly/function, by RNAi knockdown studies of the mitotic kinetics. This research was complemented with investigation of the kinetochore failure in the SAC signaling;
- 2) elucidation of the nature of the multipolar spindle formation in cells depleted of CENP-W, which we found exhibited characteristic rolling spindle phenotype. We aimed to examine the mechanism of the pole fragmentation observed in these cells by applying drug-based methods and molecular biology modifications towards the disturbed kinetochore force generation alteration in order to re-establish the initial distribution;
- 3) evaluation of caspase-3 involvement in normal mitosis of the HeLa cell cycle, by investigating its modulation in populations fractionated according to the cell cycle phase and/or by small interference RNA-based (siRNA) depletion of this specific apoptotic protein and examining mitotic kinetics in otherwise untreated HeLa H2B-GFP cells.

1.6. REFERENCES

- Allan L. A., Clarke P. R. (2007). Phosphorylation of caspase-9 by CDK1/cyclin B1 protects mitotic cells against apoptosis. *Molecular Cell* **26**(2): 301-310.
- Allshire R. C., Karpen G. H. (2008). Epigenetic regulation of centromeric chromatin: old dogs, new tricks? *Nature Reviews Genetics* **9**(12): 923-937.
- Alnemri E. S., Livingston D. J., Nicholson D. W., Salvesen G., Thornberry N. A., Wong W. W., Yuan J. (1996). Human ICE/CED-3 protease nomenclature. *Cell* **87**(2): 171-172.
- Amano M., Suzuki A., Hori T., Backer C., Okawa K., Cheeseman I. M., Fukagawa T. (2009). The CENP-S complex is essential for the stable assembly of outer kinetochore structure. *Journal of Cell Biology* **186**(2): 173-82.
- Amaro A. C., Samora C. P., Holtackers R., Wang E., Kingston I. J., Alonso M., Lampson M., McAinsh A. D., Meraldi P. (2010). Molecular control of kinetochore-microtubule dynamics and chromosome oscillations. *Nature Cell Biology* **12**(4): 319-329.
- Andersen J. L., Johnson C. E., Freel C. D., Parrish A. B., Day J. L., Buchakjian M. R., Nutt L. K., Thompson J. W., Moseley M. A., Kornbluth S. (2009). Restraint of apoptosis during mitosis through interdomain phosphorylation of caspase-2. *The EMBO Journal* **28**(20): 3216-3227.
- Andrews P. D., Ovechkina Y., Morrice N., Wagenbach M., Duncan K., Wordeman L., Swedlow J. R. (2004). Aurora B regulates MCAK at the mitotic centromere. *Developmental Cell* **6**(2): 253-268.
- Babu J. R., Jeganathan K. B., Baker D. J., Wu X., Kang-Decker N., Van Deursen J. M. (2003). Rae1 is an essential mitotic checkpoint regulator that cooperates with Bub3 to prevent chromosome missegregation. *The Journal of Cell Biology* **160**(3): 341-353.
- Baek K., Shin H., Jeong S., Park J., McKeon F., Lee C., Kim C. (2005). Caspases-dependent cleavage of mitotic checkpoint proteins in response to microtubule inhibitor. *Oncology Research* **15**(3): 161-168.
- Baker D. J., Jeganathan K. B., Cameron J. D., Thompson M., Juneja S., Kopecka A., Kumar R., Jenkins R. B., De Groen P. C., Roche P. (2004). BubR1 insufficiency causes early onset of aging-associated phenotypes and infertility in mice. *Nature Genetics* **36**(7): 744-749.
- Bernard P., Maure J. F., Partridge J. F., Genier S., Javerzat J. P., Allshire R. C. (2001). Requirement of heterochromatin for cohesion at centromeres. *Science* **294**(5551): 2539.
- Bertran M. T., Sdelci S., Regue L., Avruch J., Caelles C., Roig J. (2011). Nek9 is a Plk1-activated kinase that controls early centrosome separation through Nek6/7 and Eg5. *The EMBO Journal* **30**(13): 2634-2647.
- Bharadwaj R., Qi W., Yu H. (2004). Identification of two novel components of the human NDC80 kinetochore complex. *Journal of Biological Chemistry* **279**(13): 13076.
- Biggins S., Murray A. W. (2001). The budding yeast protein kinase Ipl1/Aurora allows the absence of tension to activate the spindle checkpoint. *Genes & Development* **15**(23): 3118.
- Black B. E., Brock M. A., Bårdard S., Woods V. L., Cleveland D. W. (2007). An epigenetic mark generated by the incorporation of CENP-A into centromeric nucleosomes. *Proceedings of the National Academy of Sciences* **104**(12): 5008.
- Black B. E., Foltz D. R., Chakravarthy S., Luger K., Woods V. L., Cleveland D. W. (2004). Structural determinants for generating centromeric chromatin. *Nature* **430**(6999): 578-582.
- Bobinnec Y., Khodjakov A., Mir L., Rieder C., Edde B., Bornens M. (1998). Centriole disassembly in vivo and its effect on centrosome structure and function in vertebrate cells. *The Journal of Cell Biology* **143**(6): 1575-1589.
- Bornens M. (2002). Centrosome composition and microtubule anchoring mechanisms. *Current Opinion in Cell Biology* **14**(1): 25-34.
- Boveri T. (2008). Concerning the origin of malignant tumours by Theodor Boveri. Translated and annotated by Henry Harris. *Journal of Cell Science* **121**(Supplement 1): 1-84.
- Burke D. J., Stukenberg P. T. (2008). Linking Kinetochore-Microtubule Binding to the Spindle Checkpoint. *Developmental Cell* **14**(4): 474-479.
- Cahill D. P., Lengauer C., Yu J., Riggins G. J., Willson J. K. V., Markowitz S. D., Kinzler K. W., Vogelstein B. (1998). Mutations of mitotic checkpoint genes in human cancers. *Nature* **392**(6673): 300-303.
- Carroll C. W., Straight A. F. (2006). Centromere formation: from epigenetics to self-assembly. *Trends in Cell Biology* **16**(2): 70-8.

- Cheeseman I., Desai A. (2008). Molecular architecture of the kinetochore–microtubule interface. *Nature Reviews Molecular Cell Biology* **9**(1): 33-46.
- Cheeseman I. M., Chappie J. S., Wilson-Kubalek E. M., Desai A. (2006). The conserved KMN network constitutes the core microtubule-binding site of the kinetochore. *Cell* **127**(5): 983-997.
- Cheeseman I. M., Hori T., Fukagawa T., Desai A. (2008). KNL1 and the CENP-H/I/K complex coordinately direct kinetochore assembly in vertebrates. *Mol Biol Cell* **19**(2): 587-94.
- Cheeseman I. M., Niessen S., Anderson S., Hyndman F., Yates J. R., Oegema K., Desai A. (2004). A conserved protein network controls assembly of the outer kinetochore and its ability to sustain tension. *Genes & Development* **18**(18): 2255.
- Ciferri C., De Luca J., Monzani S., Ferrari K. J., Ristic D., Wyman C., Stark H., Kilmartin J., Salmon E. D., Musacchio A. (2005). Architecture of the human ndc80-hec1 complex, a critical constituent of the outer kinetochore. *Journal of Biological Chemistry* **280**(32): 29088-29095.
- Ciferri C., Musacchio A., Petrovic A. (2007). The Ndc80 complex: hub of kinetochore activity. *FEBS letters* **581**(15): 2862-2869.
- Ciferri C., Pasqualato S., Screpanti E., Varetto G., Santaguida S., Dos Reis G., Maiolica A., Polka J., De Luca J. G., De Wulf P. (2008). Implications for kinetochore-microtubule attachment from the structure of an engineered Ndc80 complex. *Cell* **133**(3): 427-439.
- Cimini D. (2008). Merotelic kinetochore orientation, aneuploidy, and cancer. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer* **1786**(1): 32-40.
- Cimini D., Cameron L. A., Salmon E. (2004). Anaphase spindle mechanics prevent mis-segregation of merotelically oriented chromosomes. *Current Biology* **14**(23): 2149-2155.
- Cimini D., Fioravanti D., Salmon E., Degraffi F. (2002). Merotelic kinetochore orientation versus chromosome mono-orientation in the origin of lagging chromosomes in human primary cells. *Journal of Cell Science* **115**(3): 507-515.
- Civril F., Musacchio A. (2008). Spindly attachments. *Genes & Development* **22**(17): 2302-2307.
- Clarke P. R., Karsenti E. (1991). Regulation of p34cdc2 protein kinase: new insights into protein phosphorylation and the cell cycle. *Journal of Cell Science* **100**(3): 409-414.
- Cleveland D. W., Mao Y., Sullivan K. F. (2003). Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling. *Cell* **112**(4): 407-421.
- Cooke C. A., Schaar B., Yen T. J., Earnshaw W. C. (1997). Localization of CENP-E in the fibrous corona and outer plate of mammalian kinetochores from prometaphase through anaphase. *Chromosoma* **106**(7): 446-455.
- Dai W., Wang Q., Liu T., Swamy M., Fang Y., Xie S., Mahmood R., Yang Y. M., Xu M., Rao C. V. (2004). Slippage of mitotic arrest and enhanced tumor development in mice with BubR1 haploinsufficiency. *Cancer Research* **64**(2): 440.
- Dalal Y. D. Y. (2009). Epigenetic specification of centromeres. *Biochemistry and Cell Biology* **87**(1): 273-282.
- Daniel N. N., Korsmeyer S. J. (2004). Cell death: critical control points. *Cell* **116**(2): 205-219.
- DeLuca J. G., Gall W. E., Ciferri C., Cimini D., Musacchio A., Salmon E. (2006). Kinetochore microtubule dynamics and attachment stability are regulated by Hec1. *Cell* **127**(5): 969-982.
- DeLuca J. G., Howell B. J., Canman J. C., Hickey J. M., Fang G., Salmon E. (2003). Nuf2 and Hec1 are required for retention of the checkpoint proteins Mad1 and Mad2 to kinetochores. *Current Biology* **13**(23): 2103-2109.
- Desai A., Rybina S., Moller-Reichert T., Shevchenko A., Hyman A., Oegema K. (2003). KNL-1 directs assembly of the microtubule-binding interface of the kinetochore in *C. elegans*. *Genes & Development* **17**(19): 2421.
- Dionne M. A., Howard L., Compton D. A. (1999). NuMA is a component of an insoluble matrix at mitotic spindle poles. *Cell Motility and the Cytoskeleton* **42**(3): 189-203.
- Doxsey S. (2001). Re-evaluating centrosome function. *Nature Reviews Molecular Cell Biology* **2**(9): 688-698.
- Dunleavy E. M., Almouzni G., Karpen G. H. (2011). H3. 3 is deposited at centromeres in S phase as a placeholder for newly assembled CENP-A in G1 phase. *Nucleus* **2**(2): 146-157.
- Earnshaw W. C., Rothfield N. (1985). Identification of a family of human centromere proteins using autoimmune sera from patients with scleroderma. *Chromosoma* **91**(3): 313-321.
- Emre D., Terracol R., Poncet A., Rahmani Z., Karess R. E. (2011). A mitotic role for Mad1 beyond the spindle checkpoint. *Journal of Cell Science* **124**(10): 1664.

- Fadeel B. (2003). Programmed cell clearance. *Cellular and Molecular Life Sciences* **60**(12): 2575-2585.
- Fadok V. A., Henson P. M. (2003). Apoptosis: giving phosphatidylserine recognition an assist-with a twist. *Current Biology* **13**(16): R655-R657.
- Fitzgerald-Hayes M., Clarke L., Carbon J. (1982). Nucleotide sequence comparisons and functional analysis of yeast centromere DNAs. *Cell* **29**(1): 235-244.
- Foltz D. R., Jansen L. E. T., Black B. E., Bailey A. O., Yates J. R., Cleveland D. W. (2006). The human CENP-A centromeric nucleosome-associated complex. *Nature Cell Biology* **8**(5): 458-469.
- Fukasawa K. (2005). Centrosome amplification, chromosome instability and cancer development. *Cancer letters* **230**(1): 6-19.
- Furuyama T., Henikoff S. (2009). Centromeric nucleosomes induce positive DNA supercoils. *Cell* **138**(1): 104-113.
- Gaitanos T. N., Santamaria A., Jeyaprakash A. A., Wang B., Conti E., Nigg E. A. (2009). Stable kinetochore-microtubule interactions depend on the Ska complex and its new component Ska3/C13Orf3. *The EMBO Journal* **28**(10): 1442-1452.
- Ganem N. J., Godinho S. A., Pellman D. (2009). A mechanism linking extra centrosomes to chromosomal instability. *Nature* **460**(7252): 278-282.
- Garrett S., Auer K., Compton D. A., Kapoor T. M. (2002). hTPX2 is required for normal spindle morphology and centrosome integrity during vertebrate cell division. *Current Biology* **12**(23): 2055-2059.
- Gascoigne K. E., Takeuchi K., Suzuki A., Hori T., Fukagawa T., Cheeseman I. M. (2011). Induced ectopic kinetochore assembly bypasses the requirement for CENP-A nucleosomes. *Cell* **145**(3): 410-422.
- Gascoigne K. E., Taylor S. S. (2008). Cancer cells display profound intra-and interline variation following prolonged exposure to antimetabolic drugs. *Cancer Cell* **14**(2): 111-122.
- Gassmann R., Essex A., Hu J. S., Maddox P. S., Motegi F., Sugimoto A., O'Rourke S. M., Bowerman B., McLeod I., Yates III J. R. (2008). A new mechanism controlling kinetochore-microtubule interactions revealed by comparison of two dynein-targeting components: SPDL-1 and the Rod/Zwilch/Zw10 complex. *Genes & Development* **22**(17): 2385-2399.
- Gassmann R., Holland A. J., Varma D., Wan X., Civril F., Cleveland D. W., Oegema K., Salmon E. D., Desai A. (2010). Removal of Spindly from microtubule-attached kinetochores controls spindle checkpoint silencing in human cells. *Genes & Development* **24**(9): 957-971.
- Glynn M., Kaczmarczyk A., Prendergast L., Quinn N., Sullivan K. F. (2010). Centromeres: assembling and propagating epigenetic function. *Genome Stability and Human Diseases*: 223-249.
- Griffis E. R., Stuurman N., Vale R. D. (2007). Spindly, a novel protein essential for silencing the spindle assembly checkpoint, recruits dynein to the kinetochore. *The Journal of Cell Biology* **177**(6): 1005-1015.
- Gruss O. J., Wittmann M., Yokoyama H., Pepperkok R., Kufer T., Silje H., Karsenti E., Mattaj J. W., Vernos I. (2002). Chromosome-induced microtubule assembly mediated by TPX2 is required for spindle formation in HeLa cells. *Nature Cell Biology* **4**(11): 871-879.
- Guimaraes G. J., DeLuca J. G. (2009). Connecting with Ska, a key complex at the kinetochore-microtubule interface. *The EMBO Journal* **28**(10): 1375-1377.
- Gustafson L. M., Gleich L. L., Fukasawa K., Chadwell J., Ann Miller M., Stambrook P. J., Gluckman J. L. (2000). Centrosome hyperamplification in head and neck squamous cell carcinoma: a potential phenotypic marker of tumor aggressiveness. *The Laryngoscope* **110**(11): 1798-1801.
- Hanks S., Coleman K., Reid S., Plaja A., Firth H., FitzPatrick D., Kidd A., Mehes K., Nash R., Robin N. (2004). Constitutional aneuploidy and cancer predisposition caused by biallelic mutations in BUB1B. *Nature Genetics* **36**(11): 1159-1161.
- Harley M. E., Allan L. A., Sanderson H. S., Clarke P. R. (2010). Phosphorylation of Mcl-1 by CDK1-cyclin B1 initiates its Cdc20-dependent destruction during mitotic arrest. *The EMBO Journal* **29**(14): 2407-2420.
- Heun P., Erhardt S., Blower M. D., Weiss S., Skora A. D., Karpen G. H. (2006). Mislocalization of the *Drosophila* centromere-specific histone CID promotes formation of functional ectopic kinetochores. *Dev Cell* **10**(3): 303-15.
- Holland A. J., Cleveland D. W. (2009). Boveri revisited: chromosomal instability, aneuploidy and tumorigenesis. *Nature Reviews Molecular Cell Biology* **10**(7): 478-487.
- Holzbaun E., Vallee R. (1994). Dyneins: molecular structure and cellular function. *Annual Review of Cell Biology* **10**(1): 339-372.

- Hori T., Amano M., Suzuki A., Backer C., Welburn J., Dong Y., McEwen B., Shang W., Suzuki E., Okawa K. (2008). CCAN makes multiple contacts with centromeric DNA to provide distinct pathways to the outer kinetochore. *Cell* **135**(6): 1039-1052.
- Hori T., Okada M., Maenaka K., Fukagawa T. (2008b). CENP-O class proteins form a stable complex and are required for proper kinetochore function. *Molecular Biology of the Cell* **19**(3): 843-854.
- Howell B., McEwen B., Canman J., Hoffman D., Farrar E., Rieder C., Salmon E. (2001). Cytoplasmic dynein/dynactin drives kinetochore protein transport to the spindle poles and has a role in mitotic spindle checkpoint inactivation. *The Journal of Cell Biology* **155**(7): 1159-1172.
- Hoyt M. A., Totis L., Roberts B. T. (1991). *S. cerevisiae* genes required for cell cycle arrest in response to loss of microtubule function. *Cell* **66**(3): 507-517.
- Hsu S., Yu C., Yin S., Tang M., Tien A., Wu Y., Huang C. (2006). Caspase 3, periodically expressed and activated at G2/M transition, is required for nocodazole-induced mitotic checkpoint. *Apoptosis* **11**(5): 765-771.
- Hut H. M. J., Lemstra W., Blaauw E. H., Van Cappellen G. W. A., Kampinga H. H., Sibon O. C. M. (2003). Centrosomes split in the presence of impaired DNA integrity during mitosis. *Molecular Biology of the Cell* **14**(5): 1993-2004.
- Hutchins J. R. A., Toyoda Y., Hegemann B., Poser I., Heriche J. K., Sykora M. M., Augsborg M., Hudecz O., Buschhorn B. A., Bulkescher J. (2010). Systematic analysis of human protein complexes identifies chromosome segregation proteins. *Science* **328**(5978): 593-599.
- Hwang L. H., Lau L. F., Smith D. L., Mistrot C. A., Hardwick K. G., Hwang E. S., Amon A., Murray A. W. (1998). Budding yeast Cdc20: a target of the spindle checkpoint. *Science* **279**(5353): 1041.
- Inanc B., Dodson H., Morrison C. G. (2010). A centrosome-autonomous signal that involves centriole disengagement permits centrosome duplication in G2 phase after DNA damage. *Molecular Biology of the Cell* **21**(22): 3866-3877.
- Izuta H., Ikeno M., Suzuki N., Tomonaga T., Nozaki N., Obuse C., Kisu Y., Goshima N., Nomura F., Nomura N. (2006). Comprehensive analysis of the ICEN (Interphase Centromere Complex) components enriched in the CENP-A chromatin of human cells. *Genes Cells* **11**(6): 673-684.
- Janicke R. U., Sprengart M. L., Wati M. R., Porter A. G. (1998). Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *Journal of Biological Chemistry* **273**(16): 9357-9360.
- Jansen L. E. T., Black B. E., Foltz D. R., Cleveland D. W. (2007). Propagation of centromeric chromatin requires exit from mitosis. *The Journal of Cell Biology* **176**(6): 795-805.
- Jokelainen P. T. (1967). The ultrastructure and spatial organization of the metaphase kinetochore in mitotic rat cells. *Journal of Ultrastructure Research* **19**(1-2): 19-44.
- Karess R. (2005). Rod-Zw10-Zwilch: a key player in the spindle checkpoint. *Trends in Cell Biology* **15**(7): 386-392.
- Kerr J. F., Wyllie A. H., Currie A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26**: 239-257.
- Khodjakov A., Rieder C. L. (2001). Centrosomes enhance the fidelity of cytokinesis in vertebrates and are required for cell cycle progression. *The Journal of Cell Biology* **153**(1): 237-242.
- Khodjakov A., Rieder C. L., Sluder G., Cassels G., Sibon O., Wang C. L. (2002). De novo formation of centrosomes in vertebrate cells arrested during S phase. *The Journal of Cell Biology* **158**(7): 1171-1181.
- Kim M., Murphy K., Liu F., Parker S., Dowling M., Baff W., Kao G. (2005). Caspase-mediated specific cleavage of BubR1 is a determinant of mitotic progression. *Molecular and Cellular Biology* **25**(21): 9232-9248.
- Kiyomitsu T., Obuse C., Yanagida M. (2007). Human Blinkin/AF15q14 is required for chromosome alignment and the mitotic checkpoint through direct interaction with Bub1 and BubR1. *Developmental Cell* **13**(5): 663-676.
- Kline S. L., Cheeseman I. M., Hori T., Fukagawa T., Desai A. (2006). The human Mis12 complex is required for kinetochore assembly and proper chromosome segregation. *The Journal of Cell Biology* **173**(1): 9.
- Kops G. J. P. L., Foltz D. R., Cleveland D. W. (2004). Lethality to human cancer cells through massive chromosome loss by inhibition of the mitotic checkpoint. *Proceedings of the National Academy of Sciences of the United States of America* **101**(23): 8699.

- Kops G. J. P. L., Kim Y., Weaver B. A. A., Mao Y., McLeod I., Yates III J. R., Tagaya M., Cleveland D. W. (2005). ZW10 links mitotic checkpoint signaling to the structural kinetochore. *The Journal of Cell Biology* **169**(1): 49-60.
- Kops G. J. P. L., Weaver B. A. A., Cleveland D. W. (2005). On the road to cancer: aneuploidy and the mitotic checkpoint. *Nature Reviews Cancer* **5**(10): 773-785.
- Kramer A., Schweizer S., Neben K., Giesecke C., Kalla J., Katzenberger T., Benner A., Muller-Hermelink H., Ho A., Ott G. (2003). Centrosome aberrations as a possible mechanism for chromosomal instability in non-Hodgkin's lymphoma. *Leukemia* **17**(11): 2207-2213.
- Kufer T. A., Sillje H. H. W., Korner R., Gruss O. J., Meraldi P., Nigg E. A. (2002). Human TPX2 is required for targeting Aurora-A kinase to the spindle. *The Journal of Cell Biology* **158**(4): 617-623.
- Kulukian A., Han J., Cleveland D. (2009). Unattached Kinetochores Catalyze Production of an Anaphase Inhibitor that Requires a Mad2 Template to Prime Cdc20 for BubR1 Binding. *Developmental Cell* **16**(1): 105-117.
- Kuo K. K., Sato N., Mizumoto K., Maehara N., Yonemasu H., Ker C. G., Sheen P. C., Tanaka M. (2000). Centrosome abnormalities in human carcinomas of the gallbladder and intrahepatic and extrahepatic bile ducts. *Hepatology* **31**(1): 59-64.
- Kwon M., Godinho S. A., Chandhok N. S., Ganem N. J., Azioune A., Thery M., Pellman D. (2008). Mechanisms to suppress multipolar divisions in cancer cells with extra centrosomes. *Genes & Development* **22**(16): 2189-2203.
- Lee E. A., Keutmann M. K., Dowling M. L., Harris E., Chan G., Kao G. D. (2004). Inactivation of the mitotic checkpoint as a determinant of the efficacy of microtubule-targeted drugs in killing human cancer cells. *Molecular Cancer Therapeutics* **3**(6): 661.
- Lee K., Kenny A. E., Rieder C. L. (2011). Caspase activity is not required for the mitotic checkpoint or mitotic slippage in human cells. *Molecular Biology of the Cell* **22**(14): 2470-2479.
- Lengauer C., Kinzler K. W., Vogelstein B. (1998). Genetic instabilities in human cancers. *Nature* **396**(6712): 643-643.
- Li R., Murray A. W. (1991). Feedback control of mitosis in budding yeast. *Cell* **66**(3): 519-531.
- Lingle W. L., Barrett S. L., Negron V. C., D'Assoro A. B., Boeneman K., Liu W., Whitehead C. M., Reynolds C., Salisbury J. L. (2002). Centrosome amplification drives chromosomal instability in breast tumor development. *Proceedings of the National Academy of Sciences* **99**(4): 1978.
- Lingle W. L., Lukasiewicz K., Salisbury J. L. (2005). Deregulation of the centrosome cycle and the origin of chromosomal instability in cancer. *Genome Instability in Cancer Development*: 393-421.
- Lingle W. L., Lutz W. H., Ingle J. N., Maihle N. J., Salisbury J. L. (1998). Centrosome hypertrophy in human breast tumors: implications for genomic stability and cell polarity. *Proceedings of the National Academy of Sciences* **95**(6): 2950.
- Liu D., Vader G., Vromans M. J. M., Lampson M. A., Lens S. (2009). Sensing chromosome bi-orientation by spatial separation of Aurora B kinase from kinetochore substrates. *Science's STKE* **323**(5919): 1350.
- Liu S., Hittle J., Jablonski S., Campbell M., Yoda K., Yen T. (2003). Human CENP-I specifies localization of CENP-F, Mad1 and Mad2 to kinetochores and is essential for mitosis. *Nature Cell Biology* **5**: 341-345.
- Liu S., Rattner J., Jablonski S., Yen T. (2006). Mapping the assembly pathways that specify formation of the trilaminar kinetochore plates in human cells. *Journal of Cell Biology* **175**(1): 41-53.
- Lockshin R. A., Zakeri Z. (2007). Cell death in health and disease. *Journal of Cellular and Molecular Medicine* **11**(6): 1214-1224.
- Logarinho E., Bousbaa H. (2008). Kinetochore-microtubule interactions "in check" by Bub1, Bub3 and BubR1: The dual task of attaching and signalling. *Cell Cycle* **7**(12): 1763-1768.
- Loncarek J., Hergert P., Magidson V., Khodjakov A. (2008). Control of daughter centriole formation by the pericentriolar material. *Nature Cell Biology* **10**(3): 322-328.
- Luders J., Stearns T. (2007). Microtubule-organizing centres: a re-evaluation. *Nature Reviews Molecular Cell Biology* **8**(2): 161-167.
- Luo X., Tang Z., Rizo J., Yu H. (2002). The Mad2 spindle checkpoint protein undergoes similar major conformational changes upon binding to either Mad1 or Cdc20. *Molecular Cell* **9**(1): 59-71.

- Ma N., Tulu U., Ferenz N. P., Fagerstrom C., Wilde A., Wadsworth P. (2010). Poleward transport of TPX2 in the mammalian mitotic spindle requires dynein, Eg5, and microtubule flux. *Molecular Biology of the Cell* **21**(6): 979-988.
- Majer B., Laky B., Knasmuller S., Kassie F. (2001). Use of the micronucleus assay with exfoliated epithelial cells as a biomarker for monitoring individuals at elevated risk of genetic damage and in chemoprevention trials. *Mutation Research/Reviews in Mutation Research* **489**(2-3): 147-172.
- Maney T., Hunter A. W., Wagenbach M., Wordeman L. (1998). Mitotic centromere-associated kinesin is important for anaphase chromosome segregation. *The Journal of Cell Biology* **142**(3): 787-801.
- Mardin B. R., Lange C., Baxter J. E., Hardy T., Scholz S. R., Fry A. M., Schiebel E. (2010). Components of the Hippo pathway cooperate with Nek2 kinase to regulate centrosome disjunction. *Nature Cell Biology* **12**(12): 1166-1176.
- Maresca T. J., Salmon E. (2010). Welcome to a new kind of tension: translating kinetochore mechanics into a wait-anaphase signal. *Journal of Cell Science* **123**(6): 825-835.
- Maresca T. J., Salmon E. D. (2009). Intrakinetochore stretch is associated with changes in kinetochore phosphorylation and spindle assembly checkpoint activity. *The Journal of Cell Biology* **184**(3): 373-381.
- Marshall O. J., Chueh A. C., Wong L. H., Choo K. (2008). Neocentromeres: new insights into centromere structure, disease development, and karyotype evolution. *The American Journal of Human Genetics* **82**(2): 261-282.
- Martin-Lluesma S., Stucke V. M., Nigg E. A. (2002). Role of Hec1 in spindle checkpoint signaling and kinetochore recruitment of Mad1/Mad2. *Science* **297**(5590): 2267.
- Masuda A., Maeno K., Nakagawa T., Saito H., Takahashi T. (2003). Association between mitotic spindle checkpoint impairment and susceptibility to the induction of apoptosis by anti-microtubule agents in human lung cancers. *The American Journal of Pathology* **163**(3): 1109-1116.
- Masumoto H., Masukata H., Muro Y., Nozaki N., Okazaki T. (1989). A human centromere antigen (CENP-B) interacts with a short specific sequence in alphoid DNA, a human centromeric satellite. *The Journal of Cell Biol* **109**(5): 1963-73.
- Matthess Y., Raab M., Sanhaji M., Lavrik I. N., Strebhardt K. (2010). Cdk1/cyclin B1 controls Fas-mediated apoptosis by regulating caspase-8 activity. *Molecular and Cellular Biology* **30**(24): 5726.
- Mattiuzzo M., Vargiu G., Totta P., Fiore M., Ciferri C., Musacchio A., Degraffi F. (2011). Abnormal kinetochore-generated pulling forces from expressing a N-terminally modified Hec1. *PLoS One* **6**(1): e16307.
- McClelland S. E., Borusu S., Amaro A. C., Winter J. R., Belwal M., McAinsh A. D., Meraldi P. (2007). The CENP-A NAC/CAD kinetochore complex controls chromosome congression and spindle bipolarity. *The EMBO Journal* **26**(24): 5033-5047.
- McEwen B. F., Dong Y. (2009). Releasing the spindle assembly checkpoint without tension. *The Journal of Cell Biology* **184**(3): 355-356.
- McIntosh J. R., McDonald K. L. (1989). The mitotic spindle. *Scientific American* **261**(4): 48.
- Meluh P. B., Yang P., Glowczewski L., Koshland D., Smith M. M. (1998). Cse4p is a component of the core centromere of *Saccharomyces cerevisiae*. *Cell* **94**(5): 607-613.
- Mendiburo M. J., Padeken J., Fulop S., Schepers A., Heun P. (2011). *Drosophila* CENH3 is sufficient for centromere formation. *Science* **334**(6056): 686-690.
- Milks K. J., Moree B., Straight A. F. (2009). Dissection of CENP-Câ€‘directed Centromere and Kinetochore Assembly. *Molecular Biology of the Cell* **20**(19): 4246-4255.
- Mitchison T., Kirschner M. (1984). Dynamic instability of microtubule growth. *Nature* **312**(5991): 237-242.
- Morris C. A., Moazed D. (2007). Centromere assembly and propagation. *Cell* **128**(4): 647-650.
- Musacchio A., Hardwick K. G. (2002). The spindle checkpoint: structural insights into dynamic signalling. *Nature Reviews Molecular Cell Biology* **3**(10): 731-741.
- Musacchio A., Salmon E. (2007). The spindle-assembly checkpoint in space and time. *Nature Reviews Molecular Cell Biology* **8**: 379-393.
- Nigg E. A. (2006). Cell biology: a licence for duplication. *Nature* **442**(7105): 874.
- Nigg E. A., Stearns T. (2011). The centrosome cycle: Centriole biogenesis, duplication and inherent asymmetries. *Nature Cell Biology* **13**(10): 1154-1160.

- Nishino T., Takeuchi K., Gascoigne K. E., Suzuki A., Hori T., Oyama T., Morikawa K., Cheeseman I. M., Fukagawa T. (2012). CENP-TWSX Forms a Unique Centromeric Chromatin Structure with a Histone-like Fold. *Cell* **148**(3): 487-501.
- Nitta T., Kanai M., Sugihara E., Tanaka M., Sun B., Nagasawa T., Sonoda S., Saya H., Miwa M. (2006). Centrosome amplification in adult T-cell leukemia and human T-cell leukemia virus type 1 Tax-induced human T cells. *Cancer Science* **97**(9): 836-841.
- Norbury C., Nurse P. (1992). Animal cell cycles and their control. *Annual Review of Biochemistry* **61**(1): 441-468.
- O'Connell C. B., Khodjakov A. L. (2007). Cooperative mechanisms of mitotic spindle formation. *Journal of Cell Science* **120**(10): 1717-1722.
- Obuse C., Yang H., Nozaki N., Goto S., Okazaki T., Yoda K. (2004). Proteomics analysis of the centromere complex from HeLa interphase cells: UV-damaged DNA binding protein 1 (DDB-1) is a component of the CEN-complex, while BMI-1 is transiently co-localized with the centromeric region in interphase. *Genes to Cells* **9**(2): 105-120.
- Ohta S., Bukowski-Wills J. C., Sanchez-Pulido L., Alves F. L., Wood L., Chen Z. A., Platani M., Fischer L., Hudson D. F., Ponting C. P. (2010). The protein composition of mitotic chromosomes determined using multiclassifier combinatorial proteomics. *Cell* **142**(5): 810-821.
- Okada M., Cheeseman I. M., Hori T., Okawa K., McLeod I., Yates III J. R., Desai A., Fukagawa T. (2006). The CENP-H-I complex is required for the efficient incorporation of newly synthesized CENP-A into centromeres. *Nature Cell Biology* **8**: 446-457.
- Ou Y., Zhang M., Rattner J. B. (2004). The centrosome: The centriole-PCM coalition. *Cell Motility and the Cytoskeleton* **57**(1): 1-7.
- Paintrand M., Moudjou M., Delacroix H., Bornens M. (1992). Centrosome organization and centriole architecture: their sensitivity to divalent cations. *Journal of Structural Biology* **108**(2): 107-128.
- Palmer D. K., Margolis R. L. (1985). Kinetochore components recognized by human autoantibodies are present on mononucleosomes. *Molecular and Cellular Biology* **5**(1): 173.
- Palmer D. K., O'Day K., Trong H., Charbonneau H., Margolis R. L. (1991). Purification of the centromere-specific protein CENP-A and demonstration that it is a distinctive histone. *Proceedings of the National Academy of Sciences* **88**(9): 3734.
- Palmer D. K., O'Day K., Trong H. L., Charbonneau H., Margolis R. L. (1991). Purification of the centromere-specific protein CENP-A and demonstration that it is a distinctive histone. *Proc Natl Acad Sci U S A* **88**(9): 3734-8.
- Palmer D. K., O'Day K., Wener M. H., Andrews B. S., Margolis R. L. (1987). A 17-kD centromere protein (CENP-A) copurifies with nucleosome core particles and with histones. *The Journal of Cell Biology* **104**(4): 805-815.
- Park S. J., Wu C. H., Gordon J. D., Zhong X., Emami A., Safa A. R. (2004). Taxol induces caspase-10-dependent apoptosis. *Journal of Biological Chemistry* **279**(49): 51057-51067.
- Perez-Castro A. V., Shamanski F. L., Meneses J. J., Lovato T. A. L., Vogel K. G., Moyzis R. K., Pedersen R. (1998). Centromeric protein B null mice are viable with no apparent abnormalities. *Developmental Biology* **201**(2): 135-143.
- Perpelescu M., Fukagawa T. (2011). The ABCs of CENPs. *Chromosoma*: 1-22.
- Peters J. M. (2006). The anaphase promoting complex/cyclosome: a machine designed to destroy. *Nature Reviews Molecular Cell Biology* **7**(9): 644-656.
- Peters J. M., Tedeschi A., Schmitz J. (2008). The cohesin complex and its roles in chromosome biology. *Genes & Development* **22**(22): 3089-3114.
- Piel M., Nordberg J., Euteneuer U., Bornens M. (2001). Centrosome-dependent exit of cytokinesis in animal cells. *Science* **291**(5508): 1550-1553.
- Pihan G. A., Purohit A., Wallace J., Knecht H., Woda B., Quesenberry P., Doxsey S. J. (1998). Centrosome defects and genetic instability in malignant tumors. *Cancer Research* **58**(17): 3974.
- Pop C., Salvesen G. S. (2009). Human caspases: activation, specificity, and regulation. *Journal of Biological Chemistry* **284**(33): 21777-21781.
- Prendergast L., van Vuuren C., Kaczmarczyk A., Doering V., Hellwig D., Quinn N., Hoischen C., Diekmann S., Sullivan K. F. (2011). Premitotic Assembly of Human CENPs-T and -W Switches Centromeric Chromatin to a Mitotic State. *PLoS Biology* **9**(6): e1001082.
- Probst A. V., Dunleavy E., Almouzni G. (2009). Epigenetic inheritance during the cell cycle. *Nature Reviews Molecular Cell Biology* **10**(3): 192-206.

- Przewlaka M. R., Glover D. M. (2009). The kinetochore and the centromere: a working long distance relationship. *Annual Review of Genetics* **43**: 439-465.
- Przewlaka M. R., Venkei Z., Bolanos-Garcia V. M., Debski J., Dadlez M., Glover D. M. (2011). CENP-C is a structural platform for kinetochore assembly. *Current Biology* **21**(5): 399-405.
- Przewlaka M. R., Zhang W., Costa P., Archambault V., D'Avino P. P., Lilley K. S., Laue E. D., McAinsh A. D., Glover D. M. (2007). Molecular analysis of core kinetochore composition and assembly in *Drosophila melanogaster*. *PLoS One* **2**(5): e478.
- Ribeiro S. A., Gatlin J. C., Dong Y., Joglekar A., Cameron L., Hudson D. F., Farr C. J., McEwen B. F., Salmon E. D., Earnshaw W. C. (2009). Condensin regulates the stiffness of vertebrate centromeres. *Molecular Biology of the Cell* **20**(9): 2371-2380.
- Ricke R. M., van Ree J. H., van Deursen J. M. (2008). Whole chromosome instability and cancer: a complex relationship. *Trends in Genetics* **24**(9): 457-466.
- Rieder C. L., Maiato H. (2004). Stuck in Division or Passing through:: What Happens When Cells Cannot Satisfy the Spindle Assembly Checkpoint. *Developmental Cell* **7**(5): 637-651.
- Rieder C. L., Salmon E. (1994). Motile kinetochores and polar ejection forces dictate chromosome position on the vertebrate mitotic spindle. *The Journal of Cell Biology* **124**(3): 223.
- Riedl S. J., Shi Y. (2004). Molecular mechanisms of caspase regulation during apoptosis. *Nature Reviews Molecular Cell Biology* **5**(11): 897-907.
- Ruchaud S., Carmena M., Earnshaw W. C. (2007). Chromosomal passengers: conducting cell division. *Nature Reviews Molecular Cell Biology* **8**(10): 798-812.
- Saitoh H., Tomkiel J., Cooke C. A., Ratrie H., 3rd, Maurer M., Rothfield N. F., Earnshaw W. C. (1992). CENP-C, an autoantigen in scleroderma, is a component of the human inner kinetochore plate. *Cell* **70**(1): 115-25.
- Salisbury J. L. (2007). A mechanistic view on the evolutionary origin for centrin-based control of centriole duplication. *Journal of Cellular Physiology* **213**(2): 420-428.
- Santaguida S., Musacchio A. (2009). The life and miracles of kinetochores. *The EMBO Journal* **28**(17): 2511-2531.
- Savoian M. S., Goldberg M. L., Rieder C. L. (2000). The rate of poleward chromosome motion is attenuated in *Drosophila* zw10 and rod mutants. *Nature Cell Biology* **2**(12): 948.
- Schaar B., Chan G., Maddox P., Salmon E., Yen T. (1997). CENP-E function at kinetochores is essential for chromosome alignment. *The Journal of Cell Biology* **139**(6): 1373-1382.
- Schiebel E. (2000). [gamma]-tubulin complexes: binding to the centrosome, regulation and microtubule nucleation. *Current Opinion in Cell Biology* **12**(1): 113-118.
- Screpanti E., De Antoni A., Alushin G. M., Petrovic A., Melis T., Nogales E., Musacchio A. (2011). Direct binding of Cenp-C to the Mis12 complex joins the inner and outer kinetochore. *Current Biology* **21**(5): 391-8.
- Sharp D., Rogers G., Scholey J. (2000). Microtubule motors in mitosis. *Nature* **407**: 41-47.
- Shelby R. D., Monier K., Sullivan K. F. (2000). Chromatin assembly at kinetochores is uncoupled from DNA replication. *The Journal of Cell Biology* **151**(5): 1113-1118.
- Shelby R. D., Vafa O., Sullivan K. F. (1997). Assembly of CENP-A into centromeric chromatin requires a cooperative array of nucleosomal DNA contact sites. *The Journal of Cell Biology* **136**(3): 501-513.
- Shi J., Orth J. D., Mitchison T. (2008). Cell type variation in responses to antimetabolic drugs that target microtubules and kinesin-5. *Cancer research* **68**(9): 3269.
- Shin H. J., Baek K. H., Jeon A. H., Park M. T., Lee S. J., Kang C. M., Lee H. S., Yoo S. H., Chung D. H., Sung Y. C. (2003). Dual roles of human BubR1, a mitotic checkpoint kinase, in the monitoring of chromosomal instability. *Cancer Cell* **4**(6): 483-497.
- Singh T. R., Saro D., Ali A. M., Zheng X. F., Du C., Killen M. W., Sachpatzidis A., Wahengbam K., Pierce A. J., Xiong Y., Sung P., Meetei A. R. (2010). MHF1-MHF2, a histone-fold-containing protein complex, participates in the Fanconi anemia pathway via FANCM. *Molecular Cell* **37**(6): 879-886.
- Sironi L., Mapelli M., Knapp S., De Antoni A., Jeang K. T., Musacchio A. (2002). Crystal structure of the tetrameric Mad1-Mad2 core complex: implications of a 'safety belt' binding mechanism for the spindle checkpoint. *The EMBO Journal* **21**(10): 2496-2506.
- Sluder G., Hinchcliffe E. H. (1999). The coordination of centrosome reproduction with nuclear events during the cell cycle. *Current Topics in Developmental Biology* **49**: 267-289.

- Sotillo R., Hernando E., Diaz-Rodriguez E., Teruya-Feldstein J., Cordon-Cardo C., Lowe S. W., Benezra R. (2007). Mad2 overexpression promotes aneuploidy and tumorigenesis in mice. *Cancer Cell* **11**(1): 9-23.
- Starr D. A., Williams B. C., Hays T. S., Goldberg M. L. (1998). ZW10 helps recruit dynactin and dynein to the kinetochore. *The Journal of Cell Biology* **142**(3): 763-774.
- Steller H. (1995). Mechanisms and genes of cellular suicide. *Science* **267**(5203): 1445.
- Strnad P., Leidel S., Vinogradova T., Euteneuer U., Khodjakov A., Gonczy P. (2007). Regulated HsSAS-6 levels ensure formation of a single procentriole per centriole during the centrosome duplication cycle. *Developmental Cell* **13**(2): 203-213.
- Sullivan B. A., Karpen G. H. (2004). Centromeric chromatin exhibits a histone modification pattern that is distinct from both euchromatin and heterochromatin. *Nature Structural & Molecular Biology* **11**(11): 1076-1083.
- Sullivan K. F. (2001). A solid foundation: functional specialization of centromeric chromatin. *Current Opinion in Genetics & Development* **11**(2): 182-188.
- Sullivan K. F., Hechenberger M., Masri K. (1994). Human CENP-A contains a histone H3 related histone fold domain that is required for targeting to the centromere. *Journal of Cell Biology* **127**(3): 581-92.
- Suzuki A., Hori T., Nishino T., Usukura J., Miyagi A., Morikawa K., Fukagawa T. (2011). Spindle microtubules generate tension-dependent changes in the distribution of inner kinetochore proteins. *The Journal of Cell Biology* **193**(1): 125-140.
- Tan E. M., Rodnan G. P., Garcia I., Moroi Y., Fritzler M., Peebles C. (1980). Diversity of antinuclear antibodies in progressive systemic sclerosis. *Arthritis & Rheumatism* **23**(6): 617-625.
- Tanaka K., Hirota T. (2009). Chromosome segregation machinery and cancer. *Cancer Science* **100**(7): 1158-1165.
- Tanaka T. U., Desai A. (2008). Kinetochore-microtubule interactions: the means to the end. *Current Opinion in Cell Biology* **20**(1): 53-63.
- Tanenbaum M. E., Macuring L. (2008). Dynein, Lis1 and CLIP-170 counteract Eg5-dependent centrosome separation during bipolar spindle assembly. *The EMBO Journal* **27**(24): 3235-3245.
- Tao W., South V. J., Zhang Y., Davide J. P., Farrell L., Kohl N. E., Sepp-Lorenzino L., Lobell R. B. (2005). Induction of apoptosis by an inhibitor of the mitotic kinesin KSP requires both activation of the spindle assembly checkpoint and mitotic slippage. *Cancer Cell* **8**(1): 49-59.
- Thompson C. B. (1995). Apoptosis in the pathogenesis and treatment of disease. *Science* **267**(5203): 1456-1462.
- Thompson L. H., Jones N. J. (2010). Stabilizing and remodeling the blocked DNA replication fork: anchoring FANCM and the Fanconi anemia damage response. *Molecular Cell* **37**(6): 749-751.
- Thornberry N. A., Rano T. A., Peterson E. P., Rasper D. M., Timkey T., Garcia-Calvo M., Houtzager V. M., Nordstrom P. A., Roy S., Vaillancourt J. P. (1997). A combinatorial approach defines specificities of members of the caspase family and granzyme B. *Journal of Biological Chemistry* **272**(29): 17907-17911.
- Tomkiel J., Cooke C. A., Saitoh H., Bernat R. L., Earnshaw W. C. (1994). CENP-C is required for maintaining proper kinetochore size and for a timely transition to anaphase. *The Journal of Cell Biology* **125**(3): 531.
- Toyoda Y., Yanagida M. (2006). Coordinated requirements of human topo II and cohesin for metaphase centromere alignment under Mad2-dependent spindle checkpoint surveillance. *Molecular Biology of the Cell* **17**(5): 2287-2302.
- Tyler-Smith C., Gimelli G., Giglio S., Floridia G., Pandya A., Terzoli G., Warburton P. E., Earnshaw W. C., Zuffardi O. (1999). Transmission of a fully functional human neocentromere through three generations. *The American Journal of Human Genetics* **64**(5): 1440-1444.
- Uchida K. S. K., Takagaki K., Kumada K., Hirayama Y., Noda T., Hirota T. (2009). Kinetochore stretching inactivates the spindle assembly checkpoint. *The Journal of Cell Biology* **184**(3): 383-390.
- Vale R. D., Fletterick R. J. (1997). The design plan of kinesin motors. *Annu. Rev. Cell Dev. Biol.* **13**(1): 745-777.
- Vallee R. (1990). Mitosis: dynein and the kinetochore. *Nature* **345**(6272): 206.
- Van Hooser A. A., Ouspenski I. I., Gregson H. C., Starr D. A., Yen T. J., Goldberg M. L., Yokomori K., Earnshaw W. C., Sullivan K. F., Brinkley B. (2001). Specification of kinetochore-forming chromatin by the histone H3 variant CENP-A. *Journal of Cell Science* **114**(19): 3529.

- Vogel C., Kienitz A., Hofmann I., Muller R., Bastians H. (2004). Crosstalk of the mitotic spindle assembly checkpoint with p53 to prevent polyploidy. *Oncogene* **23**(41): 6845-6853.
- Wade C., Giulotto E., Sigurdsson S., Zoli M., Gnerre S., Imsland F., Lear T., Adelson D., Bailey E., Bellone R. (2009). Genome sequence, comparative analysis, and population genetics of the domestic horse. *Science* **326**(5954): 865-867.
- Wang S. Z., Adler R. (1995). Chromokinesin: a DNA-binding, kinesin-like nuclear protein. *The Journal of Cell Biology* **128**(5): 761-768.
- Weaver B., Cleveland D. (2005). Decoding the links between mitosis, cancer, and chemotherapy: The mitotic checkpoint, adaptation, and cell death. *Cancer Cell* **8**(1): 7-12.
- Weaver B. A. A., Bonday Z. Q., Putkey F. R., Kops G. J. P. L., Silk A. D., Cleveland D. W. (2003). Centromere-associated protein-E is essential for the mammalian mitotic checkpoint to prevent aneuploidy due to single chromosome loss. *The Journal of Cell Biology* **162**(4): 551-563.
- Weaver B. A. A., Cleveland D. W. (2006). Does aneuploidy cause cancer? *Current Opinion in Cell Biology* **18**(6): 658-667.
- Welburn J. P. I., Grishchuk E. L., Backer C. B., Wilson-Kubalek E. M., Yates III J. R., Cheeseman I. M. (2009). The human kinetochore Skl complex facilitates microtubule depolymerization-coupled motility. *Developmental Cell* **16**(3): 374-385.
- Wittmann T., Hyman A., Desai A. (2001). The spindle: a dynamic assembly of microtubules and motors. *Nature Cell Biology* **3**: E28-E34.
- Wittmann T., Wilm M., Karsenti E., Vernos I. (2000). TPX2, a novel *Xenopus* MAP involved in spindle pole organization. *The Journal of Cell Biology* **149**(7): 1405-1418.
- Wood K. W., Sakowicz R., Goldstein L. S. B., Cleveland D. W. (1997). CENP-E is a plus end-directed kinetochore motor required for metaphase chromosome alignment. *Cell* **91**(3): 357-366.
- Yamada H., Gorbsky G. (2006). Spindle checkpoint function and cellular sensitivity to antimetabolic drugs. *Molecular Cancer Therapeutics* **5**(12): 2963-2969.
- Yan Z., Delannoy M., Ling C., Dae D., Osman F., Muniandy P. A., Shen X., Oostra A. B., Du H., Steltenpool J., Lin T., Schuster B., Decaillet C., Stasiak A., Stasiak A. Z., Stone S., Hoatlin M. E., Schindler D., Woodcock C. L., Joenje H., Sen R., De Winter J. P., Li L., Seidman M. M., Whitby M. C., Myung K., Constantinou A., Wang W. (2010). A histone-fold complex and FANCM form a conserved DNA-remodeling complex to maintain genome stability. *Molecular Cell* **37**(6): 865-878.
- Yoda K., Ando S., Morishita S., Houmura K., Hashimoto K., Takeyasu K., Okazaki T. (2000). Human centromere protein A (CENP-A) can replace histone H3 in nucleosome reconstitution in vitro. *Proceedings of the National Academy of Sciences* **97**(13): 7266.

CHAPTER 2

Caspase-3 activity is not required for normal mitosis in human cells

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Key words: mitosis, apoptosis, spindle assembly checkpoint (SAC), mitotic slippage, caspase activity

2.1. SUMMARY

Biochemical studies reported that caspase-3 activity is up-regulated in normal cell cycle during G2/M transition and caspase-3 activity is increased in mitotic checkpoint activated cells. This activity has been reported to influence rates of mitotic slippage and apoptosis, suggesting the hypothesis that caspase-3 activity has a role in a normal cell cycle during mitosis. To test this hypothesis directly we synchronized HeLa cells and examined the abundance and activity of the apoptotic mediator caspase-3 across the cell cycle. Subsequently, we developed an assay to measure mitotic kinetics in an untreated HeLa cells expressing histone H2B-GFP fusion protein. Finally, we followed H2B-GFP cells through mitosis after RNAi depletion of caspase-3. We demonstrated that active caspase-3 was not present across the fractionated HeLa cell cycle. Furthermore, we showed that the loss of this protein has no detectable influence on normal mitotic progression. Taken together, our data demonstrate that caspase-3 involvement is not required for a normal mitosis, but it is activated in chronically arrested mitosis. It is thought, that the latter activation of caspase-3 following treatment with anti-mitotic drugs determines the time of delay and one of the possible outcome from the arrest: either mitotic catastrophe and death in mitosis or mitotic slippage via caspase-3 dependent degradation of mitotic checkpoints.

2.2. INTRODUCTION

Chromosome missegregation often leads to aneuploidy or polyploidy, which has been recognized as a common characteristic of cancer cells and possible contributor to tumorigenesis (Weaver and Cleveland 2005). To avoid loss of genomic information during mitosis each pair of sister chromatids connects to spindle microtubule through their kinetochores, macromolecular protein rich structures that assemble and disassemble every cell cycle on the specific sites of centromeric DNA (Liu *et al.* 2006), that monitor the attachment state and activate signaling pathways to prevent anaphase onset before all chromosomes have achieved amphitelic attachment (Cheeseman and Desai 2008). Defects in chromosome biorientation activate the spindle assembly checkpoint (SAC) which is required to block sister chromatid separation until all chromosomes are properly attached to the mitotic spindle (Musacchio and Salmon 2007).

The SAC localization at the kinetochore is hierarchical. This means recruitment of some proteins depends on prior recruitment of others (Vigneron *et al.* 2004). In a normal mitosis, unattached kinetochores engage SAC proteins and via their signaling generate a “wait anaphase signal”, which delays the irreversible transition from metaphase to anaphase until every kinetochore is attached to their spindle (Musacchio and Hardwick 2002). The SAC prevents cells entering anaphase by inhibiting the ubiquitination of cyclin B and securin by the anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase (Medema 2009). The checkpoint proteins, including BubR1, Bub1, Bub3, Mad1, Mad2 and CENP-E, target the essential APC/C activator Cdc20. BubR1 is a kinase checkpoint protein that interacts with, and phosphorylates motor protein CENP-E (Musacchio and Salmon 2007). BubR1, together with other kinases, such as MPS1, Bub1, PLK1, NEK2, MAPK and Aurora-B, recruits SAC proteins to unattached kinetochores to generate robust SAC activity. Like all checkpoints, the spindle assembly checkpoint does not arrest cells permanently, but provides necessary time to correct chromosome attachment problems (Brito and Rieder 2006).

Sustained activation of the mitotic checkpoint is an important chemotherapeutic strategy (Weaver and Cleveland 2005). Drugs that disrupt microtubules cause cells to accumulate in mitosis due to spindle checkpoint signaling from unattached kinetochores. Chronic checkpoint activation can result in mitotic slippage or adaptation, producing aneuploid or polyploid cells (Figure 2.1). Alternatively, cell death can occur via apoptosis, after failed cytokinesis and entry into G1, or execution of a death pathway directly from mitosis (Rieder and Maiato 2004; Weaver and Cleveland 2005; Yamada and Gorbsky 2006; Gascoigne and Taylor 2008).

What happens when a cell can not satisfy the SAC???

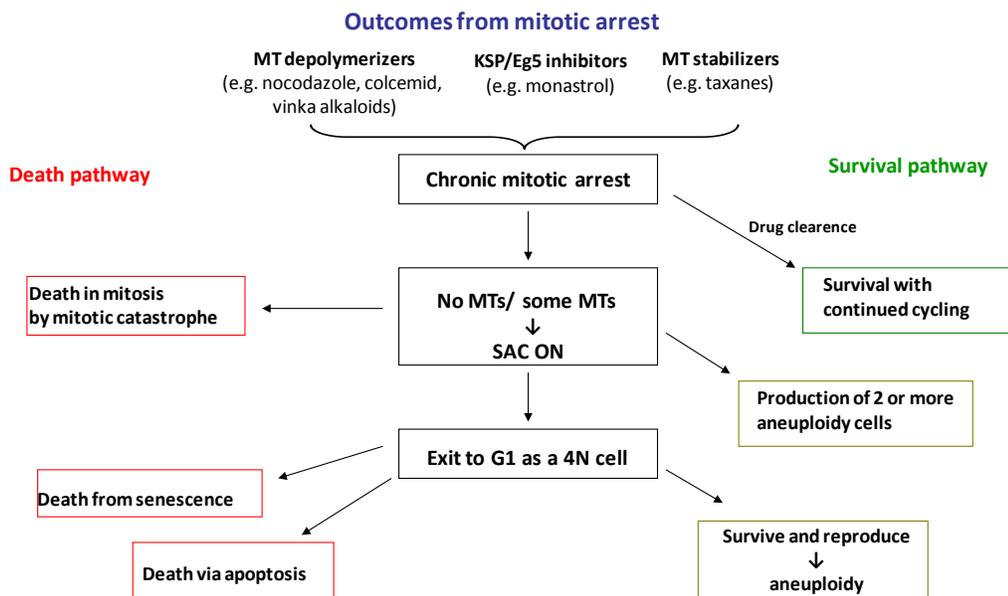


Figure 2.1. Schematic representation of the possible outcomes from mitotic arrest. There are two distinctive cell fates following treatment with anti-mitotic drugs presented: survival pathway and death pathway.

Apoptosis, or programmed cell death is a complex process that eliminates damaged or potentially harmful cells and it is directly linked to a proliferative capacity of cells (Steller 1995). There is evidence that indicates anti-mitotic drug-mediated cell death is associated with apoptotic machinery in mammalian cells (Yamada and Gorbsky 2006). It has been proposed that activation of caspase-3, the main apoptosis executor protein, might be involved in the regulation of the mitotic spindle checkpoint (Kim *et al.* 2005; Hsu *et al.* 2006). Moreover, checkpoint proteins, BubR1 and Bub1, have been reported to be caspase targets (Baek *et al.* 2005; Kim *et al.* 2005). It was shown that following microtubule disruption, time-dependent degradation of BubR1 and subsequent inactivation of SAC signaling, were associated with caspase activation, suggesting the cause-effect relationship between time of mitotic delay and apoptotic machinery activation (Gascoigne and Taylor 2008). It has been reported that caspase-3 is upregulated during G2/M transition (Whitfield *et al.* 2002), so a role for caspase-3 during normal cell cycle has been proposed. However, the mechanism coupling mitotic delay to subsequent death, in addition to what determines the timing and extent of apoptosis remains unexplored. Therefore, further analysis is needed to advance our knowledge and the link between apoptosis and spindle assembly checkpoint. To address these questions we looked at caspase activity in the cell cycle and directly examined the mitotic consequences of caspase-3 depletion.

2.3. MATERIALS AND METHODS

Unless otherwise stated, all reagents and chemicals for cell culture were purchased from Sigma-Aldrich Ireland Ltd. (Arklow, Co. Wicklow, Ireland). Sterile plastic-ware was obtained from Sarstedt Ltd (Sinnottstown Lane, Drinnagh, Wexford, Ireland), unless otherwise stated. All plastics were autoclaved at 121°C for 15 min prior to use. Protein molecular weight markers were obtained from New England Biolabs (NEB, ISIS Ltd., Unit 1 & 2, Ballywaltrim Business Centre, Boghall Road, Bray, Co. Wicklow, Ireland). Immobilon-P polyvinylidene fluoride (PVDF) membrane was obtained from Millipore Ireland B.V. (Tullagreen Carrigtwohill, County Cork Ireland) and X-ray film was obtained from Agfa (Vantage West, Great West Road, Brentford, Middlesex TW8 9AX, United Kingdom).

2.3.1. Cell culture

HeLa CCL-2 cells were purchased from ATCC (American Type Culture Collection) and were grown in Dulbecco's Modified Eagle Medium (DMEM) with 4.5g/L glucose (Lonza Biologics, Slough, United Kingdom) stable L-glutamine, 10% fetal calf serum (FCS), non essential amino acids (NEAA) and 1% penicillin streptomycin (PS). Culturing of cells was carried out in a Class III Bio-safety cabinet. Cells were typically grown in 100mm x 20mm or 150mm x 25mm culture dishes and incubated in 5% CO₂ at 37 °C. Cells were subcultured when they reached approximately 80% confluence; cells were first washed in phosphate-buffered saline (PBS) and then treated with 1X Trypsin EDTA (Gibco-Invitrogen Life Technologies, Paisley, UK) for 5 min to detach the cells. The cells were then resuspended in fresh medium and centrifuged at 1,200 r.p.m (300g) for 5 minutes. Cells were then resuspended in fresh medium, counted using a hemocytometer and an appropriate number of cells plated.

2.3.2. Synchronization and drug treatment

HeLa cells were plated in 100mm x 20mm dishes and grown to 50% confluence and treated with 5 mM thymidine (200 mM stock concentration, Sigma) for 16 hr. This first treatment causes some cells to be arrested at the G1/S transition while other cells will be at a later stage of S phase. Cells were washed twice with PBS for 5 min followed by one 5 min wash with fresh medium. Cells were then released into fresh medium for a 10 hr release during which cells will progress through the cell cycle. Following the 10 hr release, cells were blocked again for 16 hr with 5 mM thymidine. The second block arrests cells already in the G1 phase at the G1/S transition, with later cells allowed to progress through G2/M and then blocked at the G1/S

transition. Cells were released from the block as previously outlined and samples harvested every 2h over a 24 hr period for analysis by flow cytometry, western blotting and caspase activity assay.

Alternatively cells were arrested in mitosis by incubation with nocodazole (Sigma) for the indicated times. Stock solution of nocodazole at 1mM was prepared in DMSO (Sigma) and added to fresh medium to reach a final concentration of 1 μ M.

Staurosporine (Sigma) was used to induce apoptosis at 500nM final concentration for 4h before harvesting for western blotting and caspase activity assay.

2.3.3. Flow cytometry analysis

Cell cycle distribution was analysed by flow cytometry. Fixed cells were thawed on ice and centrifuged at 4000 r.p.m. for 10 minutes. The pellets were then disrupted by vortexing, 500 μ l of propidium iodide (PI/RNase Staining Buffer, BD Pharmingen, 550825) was added to the pellets. The samples were then incubated for at least 30 minutes in the dark before analysis was carried out on the FACS calibur flow cytometer (Becton Dickinson Biosciences, San Jose, California, USA). The data was analysed using the WinMDI v2.9 software. Scatter plots were generated to gate out cell doublets and DNA content was visualised by generating a histogram plotting cell count versus PI content and thus showing the progression of cells through the cell cycle.

2.3.4. Western blotting

Protein pellets were lysed in 2X LSB (Laemmli Sample Buffer) with 1M DTT (final concentration 100 mM DTT), the samples were then boiled for 10min to denature sample and facilitate separation. Typically 100,000 cells of each protein sample was loaded per well. Samples were separated during SDS-PAGE electrophoresis and transferred onto PVDF membrane. The membrane was blocked in 5% blocking solution (100mM TRIS, 68mM NaCl, 0.1% Tween -20 (TBS-T), and 5% non-fat dried milk (Marvel) for 1 hr at room temperature on a platform rocker. The membrane was probed with the primary antibody diluted in the 5% blocking solution at the appropriate dilution. The membrane was then heat sealed in a plastic bag and incubated at 4°C on a platform rocker overnight. After the primary antibody incubation period the membrane was washed three times for 10 minutes in TBS-T to remove any unbound primary antibody. The primary antibody was then detected with the addition of a secondary antibody linked to horseradish peroxidase (HRP). The primary antibodies used: rabbit anti-

Cyclin B (Upstate, 1:500), rabbit anti-H3P (Abcam, 1:1000), mouse anti-BubR1 (Millipore, 1:250), mouse anti- α -tubulin (Sigma, 1:5000), rabbit anti-caspase-3 (Cell Signalling, 1:1000). The secondary antibodies were diluted 1/10,000 in 2.5% blocking solution and the membrane was incubated 1 hr at room temperature on a platform shaker. Following the secondary antibody incubation the membrane was washed three times for 10 min each in TBS-T to remove any unbound secondary antibody.

The activity of the HRP was then detected with the addition of ECL + Western Blotting Chemoluminescent detection system from Millipore Ireland B.V. (Tullagreen Carrigtwohill, County Cork Ireland). The solutions were brought to room temperature and Solution A was mixed with Solution B in a ratio of 1:1. 2ml of the ECL was prepared for each membrane and the membrane was incubated for 5 minutes at room temperature. The membrane was then transferred to a film cassette; the remainder of the detection was carried out in a dark room. X-RAY medical film (Konica Minolta) was used to detect the light emitted by ECL. The detection was carried out using a CP1000 Automatic Film processor with Devalex X Ray Developer and FixaPlus X Ray Fixer.

2.3.5. Caspase Activity Assay

The activity of effector caspases, DEVDases, was determined fluorometrically. Effector caspases use the cysteine side chain in their active centre as a nucleophile during cleavage of peptide bond after the DEVD↓ sequence with a stringent specificity for D↓ in the cleavage site for protein substrates. This tetrapeptide motif of DEVD has been used to develop caspase-specific fluorogenic peptide substrate, such as AFC (7-amino-4-trifluoromethylcoumarin). Caspase-3 and caspase-3-like activity was measured by quantification of the artificial substrate DEVD-AFC (Sigma) which generates fluorescence when catalyzed by caspase-3. Following samples collection and processing in a lysis buffer (50 mM HEPES/KOH pH 7.2, 5 mM EGTA, 10 mM KCl, 2 mM MgCl₂, 2 mM DTT, 0.1% CHAPS), caspase activity was determined by mixing 100 μ l of extract with 100 μ l of assay buffer (same as lysis buffer, no CHAPS, DTT and 20 μ M DEVD-AFC added directly before use) and measuring the change in fluorescence (excitation at 400 nm; emission at 508 nm) at 30 °C using a CytoFluor 2000 Multi-Well Plate Reader. The rate of AFC formation was used to calculate the caspase-3-like activity in the sample extract, expressed as AFU generated per minute per μ g of protein (AFU/minute/ μ g).

2.3.6. RNAi treatment

Cells were cultured as previously described (section 2.3.1). Synthetic double stranded ON-TARGET plus SMARTpool sequences (all from Dharmacon) for caspase-3 (L-043042) or non-targeting control pool (D-001810-10) were used. Targeted duplexes were transfected at 100 nM final concentration into cells using DharmaFECT 1 reagent (Dharmacon, T-2001-02) according to the manufacturer's instruction. Cells were incubated with siRNA in a complete media for 48h before start of videomicroscopy.

2.3.7. Time-lapse live cell microscopy

HeLa cell line stably expressing a histone H2B-GFP, obtained as previously described (Kanda *et al.* 1998), was used to visualize chromosomes. Cells were transfected with siRNA for 48h before imaging. For studies on the duration of mitosis, time-lapse images were acquired at 3 min intervals for 12h. Images were captured using a DeltaVision Core system (Applied Precision) controlling an interline charge-coupled device camera (Coolsnap HQ2; Roper) mounted on an inverted microscope (IX-71; Olympus). Collected sequences were analyzed using ImagePro Plus 6.2 software and mitotic timing was scored based on tracking the object function for maximum density (as further described in results section).

2.4. RESULTS

2.4.1. Cell cycle synchronization of HeLa cells by double thymidine block and nocodazole

In this study we examined the relative protein abundance and activation of the apoptotic protease – caspase-3 to determine whether caspase-3 activity is required for a normal mitosis during cell cycle. To investigate this, we selected HeLa cells as the primary cell system. These cells, a highly transformed adenocarcinoma (Scherer *et al.* 1953), were chosen because they have been extensively used for cell cycle analysis of centromere and kinetochore assembly and function in mitosis (Foltz *et al.* 2009; Prendergast *et al.* 2011) and for human cell cycle gene expression profiles for caspases (Whitfield *et al.* 2002; Hsu *et al.* 2006).

For cell synchrony, replicate dishes were each seeded with approximately 1×10^6 cells from a uniform suspension and synchronised by a double thymidine arrest/release protocol (Bootsma *et al.* 1964) (Figure 2.2A). This protocol synchronises cells by arresting the cell cycle at the G1/S transition by exposing cells to high concentrations of thymidine, which causes an increase in deoxythymidine triphosphate (dTTP) pools. The increase in the dTTP pool has an inhibitory effect on ribonucleotide reductase, the enzyme involved in the biosynthesis of pyrimidines, which results in a reduction in the production of deoxycytidine triphosphate (dCTP). This decrease inhibits DNA synthesis, which causes cells to arrest at the G1/S transition. The cells were released from the G1/S boundary and samples were harvested at time points following release (t=0 and every 2h up to t=20).

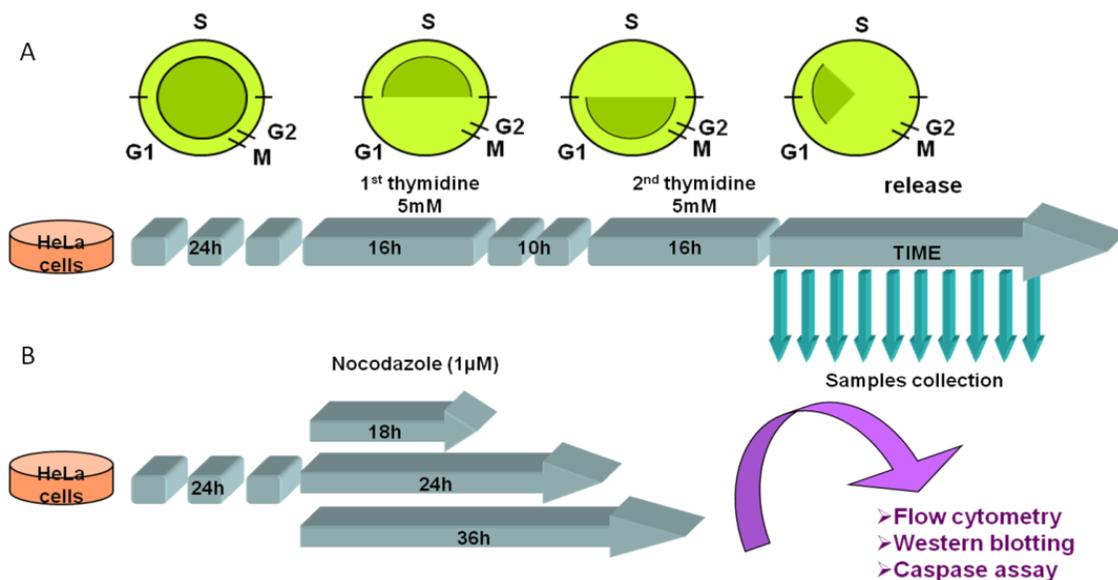
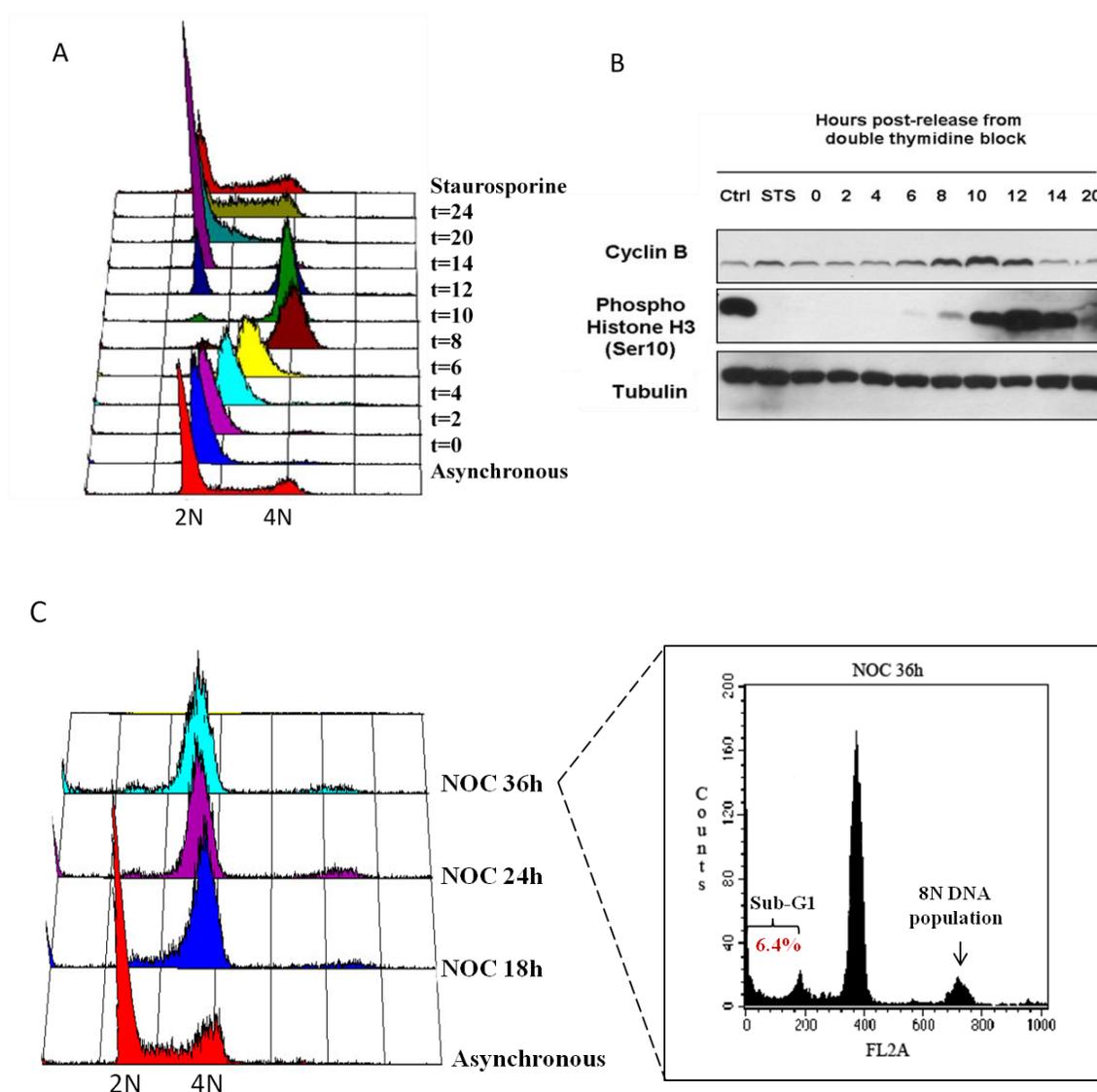


Figure 2.2. Schematic of strategies used in a synchronization and nocodazole arrest experiments. (A) A schematic of a double thymidine synchronization method with a detailed step-by-step cell cycle fractionation/arrest in the green circles above. (B) HeLa cells incubated with nocodazole for indicated amount of time were harvested for different assays analysis.

Alternatively, we obtained an enriched mitotic population by incubating with nocodazole (NOC) (Figure 2.2B), an anti-mitotic drug that arrests cells in mitosis in prometaphase-like state due to its inhibitory effect on microtubule polymerization (Jordan *et al.* 1992). Similarly to synchronized samples cells were harvested at each indicated time points (18h, 24h and 36h following nocodazole) for analysis with three independent assays: flow cytometry, western blotting and caspase activity assay to study the degree of synchrony/arrest, the appearance of cleaved caspase-3 form and caspase-3 activity, respectively.

Flow cytometry analysis of samples obtained following double thymidine arrest revealed the synchronous progression of the cells through S phase and into cells with 4N DNA content (corresponding to G2 and mitotic populations) 8 to 10 hours after release, with approximately 80% of cells executing mitosis 12 to 14 hours following release (Figure 2.3A). Western blotting with antibodies against known cell cycle markers, cyclin B (Pines and Hunter 1990) and phospho-histone H3 (Ser10) (Hendzel *et al.* 1997) were used to validate the synchrony. Cyclin B accumulates late in interphase and shows maximum level 8 to 10 hours after release (G2) while phosphorylation of histone H3 peaks 12 hours after release (Figure 2.3B) which is concurrent with mitosis and consistent with a normal progression through the cell cycle (Shelby *et al.* 2000).

On the other hand, flow cytometry analysis of nocodazole-treated cells revealed a significant accumulation of 4N DNA content population with approximately 80-90% of cells being effectively arrested in mitosis throughout duration of the experiment (Figure 2.3C). In later time points (24h and 36h) the appearance of 8N and/or sub-G1 DNA content was observed indicating cytokinesis failure and continued cycling with duplicated DNA or onset of apoptotic events, respectively (Figure 2.3C see enlargement of NOC36h). Moreover, DAPI staining on NOC-treated cells revealed accumulation of cells with condensed chromosomes characteristic of a sustained mitotic arrest, indicating that HeLa cells possess robust mitotic checkpoint response (Cahill *et al.* 1998) (Figure 2.3D).



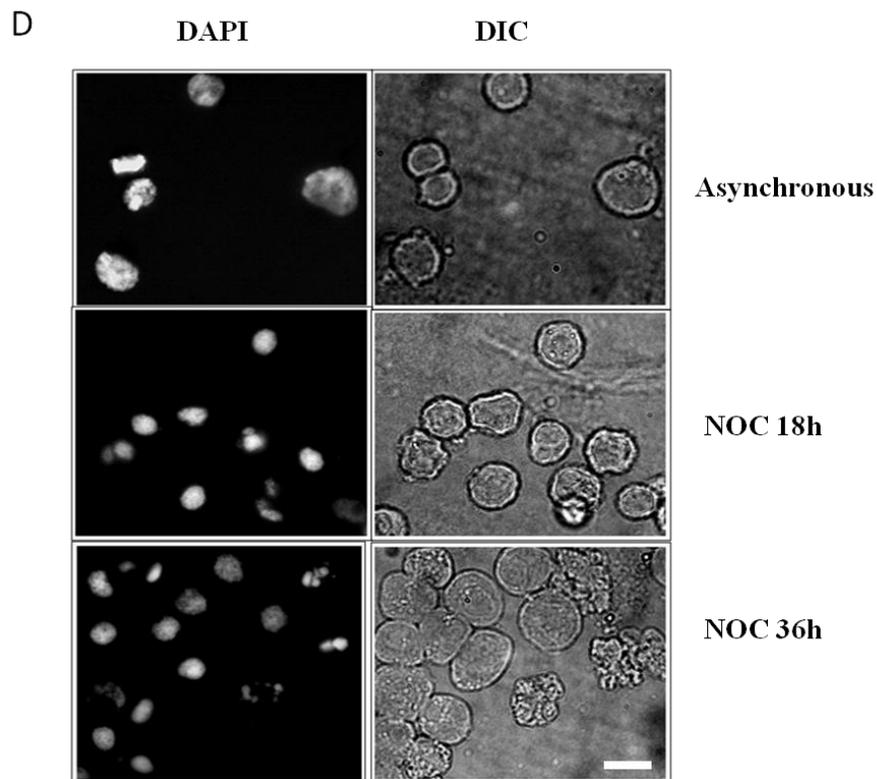


Figure 2.3. Validation of HeLa cell cycle fractionation and mitotic arrest. (A) Synchronization of HeLa cells by double thymidine block. Propidium Iodide (PI) staining followed by flow cytometry analysis indicated a synchronous progression of cells through S phase with mitosis occurring 10-12 hours after release. (B) Western blot analysis of the synchrony. Samples from the double thymidine block were probed with antibodies against cell cycle specific markers: H3P(Ser10) and cyclin B (known markers of mitosis). Bottom panel shows tubulin as loading control. (C) Flow cytometry analysis documenting 4N population accumulation following 1 μ M nocodazole (NOC) incubation for indicated time. (D) DAPI staining following nocodazole revealed a significant proportion of cells with condensed chromatin. Scale bar 20 μ m.

2.4.2. Evaluation of active caspase-3 involvement in a normal mitosis

Global analysis of mRNA expression levels for different caspases has shown that gene expression pattern of caspase-3, but not of caspase-1, -6, -7, -8, -9 or 10 undergoes periodic change in the HeLa cell cycle (Whitfield *et al.* 2002) and suggested possible role of this protein in a normal mitosis. The majority of biological processes are however mediated by proteins which are subject to translational and post-translational regulation and this cannot be observed at the mRNA level (Belle *et al.* 2006). Further, it was reported that caspase 3 activity is increased in mitotic checkpoint disrupted cells, resulting in mitotic slippage and apoptosis, indicating existence of mitotic substrates for caspase-3 (Baek *et al.* 2005). Therefore, we hypothesized that caspase-3 has a role in a normal cell cycle during mitosis.

Once a high degree of synchrony was established following double thymidine block, we examined the possible involvement of caspase-3 in the cell cycle, particularly in the G2/M transition, by biochemical methods. Total proteins were extracted from cells harvested at various times following release from G1/S block and samples were assayed for caspase-3 like activity and expression of different cell cycle specific proteins (Figure 2.3B) as well as caspase-3 activation and cleavage of a mitotic checkpoint BubR1. This particular mitotic checkpoint protein was found to possess two specific caspase cleavage sites cleaved after exposure to microtubule-targeting agents such as paclitaxel (Kim *et al.* 2005), therefore being a reasonable substrate to study caspase-3 activation in a normal mitosis.

Contrary to findings from caspase gene expression profiles and previous work reporting active caspase-3 at G2/M transition, we found no detectable levels of cleaved (active) caspase-3 as well as no evidence for BubR1 protein cleavage across the cell cycle (Figure 2.4A). These findings were further supported by direct analysis of caspase activity. Using a fluorogenic substrate-based assay we found no significant change or increase in caspase activity along cell cycle progression (Figure 2.4B). The above assays were validated by applying staurosporine (STS), a commonly used drug that at low doses (1-20nM) inhibits protein kinases such as protein kinase C, A and G, but at higher concentrations (0.2-1µM) induces cell apoptosis (Couldwell *et al.* 1994). As expected, treatment with staurosporine resulted in a pronounced cleavage of caspase-3 and over a 20-fold increase in fluorescence corresponding to a prominent caspase-3 activation and ongoing apoptosis (Figure 2.4). Taken together these findings demonstrated that in synchronized HeLa cells no detectable caspase-3 activation can be observed and therefore we speculate no involvement of this protein in normal mitosis.

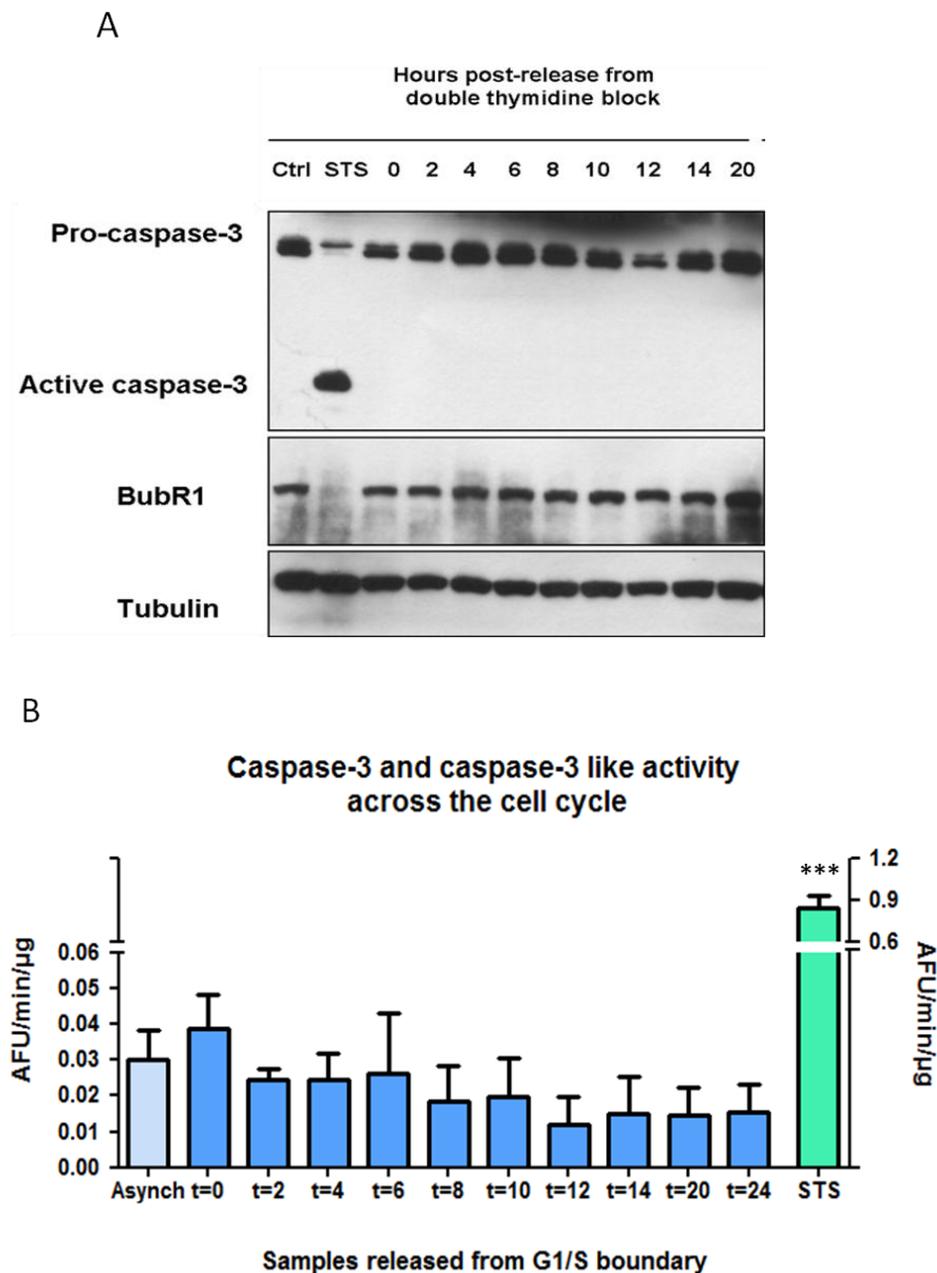
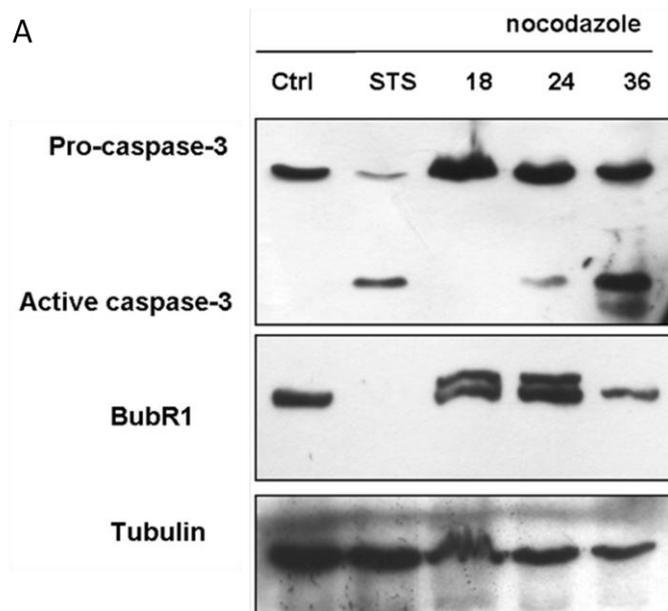


Figure 2.4. In synchronized HeLa cells no active caspase-3 is observed across the cell cycle. (A) Western blot analysis of caspase-3 activation in HeLa cell cycle. Cells were synchronized using double thymidine block and detection of cleavage of caspase-3 and BubR1 occurrence was analyzed by western blot. Tubulin was used as a loading control. (B) Graph represents mean (+SEM) of caspase activity measured in samples collected following G1/S release. Data obtained from three independent experiments. Staurosporine (STS) treatment was used as a positive control for caspase-3 activation. *** $p < 0.0001$ (one-way ANOVA followed by Dunnett's multiple comparison).

In the contrary, following nocodazole treatment mitotically arrested cells demonstrated a time dependent increase in levels of caspase-3 activity, documented both by western blotting analysis of the appearance of cleaved caspase-3 form (Figure 2.5A) and caspase activity assay (Figure 2.5B). Moreover, following microtubule disruption with high concentration nocodazole the detection of a slowly migrating form of BubR1 was observed by western blot, suggesting hyperphosphorylated forms of this protein (Elowe *et al.* 2007). In addition, in a long term nocodazole treatment (36h) BubR1 protein abundance decreased while active caspase-3 progressively increased, suggesting a caspase-dependent BubR1 degradation in a prolonged mitotic checkpoint arrested cells. However, this hypothesis would have to be confirmed by for example inhibition of caspase activity using z-VAD-fmk. Additionally, the accumulation of 8N DNA containing cells in a 24h nocodazole treatment experiment is consistent with “slippage” population re-entering cell cycle following mitotic arrest. These findings indicate that activation of caspase-3 provides a favorable environment for mitotic checkpoint deactivation and slippage following treatment with anti-mitotic drugs (Kim *et al.* 2005). However, whether or not this activation of caspase-3 is required and/or sufficient for mitotic slippage cannot be determined simply based on this experiment, since a destruction of BubR1 may also reflect a progressive increase in the number of cells dying in mitosis, and not the slippage.

Nevertheless, the combined results suggest that in our experimental conditions active caspase-3 is not a determinant of a mitotic progression and therefore it is not required for spindle assembly checkpoint activation or degradation during normal mitosis. However, its activation could be involved in resolution of mitotic arrest and thus influence the durability of the cell cycle delay following treatment with anti-mitotic drugs.



B

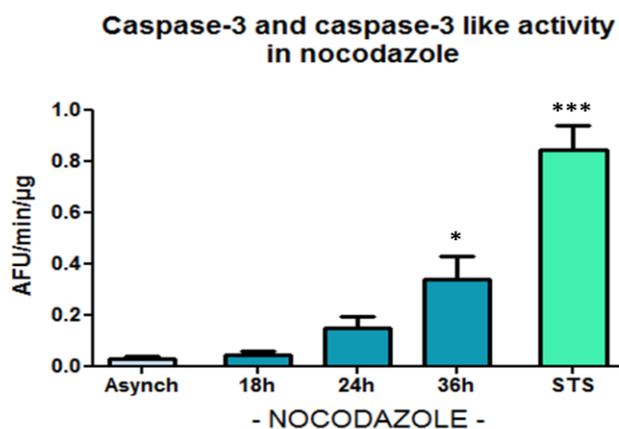


Figure 2.5. Active caspase-3 is detectable in HeLa cells with extended mitotic checkpoint activation.

(A) Cells were arrested in mitosis by incubation with $1\mu\text{M}$ nocodazole for the indicated times. Western blot analysis was used to detect the appearance of a cleaved caspase-3 band indicating the presence of active caspase. Additionally BubR1 hyperphosphorylation was detected following treatment and degradation occurred after 36h. (B) Graph represents mean (+SEM) of caspase activity measured in samples collected following nocodazole arrest. Data obtained from three independent experiments. Staurosporine (STS) treatment was used as a positive control for caspase-3 activation. * $p < 0.01$, *** $p < 0.0001$ (one-way ANOVA followed by Dunnett's multiple comparison).

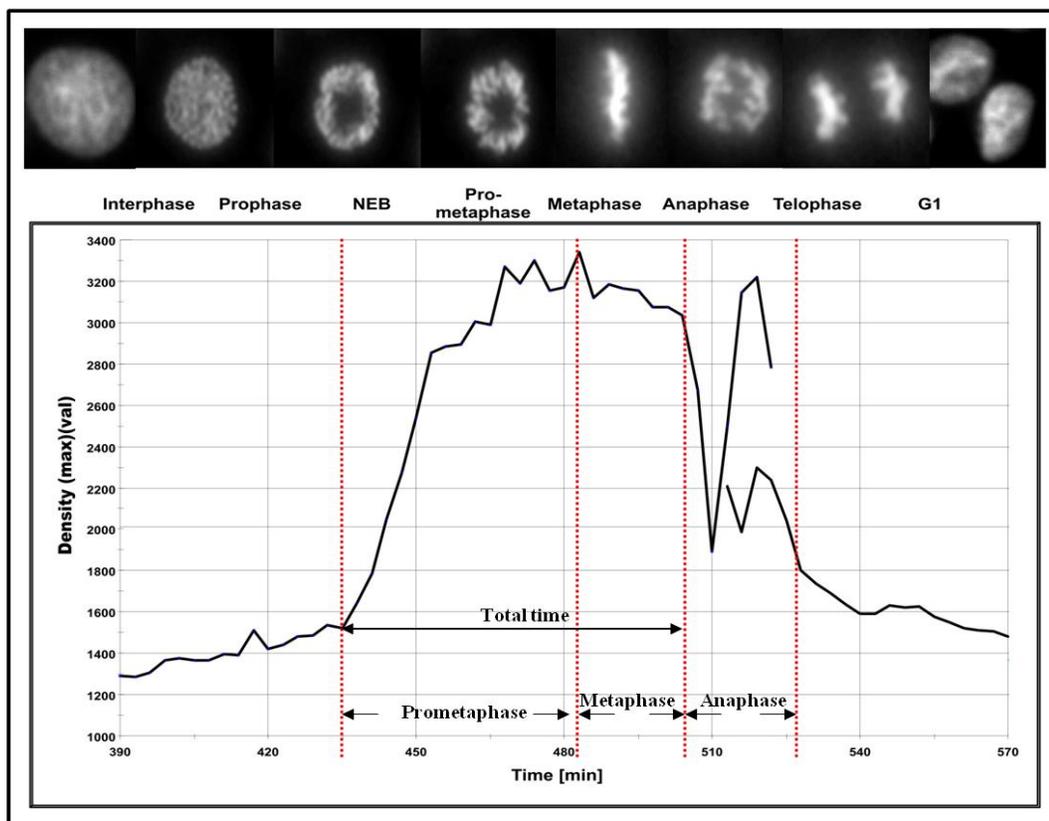
2.4.3. Mitotic kinetics in H2B-GFP cells depleted of caspase-3

The previous attempts to determine whether or not caspase-3 is active in a normal mitosis resulted in unambiguous findings disproving this hypothesis. However, the double thymidine block protocol requires a physiological perturbation of cells to induce synchrony. We therefore developed an independent assay to further investigate caspase-3 activation and requirement for mitosis in HeLa cells. We applied live cell microscopy to study mitotic progression of cells depleted of caspase-3 by siRNA treatment.

First, we developed an assay to measure mitotic kinetics in live HeLa cells. Briefly, we used a HeLa cell line expressing a histone H2B-GFP fusion to visualize chromosomes (Kanda *et al.* 1998). Exponentially growing HeLa H2B-GFP cells were plated on a glass-bottom live cell dish at least two days before imaging to allow uniform suspension and stable attachment to the glass. The sequences of images were acquired on Delta Vision fluorescent microscope for 12h with 3 minutes intervals. Movies were analyzed using ImagePro Plus 6.2 software and mitotic timing was scored based on tracking the object function for maximum density. As illustrated in the graph (Figure 2.6A), changes in the intensity features that are caused by chromosome condensation and movement on the spindles enabled us to time important stages of mitosis, from prophase increasing upon nuclear envelope breakdown (NEB) through prometaphase reaching the maximum peak at metaphase and declining following anaphase onset.

The analysis of mitotic kinetics required counting the above events giving the total time spent in mitosis as an additive amount of prometaphase and metaphase time. Following this protocol we quantified the observed mitoses. The chromosomes of the untreated cells or non-targeting control siRNA treated cells typically after NEB formed prometaphase rosette shapes, and then aligned at the metaphase plate within 40-50 minutes. After a delay of ~20-40 minutes, a complete chromosome congression was established and anaphase initiated with the chromosomes separating into two clear masses followed by their further de-condensation in telophase and finally formation of two daughter G1 cells. In order to correct for the edge effects, mitoses that initiated within 150 min (normal mitotic time plus two standard deviation value) and did not have a defined metaphase endpoint (anaphase onset or apoptosis) were excluded from the analysis.

A



B

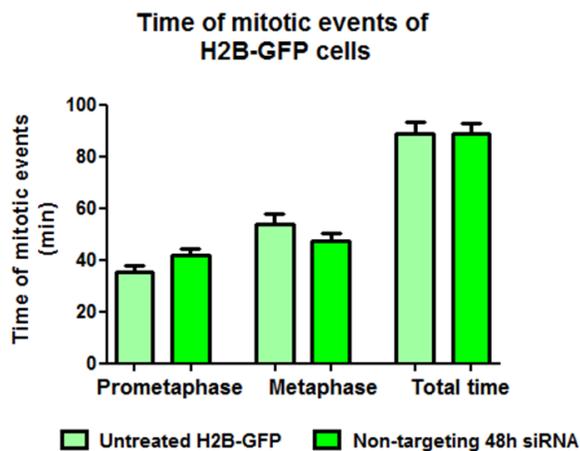


Figure 2.6. An assay developed to measure mitotic kinetics in H2B-GFP HeLa cells. (A) Graph represents changes in the fluorescent intensity features, caused by chromosome condensation and movement on the spindles, that were used to time important stages of mitosis in an individual cell undergoing mitosis. Upper panel shows images corresponding to the above intensity changes of mitotic progression in untreated H2B-GFP HeLa cells. **(B)** Graph represents mean (+SEM) of the mitotic timing quantified in the untreated and non-targeting siRNA treated H2B-GFP HeLa cells. Non-significant difference between the two populations was observed following one-way ANOVA comparison test.

To directly assess if caspase-3 has its role in normal mitosis, we monitored mitotic kinetics in H2B-GFP cells transfected with caspase-3 siRNA for 48h. At least 30 full mitotic events were scored for the control population and different siRNA treatments. As expected, mitotic timing obtained from control mitoses (non-targeting siRNA-treated cells) did not differ significantly from the untreated population (Figure 2.6B), demonstrating no impact of siRNA duplexes and transfection conditions on normal mitotic progression. Next we examined caspase-3 depleted mitoses. Using pooled siRNA we managed to effectively knock down by 80% caspase-3 from H2B-GFP cells (Figure 2.7C). Consistent with our previous biochemical studies, indicating no evidence for active caspase-3 involvement in a normal mitosis, the quantification of the mitotic timing of caspase-3 depleted H2B-GFP cells demonstrated very subtle effect. Apart from slight acceleration in metaphase, there was very little influence of caspase-3 depletion on mitotic timing in HeLa cells (Figure 2.7B). Simultaneous image analysis of cells depleted of caspase-3 revealed no major mitotic defects following the depletion (Figure 2.7A). Cells progressed through mitosis with a relatively normal kinetics, displaying typical features of each mitotic stage (from NEB to anaphase onset) with no evidence of misaligned or lagging chromosomes, a very characteristic feature of mitoses with impaired spindle assembly checkpoint activity.

Therefore we concluded that depletion of caspase-3 has no observable influence on mitotic kinetics. Taken together with biochemical results that showed no modulation of caspase-3 activity in the HeLa cell cycle, we conclude that this protein is not involved in a normal mitosis during the HeLa cell cycle.

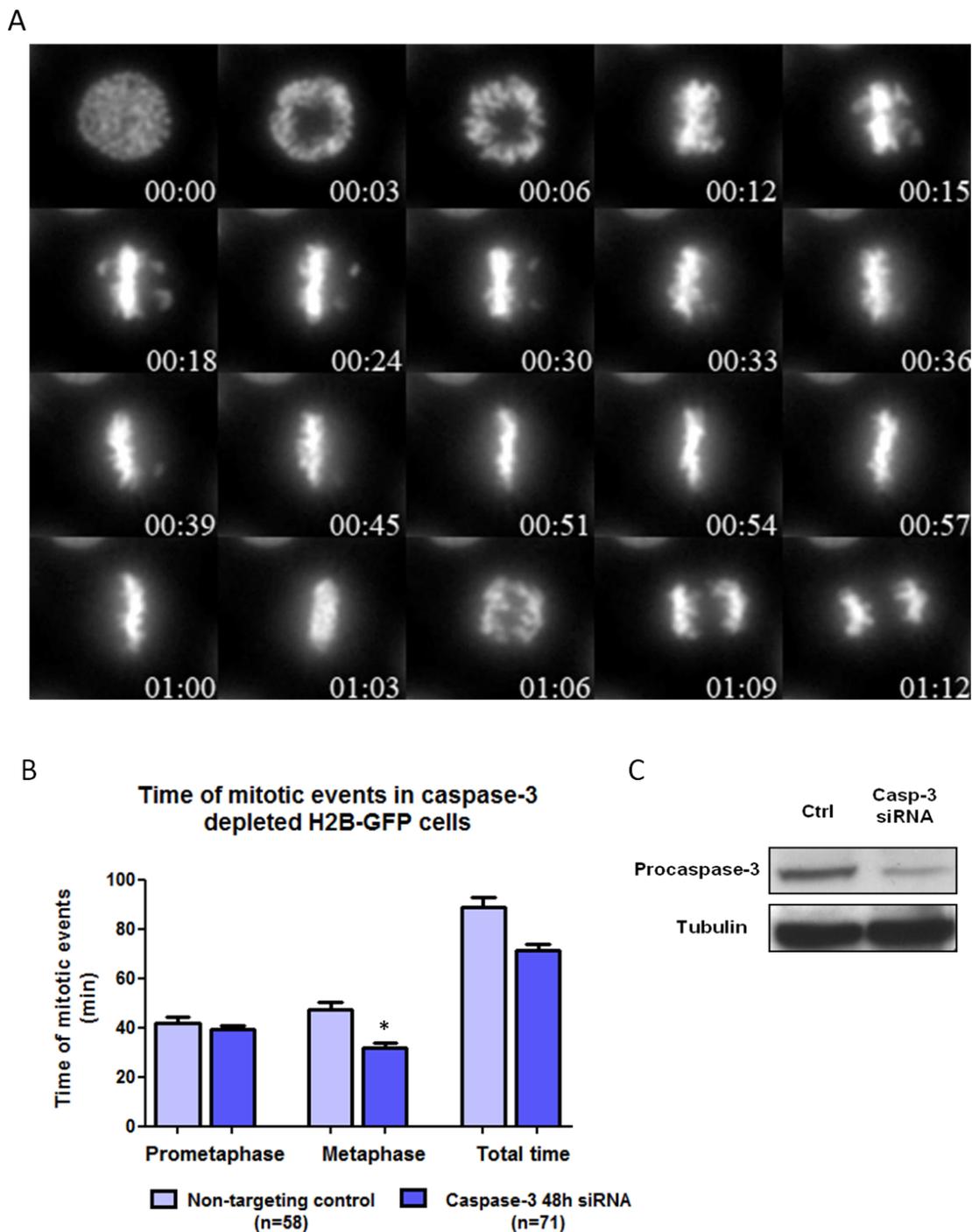


Figure 2.7. Depletion of caspase-3 does not affect normal mitotic kinetics. (A) Representing panel of H2B-GFP cell progressing through mitosis with normal kinetics following caspase-3 siRNA treatment. Time is given in minutes. (B) Graph represents mean (+SEM) of the mitotic timing quantify in the untreated and caspase-3 depleted H2B-GFP cells. * $p < 0.05$ (one-way ANOVA followed by Dunnett's multiple comparison). (C) Western blot analysis of siRNA depleted cells. Expression of caspase-3 was knocked down by 80% following siRNA treatment for 48h in asynchronous H2B-GFP cells.

2.5. DISCUSSION

In this study we investigated the requirement for function of caspase-3 apoptotic protein in normal mitosis in HeLa cell cycle. Originally, the idea that caspase activity is required for the normal mitotic checkpoint (Hsu *et al.* 2006) and mitotic slippage came from biochemical and FACS studies of HeLa and other cancer cells harvested after a prolonged drug-induced block in mitosis. These observations suggested that in addition to its role in programmed cell death, caspase activity is required for timely mitotic slippage following treatment with anti-mitotic drugs (Baek *et al.* 2005; Kim *et al.* 2005). It is well established that spindle poisons like nocodazole, taxol or Eg5 inhibitors significantly prolong mitosis by preventing satisfaction of the spindle assembly checkpoint (SAC), also called mitotic checkpoint (Brito and Rieder 2006; Musacchio and Salmon 2007). Following prolonged arrest, cells either die in mitosis (via apoptosis or mitotic catastrophe) or slip into the next G1 as 4N cells (Rieder and Maiato 2004), where, depending on their p53 status may continue to cycle (Lanni and Jacks 1998). Despite their widespread use in chemotherapy, the mechanism by which anti-mitotic drugs kill cancer cells and factors that dictate cell fate following prolonged arrest are not completely understood. The most favorable model fosters the idea that the outcome from spindle poisons is governed by a temporal mechanism that consists of two competing networks, one modulating the balance between pro- and antiapoptotic proteins towards caspase activation, and the other protecting cyclin B1 from degradation (Gascoigne and Taylor 2008). In addition, cyclin B1/CDK1 activity was found to restrain certain apoptotic routes in nocodazole-treated cells, by directly inhibiting caspase-2 (Andersen *et al.* 2009), caspase-8 (Matthess *et al.* 2010) or caspase-9 (Allan and Clarke 2007). Still, to date, most of the work on cells treated with spindle poisons reveals that apoptosis during mitosis occurs primarily via the intrinsic pathway involving caspase-9 as the main initiator and caspase-3 as the main executioner (Shi *et al.* 2008; Pop and Salvesen 2009).

The idea that caspase activity is required for mitotic slippage, supported by indirect (Kim *et al.* 2005) and direct (Gascoigne and Taylor 2008) data, both revealing prolongation of mitotic arrest in the presence of pan-caspase inhibitors, as well as the requirement for BubR1 being destroyed in a caspase-dependent manner (Baek *et al.* 2005), led to a hypothesis that caspase-3 activity is indeed involved in a normal mitosis of human cells. To examine this proposition, we first synchronized HeLa cell cycle by double thymidine block and tested the released fractions, representing each of the cell cycle phases (G1, S, G2 and M phase) for the appearance of the cleaved caspase-3 form (by western blot detection) or increase in a caspase-3 activity across the untreated HeLa cell cycle (by caspase activity assay). Our biochemical-based approach revealed

no significant modulation in caspase-3 activity or any evidence for caspase-3 cleavage, corresponding to activation neither in mitotic population released after synchronization nor elsewhere in the cell cycle (Figure 2.4). These findings were inconsistent with a previous reports documenting both caspase-3 mRNA profile and protein being up-regulated prior mitosis (Whitfield *et al.* 2002; Hsu *et al.* 2006). As far as the discrepancy between the mRNA expression profile for caspase-3 and our biochemical based assay can be explained by variation in translational and post-translational regulation and this cannot be observed at the mRNA level (Belle *et al.* 2006); the striking complete opposite result obtained by Hsu *et al.* group as caspase-3 being periodically expressed and activated in G2/M transition of the synchronized HeLa cells, cannot be easily clarified. It is possible that a population-based studies can lead to a confusing interpretations (Rieder and Maiato 2004), e.g. sensitive immunoblotting techniques can easily detect activation of a particular signaling pathway, even if only a small fraction of cells within population have triggered that pathway. This highlights the need of a cell-based assay development that would be less invasive on the cellular biochemistry in a process of investigating the impact of that cellular responses on cell fate.

Alternatively to synchronized HeLa cells, we investigated caspase-3 activation and involvement in nocodazole-treated population. Consistent with previous findings (Baek *et al.* 2005; Kim *et al.* 2005; Kim *et al.* 2008), we observed a time-dependent increase in caspase-3 activation and cleavage (respectively measured by caspase activity assay and western blot detection), correlated with a progressive BubR1 degradation, suggesting caspase-dependent mitotic checkpoint cleavage and mitotic slippage. The role of the SAC in cell death in response to spindle disruption is complicated (Bekier *et al.* 2009). In accordance with a favorable notion indicating caspase-3 dependent release from mitotic arrest following BubR1 degradation, there are findings of a dominant-negative Bub1 that reduced significantly cell death in HeLa cells exposed to nocodazole (Taylor and McKeon 1997). In contrast, it has been shown that depletion of Mad2 or Bub1 with siRNA might have a distinctive effect on the cell survival following exit from mitotic arrest, depending on the concentration of the drug and cell line used, reducing the short-term survival in MCF-7 cells (lacking caspase-3) exposed to 100 nM taxol (Sudo *et al.* 2004), or increasing long-term survival of HeLa cells exposed to 10 nM taxol (Niikura *et al.* 2007). Therefore, the distinct role of the SAC has been assigned to their checkpoint activity in determining the short- and long-term cell viability following treatment with anti-mitotic drugs. The idea of the length of the mitotic arrest dictating cell fate after mitotic exit (Bekier *et al.* 2009) is opposed to the previous study demonstrating that the duration of the mitotic arrest does

not dictate cell fate, neither does so genetically predetermined background of the cells (Gascoigne and Taylor 2008). However, both studies emphasize the notion that cell death or survival is linked to gradual changes in cellular biochemistry, in particular gradual accumulation of prodeath signals (Blagosklonny 2007; Gascoigne and Taylor 2008; Bekier *et al.* 2009).

Finally, to directly address the question of caspase-3 activity involvement in a normal mitosis, we took the time-lapse approach based on tracking of the individual living cells expressing GFP-H2B fusion protein, which allowed us to directly correlate mitotic timing in the absence of caspase-3 protein. We reasoned that if caspase-3 activity (or protein) is engaged in a modulation of either mitotic checkpoint or any other pre-mitotic or mitotic process it should have a reflection in the mitotic kinetics of depleted cells. In accordance with our previous biochemical findings, the analysis of mitotic kinetics of cells depleted of caspase-3 with siRNA, revealed no major mitotic defects associated with this depletion, such as chromosome alignment and congression problems or the appearance of lagging chromosomes and subsequent formation of anaphase bridges, features characteristic for an impaired spindle checkpoint function across all the species (Ditchfield *et al.* 2003; Johnson *et al.* 2004; Morrow *et al.* 2005; Musacchio and Salmon 2007). Indeed, the depletion of caspase-3 did not affect the duration or fidelity of mitosis of otherwise untreated HeLa cells, with the only observable dissimilarity to control cells being the slight acceleration in the length of metaphase in cells lacking caspase-3. This noticeable difference in metaphase time could be explained indirectly by the changes in the cellular biochemistry and balance between the levels of the pro-apoptotic versus anti-apoptotic proteins in the absence of one of the main apoptotic executor caspase-3. It has been shown that following prolonged mitotic arrest, factors influencing caspase activation are both anti-apoptotic proteins, such as Mcl-1 as well as pro-apoptotic proteins, such as caspase-9. These proteins were found to be implicated, to a different extent, through their CDK-1 cyclin B1 dependent phosphorylation status, in processes controlling the balance of ubiquitination and deubiquitination of cyclin B1 (Allan and Clarke 2007; Harley *et al.* 2010). Therefore, we hypothesize that the accelerated progression through the metaphase chromosome alignment observed in cells depleted of caspase-3, could result from the unbalance of the apoptotic machinery towards the faster degradation of cyclin B1 and subsequent exit from mitosis. However, to prove this hypothesis further analysis is required, and the easiest could be the application of the pan-caspase inhibitors, that in a sense would give the same content-related answer to the question of caspase-3 activity involvement in mitosis, without physically changing the abundance of the caspase-3 protein in the cell.

Our findings from live cell studies were confirmed by the work of Lee *et al.* (Lee *et al.* 2011). Their large scale analysis of mitotic timing performed in non-transformed human telomerase immortalized retinal pigment epithelia (RPE-1) cells demonstrated, that inhibition or depletion of selected caspases (caspase-3, caspase-8 and caspase-9) led to no defects in mitosis neither had no influence on mitotic timing. Moreover, their findings argued the long-lived notion of caspase activity being required for a mitotic checkpoint deactivation and slippage following prolonged mitosis. Contrary, they showed that nocodazole-treated cells that recently slipped through mitosis in the presence or absence of pan-caspase inhibitors still contained a numerous BubR1 foci in their nuclei and depletion of caspase-3 and caspase-9 accelerated the rate of slippage. They concluded that caspase activity is not required for a functional mitotic checkpoint, and that inhibiting caspase activity in toto with pan-caspase inhibitors or after simultaneously depleting caspase-3 and caspase-9 does not prolong mitosis in RPE-1 cells when the mitotic checkpoint cannot be satisfied following treatment with anti-mitotic drugs (Lee *et al.* 2011). The discrepancies between these findings and the previous report documenting from live cell analyses that caspase inhibition often prolongs the duration of the mitotic arrest (Gascoigne and Taylor 2008), could be explained primarily by differences in cell lines used, since the latter work focused mainly on transformed tumor cell lines. It is also worth noticing that the prolongation of mitosis by pan-caspase inhibitor in cells arrested with spindle poisons was seen primarily in cells that ultimately die in mitosis (Huang *et al.* 2010), since it has been shown previously that many tumor lines lost the ability to slip in a timely manner, for example to degrade cyclin B1 below the threshold level (Brito and Rieder 2009).

However, regardless of the explanation(s) of the nature of the mitotic slippage following spindle poison treatment, our combined results, from biochemical and individual cell-based approaches are coherent with reports to date, and demonstrate unambiguously that active caspase-3 is not required for a normal mitosis progression and for mitotic checkpoint stimulation. In support to this notion, there are cell lines available that lack caspase-3 (MCF-7) or both caspase-3 and caspase-7 (Lakhani *et al.* 2006) and even more, viable mice can be produced lacking either caspase (Kuida *et al.* 1996). Further investigation in detailed control manner of the mitotic responses to depletion of caspase-3 of the untreated (i.e. metaphase acceleration) or spindle poison treated HeLa cells was beyond the scope of this work. Nevertheless, the assay developed during the work on this project was further applied for investigation of mitotic kinetics in cells depleted of CENP-W/T complex.

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2.6. REFERENCES

- Allan L. A., Clarke P. R. (2007). Phosphorylation of caspase-9 by CDK1/cyclin B1 protects mitotic cells against apoptosis. *Molecular Cell* **26**(2): 301-310.
- Andersen J. L., Johnson C. E., Freel C. D., Parrish A. B., Day J. L., Buchakjian M. R., Nutt L. K., Thompson J. W., Moseley M. A., Kornbluth S. (2009). Restraint of apoptosis during mitosis through interdomain phosphorylation of caspase-2. *The EMBO Journal* **28**(20): 3216-3227.
- Baek K., Shin H., Jeong S., Park J., McKeon F., Lee C., Kim C. (2005). Caspases-dependent cleavage of mitotic checkpoint proteins in response to microtubule inhibitor. *Oncology Research* **15**(3): 161-168.
- Bekier M. E., Fischbach R., Lee J., Taylor W. R. (2009). Length of mitotic arrest induced by microtubule-stabilizing drugs determines cell death after mitotic exit. *Molecular Cancer Therapeutics* **8**(6): 1646-1654.
- Belle A., Tanay A., Bitincka L., Shamir R., O'Shea E. K. (2006). Quantification of protein half-lives in the budding yeast proteome. *Proceedings of the National Academy of Sciences* **103**(35): 13004.
- Blagosklonny M. V. (2007). Mitotic arrest and cell fate: why and how mitotic inhibition of transcription drives mutually exclusive events. *Cell Cycle* **6**(1): 70-74.
- Bootsma D., Budke L., Vos O. (1964). Studies on synchronous division of tissue culture cells initiated by excess thymidine. *Experimental Cell Research* **33**(1): 301-309.
- Brito D., Rieder C. (2006). Mitotic checkpoint slippage in humans occurs via cyclin B destruction in the presence of an active checkpoint. *Current Biology* **16**(12): 1194-1200.
- Brito D. A., Rieder C. L. (2009). The ability to survive mitosis in the presence of microtubule poisons differs significantly between human nontransformed (RPE-1) and cancer (U2OS, HeLa) cells. *Cell Motility and the Cytoskeleton* **66**(8): 437-447.
- Cahill D. P., Lengauer C., Yu J., Riggins G. J., Willson J. K. V., Markowitz S. D., Kinzler K. W., Vogelstein B. (1998). Mutations of mitotic checkpoint genes in human cancers. *Nature* **392**(6673): 300-303.
- Cheeseman I., Desai A. (2008). Molecular architecture of the kinetochore-microtubule interface. *Nature Reviews Molecular Cell Biology* **9**(1): 33-46.
- Couldwell W. T., Hinton D. R., He S., Chen T. C., Sebat I., Weiss M. H., Law R. E. (1994). Protein kinase C inhibitors induce apoptosis in human malignant glioma cell lines. *FEBS letters* **345**(1): 43-46.
- Ditchfield C., Johnson V. L., Tighe A., Ellston R., Haworth C., Johnson T., Mortlock A., Keen N., Taylor S. S. (2003). Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores. *The Journal of Cell Biology* **161**(2): 267.
- Elowe S., Hummer S., Uldschmid A., Li X., Nigg E. A. (2007). Tension-sensitive Plk1 phosphorylation on BubR1 regulates the stability of kinetochore-microtubule interactions. *Genes & Development* **21**(17): 2205.
- Foltz D. R., Jansen L. E. T., Bailey A. O., Yates III J. R., Bassett E. A., Wood S., Black B. E., Cleveland D. W. (2009). Centromere-specific assembly of CENP-A nucleosomes is mediated by HJURP. *Cell* **137**(3): 472-484.
- Gascoigne K. E., Taylor S. S. (2008). Cancer cells display profound intra-and interline variation following prolonged exposure to antimetabolic drugs. *Cancer Cell* **14**(2): 111-122.
- Harley M. E., Allan L. A., Sanderson H. S., Clarke P. R. (2010). Phosphorylation of Mcl-1 by CDK1-cyclin B1 initiates its Cdc20-dependent destruction during mitotic arrest. *The EMBO Journal* **29**(14): 2407-2420.

- Hendzel M. J., Wei Y., Mancini M. A., Van Hooser A., Ranalli T., Brinkley B., Bazett-Jones D. P., Allis C. D. (1997). Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* **106**(6): 348-360.
- Hsu S., Yu C., Yin S., Tang M., Tien A., Wu Y., Huang C. (2006). Caspase 3, periodically expressed and activated at G2/M transition, is required for nocodazole-induced mitotic checkpoint. *Apoptosis* **11**(5): 765-771.
- Huang H. C., Mitchison T. J., Shi J. (2010). Stochastic competition between mechanistically independent slippage and death pathways determines cell fate during mitotic arrest. *PLoS One* **5**(12): e15724.
- Johnson V. L., Scott M. I. F., Holt S. V., Hussein D., Taylor S. S. (2004). Bub1 is required for kinetochore localization of BubR1, Cenp-E, Cenp-F and Mad2, and chromosome congression. *Journal of Cell Science* **117**(8): 1577.
- Jordan M. A., Thrower D., Wilson L. (1992). Effects of vinblastine, podophyllotoxin and nocodazole on mitotic spindles. Implications for the role of microtubule dynamics in mitosis. *Journal of Cell Science* **102**(3): 401.
- Kanda T., Sullivan K. F., Wahl G. M. (1998). Histone-GFP fusion protein enables sensitive analysis of chromosome dynamics in living mammalian cells. *Current Biology* **8**(7): 377-385.
- Kim M., Liao J., Dowling M. L., Voong K. R., Parker S. E., Wang S., El-Deiry W. S., Kao G. D. (2008). TRAIL inactivates the mitotic checkpoint and potentiates death induced by microtubule-targeting agents in human cancer cells. *Cancer Research* **68**(9): 3440.
- Kim M., Murphy K., Liu F., Parker S., Dowling M., Baff W., Kao G. (2005). Caspase-mediated specific cleavage of BubR1 is a determinant of mitotic progression. *Molecular and Cellular Biology* **25**(21): 9232-9248.
- Kuida K., Zheng T. S., Na S., Kuan C. Y., Yang D., Karasuyama H., Rakic P., Flavell R. A. (1996). Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* **384**(6607): 368-372.
- Lakhani S. A., Masud A., Kuida K., Porter G. A., Booth C. J., Mehal W. Z., Inayat I., Flavell R. A. (2006). Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. *Science* **311**(5762): 847.
- Lanni J. S., Jacks T. (1998). Characterization of the p53-dependent postmitotic checkpoint following spindle disruption. *Molecular and Cellular Biology* **18**(2): 1055.
- Lee K., Kenny A. E., Rieder C. L. (2011). Caspase activity is not required for the mitotic checkpoint or mitotic slippage in human cells. *Molecular Biology of the Cell* **22**(14): 2470-2479.
- Liu S., Rattner J., Jablonski S., Yen T. (2006). Mapping the assembly pathways that specify formation of the trilaminar kinetochore plates in human cells. *Journal of Cell Biology* **175**(1): 41-53.
- Matthess Y., Raab M., Sanhaji M., Lavrik I. N., Strebhardt K. (2010). Cdk1/cyclin B1 controls Fas-mediated apoptosis by regulating caspase-8 activity. *Molecular and Cellular Biology* **30**(24): 5726.
- Medema R. (2009). Relaying the Checkpoint Signal from Kinetochore to APC/C. *Developmental Cell* **16**(1): 6-8.
- Morrow C. J., Tighe A., Johnson V. L., Scott M. I. F., Ditchfield C., Taylor S. S. (2005). Bub1 and aurora B cooperate to maintain BubR1-mediated inhibition of APC/CCdc20. *Journal of Cell Science* **118**(16): 3639.
- Musacchio A., Hardwick K. G. (2002). The spindle checkpoint: structural insights into dynamic signalling. *Nature Reviews Molecular Cell Biology* **3**(10): 731-741.
- Musacchio A., Salmon E. (2007). The spindle-assembly checkpoint in space and time. *Nature Reviews Molecular Cell Biology* **8**: 379-393.
- Niikura Y., Dixit A., Scott R., Perkins G., Kitagawa K. (2007). BUB1 mediation of caspase-independent mitotic death determines cell fate. *The Journal of Cell Biology* **178**(2): 283-296.
- Pines J., Hunter T. (1990). Human cyclin A is adenovirus E1A-associated protein p60 and behaves differently from cyclin B. *Nature* **346**: 760-763.
- Pop C., Salvesen G. S. (2009). Human caspases: activation, specificity, and regulation. *Journal of Biological Chemistry* **284**(33): 21777-21781.
- Prendergast L., van Vuuren C., Kaczmarczyk A., Doering V., Hellwig D., Quinn N., Hoischen C., Diekmann S., Sullivan K. F. (2011). Premitotic Assembly of Human CENPs-T and -W Switches Centromeric Chromatin to a Mitotic State. *PLoS Biology* **9**(6): e1001082.

- Rieder C. L., Maiato H. (2004). Stuck in Division or Passing through:: What Happens When Cells Cannot Satisfy the Spindle Assembly Checkpoint. *Developmental Cell* **7**(5): 637-651.
- Scherer W. F., Syverton J. T., Gey G. O. (1953). Studies on the propagation in vitro of poliomyelitis viruses. *The Journal of Experimental Medicine* **97**(5): 695.
- Shelby R. D., Monier K., Sullivan K. F. (2000). Chromatin assembly at kinetochores is uncoupled from DNA replication. *The Journal of Cell Biology* **151**(5): 1113-1118.
- Shi J., Orth J. D., Mitchison T. (2008). Cell type variation in responses to antimetabolic drugs that target microtubules and kinesin-5. *Cancer Research* **68**(9): 3269.
- Steller H. (1995). Mechanisms and genes of cellular suicide. *Science* **267**(5203): 1445.
- Sudo T., Nitta M., Saya H., Ueno N. T. (2004). Dependence of paclitaxel sensitivity on a functional spindle assembly checkpoint. *Cancer Research* **64**(7): 2502.
- Taylor S. S., McKeon F. (1997). Kinetochores localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage. *Cell* **89**(5): 727-735.
- Vigneron S., Prieto S., Bernis C., Labbe J., Castro A., Lorca T. (2004). Kinetochores localization of spindle checkpoint proteins: Who controls whom? *Molecular Biology of the Cell* **15**(10): 4584-4596.
- Weaver B., Cleveland D. (2005). Decoding the links between mitosis, cancer, and chemotherapy: The mitotic checkpoint, adaptation, and cell death. *Cancer Cell* **8**(1): 7-12.
- Whitfield M. L., Sherlock G., Saldanha A. J., Murray J. I., Ball C. A., Alexander K. E., Matese J. C., Perou C. M., Hurt M. M., Brown P. O. (2002). Identification of genes periodically expressed in the human cell cycle and their expression in tumors. *Molecular Biology of the Cell* **13**(6): 1977-2000.
- Yamada H., Gorbsky G. (2006). Spindle checkpoint function and cellular sensitivity to antimetabolic drugs. *Molecular Cancer Therapeutics* **5**(12): 2963-2969.

CHAPTER 3

The CENP-W/T complex is required in each mitosis

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Key words: centromere, CCAN, kinetochore assembly, mitosis, spindle assembly checkpoint (SAC)

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Premitotic assembly of human CENPs -T and -W switches centromeric chromatin to a mitotic state.

3.1. SUMMARY

The CENP-W/T is a recently identified complex of the CCAN, which consists of a histone fold dimer and is associated with a canonical histone H3 rather than CENP-A nucleosomes. The CENP-W/T complex was found to be critical for mitosis in vertebrate cells (Foltz *et al.* 2006; Hori *et al.* 2008). Here, we investigated the requirement for function of CENP-W and – T in the cell cycle in HeLa cells. Using time-lapse imaging of cells expressing H2B-GFP fusion and immunostaining we respectively examined mitotic kinetics and spindle structure following RNAi depletion of CENP-W and CENP-T. Quantitative analysis of mitotic timing revealed distinct phenotypic response to depletion, though both knockdowns resulted in mitotic delay. This delay, shown to occur in the first mitosis of depleted populations was more severe in CENP-W depleted cells with characteristic multipolar spindles, extended prometaphase and pronounced spindle rolling phenotype, which was further confirmed by live cell studies on fluorescently-tagged mCherry-tubulin H2B-GFP cells. The depletion of CENP-T protein resulted in a more subtle effects, yet cells exhibited mitotic defects, the major being difficulties in a proper chromosome congression, frequent metaphase laggards and formation of anaphase bridges. Subsequently, we examined spindle assembly checkpoint (SAC) response upon loss of CENP-W/T complex. We found, that kinetochores depleted of CENP-W/T complex lacked Mad1/Mad2 checkpoint proteins, but did not affect localization of the other checkpoint proteins Bub1, BubR1, Zwint-1 or Aurora B. Moreover, we discovered that in CENP-W depleted cells the lack of stable end-on attachments associated with loss of intrakinetochore stretch and permanent Aurora B activation were the primary cause of the significant mitotic arrest in these cells, rescued by the inhibition of Aurora B kinase activity. Taken together, our data demonstrated that CENP-W/T complex is required for mitosis in each cell cycle. We propose that within centromere assembly CENP-W/T complex is specialized for kinetochore activities, presumably via providing the connection between the centromere and outer kinetochore proteins and mediating the attachments of the chromosomes and spindle microtubules.

3.2. INTRODUCTION

Centromeres are differentiated chromatin domains, present in a single copy on each chromosome, that direct segregation of the genome in mitosis and meiosis by specifying assembly of kinetochore (Cleveland *et al.* 2003). Kinetochores are built upon centromeric chromatin, which contains the histone H3 variant CENP-A, found exclusively at centromeres of all eukaryotes, and the constitutive centromere-associated network (CCAN) (Hori *et al.* 2008). To date, 16 centromere proteins have been identified as members of the CCAN (Perpelescu and Fukagawa 2011), and despite of the great effort, the role that many of these proteins play in the establishment of centromeric chromatin is still unknown. Therefore the main interest of our group was focused primarily on testing the functional role of particularly two of them – CENP-W and CENP-T proteins.

Within the CCAN there are four additional histone fold domain (HFD) containing proteins: the CENP-W/T complex and the CENP-S/X complex. CENP-T was originally identified as a member of the CENP-A NAC (Foltz *et al.* 2006). Further purification of complexes containing CENP-T identified CENP-W protein, previously named CUG2 (Lee *et al.* 2007) associating with CENP-T in a complex, for the first time in chicken DT40 cells (Hori *et al.* 2008), and next the human homologue of CENP-W was found to also localize to centromeres as a constitutive component through the cell cycle (Kim *et al.* 2009). The CENP-W/T complex itself associates with a population of histone H3-containing nucleosomes within centromeric chromatin and knockout experiments in DT40 cells revealed that CENP-T is recruited upstream of CENP-H and CENP-O class of the CCAN members but parallel to CENP-C in centromere assembly (Foltz *et al.* 2006). Therefore, it was suggested that CENP-W/T complex plays a critical role in mitosis, in centromere assembly.

Although all CCAN members assemble downstream of CENP-A, in human cells the presence of overexpressed CENP-A does not appear to be sufficient for kinetochore assembly (Van Hooser *et al.* 2001). A recent work has shown that induced ectopic kinetochore assembly, governed by CENP-C and CENP-T, bypasses the requirement for CENP-A nucleosomes (Gascoigne *et al.* 2011), indicating an exclusive role for CENP-T/W complex in this process. Moreover, consistent with a hierarchical relationship between the CCAN and the outer kinetochore components (Cheeseman and Desai 2008), cells can counteract the loss of some of the CENPs more than the others. As mentioned above, the CCAN makes a platform for kinetochore assembly, and its main function is to mediate the microtubule dependent movement of

chromosomes during mitosis and sensing errors in chromosome attachments and activate the spindle assembly checkpoint (SAC), which delays cells at the metaphase-anaphase transition until correct bipolar attachment is achieved (Musacchio and Hardwick 2002). Several CCAN components have been implicated in the establishment of CENP-A chromatin as well as in kinetochore function and microtubule modulation (Cheeseman and Desai 2008). Based on the functional studies carried out on different CCAN members depletion, in several different systems, it is clear now that some of the CCAN components are more essential for cell viability than the other. For example, it has been shown, in both human and chicken cells, that depletion of CENP-A led to a gradual decrease in CENP-A protein levels, without any observable influence on mitosis, until critical depletion, consistent with CENP-A being a long-lived protein (Shelby *et al.* 2000). This behaviour is similar to early results obtained by microinjections of antibodies to CENP-C, showing that cells can accommodate the loss of activity of these two core inner centromere components by making smaller but functional kinetochores (Tomkiel *et al.* 1994). However, reaching the critical threshold of about 10%, analysis of CENP-A depleted DT40 chicken cells revealed a severe mitotic defects, compromising the localization of the inner kinetochore components, like CENP-C, CENP-H and CENP-I, as well as the outer kinetochore components Nuf2/Hec1, CENP-E which are responsible for kinetochore-microtubule attachments. It was also found that CENP-A depletion affects the kinetochore localization of the checkpoint components Mad2 and BubR1 (Regnier *et al.* 2005).

The functional studies on the multi-subunit CENP-H-I complex showed that these proteins have multiple functions in facilitating proper chromosome segregation (Okada *et al.* 2006). The CENP-H-I complex was shown to contribute to the efficient incorporation of newly synthesized CENP-A into centromeres, as well as to have functions independent of that process. Its depletion resulted in a reduction in levels of outer kinetochore protein Hec1 with still detectable wild-type levels of CENP-A at the same timepoint, suggesting that CENP-H-I associated complex is also required for interaction with other centromere complexes to establish the full range of kinetochore function (Okada *et al.* 2006). Independent study of the function of CENP-I protein in human cells, showed that kinetochores depleted of CENP-I lacked CENP-F and one of the major mitotic checkpoint protein – Mad2 (Liu *et al.* 2003). Some of the CCAN components were found to be periodically expressed during the cell cycle, e.g. CENP-E and CENP-F, that localize in a fibrous corona from G2/M transition, coincident with kinetochore assembly and afterwards travel to the midzone and diminished from the kinetochore in the subsequent G1 entry (Yen *et al.* 1992; Rattner *et al.* 1993). However, their requirement for function was

reflected in a severe mitotic delay, metaphase arrest and a frequent formation of multipolar spindles following inhibition or depletion of CENP-E protein (Yen *et al.* 1991; Martin-Lluesma *et al.* 2002). CENP-V, which localizes to the inner kinetochore and mediates between the centromere chromatin and centromere passenger complex (CPC) was also found to be essential for cell viability, since its depletion leads to hypercondensation of the pericentromeric chromatin, compromises proper localization of the CPC, perturbs nuclear distribution of H2K9me and induces severe mitotic abnormalities (Tadeu *et al.* 2008).

Taken together, these data suggest that there might be a subset of the conserved CCAN components that possess an intrinsic role to stimulate proper kinetochore assembly independently of CENP-A based centromere assembly. Following the discovery of CENP-W/T complex in DT40 chicken cells (Hori *et al.* 2008), very little was known about its role in centromere assembly in human cells. Therefore, in this work we investigated the requirement for function of the two HFD proteins, CENPs – T and – W in HeLa cell cycle and examined how the interference with the function of the inner kinetochore plate components might affect the SAC checkpoint function.

3.3. MATERIALS AND METHODS

3.3.1. Cell culture, RNAi and drug treatment

HeLa cells were cultured as previously described (Chapter 2, section 2.3.1). For cells stably expressing mCherry-tubulin a medium with a selective antibiotic G418 (300mg/mL) was used. In siRNA experiments, synthetic double stranded ON-TARGET plus SMARTpool sequences (Dharmacon) for CENP-W (L-032901-01), CENP-T (L-014577-01), CENP-A (L-003249-00) and non-targeting control pool (D-001810-10) were used. Targeted duplexes were transfected at 100 nM concentration into cells using DharmaFECT 1 reagent (Dharmacon), according to the manufacturer's instruction. Cells were analyzed 24h and/or 48h post-transfection.

To inhibit microtubule dynamics and induce maximum spindle assembly checkpoint binding to the kinetochores cells were incubated with 1 μ M nocodazole for 12h before immunostaining for indicated checkpoint proteins. To inhibit Aurora B kinase activity cells were treated with ZM447439 inhibitor (JS Research Chemicals Trading, Schleswig Holstein, Germany) at final concentration of 2 μ M directly before the onset of live cell imaging.

3.3.2. Immunofluorescence

Cells were rinsed in 1X PBS, fixed for 20 min with 4% paraformaldehyde (PFA) or for 10 min in 1X PTEMF extraction buffer (20mM PIPES, pH 6.8, 10mM EGTA, 1mM MgCl₂, 0.2% Triton X-100 and 4% PFA) prepared fresh from 4X stock solution (for all SAC antibodies). Cells were permeabilized by 2x3min wash in 0.1% Triton-X-PBS (PBS-TX), followed by blocking in PBS-TX containing 1% BSA for 30 min at room temperature, before being processed for immunofluorescence. Primary antibodies were diluted in 1% BSA in 1X PBS-TX in a 37 °C incubator for 1hr. Primary antibody dilutions were as follows: mouse anti- α -tubulin (DM1 α , Sigma, 1:1000), human anti-centromere antibody (ACA, 1:2000), mouse anti-Aurora B (1:100), mouse anti-BubR1 (Millipore, 1:50), mouse anti-Mad1 (a kind gift from Dr. Andrea Musacchio, 1:300), sheep anti-Mad2 (a kind gift from Dr. Steven Taylor's Laboratory, 1:200), mouse anti-Bub1 (a kind gift from Dr. Steven Taylor's Laboratory, 1:1000), rabbit anti-Zwint-1 (Bethyl, 1:100). Cells were washed twice in 1X PBS-TX for 10 min each. Fluorescently conjugated secondary antibodies (FITC, TRITC or Cy5, Jackson Laboratories) were chosen as appropriate, diluted in 1% BSA in 1X PBS-TX and applied on cells in the dark in a 37 °C incubator for 1hr. Cells were washed once in 1X PBS-TX for 10min, followed by 1 wash in 1X PBS for 10min. Finally cells were washed once in distilled H₂O for 1min. Coverslips were then allowed to completely dry before being mounted in slowfade with 1% 4'-6-Diamidino-2-

phenylindole (DAPI) (if required, for chromatin visualization). Coverslips were sealed with nail varnish and stored at 4°C in the dark.

Microscopy was carried out using a Deltavision Core system (Applied Precision) controlling an interline charge-coupled device camera mounted on an inverted microscope (Olympus). For each sample images were collected at 2x2 binning using a 100x oil objective at 0.2 μm z sections. Immunofluorescence images were subject to iterative constrained deconvolution and maximum intensity projection using the SoftWoRx software (Applied Precision).

Following immunofluorescence and data collection, deconvolved and projected images were used in Image Pro Plus software. Using its analysis tool, the threshold of the intensity range for each image of each treatment in the reference channel was defined (a process called segmentation) and based on that kinetochores were scored as individual objects. Next, the masks – saved outlines of each object, were applied from reference channel into the experimental channel (checkpoint proteins signal) staining and mean intensity values for each object were recorded. Finally, the collected fluorescence intensity values of both reference and experimental channel were background corrected for further analysis.

3.3.3. Live cell microscopy

HeLa cell line expressing a histone H2B-GFP was used to visualize chromosomes. Cells were transfected with siRNA for 24h or 48h before imaging. Time-lapse live cell imaging and analysis was performed as previously described (Chapter 2, section 2.3.7).

3.3.4. Western blotting

Protein samples were processed as previously described (Chapter 2, section 2.3.4.). The membranes were incubated with primary antibodies: rabbit anti-CENP-W (Abcam, 1:1000), rabbit anti-CENP-T (Bethyl, 1:1000), mouse anti-GFP (Roche, 1:1000), mouse anti-Mad1 (1:100) and mouse anti-tubulin (Sigma, 1:5000). Goat anti-mouse or goat anti-rabbit HRP antibodies (Jackson Laboratories) were successively applied. Antigens on the membrane were revealed by enhanced chemiluminescence (ECL, Milipore). Densitometric analysis of immunoblots were determined using the Gene Snap software from Syngene.

3.3.5. DNA plasmid preparation and transfection

Construct of mCherry-tubulin was generated using Gateway Cloning System (Invitrogen, see more details in section 3.4.3). Midi plasmid DNA preparations of the construct was generated using Qiagen Mini Prep kit following the manufacturer's instructions. Transfection of H2B-GFP HeLa cells with mCherry-tubulin plasmid construct was performed using a Lipofectamine2000

(Invitrogen) transfection reagent in accordance with the manufacturer's recommendations. Following transfection cells were incubated in a selective medium containing G418 antibiotic to screen for a stably expressing mCherry-tubulin clone. Selected clones were checked for the expression of a desired gene using fluorescent microscopy and direct sequencing.

3.3.6. cDNA synthesis

Total RNA was reverse transcribed using random nonamer primers to generate cDNA using the Precision qScript Reverse Transcript Kit supplied by Primer Design (Southampton, UK). 1µg of total RNA was combined with 1µl of random nonamer primers and were annealed at 65°C for 5min. Samples were placed on ice to chill following the incubation period. 2µl of 10X qScript Buffer, 1µl of dNTP mix (10mM each), 2µl of 100 mM DTT, 1µl of qScript enzyme and 4µl of DNase/RNase free water was then added and the mixture was vortexed followed by a pulse spin. The mixture was incubated at 25°C for 5min followed by 20 min incubation at 55°C. The extension reaction was stopped by heat inactivation (15min, 75°C). cDNA was stored at -20°C.

3.3.7. qRT-PCR

Relative transcript abundance was measured using real-time PCR (qPCR) in conjunction with SYBR green dye. qPCR measures the amount of amplified product at the end of each cycle. The changes in product concentration at the end of each cycle are calculated by measuring the levels of SYBR green fluorescence, which is proportional to the amount of product formed. SYBR green dye binds to double stranded DNA which results in an increase in fluorescence. The qPCR were carried out in 96 well optical plates on a StepOnePlus™ Real-Time PCR System (Applied Biosystems supplied through Life Technologies, Paisley, PA4 9RF United Kingdom). Primers for specific genes were resuspended in RNase/DNase free water. cDNA as prepared in section 2.2.7 was used as template for each reaction. Each 20µl reaction contained 5µl cDNA, 1µl primer mix (300nM, 150nM, and 75nM), 10µl SYBR Green master mix (Primer Design, Southampton, UK) and 4µl of RNase free water. The cycling conditions were as follows 95°C for 10min, 1 cycle, and then 95°C for 15sec, 60°C for 1min, 40 cycles.

3.3.8. Caspase Activity Assay and Flow Cytometry analysis

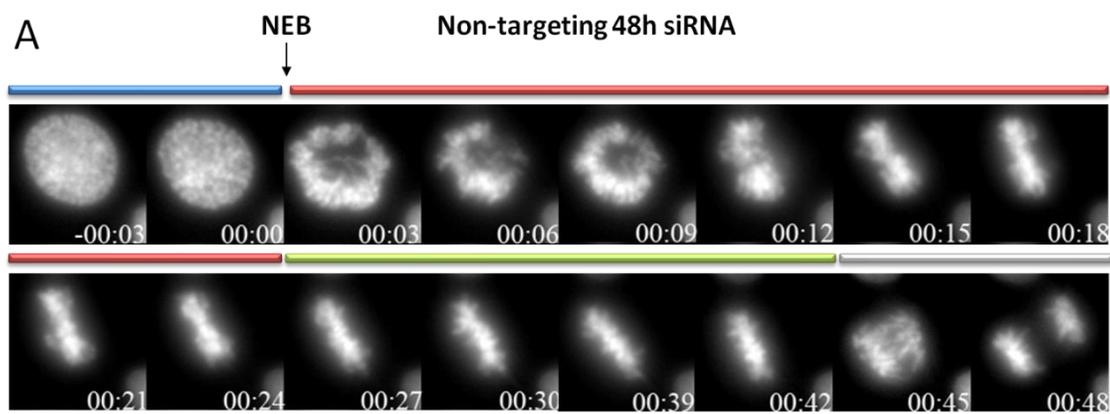
Caspase-3 activity assay was performed as previously described (Chapter 2, section 2.3.5). Flow cytometry analysis was performed as previously described (Chapter 2, section 2.3.3).

3.4. RESULTS

3.4.1. Mitotic kinetics in GFP-H2B cells depleted of CENP-W/T complex

To examine the functional requirements of CENP-W/T complex within the HeLa cell cycle, we performed a quantitative analysis of the mitotic kinetics measured in cells expressing histone H2B-GFP fusion following RNAi depletion. Using live cell microscopy we carried out 12h long with 3min intervals time-lapse imaging of cells progressing through mitosis upon treatment with pooled siRNA against either CENP-W or CENP-T protein, as previously described (Chapter 2).

As expected, the chromosomes of control treated cells (non-targeting control siRNA) typically after NEB formed prometaphase horseshoe shapes, and then aligned at the metaphase plate within 30-50 minutes (Figure 3.1A). After a delay of ~20 minutes, anaphase initiated with the chromosomes separating into two clear masses. Following RNAi, time-lapse analysis of CENP-W and -T-RNAi treated mitotic cells resulted in a very different mitotic behavior. In particular, chromosome alignment and anaphase onset were frequently delayed (Figure 3.1). Depletion of CENP-W for 48h resulted in a severe disruption of mitosis comparable to that of a conditional knockout in chicken cells (Hori *et al.* 2008). Cells exhibited extended prometaphase with numerous misaligned chromosomes, mono-oriented chromosomes or metaphase-like configuration with chromosomes scattered near the spindle poles. In addition to failure of congression, many cells exhibited a persistent movement of the chromosome mass, rotating within the cell in one principle direction for 20-30min at a time, suggesting a pronounced spindle rolling phenotype (Figure 3.1B). Depletion of CENP-T for 48h using pooled siRNA resulted relatively subtle effects, with high frequencies of misaligned chromosomes, congression defects and lagging chromosomes, which resulted in a formation of numerous anaphase bridges (Figure 3.1C).



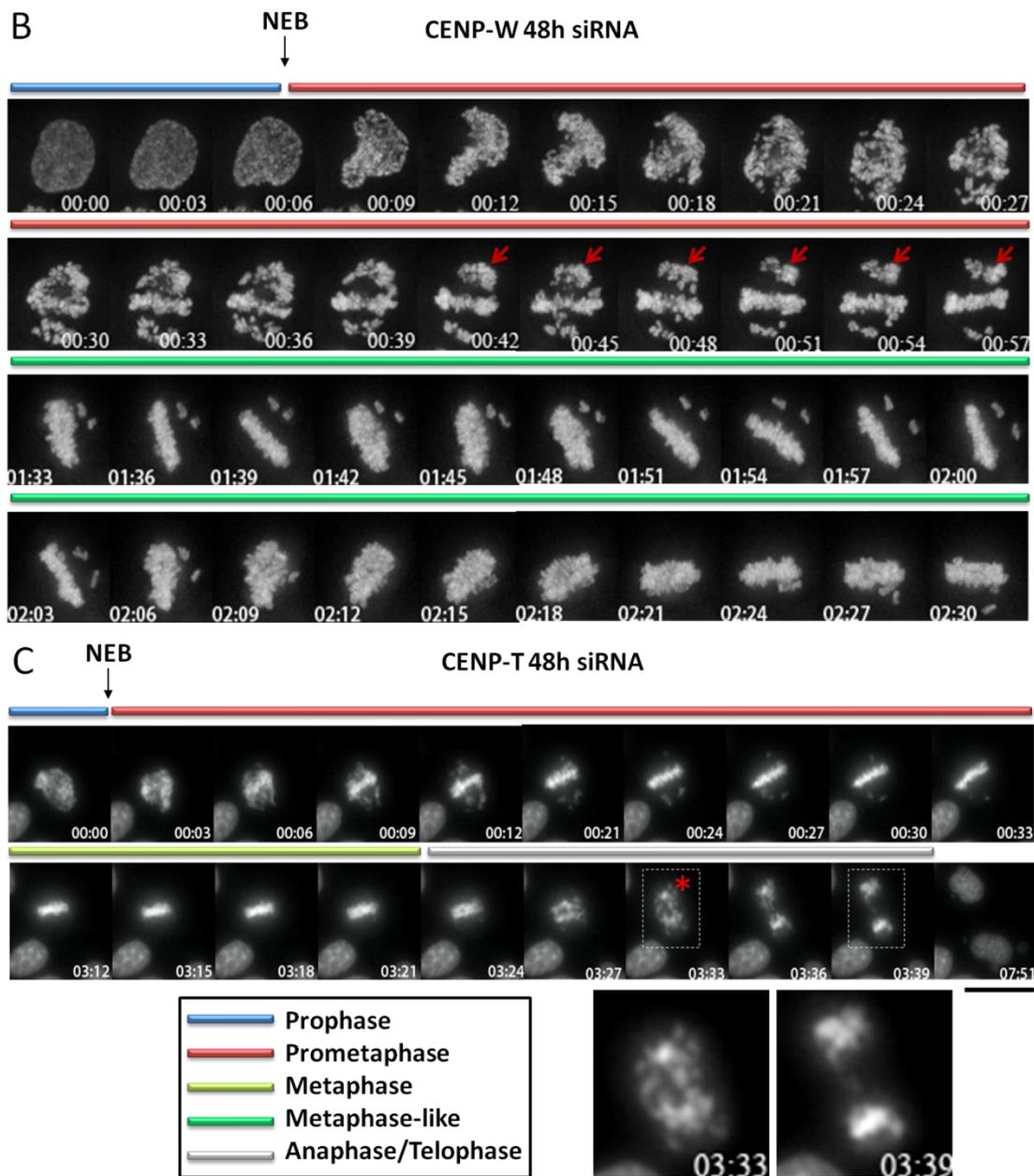


Figure 3.1. CENP-W and CENP-T depleted cells fail to congress their chromosomes and enter anaphase with unaligned chromosomes. (A) Representative panel of mitotic progression in control (non-targeting siRNA treated) H2B-GFP cells. (B) Representative panel of the CENP-W phenotype. Upon 48h siRNA depletion of CENP-W the mitotic cell exhibited severe problems with chromosome congression leading to a metaphase-like configuration with a great portion of chromosome mass scattered along the spindle poles (see red arrows) and undergoing constant rolling motion. (C) Representative panel of the CENP-T phenotype. Mitotic cell depleted of CENP-T for 48h displayed a pronounced misaligned and lagging chromosomes that formed anaphase bridges visualize as a chromosome mass dispersed between two daughter cells (see red asterisk and enlargements). NEB=Nuclear Envelope Breakdown (defined as onset of deformations in nuclear chromatin) is depicted for each siRNA treatment. Scale bar 10 μ m, time = hr:min.

Considering that HeLa cell cycle is accomplished between 22-24h, many cells observed at 48-60 hr following siRNA transfection may be executing their second mitosis. It has been shown that cells depleted of CENP-A can maintain normal kinetochore function for 2-3 cell cycles (Liu *et al.* 2006), delaying the onset of mitotic defects until a critical threshold of about 10% of normal levels is reached and mitosis fails (Figure S3.5). CENP-A example shows that cells can accommodate the loss of activity of certain CENPs by making smaller yet functional kinetochores (Tomkiel *et al.* 1994). On the other hand, some CENPs have been shown to be required in each single mitosis, a behavior characteristic e.g. for CENP-E motor protein that is used and discarded in each mitosis and yet required for its proper progression (Yen *et al.* 1992; Wood *et al.* 1997). Taking into account the complexity of the centromere and kinetochore assembly and differential outcomes following different CENPs depletion, we concluded that to examine the real biological requirement for function of CENP-W/T complex we must observe the first mitosis following administration of siRNA (Figure 3.2A). Therefore, by observing cells between 24-36 hr after initiation of transfection, the consequences of CENP-W/T loss on the first mitosis following depletion could be examined.

Following live cell imaging, using Image Pro Plus software and its function for tracking the objects, we measured the changes of the GFP fluorescence intensity caused by chromatin condensation as a function of time (which corresponds to time between NEB and chromosome de-condensation following anaphase onset). Subsequently, we defined a delay in mitosis as any cell that does not initiate anaphase within the mean mitotic time + one standard deviation (1SD). The quantitative data for the non-targeting siRNA treated H2B-GFP cells showed an average pre-anaphase time of 89 +/- 38 minutes (Figure 3.2). Following RNAi, over 35% of CENP-W depleted cells showed a mitotic delay with some cells spending an excess of up to 5h in mitosis following 24h CENP-W depletion (Figure 3.2B and C). These defects were much more severe 48h post-transfection, with a population of nearly 80% of cells displaying the above mitotic defects and an average delay of 173 minutes. However, the quantitative results of mitotic timing of CENP-T depleted cells (both 24h and 48h siRNA treated) demonstrated mildness of this phenotype and did not differ significantly from untreated cells (Figure 3.2D). The differences in phenotypes after depletion of CENP-W/T complex subunits were unexpected and could be related to a partial knockdown of CENP-T under our experimental conditions (Figure S3.1). Despite great effort, it was not possible to achieve definitive protein depletion in a time course of 24-48 hr siRNA experiments. However, these results indicate that deposition of CENP-W is required in each cell cycle for a robust mitosis.

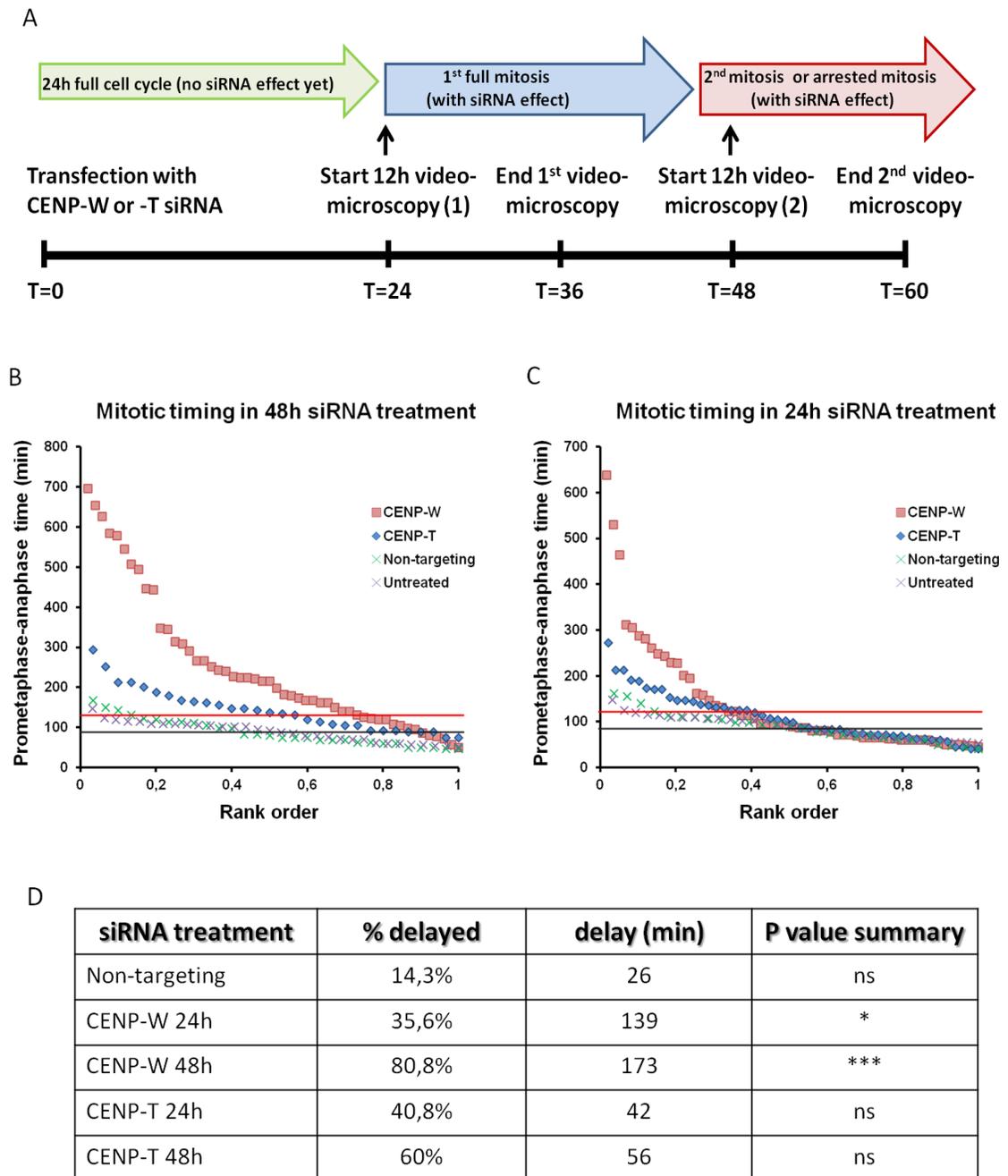


Figure 3.2. Mitotic progression is severely delayed in cells depleted of CENP-W or CENP-T. (A) Schematic representation of siRNA treatment and video-microscopy protocol. (B-C) Mitotic kinetics were assayed using histone H2B-GFP HeLa cells treated with CENP-W (red squares) and CENP-T (blue rhombus) siRNA for 48 (B) or 24 h (C), scoring time between NEB and anaphase onset and are plotted by rank order of individual cells. Mean time for non-targeting siRNA control (green X) and untreated cells (purple X) is shown as a black line, with +1 standard deviation (SD) marked with a red line. At least 30 mitoses were scored for each treatment, in duplicate assays. (D) Table represents cumulative data of percentage of cells with NEB-anaphase times in excess of +1 SD and the average delay in anaphase onset (in minutes), following indicated siRNA treatment. * $p < 0.01$, *** $p < 0.0001$, ns = non-significant (one-way ANOVA followed by Dunnetts multiple comparison).

3.4.2. Distinct spindle pole phenotypes following depletion of CENP-W and CENP-T

As described above, we demonstrated that CENP-W/T complex is critical for mitosis and its depletion induced aberrant cell division. To further investigate mitotic defects associated with loss of CENP-W/T complex we monitored mitotic spindles in fixed cells. Wild type HeLa cells transfected with CENP-W and CENP-T siRNAs were immunostained with anti- α -tubulin and ACA serum for detection of the spindle microtubules and centromeres, respectively. Consistent with findings from live cell studies immunofluorescence staining revealed a profound defects in mitotic spindle organization in siRNA affected cells (Figure 3.3).

In a normal mitosis (non-targeting control treated cells) upon complete chromosome congression metaphase spindles are uniformly established in a bipolar manner, tightly connecting with the kinetochores of each sister chromatid and therefore subjecting them to tension and microtubule-dependent poleward movement following anaphase onset. This characteristic tension dependent centromere stretch can be easily observed under fluorescent microscopy following centromere staining with ACA (Figure 3.3A, top panel). Occasionally, the formation of a tripolar spindle in mitotic cells of control population can be observed (3.6%). However, following RNAi treatment distinct aberrations of a mitotic spindle structure were observed. As expected, both siRNA treatments for CENP-W and CENP-T resulted in an increase of mitotic index. Spindles of CENP-W depleted cells were frequently multipolar (Figure 3.3A and B) with disorganized prometaphase chromosomes and centromeres not exhibiting characteristic stretch. Nevertheless, centromeres were still positive for ACA staining, indicating that CENP-W and/or CENP-T assembly is downstream of CENP-A and CENP-B protein deposition since these are the main components of the ACA serum. Depletion of CENP-T demonstrated a different effect on the spindle. In spite of an increased formation of a tripolar spindles (Figure 3.3B), most of the mitotic cells possessed bipolar configuration with characteristic fusiform spindle morphology, individual misaligned chromosomes and stretched centromeres, suggesting proper MT-kinetochore attachments in these cells (Figure 3.3A).

Taken together, these results are consistent with findings from live cell studies on depleted of CENPs – W and – T cells and demonstrate a critical role for CENP-W during mitotic cell division, possibly through mediation of the attachment of the chromosome and spindle microtubules, which will be further elaborated in Chapter 4 of this thesis.

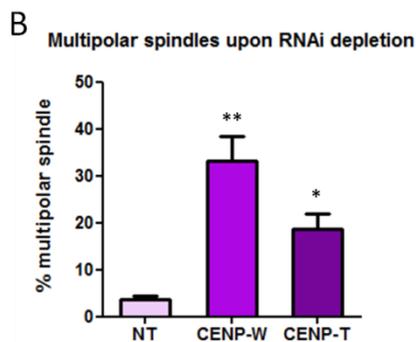
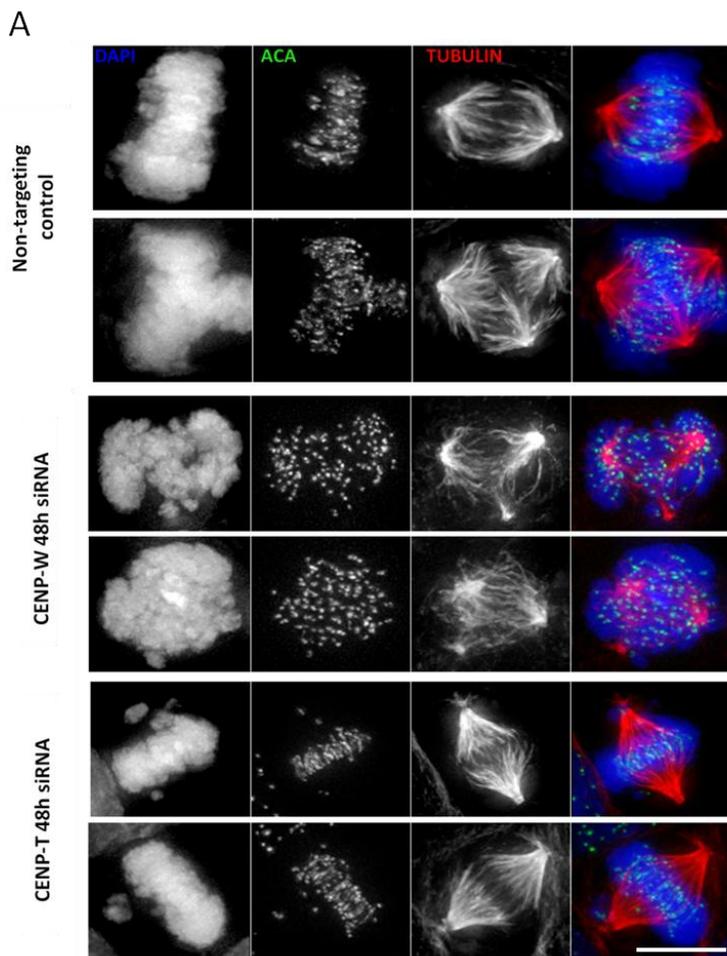


Figure 3.3. Multipolar spindles are frequently observed in fixed specimens of CENP-W siRNA treated cells. (A) Representative panel of HeLa cells transfected with pooled siRNA for CENP-W, CENP-T and non-targeting siRNA (NT) control and immunostained for visualization of centromeres, MTs and chromosomes with ACA (anti-centromere antibody, green), α -tubulin (red) and DAPI (blue), respectively. Scale bar 10 μ m. (B) Graph represents mean percentage (+SEM) of the multipolar spindle pole occurring in mitotic cells following CENP-W and CENP-T depletion (at least 100 mitotic cells were scored, in triplicate assays). * $p < 0.01$, ** $p < 0.001$ (one-way ANOVA followed by Dunnett's multiple comparison).

3.4.3. Spindle rolling phenotype of CENP-W depleted cells

As described above, following CENP-W depletion many cells exhibited a persistent rotation and 3D observation of affected cells confirmed the whole chromosome mass spinning within the cell cortex (Prendergast *et al.* 2011). To directly visualize the rolling spindles, we generated a cell line stably expressing mCherry- α -tubulin in H2B-GFP cells, according to an experimental strategy illustrated on Figure 3.4A. Briefly, using a standard PCR reaction conditions with gateway adapted primers (Figure S3.3A) we amplified α -tubulin 1b (TUBA1B) isoform from a cDNA of HeLa cells. Next, we applied Gateway cloning system and by performing two-step reaction with an intermediate entry vector (BP followed by LR) we successfully cloned the amplified tubulin coding sequence into a destination pmCherry vector, gateway adapted previously using SacII restriction enzyme. The correct sequence of the obtained plasmid was confirmed both by evaluating the sizes of DNA fragments following double digestion with restriction enzymes and by direct sequencing (Figure S3.3B and Appendix A). Transient transfection with pmCherry-tubulin resulted in an appropriate incorporation of fusion protein into cellular microtubule networks. Cells were further cultured in a media containing selective marker – G418 antibiotic (300mg/mL) to select for stably transfected clonal cell lines and to maintain recombinant protein expression throughout multiple passages.

Once established a stable cell line expressing mCherry- α -tubulin, we used time-lapse live cell microscopy to monitor the dynamics of fluorescently labeled spindles following CENP-W depletion. Consistent with previous findings tubulin labeling confirmed persistent spindle rolling phenotype in cells depleted of CENP-W (Figure 3.4B).

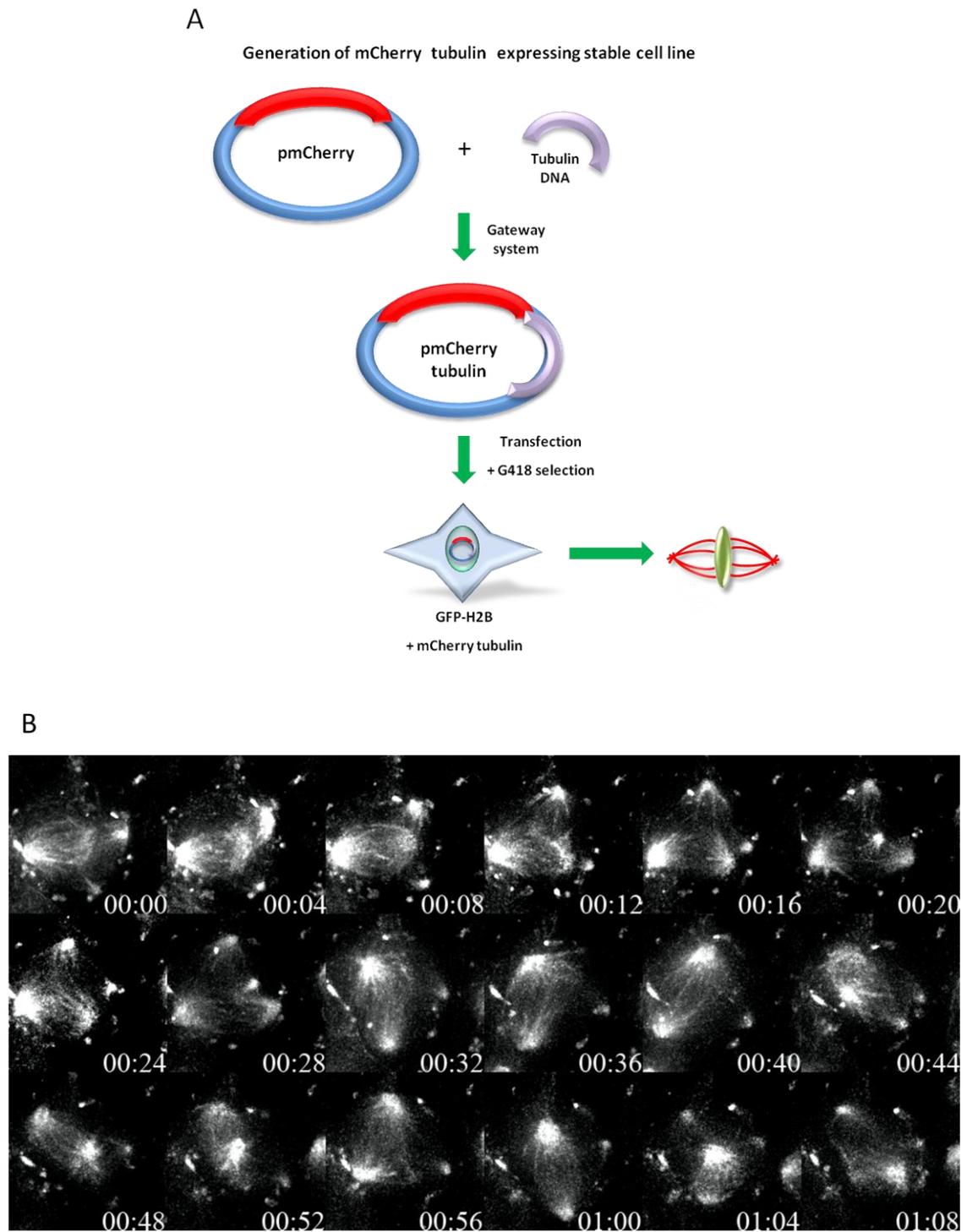


Figure 3.4. Tubulin labeling confirms whole spindle motion in CENP-W depleted cells. (A) Schematic experimental strategy used to construct stable mCherry- α -tubulin expressing cell line. **(B)** HeLa H2B-GFP cells were transfected with mCherry- α -tubulin to directly visualize spindle MTs motion following transfection with pooled siCENP-W for 48h. It is clear that the entire spindle is moving within cells that exhibit rolling, time = hr:min.

3.4.4. The SAC and Kinetochores response upon depletion of CENP-W/T complex

In vertebrates, the centromere is the foundation on which the kinetochore and SAC assembly occurs (Sullivan 2001). Within the CCAN some of the CENPs are more essential for viability of the cells than the other (Cheeseman and Desai 2008). There are evidence supporting a notion of ‘epistatic’ relationships in which the inner kinetochore components are required for the outer kinetochore components, e.g. CENP-A, CENP-T/W, CENP-C and CENP-H/I/K proteins all contribute, to different extents, to the recruitments of the KMN complex and associated proteins, that are necessary for proper kinetochore attachments to the spindle (Liu *et al.* 2003; Hayashi *et al.* 2004; Liu *et al.* 2006; Cheeseman *et al.* 2008).

It has been reported that kinetochores depleted of CENP-I lacked CENP-F and the checkpoint protein Mad2, but did not affect localization of the other checkpoint proteins such as Bub1, BubR1, Zw10, Zwint-1 or Rod, showing that CENP-I specifies a discrete branch of the kinetochore assembly pathway (Liu *et al.* 2003). Our previous quantitative analysis of mitotic kinetics have shown that CENP-W/T complex, part of histone fold domain (HFD) - family proteins of the CCAN, is important for kinetochore function. Thus, we focused on determining the influence of depletion of CENP-W and CENP-T proteins on SAC protein activity, e.g. cell cycle arrest. This work is aimed at understanding whether defects or differences in SAC proteins assembly are associated with the HFD CENPs knockdown.

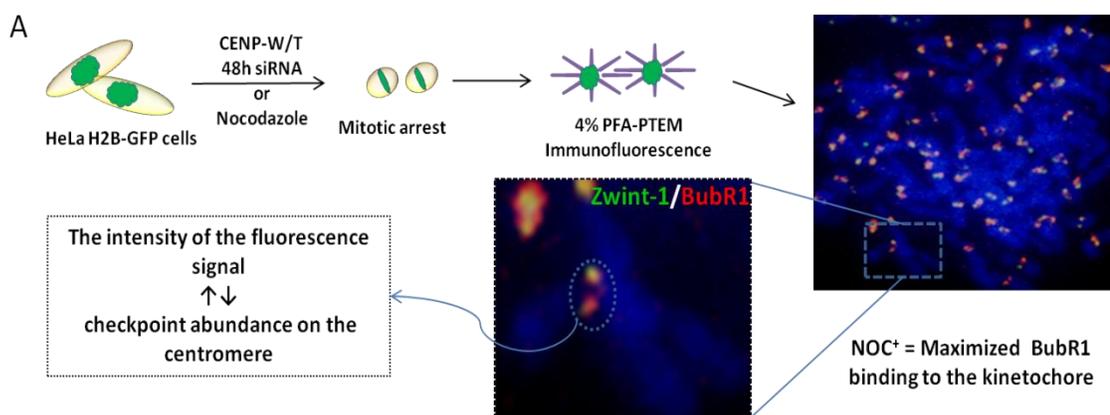
3.4.4.1. Differential binding of mitotic checkpoint proteins to kinetochores depleted of CENP-W/T complex

An essential function of centromere proteins is to provide a platform for kinetochore mediated checkpoint signal transmission (Cheeseman and Desai 2008). Disruption of that platform can result in mitotic arrest that is always mediated by the Mad/Bub-dependent pathway that operates in vertebrate to delay progression through mitosis (Rieder and Maiato 2004). However, one possible consequence of the CCAN components depletion is an impairment of the ability of checkpoint proteins to bind to kinetochores (Liu *et al.* 2003). As describe above, HeLa cells depleted of CENP-W and CENP-T demonstrated differential mitotic defects and delay in progression. Therefore, we hypothesized that there might be a distinctive checkpoint protein response following depletion of these two different CENPs under our experimental conditions.

To address that question, we depleted H2B-GFP cells of CENP-W and CENP-T using siRNA and processed for indirect immunofluorescence to visualize key components of the SAC, such as Bub1, BubR1, Mad1, Mad2, Aurora B and Zwint-1 (Musacchio and Salmon 2007). Next, we

established a quantitative method to examine the checkpoint protein binding capacity of the kinetochore. Using image analysis tools, fluorescence intensities of checkpoint proteins at kinetochore sites, corresponding to checkpoint abundance and activation of the SAC on the centromere was determined. The proportion of centromeres exhibiting checkpoint protein binding and the intensities of that binding was measured (Figure 3.5A).

First, we performed a quantitative analysis of BubR1 checkpoint protein binding capacity on the fluorescent images collected from untreated population. Briefly, HeLa H2B-GFP cells were used to visualize chromosomes and define an appropriate mitotic stage. Following pre-extraction and fixation method, cells were immunostained with ACA for centromere visualization, BubR1 for checkpoint protein detection and Zwint-1 for outer kinetochore component marker. Human Zwint-1 protein was shown to be recruited to kinetochores in early prophase and persists into a mid-anaphase (Starr *et al.* 2000), which makes this protein a marker of the outer kinetochore. Data were collected in 3-dimensional stacks and subject to constrained interative deconvolution. Using maximum projection images, kinetochores were defined by threshold segmentation of Zwint-1 signals and the resulting object masks were applied to the experimental channel, e.g. BubR1, to determine the intensity of staining at each kinetochore. For each cell analyzed, background was determined in each channel and used to correct intensity values, in particular mean intensity values (Figure 3.5B). These kinetochore intensities were then analyzed as discussed below.



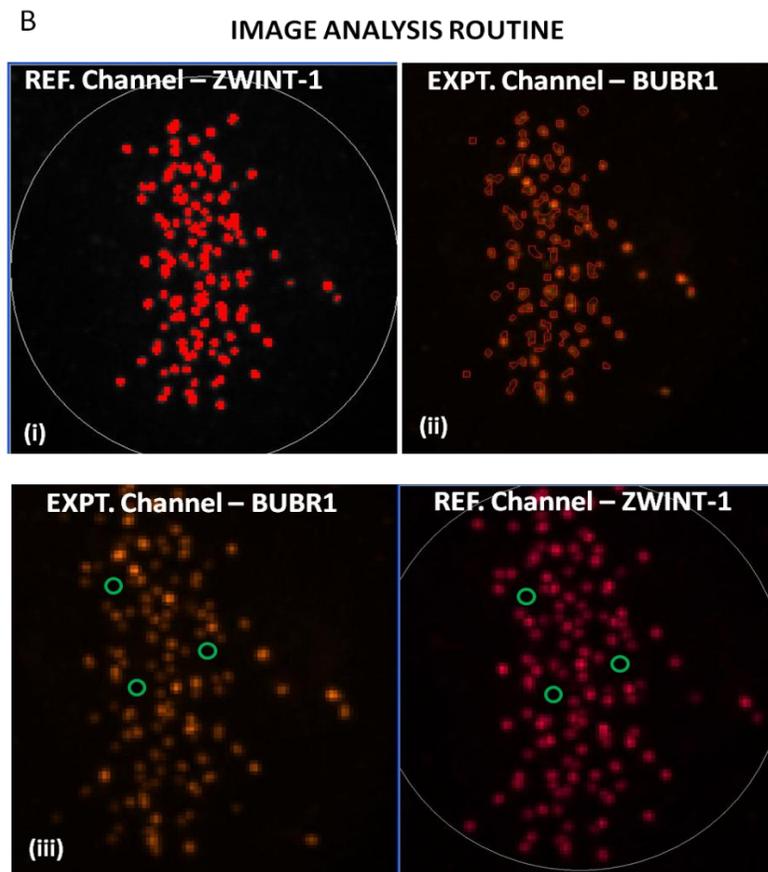


Figure 3.5. Application of quantitative microscopy for mitotic checkpoint studies. (A) Schematic experimental strategy used to examine SAC abundance at kinetochores. (B) The routine of image analysis used to measure the intensity of SAC signal at kinetochores, (i) segmentation, (ii) mask application on experimental channel signals, (iii) background correction on both channels. See more details in Materials&Methods (section 3.3.2).

This method was applied to examination of BubR1 checkpoint protein occupancy at kinetochores of non-targeting control cells. As previously described, BubR1 protein started to localize to kinetochores after nuclear envelope breakdown (NEB), unlike other checkpoint proteins (Mad1, Mad2 or Bub1) that localize to kinetochores already in prophase (Howell *et al.* 2004). It occupies the unattached kinetochores in prometaphase, followed by depletion from kinetochores in late metaphase and complete disappearance upon anaphase onset (Figure 3.6A). Consistent with immunofluorescence staining for BubR1 localization, the quantitative analysis of BubR1 fluorescence intensities revealed the same trend, corresponding to the checkpoint protein abundance at kinetochores (Figure 3.6B). The signal intensity varied substantially, depending on the mitotic stage, dividing into two sub-populations in prometaphase signal strength reflecting the microtubule attachment status of the kinetochores occupied with the checkpoint proteins (Figure 3.6C).

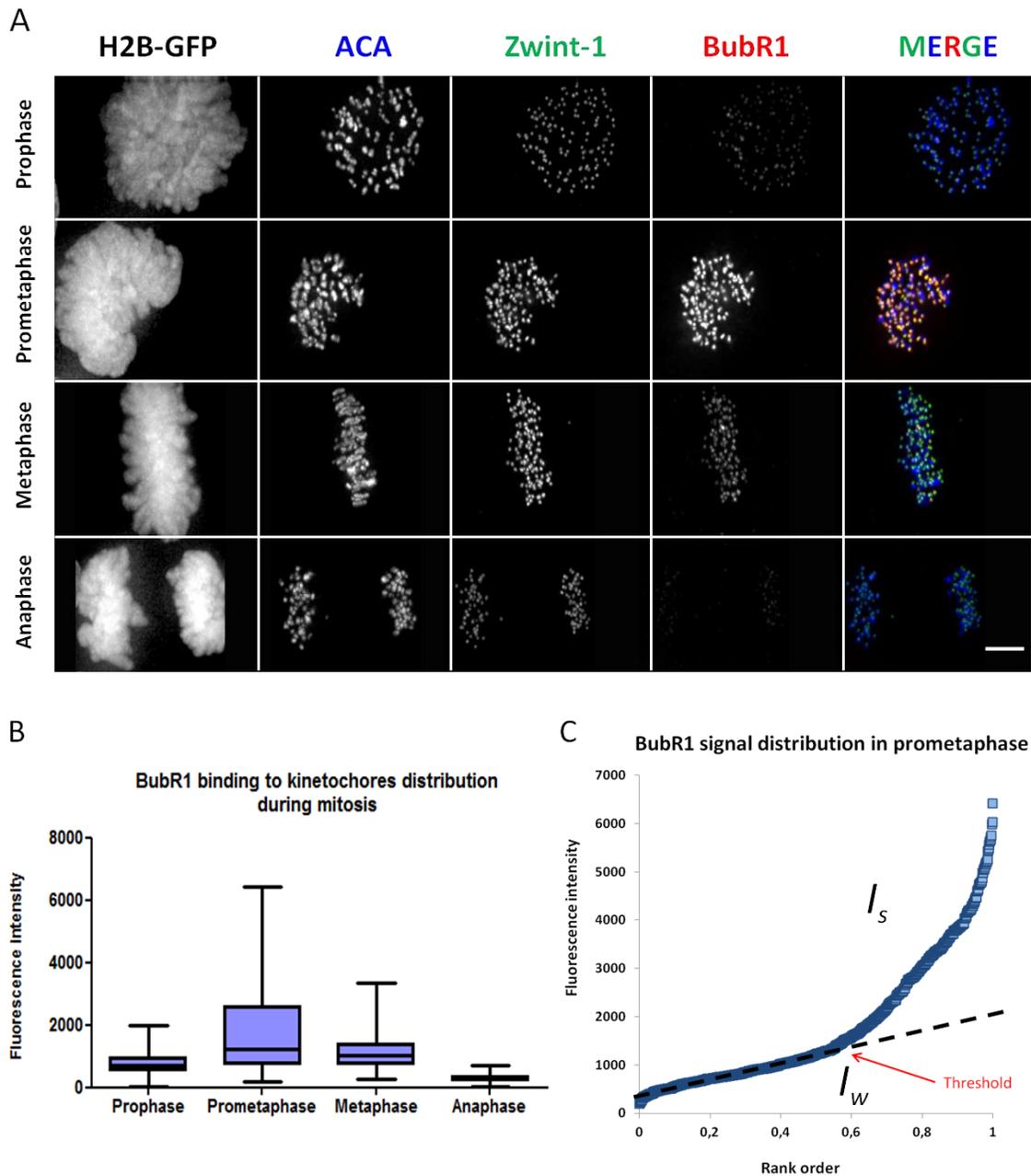


Figure 3.6. BubR1 binding to the kinetochores across different stages of mitosis. (A) Representative panel of differential BubR1 abundance at the kinetochores according to the indicated mitotic stage. Untreated HeLa H2B-GFP cells were immunostained for visualization of centromeres, outer kinetochores and checkpoint protein with ACA (anti-centromere antibody, blue), Zwint-1 (green) and BubR1 (red), respectively. GFP channel was used for chromosome visualization and mitotic stage recognition. The signal variation in BubR1 channel following uniform display settings across different stages of mitosis can be observed. Scale bar 5 μ m. (B) A relative BubR1 fluorescence signal intensity was quantified and plotted as whiskers plot versus mitotic stage. (C) Graph represents mean intensities of the prometaphase BubR1 staining signal plotted in a rank order to determine the threshold for signal intensity classifications. Kinetochores of a weak (I_w) and a strong (I_s) signal intensity are depicted.

Considering the high degree of checkpoint protein signal diversity of prometaphase cells it could be unfeasible to distinguish between the late prometaphase-lack of signal and the real biological impairment caused by defective kinetochore signaling. Therefore, based on intensity variation of the prometaphase cells checkpoint signals plotted (min. to max. in rank order) on the graph we defined a threshold exactly where the break in a curve occurred, classifying the fluorescent intensities (I) as ‘strong’ ($I_s > \text{threshold}$), ‘weak’ ($\text{threshold} > I_w > 0$) or ‘none’ ($I_n < 0$) signal positive = active kinetochores for each individual checkpoint protein (Figure 3.6C).

In order to assess the proportion of kinetochores positive for the checkpoint protein binding subjected to different siRNA treatments, we immunostained HeLa H2B-GFP cells for indicated checkpoint proteins and applied the above strategy. For simplicity, data presented here are reporting only the proportions of I_s – kinetochores. To obtain a reference state corresponding to maximum signal for checkpoint proteins at the kinetochores, we treated the cells with 1 μM nocodazole for 12h. This represents a positive control for checkpoint protein binding capacity, since it has been reported that microtubule poison drugs cause cells to accumulate in mitosis via SAC-dependent prolonged signaling due to lack of tension and microtubule attachment at kinetochores (Shannon *et al.* 2002).

As expected, immunofluorescence with BubR1 antibody revealed a uniform fluorescence signal detection at kinetochores following nocodazole treatment, corresponding to a sustained checkpoint activation in these cells (Figure 3.7A). Subsequent analysis of CENP-W or CENP-T depleted mitotic cells did not reveal any major defects in BubR1 protein recruitment and binding capacity to the kinetochores (Figure 3.7A). Similarly, we found that kinetochore localization of Bub1 checkpoint protein was not prevented in these cells (Figure S3.4), consistent with a known function of Bub1 protein as being a required precursor for BubR1 recruitment to the kinetochores (Johnson *et al.* 2004). Consistent with live cell studies, the quantitative analysis revealed a substantial increase in proportion of BubR1 positive kinetochores (exhibiting ‘strong’ checkpoint protein signal), comparable to nocodazole-arrested population in cells depleted of CENP-W, but not of CENP-T, indicating a sustained BubR1-dependent mitotic arrest and inability to satisfy the SAC in these cells (Figure 3.7B). However, the quantitative measurements of fluorescence intensity distribution of the checkpoint proteins in mitotic cells revealed a distinctive kinetochore defect. In spite of relatively normal localization of Zwint-1 at kinetochores, following CENP-T depletion we observed a significant decrease of relative

fluorescent intensity signal, indicating that Zwint-1 localization at kinetochores is not prevented in these cells, but severely impaired (Figure 3.7C).

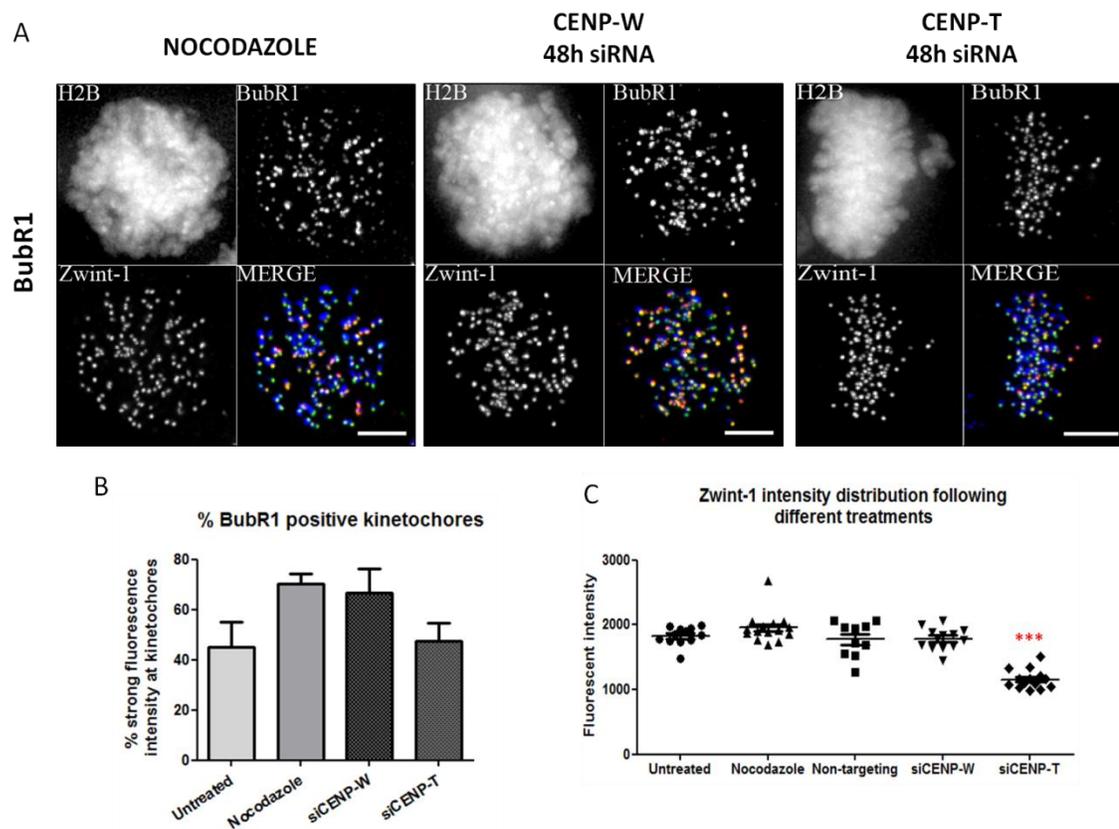


Figure 3.7. Kinetochores depleted of CENP-W/T complex exhibit normal BubR1 checkpoint protein occupancy. (A) Representative panel of HeLa H2B-GFP cells treated with 1 μ M nocodazole for 12h (for maximized mitotic checkpoint protein induction) or cells depleted of CENP-W/T with siRNA for 48h. GFP channel was used to visualize chromosomes of cells stained for Zwint-1 (green) and BubR1 (red) detection. In the overlay, blue signal corresponds to ACA staining for centromeres visualization and yellow indicates co-localization of the two outer kinetochore proteins. Scale bar 5 μ m. (B) Graph represents mean percentage (+SEM) of BubR1 positive kinetochores exhibiting strong fluorescence intensities (at least 1000 kinetochores were scored, in duplicate assays). (C) Graph represents quantitative measurements of the mean (+SEM) of relative Zwint-1 fluorescence signal intensities distribution in mitotic cells following indicated treatments. *** $p < 0.0001$ (one-way ANOVA followed by Dunnett's multiple comparison).

Sustained activation of the checkpoint proteins and following prolonged SAC-dependent arrest in mitosis, as a commonly used chemotherapeutic strategy can lead to variety of outcomes (Figure 2.1). It was frequently found to be associated with an activation of the caspase-3 apoptotic protein. To examine whether the sustained BubR1 binding to the kinetochores depleted of CENP-W and CENP-T causes the mitotic arrest capable of caspase-3 activation, similarly to the one of nocodazole treatment, we performed caspase activity assay on cells depleted of the two CENPs. Surprisingly, CENP-W depleted cells with the increased proportion

of kinetochores with strong BubR1 signal (Figure 3.7B) demonstrated no substantial increase in caspase-3 activity as compared to nocodazole-arrested cells (Figure 3.8). We concluded, that CENP-W depletion arrested cells behave differently than nocodazole cells, which distinguishes from the impact on the SAC activity by mechanically lack of kinetochore-MT attachments caused by drug treatment from the one caused by kinetochore failure.

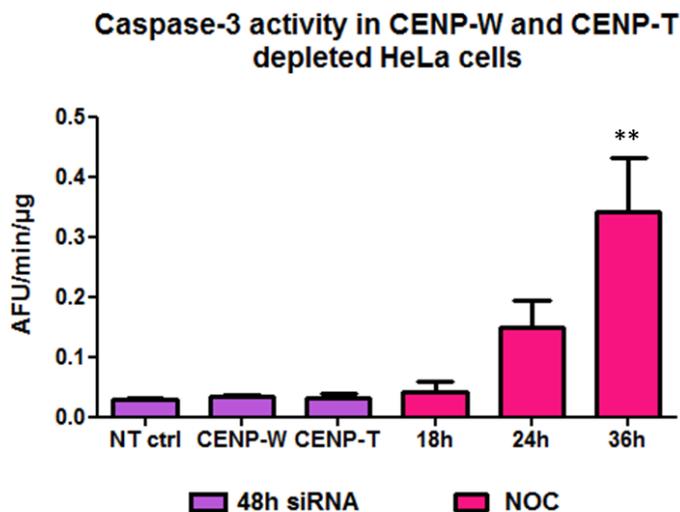


Figure 3.8. HeLa cells delayed in mitosis due to depletion of CENP-W/T complex do not exhibit increase caspase-3 activity. Graph represents mean (+SEM) of caspase-3 activity measured in samples collected following depletion of CENP-W/T complex with 48h siRNA or nocodazole arrest. Data obtained from three independent experiments. ** $p < 0.001$ (one-way ANOVA followed by Dunnetts multiple comparison).

As described above, kinetochores depleted of CENP-W or CENP-T were positive for BubR1 detection and displayed an increased level of this mitotic checkpoint protein signal, comparable to the one of nocodazole-arrested kinetochores. However, as shown previously, in mammalian cells another checkpoint protein – Mad2 is essential for spindle checkpoint activity and Mad2 recruitment is required at unattached kinetochores for catalyzing the formation of inhibitory Mad2-Cdc20 complexes and releasing them into the cytosol where they can prevent anaphase onset by inhibition of the APC/C (Waters *et al.* 1998; Dobles *et al.* 2000; Howell *et al.* 2000). Therefore, we quantified the relative Mad2 abundance and its binding partner Mad1 at kinetochores depleted of CENP-W and CENP-T.

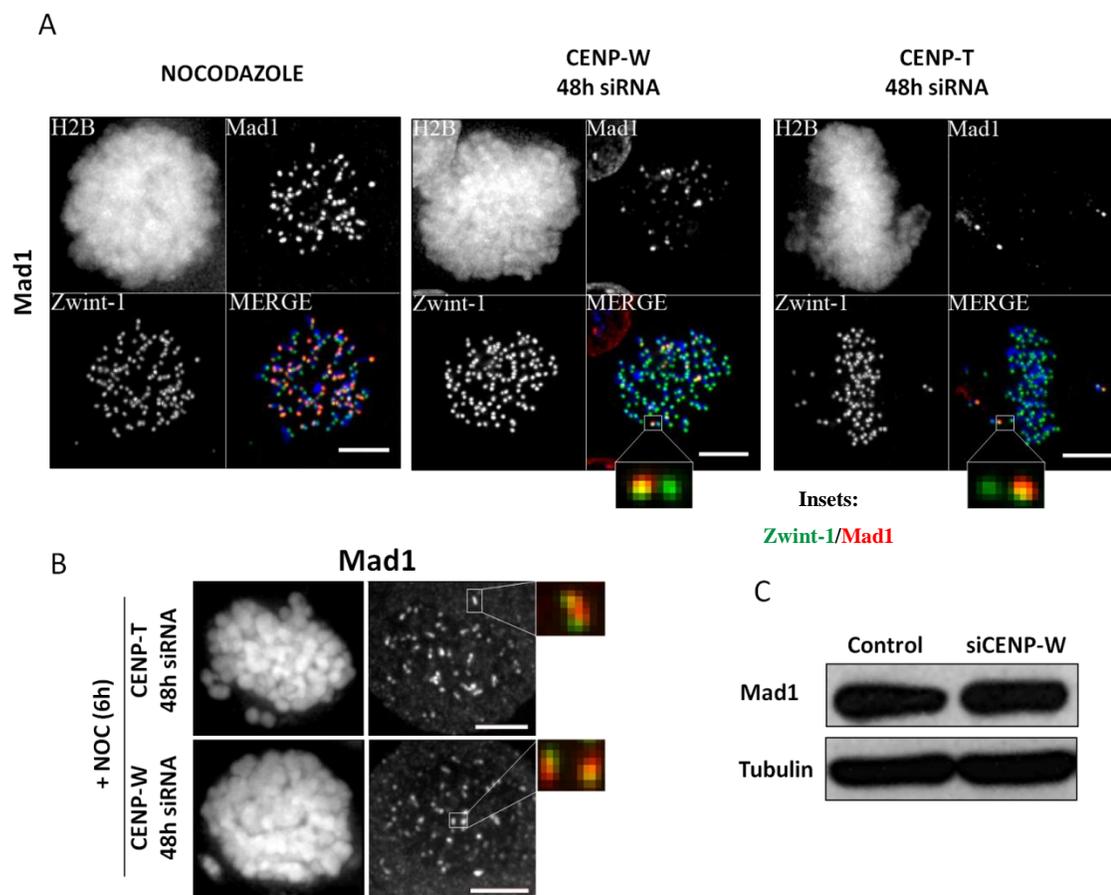
Unlike BubR1 protein, both Mad1 and Mad2 proteins are associated with nuclear pore complexes in interphase and begin to localize to unattached kinetochores already in prophase (Campbell *et al.* 2001), remain detectable throughout prometaphase with a prominent spindle pole localization and finally are almost completely depleted from metaphase bioriented

kinetochores via dynein/dynactin poleward transport (Howell *et al.* 2001). Following nocodazole treatment immunofluorescence data for Mad1 and Mad2 revealed a prominent Mad1/Mad2 kinetochore localization of mitotic cells depleted of spindle microtubules (Figure 3.9A and D), consistent with their checkpoint role of sensing the unattached kinetochores. Occasionally, the mitotic cells with an additional spindle pole accumulation of Mad2 protein were observed, presumably indicating the residual mitotic spindle activity and associated dynein/dynactin poleward transport of the checkpoint protein.

However, a distinctive phenotype was observed following depletion of CENP-W and CENP-T. Mitotic cells depleted of CENP-W/T complex exhibited severe defects in kinetochore localization of Mad1 and Mad2 checkpoint proteins (Figure 3.9A and C) with a very subtle but, based on mitotic kinetics studies, biologically important differences between the two knockdowns. Unlike in CENP-T depleted cells, in which Mad1 localization at kinetochores was almost completely abolished, only residually and unequally distributed between sister kinetochores (Figure 3.9A and D), CENP-W depletion only partially prevented from Mad1 recruitment to the kinetochores, without affecting the soluble cytoplasmic pool (Figure 3.9C). Overall, over 30% kinetochores of CENP-W depleted mitotic cells still exhibited strong fluorescent intensity signal, indicating the ongoing yet limited checkpoint protein recruitment, in comparison to 2% active kinetochores of CENP-T depleted cells (Figure 3.9E). However, the ability to sustain Mad1 dependent arrest in mitosis in both CENPs knockdowns was restored following subsequent 6h incubation with nocodazole (Figure 3.9B), indicating the involvement of the mitotic spindle in the lack of Mad1 binding to the kinetochores, perhaps by premature shedding off the checkpoint from still unattached kinetochores. Another possible explanation arises from the dual role of Mad1 checkpoint protein which has been recently suggested to contribute directly or indirectly to the establishment of proper kinetochore-MT attachments (Emre *et al.* 2011).

Subsequently, we examined Mad2 binding capacity and abundance at the perturbed with siRNA kinetochores. As expected, Mad2 checkpoint protein was absent from kinetochores depleted of CENP-W and CENP-T (Figure 3.9D and F), which is consistent with a prior requirement of Mad1 binding to the kinetochores in order to recruit and activate Mad2 protein (Martin-Lluesma *et al.* 2002; Emre *et al.* 2011). Moreover, in both CENP-W and CENP-T knockdown mitotic cells the characteristic prominent accumulation of Mad2 checkpoint protein at spindle poles was observed (Figure 3.9D, insets).

Taken together, our findings demonstrated that depletion of CENP-W and CENP-T protein can differentially affect the kinetochore association of various SAC components (Figure 3.9G), resulting in most profound impairment of Mad1/Mad2 recruitment to the perturbed kinetochores. These findings are in agreement with live cell studies of the mitotic kinetics, documenting a significant differences of mitotic phenotype and delay following different CENPs depletion, suggesting inability to sustain SAC-dependent mitotic arrest via Mad1/Mad2 pathway and providing the possible explanation for the observe phenotype, especially for a frequent laggards and anaphase bridges formation in cells depleted of CENP-T.



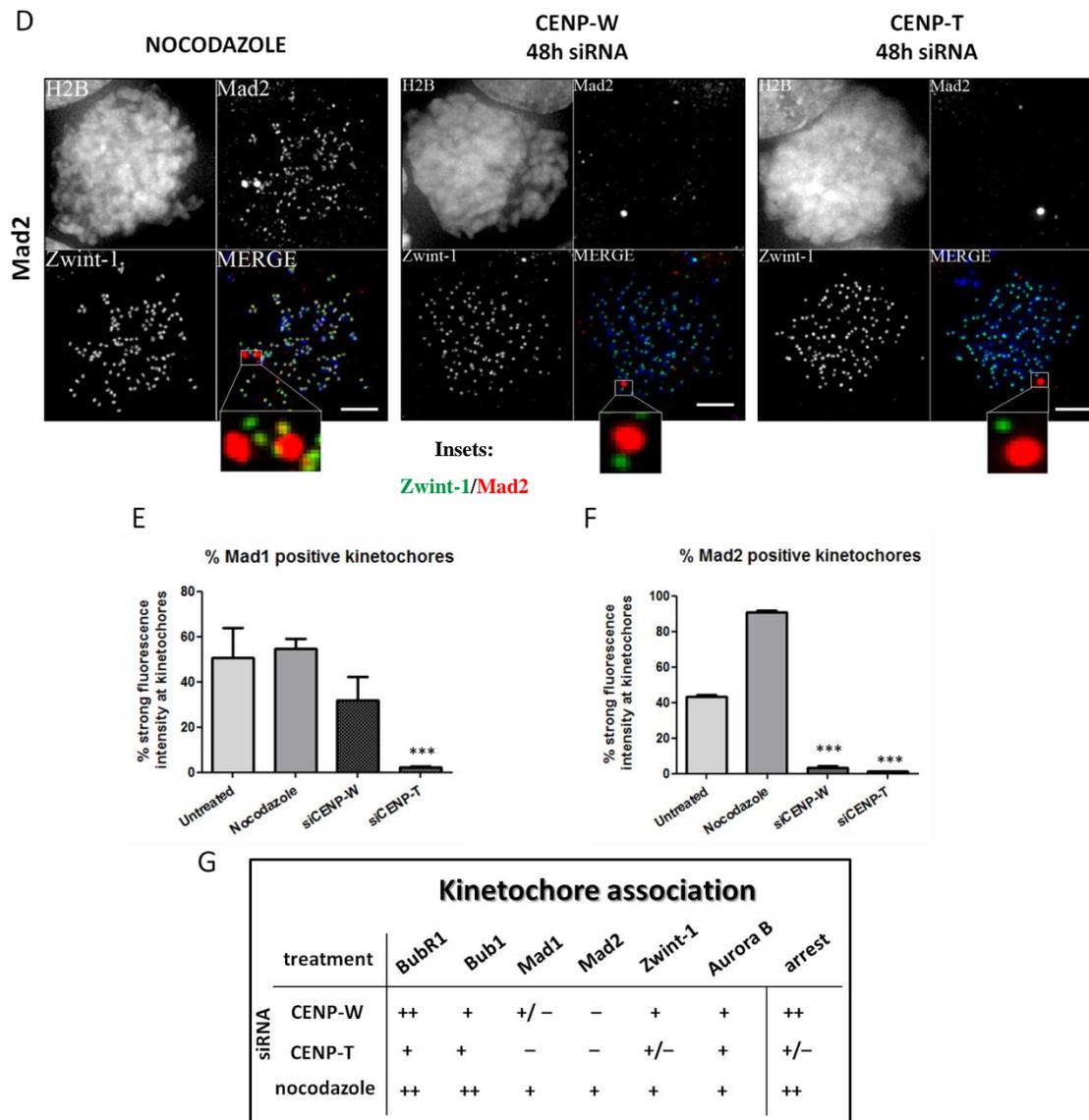


Figure 3.9. Mad1/Mad2 complex is depleted from the kinetochores following siCENP-W/T. (A) Representative panel of HeLa H2B-GFP cells depleted of CENP-W/T with siRNA for 48h. GFP channel was used to visualize chromosomes of cells stained for Zwint-1 (green) and Mad1 (red) detection at kinetochores. The boxed enlargements show co-localization (or lack) of the two outer kinetochores proteins. In the overlay, blue signal represents ACA staining for centromeres visualization. (B) Representative panel of Mad1 positive kinetochores following depletion of CENP-W/T complex for 48h and subsequent 6h treatment with 1 μ M nocodazole. (C) Western blotting detection of Mad1 checkpoint protein indicates no significant depletion of the soluble Mad1 pool from the cytoplasm following CENP-W protein knock down. (D) Representative panel of Mad2 checkpoint protein depletion from the kinetochores following indicated siRNAs treatments. The boxed enlargements show accumulation of Mad2 checkpoint protein (red) at the spindle poles. (E-F) Graph represents mean percentage (+SEM) of Mad1(D) or Mad2(C) positive kinetochores exhibiting strong fluorescence intensities. (G) Table represents cumulative results of the mitotic checkpoint experiments. Following depletion with siRNA of CENP-W and CENP-T, kinetochores association for listed proteins was tested. Nocodazole treatment was used as a positive control for SAC activation. + denotes persistent kinetochores association; - denotes loss of kinetochores association. The presence or absence of a mitotic arrest is indicated by + or -, respectively. *** $p < 0.0001$ (one-way ANOVA followed by Dunnett's multiple comparison). Scale bar 5 μ m.

3.4.4.2. The effect of Aurora B inhibition

The findings obtained from quantitative SAC analysis following depletion of CENP-W and CENP-T, indicating a severe loss of kinetochore associated Mad1/Mad2 complex were consistent with live cell microscopy data to some extent. The results provided the possible reason for a little mitotic delay of CENP-T depleted mitotic cells, due to the complete lack of Mad1 binding to the kinetochores and therefore inability to sustain the arrest, in contrast to only limited Mad1 binding and increased BubR1 signal of CENP-W depleted kinetochores. The reduction of Mad1/Mad2 binding to the kinetochores could be caused by impaired Ndc80 complex assembly on the kinetochore since it has been shown that depletion of Ndc80 compromises Mad1/Mad2 localization to the kinetochores in several systems (Martin-Lluesma *et al.* 2002; DeLuca *et al.* 2003; McClelland *et al.* 2003). However, the lack of one microtubule binding factor Ndc80 and Mad1/Mad2 checkpoint proteins would not simply explain the phenotype of a profound mitotic arrest observed in CENP-W depleted cells, since it would have to only rely on BubR1 checkpoint signaling. The alternative explanation could arise from taxol studies, in which the widely used microtubule stabilizing drug was shown to reduce tension at kinetochores, but did not cause a significant decrease in kinetochore microtubule number (McEwen *et al.* 1997). Moreover, in the presence of low doses of taxol the SAC is satisfied with only a few metaphase kinetochores harboring bound Mad2, suggesting that the checkpoint protein responds to the lack of attachment rather than the lack of tension (Waters *et al.* 1998; Maresca and Salmon 2009). Indeed, vertebrate cells possess another checkpoint protein that is responsible for sensing and responding to the lack of tension, the member of a chromosome passenger complex (CPC) – Aurora B kinase (Biggins and Murray 2001). There is evidence that Aurora B phosphorylates kinetochore proteins that bind microtubules and reduces their binding affinity (Cheeseman *et al.* 2002; DeLuca *et al.* 2006). Therefore, Aurora B is thought to primarily contribute to the SAC pathway, because by destabilizing erroneous microtubule attachments in the absence of tension can create unattached kinetochores (Pinsky *et al.* 2005). It has been proposed that this checkpoint activity of Aurora B is regulated by intrakinetochore stretch achieved upon chromosome biorientation, by spatially positioning Aurora B substrates within the kinetochore away from the range of Aurora B phosphorylation gradient, and/or directly by regulating its kinase activity through the conformational changes of the CPC (Santaguida and Musacchio 2009; Maresca and Salmon 2010).

Therefore, we hypothesize that the mitotic arrest of CENP-W depleted cells is sustained primarily not due to the SAC activity, but the lack of intrakinetochore stretch and tension-

dependent Aurora B activity. Thus, inhibition of its kinase activity should result in release from the arrest and subsequent mitotic exit.

To address this question, we used a small-molecule inhibitor of Aurora B kinase ZM447439 (ZM) and observed H2B-GFP siCENP-W and ZM-treated mitotic cells under live fluorescent microscopy. ZM447439 was found to inhibit both Aurora A and B with IC_{50} of 0.11 and 0.13 μ M, respectively, without affecting their appropriate localization at spindle poles (for Aurora A) or centromeres (for Aurora B) (Ditchfield *et al.* 2003). The inhibition of Aurora B kinase activity by ZM447439 was suggested to compromise the spindle checkpoint function since ZM-treated cells exited mitosis with a normal kinetics, despite of presence of maloriented chromosomes and fail to arrest in mitosis following microtubule poison drug exposure. Indeed, in the absence of Aurora B function kinetochore localization of spindle checkpoint components BubR1, Mad2 and CENP-E was diminished, leading to the conclusion that by targeting checkpoint proteins to kinetochores, Aurora B couples chromosome alignment with anaphase onset (Ditchfield *et al.* 2003).

As expected, the examination of H2B-GFP cells depleted of CENP-W and subsequently treated with ZM447439 inhibitor 48h later, directly before the beginning of video-microscopy, revealed an immediate exit from mitotic arrest. Within the first 45 min of imaging in the presence of ZM inhibitor, 96% of mitotically arrested siCENP-W cells de-condensed their chromosomes and flattened down to form two, three or even four uniform nucleus of the daughter cells, corresponding to the mitotic exit with more than two centrosomes (Figure 3.10A). These findings were confirmed by flow cytometry analysis following siRNA and ZM treatment, documenting 8N DNA and sub-G1 DNA content populations, indicating exit from mitosis with duplicated DNA (due to cytokinesis failure) and onset of apoptotic events, respectively (Figure 3.11A). The observed phenotype was considered to be due to the lack of Aurora B kinase activity rather than the loss of Aurora B protein targeting to the kinetochores, since immunofluorescence staining of H2B-GFP cells depleted of CENP-W (and CENP-T) for Aurora B detection revealed a relatively normal localization of this kinase at kinetochore sites (Figure 3.11B). Despite the fact that ZM447439 was reported to inhibit both kinases, Aurora A and B, the observed phenotype was also thought to be due to the inhibition of Aurora B per se, rather than Aurora A. It was previously reported that, in human cells, Aurora B RNAi induces similar phenotype as Hesperadin, another potent and specific inhibitor of Aurora B (Hauf *et al.* 2003), such as lack of histone H3 phosphorylation (which strictly depends on Aurora B kinase activity), midspindle defects, delay in chromosome congregation and alignment, formation of the anaphase

brigdes and micronuclei (Hauf *et al.* 2003). The above mentioned phenotypic responses were also very characteristic for cells treated with ZM inhibitor, both in control or siCENP-W depleted cells (data not shown), which supports the conclusion, that the effects of ZM treatment are due to inhibition of Aurora B kinase activity and not Aurora A.

In addition to the immediate release from mitotic arrest, Aurora B inhibition resulted in a significant decrease of the mitotic timing of cells depleted of CENP-W and a major increase in cell death (Figure 3.10B and C). In contrast to the previous findings for ZM447439 treatment (Ditchfield *et al.* 2003), the control H2B-GFP cells treated with ZM inhibitor did not exit mitosis with the normal kinetics, but experienced profound difficulties in metaphase chromosome alignment (data not shown) leading to a significant lengthening of the mitotic timing (Figure 3.10B). This indicates functional SAC activity in these cells. However, in accordance with our previous results from the SAC analysis, siCENP-W+ZM treated cells progressed through mitosis with a relatively normal kinetics and exited mitosis in the presence of misaligned chromosomes, indicative of impaired spindle checkpoint response in these cells, presumably correlated with CENP-W depletion and independent of Aurora B activity. The differences in SAC activity and its requirement for normal mitosis was also reflected in the comparison of the subsequent cell fates following ZM treatment (Figure 3.10C). Over 60% of CENP-W depleted mitotic cells, which completed mitosis in the presence of the drug, underwent apoptosis following exit from mitosis, recognized as the appearance of highly condensed chromatin and pycnotic nuclei. In comparison, only 15% of control mitotic cells executed apoptosis following exit from mitosis in the presence of Aurora B inhibitor. Surprisingly, the significant difference in death between the two distinct assays was reported – only 9% of sub-G1 DNA population was detected on the PI profile by flow cytometry (Figure 3.11A) in comparison to 60% observed apoptotic events within the 12h video-microscopy (Figure 3.10C). These discrepancies can be explained by the differential time-course of a drug treatment between these two assays. In the live cell approach, the effect of ZM-treatment is observed immediately following its addition, whereas the samples harvested for flow cytometry were incubated with a drug for 18h following siRNA depletion, which could cause death in cells that were then washed out during sample preparation and therefore not detected by flow cytometry. However, this confusing result should be addressed by the application of more cell death methods (e.g. active caspase-3 detection or caspase-3 activity assay) to confirm the effect of ZM-treatment on cell fate following CENP-W depletion.

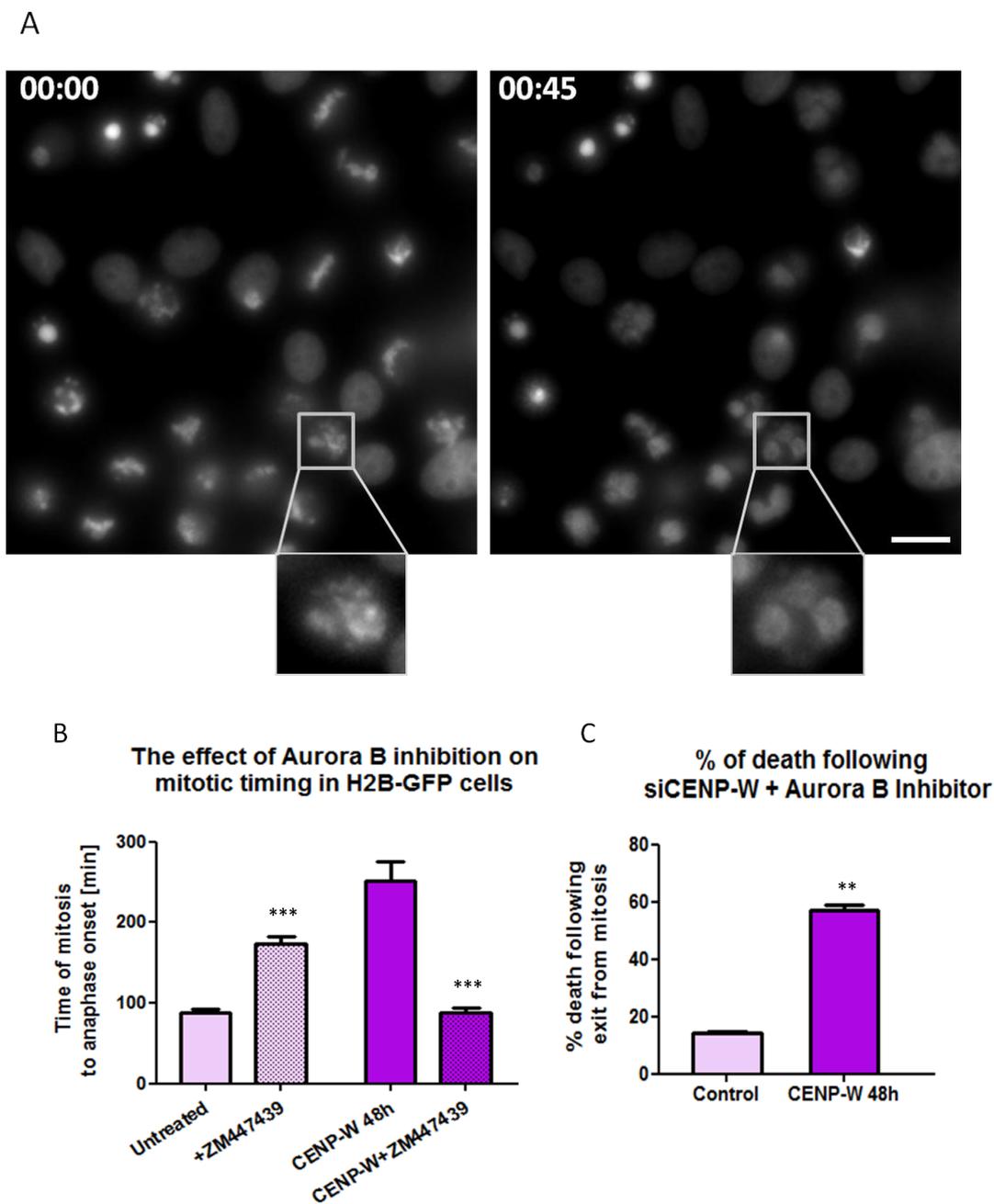


Figure 3.10. Inhibition of Aurora B rescues mitotic arrest and results in subsequent death of cells depleted of CENP-W. (A) Representative panel of H2B-GFP cells depleted of CENP-W for 48h followed by Aurora B inhibition with 2 μ M ZM447439. The boxed enlargements show siCENP-W arrested mitotic cell and the same cell released from arrest following ZM447439 treatment, respectively. Time is given in minutes. Scale bar 10 μ m. (B) Graph represents mean (+SEM) of the mitotic timing quantify in the control siRNA and siCENP-W treated H2B-GFP HeLa cells following incubation with Aurora B inhibitor (at least 100 mitoses were scored for each treatment).*** p<0.0001 (one-way ANOVA followed by Dunnetts multiple comparison). (C) Graph represents mean percentage (+SEM) of dead siCENP-W treated cells following exit from mitosis induced by Aurora B inhibition. ** p<0.005 (t-test).

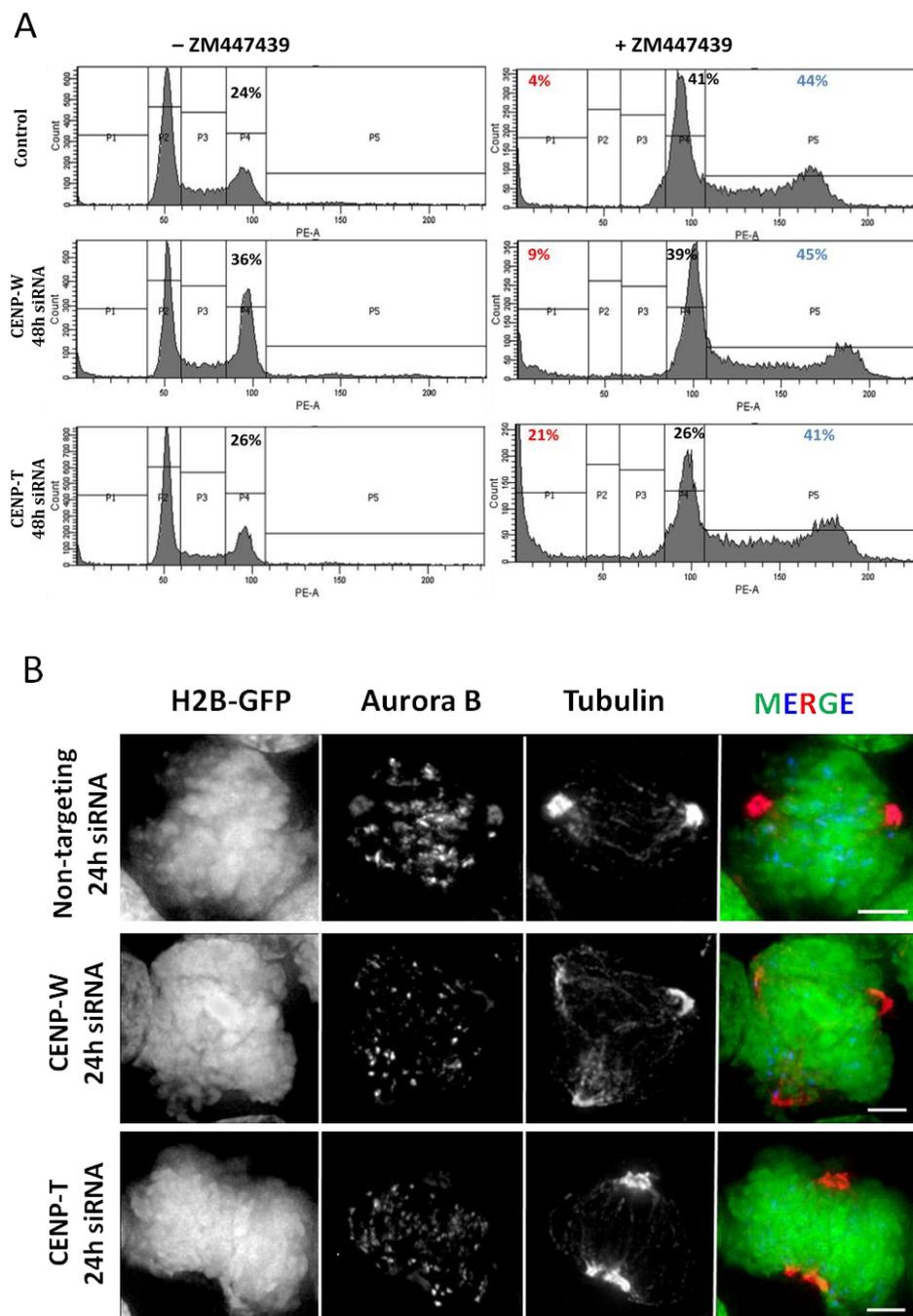


Figure 3.11. Inhibition of Aurora B results in immediate mitotic exit and accumulation of dead cells following CENP-W/T depletion. (A) Propidium Iodide (PI) staining followed by flow cytometry analysis revealed an accumulation of G2 and mitotic populations (4N DNA content) following CENP-W/T depletion (– ZM447439 cell cycle profiles, left hand panel). Subsequent treatment with Aurora B inhibitor for 18h resulted in 8N DNA and sub-G1 DNA content populations accumulation indicating exit from mitosis with duplicated DNA (due to cytokinesis failure) and onset of apoptotic events, respectively (+ZM447439 cell cycle profiles, right hand panel). (B) Aurora B localizes to kinetochores depleted of CENP-W or CENP-T. HeLa H2B-GFP cells were transfected with indicated siRNA and immunostained for Aurora B and α -tubulin detection. Scale bar 5 μ m.

Taken together, these findings demonstrated that inhibition of Aurora B kinase activity with ZM447439 treatment rescues mitotic arrest of CENP-W depleted cells and supports the previous findings of the severe impairment in targeting of the mitotic checkpoint proteins, such as Mad1 and Mad2, to the disturbed kinetochores, since the loss of CENP-W significantly decreased a mitotic delay caused by Aurora B inhibition. Therefore, we postulate that in CENP-W depleted cells the lack of proper kinetochore microtubule attachments resulting in loss of intrakinetochore tension and therefore permanent Aurora B activity at kinetochores could be the primary reason for a significant mitotic delay in these cells.

3.5. DISCUSSION

Despite many functional studies, it remains uncertain how many kinetochore components contribute to full kinetochore formation. The aim of this study was to determine whether the program of kinetochore assembly and function, independent of CENP-A based centromere assembly could be assigned to the specific centromere proteins of the CCAN, in particular to those containing histone fold domain and associate with histone H3 nucleosomes – CENPs – W and – T. The dimeric complex of these two CENPs have been previously found to be recruited in centromere assembly upstream of most of the CCAN class members, but parallel to CENP-C (Foltz *et al.* 2006; Hori *et al.* 2008) and therefore implicating a critical role for CENP-W/T complex in mitosis. Consistent with this notion, HeLa cells depleted with siRNA of CENP-W/T complex displayed a distinct phenotypic response to the one of CENP-A protein loss (Figure S3.3), depletion of which is acceptable in a cell until the critical threshold is achieved, about three cell cycles later (Liu *et al.* 2006). Our quantitative analysis of the mitotic kinetics revealed profound mitotic defects and delay in metaphase chromosome congression of both CENP-W and CENP-T depleted cells, emerging already in the first mitosis following administration of the siRNA, exclusively indicating the requirement of the complex assembly in each mitosis. This delay in mitosis was significantly increased in CENP-W depleted cells, with a characteristic rolling spindle phenotype, a feature not observed for the more subtle phenotype of CENP-T depletion. These surprising phenotypic differences between the two proteins depletion were unexpected, and we believe were related to the insufficient knockdown of CENP-T protein following siRNA. Despite of a great effort and the application of additional methods (shRNA constructs, data not shown), in our experimental conditions, we were not able to reproducibly and efficiently reduce CENP-T protein expression, particularly in a 24h experiments (Figure S3.1), down to the level that was published using similar siRNA approach for this protein (Gascoigne *et al.* 2011). These discrepancies, highlight the need to apply in the future, new generation approaches, such as inducible Degron System for rapid protein depletion, in order to examine different proteins requirement for each cell cycle. Conversely, another explanation for the differences in phenotypes after depletion of CENP-W/T complex subunits may arise from the real biological differences between their function. Such conclusion is supported by findings from studies of another kinetochore associated complex Mis12 of *C.elegans*, where it was found that only depletion of one subunit of this heterotetramer, Dsn1 leads to severe “kinetochore null” phenotype, whereas the loss of remaining three subunits triggers only subtle mitotic defects (Desai *et al.* 2003; Cheeseman *et al.* 2004). However, the lack of efficient knockdown of CENP-T following siRNA, appears to be a more realistic reason for phenotypic diversity, given the

findings of comparable profound mitotic defects observed in DT40 knockout cell lines for both CENP-T and -W depletion (Hori *et al.* 2008). The latter findings combined with our mitotic kinetic studies of HeLa cells clearly indicate that CENP-W/T complex deposition is required for each mitosis for a robust cell division (Prendergast *et al.* 2011).

Moreover, the CENP-W/T complex has been suggested to be the connection between the centromere and the outer kinetochore in the vertebrate cells (Hori *et al.* 2008; Gascoigne *et al.* 2011). A recent study of our group which examined the heritability and the timing of assembly of the HFD proteins CENP-T and CENP-W, found that both proteins show maximum protein levels during S phase and assemble at centromeres late S/G2 phase of the cell cycle, however the complex is not stably associated with centromeres over multiple generations (Prendergast *et al.* 2011). Unlike CENP-A which is required to specify centromere identity, assembly of CENP-W/T has been proposed to act as a trigger for kinetochore assembly before cells enter mitosis (Prendergast *et al.* 2011). Support for this model comes from experiments in which CENP-T was artificially tethered to a lac operator array along with CENP-C, creating ectopic sites of kinetochore assembly (Gascoigne *et al.*, 2011). The latter work also demonstrated that CENP-T makes a direct contact with Ndc80 complex via its flexible N-terminus tail, which facilitates its role in a formation of stable kinetochore-microtubule attachments. This was recently confirmed by Suzuki *et al.* (2011), where based on their EM analysis of DT40 kinetochores they proposed that the inner kinetochore stretch (following tension applied from spindle microtubules) is due in part to CENP-T, which appears to be a stretchable molecule (Suzuki *et al.* 2011).

Our findings suggest that interference with CENP-W/T function results in an overall destabilization of the kinetochore, possibly altering the nature of the attachment of microtubules to the outer plate. Kinetochores making such improper or unstable microtubule attachments may continue to transmit the inhibitory signal even after the chromosomes have achieved a bipolar orientation and congressed to the spindle equator and consequently arrest cells in a SAC-dependent manner (Tomkiel *et al.* 1994; Schaar *et al.* 1997). Therefore, we examined different SAC proteins localization at the prometaphase kinetochores. Surprisingly, our quantitative analysis of the SAC checkpoint proteins binding capacity revealed a severe impairment in SAC signaling, following both siRNA treatments. As long as, mitotic cells depleted of CENP-W/T complex exhibited relatively normal BubR1 (and Bub1) binding to the unattached kinetochores, with a significantly increased levels of BubR1 checkpoint protein in CENP-W depleted cells; the unattached prometaphase kinetochores of CENP-W/T depleted cells were almost completely depleted of the Mad1/Mad2 checkpoint proteins. This striking result was in contrast with a well

known dogma of Mad1/Mad2 complex being the main ‘executioner’ of the SAC signaling (Luo *et al.* 2002; Sironi *et al.* 2002). Therefore, we postulate two possible scenarios. First, because of the existence of two discrete branches of the spindle checkpoint proteins, one specified by Bub- and other by Mad-proteins, that can act independently of each other to maintain the mitotic arrest until all the kinetochores are properly attached to the bioriented poles (Liu *et al.* 2003), the extensive signaling from only one can sustain the mitotic arrest. In our case that would be attributed to increased levels of BubR1 at the CENP-W depleted kinetochores, triggering mitotic delay. Second situation, equally possible, is that following CENP-W/T complex depletion, the kinetochores are incapable for a proper end-on MT attachment formation, possibly due to the lack of one (or more) of the KMN complex components and therefore privileging the formation of the unstable lateral or maloriented microtubule attachments, which cannot be sense by SAC proteins, but activate a third branch of checkpoint signaling, mediated by Aurora B kinase activity (Cimini *et al.* 2004).

Indeed, a recent study of CENP-T depleted cells has shown that Ndc80/Hec1 was mislocalized from the kinetochores in these cells (Gascoigne *et al.* 2011). Our analysis of Hec1 binding to the kinetochores following CENP-W depletion in GFP-CENP-W expressing cells revealed similar impairment in Hec1 recruitment (Chapter IV, Figure 4.4.). In agreement with these findings, Mad1/Mad2 complex was diminished from CENP-W/T depleted kinetochores, since its localization is dependent on prior Ndc80/Hec1 localization to the kinetochores (Martin-Lluesma *et al.* 2002; Meraldi *et al.* 2004; Guimaraes *et al.* 2008). These findings were also consistent with another work documenting premature depletion of Mad2 from kinetochores in Hec1-depleted PtK1 cells (Guimaraes *et al.* 2008). In the latter work, Mad2 was found to localize normally to the kinetochores in Ndc80-depleted PtK1 cells following nocodazole treatment, and these cells remained arrested in mitosis for as long as control cells (Guimaraes *et al.* 2008). Similarly, we found that Mad1 (and presumably Mad2) was present at the kinetochores depleted of CENP-W/T complex in the absence of spindle microtubules. This finding indicates the existence of two distinct pools of Mad1/Mad2 complexes at the kinetochore and microtubule involvement in the premature SAC silencing. Since Mad1/Mad2 localization depends also on RZZ complex recruitment (Buffin *et al.* 2005; Gassmann *et al.* 2008), therefore the RZZ complex or another kinetochore component Spindly, might be the additional to Hec1, Mad1/Mad2 receptor at the kinetochore, which was already reported for Spindly homologue in *C.elegans* (Yamamoto *et al.* 2008). Moreover, in majority of the examined cells depleted of CENP-W/T complex, Mad2 was found to accumulate at spindle poles, providing an additional

support for the notion of premature Mad1/Mad2 depletion from the kinetochores and subsequent dynein-dependent poleward transport of the checkpoint complex (Vallee 1990; Griffis *et al.* 2007). This can be observed even in the presence of unattached or weakly attached kinetochores during the disturbed prometaphases of the depleted cells (Guimaraes *et al.* 2008). On the other hand, such a strong accumulation of Mad2 at spindle poles could be caused by a complete loss of cytoplasmic dynein recruitment to the kinetochores following CENP-W and CENP-T depletion. It has been shown that interactions between microtubules and dynein/dynactin complex are required for outer kinetochore proteins (including Mad1/Mad2 and RZZ components) transport from the spindle poles and their kinetochore localization recovery, phenotype observed following the previous ATP inhibition (Howell *et al.* 2001). Thus, the depletion of the two centromere proteins, CENP-W and CENP-T, could result in a loss of kinetochore dynein recruitment on one hand, and on the other, somehow mimic the conditions of ATP inhibition and therefore pulling the Mad1/Mad2 checkpoint proteins towards spindle poles. Nevertheless, it is clear from our findings that the loss of these two centromeric proteins results in a severe impairment in Mad1/Mad2 mediated branch of mitotic checkpoint signaling, indicating a specific role for CENP-W/T complex in a proper kinetochore function.

Finally, we examined the last possible cause of such a strong mitotic delay following the depletion of CENP-W/T complex and mediated by Aurora B tension dependent pathway. As mentioned above, disturbed kinetochores are more prone for formation of the erroneous attachments with spindle microtubules, as observed in many functional studies of cells with modified or depleted with any of the KMN complex components (Cheeseman *et al.* 2004; Ciferri *et al.* 2005; DeLuca *et al.* 2005; Kline *et al.* 2006; Mattiuzzo *et al.* 2011). In vertebrate cells, Aurora B kinase, a component of the conserved chromosomal passenger complex (CPC) is a key player in regulating kinetochore function (Ruchaud *et al.* 2007). A number of studies suggested that Aurora B is crucial for the microtubule attachment correction mechanism, since its inhibition significantly increases the incidence of both merotelic and syntelic attachments (Lampson *et al.* 2004; Cimini *et al.* 2006; Knowlton *et al.* 2006). Over the last decade, great effort was put towards understanding the precise mechanism of this regulation, which resulted in identifying N-terminus of the Ndc80 as the major conserved substrate of Aurora B at the outer kinetochore (Cheeseman *et al.* 2006; DeLuca *et al.* 2006; Ciferri *et al.* 2008). The positively charged N-terminal domain of Hec1 is a substrate for Aurora B kinase phosphorylation *in vitro*, and these phosphorylations neutralize its positive charge, thereby reducing Hec1 affinity for microtubules and destabilizing them exclusively at incorrect attachment sites (DeLuca *et al.*

2006). The non-phosphorylatable mutants are viable, but cells suffer from frequent microtubule attachment errors and hyperstretching of centromeres, as a result of hyperstabilization of the erroneous microtubule binding (Cheeseman *et al.* 2002). Consistently, HeLa cells depleted of CENP-W/T complex demonstrated impaired Hec1 kinetochore binding and therefore, presumably frequent formation of the merotelic attachments, which, when not corrected resulted in frequent lagging chromosomes during cell division and creation of the anaphase bridges, very characteristic for CENP-T depleted cells.

Alternatively, Aurora B kinase activity has been shown to phosphorylate other members of the KMN complex (Welburn *et al.* 2010), which can contribute to destabilization of the microtubule attachments as well. In addition Aurora B kinase activity gradient was shown to be involved in temporal control of the two microtubule-depolymerizing kinesins, Kif2b and MCAK (Bakhoun *et al.* 2008). Therefore, the complex Aurora B activity and its phosphorylation gradient at low inter-kinetochore tension (in the presence of merotelic attachments) will continue to signal mitotic arrest, via altering microtubule dynamics at disturbed kinetochores. Indeed, in the absence of the Aurora B kinase activity, CENP-W depleted cells immediately exited mitosis, even in the presence of misaligned chromosomes and underwent cell division with more than two spindle poles, reflected in the formation of poliploid progeny. The latter, however, were not viable, presumably due to activation of the conserved checkpoint pathways (other than p53-dependent), that are responsible for genome stability and trigger apoptotic pathway to eliminate potentially dangerous aneuploid cells.

Taken together, we postulate that the loss of CENP-W/T complex causes severe defects in kinetochore recruitment of Ndc80/Hec1 component of the KMN network, resulting in loss of tension and intrakinetochore stretch due to frequent formation of the merotelic attachments. These in turn, activate Aurora B pathway which can preferentially destabilize erroneous attachments at the outer kinetochore sites. Since in CENP-W/T depleted cells the rate of these attachments formation is significantly increased, Aurora B kinase permanent activation primarily contributes to the significant mitotic delay. That delay is a consequence of the improper kinetochore assembly and inability to achieve stable end-on attachments, facilitating chromosome congression and bi-orientation, following depletion of the two HFD containing CENPs. The above mentioned mitotic defects and the mechanism underlying their origin, emphasize the extremely important role for CENP-W/T complex in each mitosis for propagating a healthy kinetochore platform formation and therefore accurate chromosome segregation on the mitotic spindle.

3.6. SUPPLEMENTAL MATERIALS

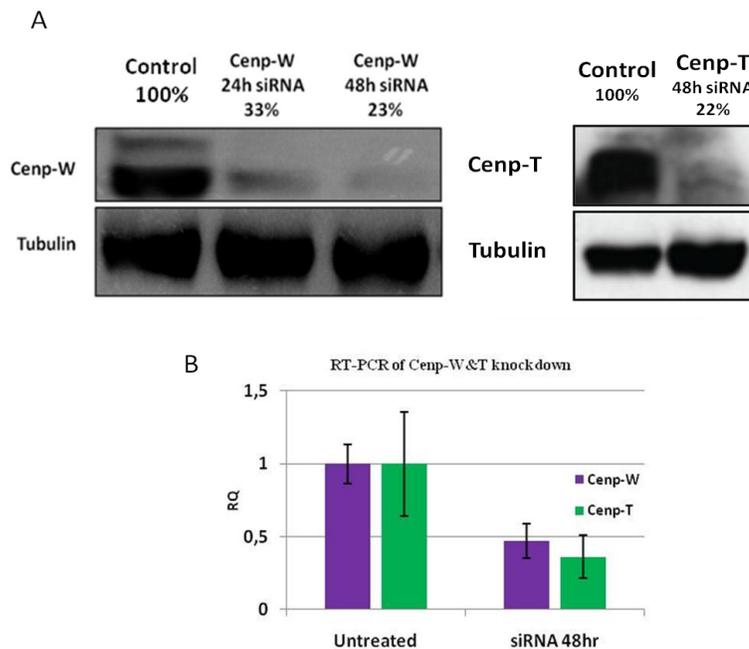


Figure S3.1. Quantification of knockdown efficiency measured by Western blotting and RT-PCR. (A) Corresponding Western blots demonstrate comparable levels of CENP-W and CENP-T protein depletion. Numbers represent relative protein abundance measured by quantification of chemiluminescence signal and normalized to tubulin. (B) Quantitative reverse transcript (RT)-PCR was used to measure the levels of depletion of both CENP-T and CENP-W transcripts following a 48h siRNA treatment. CENP-W was depleted to approximately 48% while CENP-T transcripts were reduced to approximately 36%.

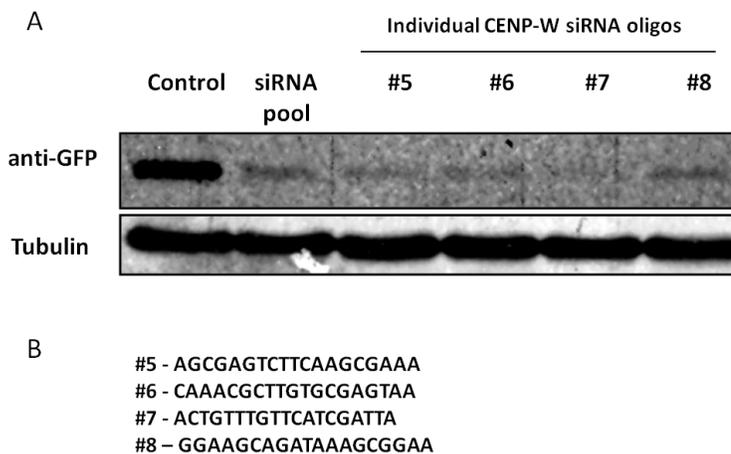


Figure S3.2. CENP-W phenotype is unlikely to be due to off-target effect. (A) HeLa GFP-CENP-W cells were depleted of CENP-W using pooled siRNA and an individual oligonucleotides alone for 48h. A comparable depletion of exogenous version of the protein with each individual oligonucleotide indicates that the observed phenotype is the real biological effect, not off-target consequence of pooled siRNA transfection. (B) Sequences of the individual CENP-W siRNA oligonucleotides.

A

Tubulin primers:

FORWARD:

5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT C = BP site
at gcg tga gtg cat ctc cat

REVERSE (CFUS):

5' -GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC = BP site
gta ttc ctc tcc ttc ttc ctc ac

B

**Double digest of LR product
(BamHI and XmnI)**

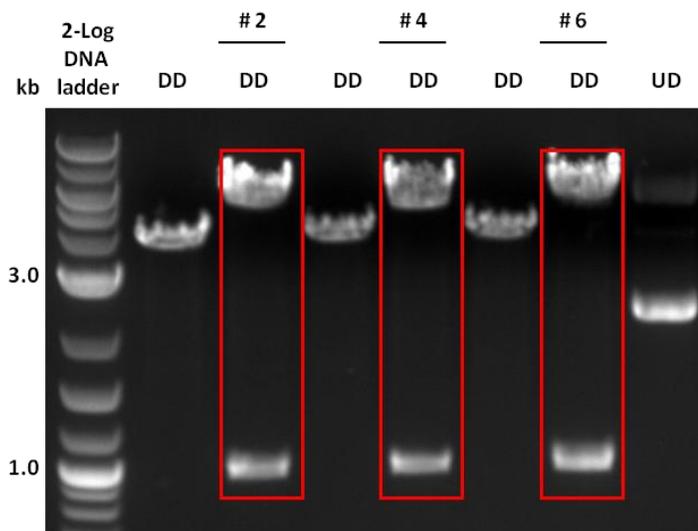


Figure S3.3. The correct sequence of the full length destination vector (pmCherry-C1) with cloned α -tubulin cDNA was confirmed by double digestion with the restriction enzymes. (A) Primer sequences used in PCR reaction for α -tubulin amplification from HeLa cDNA. Red writing represents the overhangs for BP reaction. **(B)** Visualization of the DNA bands (run on 0.7% agarose gel) following double digest (DD, with BamHI and XmnI restriction enzymes) of the LR reaction cloning products. BamHI cleaves only the sequence in the destination vector (pmCherry-C1), whereas XmnI cleaves only in α -tubulin site. Therefore, only those constructs that successfully incorporated α -tubulin fragment into destination vector would demonstrate two bands of the appropriate size (955bp and 5180bp) following double digest (colonies: 2, 4 and 6). The last line represents undigested (UD) control of the destination vector alone.

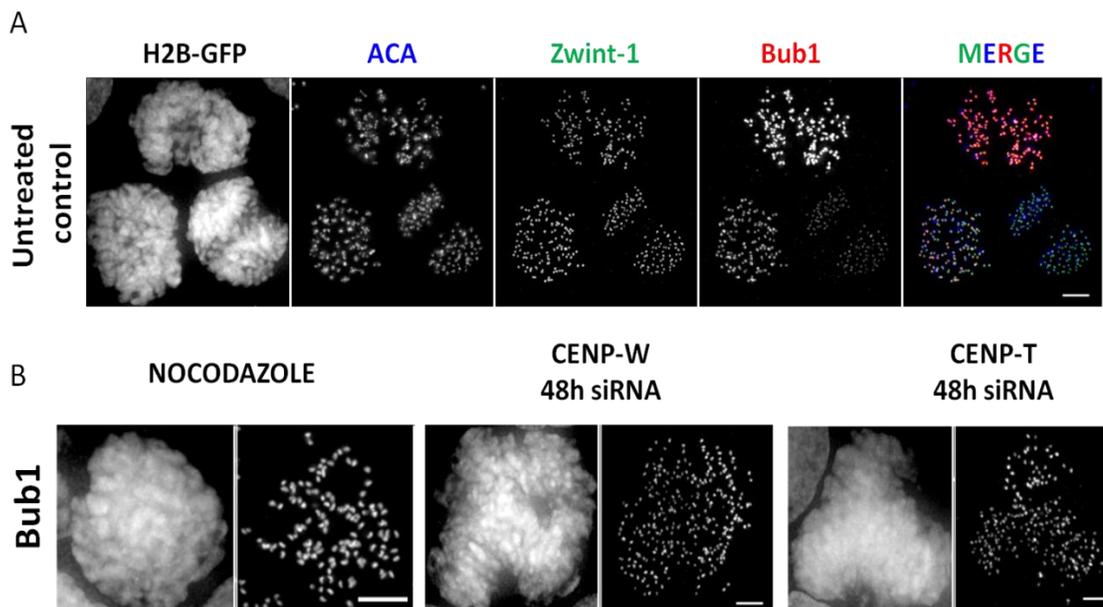


Figure S3.4. Bub1 checkpoint protein binding to the kinetochores depleted of CENP-W/T complex. (A) Representative panel of differential Bub1 abundance at the kinetochores according to the mitotic stage. Untreated HeLa H2B-GFP cells were processed for immunofluorescence for Bub1 detection, as previously described (Figure 3.5A). Scale bar 5 μ m. (B) Kinetochores of H2B-GFP cells depleted of CENP-W/T complex display relatively normal Bub1 checkpoint protein abundance. Scale bar 5 μ m.

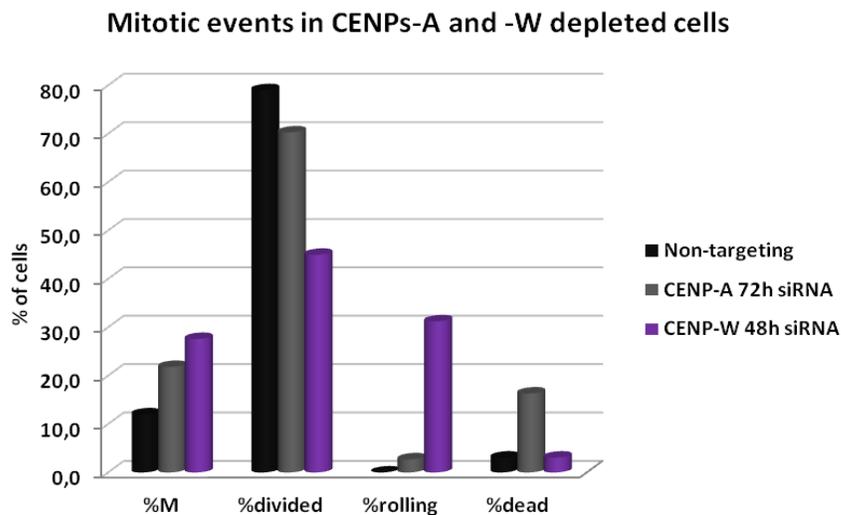


Figure S3.5. CENP-A and CENP-W/T complex proteins are proposed to possess differential functions in centromere assembly. Depletion of two different HFD centromere proteins resulted in a very distinctive phenotypic outcomes. Time-lapse live cell image analysis of H2B-GFP cells reflected the differences of assessed mitotic events (e.g. % of initiated mitosis, % of normal cell division, % of rolling cells and % of dead cells) following transfection with a pooled siRNA for 72h and 48h for CENP-A and CENP-W depletion, respectively.

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3.7. REFERENCES

- Bakhom S. F.,Thompson S. L.,Manning A. L., Compton D. A. (2008). Genome stability is ensured by temporal control of kinetochore-microtubule dynamics. *Nature Cell Biology* **11**(1): 27-35.
- Biggins S., Murray A. W. (2001). The budding yeast protein kinase Ipl1/Aurora allows the absence of tension to activate the spindle checkpoint. *Genes & Development* **15**(23): 3118.
- Buffin E.,Lefebvre C.,Huang J.,Gagou M. E., Karess R. E. (2005). Recruitment of Mad2 to the kinetochore requires the Rod/Zw10 complex. *Current Biology* **15**(9): 856-861.
- Campbell M. S.,Chan G., Yen T. J. (2001). Mitotic checkpoint proteins HsMAD1 and HsMAD2 are associated with nuclear pore complexes in interphase. *Journal of Cell Science* **114**(5): 953.
- Cheeseman I., Desai A. (2008). Molecular architecture of the kinetochore–microtubule interface. *Nature Reviews Molecular Cell Biology* **9**(1): 33-46.
- Cheeseman I. M.,Anderson S.,Jwa M.,Green E. M.,Kang J.,Yates J. R.,Chan C. S. M.,Drubin D. G., Barnes G. (2002). Phospho-regulation of kinetochore-microtubule attachments by the Aurora kinase Ipl1p. *Cell* **111**(2): 163-172.
- Cheeseman I. M.,Chappie J. S.,Wilson-Kubalek E. M., Desai A. (2006). The conserved KMN network constitutes the core microtubule-binding site of the kinetochore. *Cell* **127**(5): 983-997.
- Cheeseman I. M.,Hori T.,Fukagawa T., Desai A. (2008). KNL1 and the CENP-H/I/K complex coordinately direct kinetochore assembly in vertebrates. *Molecular Biology of the Cell* **19**(2): 587-594.
- Cheeseman I. M.,Niessen S.,Anderson S.,Hyndman F.,Yates J. R.,Oegema K., Desai A. (2004). A conserved protein network controls assembly of the outer kinetochore and its ability to sustain tension. *Genes & Development* **18**(18): 2255.
- Ciferri C.,De Luca J.,Monzani S.,Ferrari K. J.,Ristic D.,Wyman C.,Stark H.,Kilmartin J.,Salmon E. D., Musacchio A. (2005). Architecture of the human ndc80-hec1 complex, a critical constituent of the outer kinetochore. *Journal of Biological Chemistry* **280**(32): 29088-29095.
- Ciferri C.,Pasqualato S.,Screpanti E.,Varetti G.,Santaguida S.,Dos Reis G.,Maiolica A.,Polka J.,De Luca J. G., De Wulf P. (2008). Implications for kinetochore-microtubule attachment from the structure of an engineered Ndc80 complex. *Cell* **133**(3): 427-439.
- Cimini D.,Cameron L. A., Salmon E. (2004). Anaphase spindle mechanics prevent mis-segregation of merotelically oriented chromosomes. *Current Biology* **14**(23): 2149-2155.
- Cimini D.,Wan X.,Hirel C. B., Salmon E. (2006). Aurora kinase promotes turnover of kinetochore microtubules to reduce chromosome segregation errors. *Current Biology* **16**(17): 1711-1718.
- Cleveland D. W.,Mao Y., Sullivan K. F. (2003). Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling. *Cell* **112**(4): 407-421.
- DeLuca J. G.,Dong Y.,Hergert P.,Strauss J.,Hickey J. M.,Salmon E., McEwen B. F. (2005). Hec1 and Nuf2 are core components of the kinetochore outer plate essential for organizing microtubule attachment sites. *Molecular Biology of the Cell* **16**(2): 519-531.
- DeLuca J. G.,Gall W. E.,Ciferri C.,Cimini D.,Musacchio A., Salmon E. (2006). Kinetochore microtubule dynamics and attachment stability are regulated by Hec1. *Cell* **127**(5): 969-982.
- DeLuca J. G.,Howell B. J.,Canman J. C.,Hickey J. M.,Fang G., Salmon E. (2003). Nuf2 and Hec1 are required for retention of the checkpoint proteins Mad1 and Mad2 to kinetochores. *Current Biology* **13**(23): 2103-2109.
- Desai A.,Rybina S.,Moller-Reichert T.,Shevchenko A.,Hyman A., Oegema K. (2003). KNL-1 directs assembly of the microtubule-binding interface of the kinetochore in *C. elegans*. *Genes & Development* **17**(19): 2421.

- Ditchfield C., Johnson V. L., Tighe A., Ellston R., Haworth C., Johnson T., Mortlock A., Keen N., Taylor S. S. (2003). Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores. *The Journal of Cell Biology* **161**(2): 267.
- Dobles M., Liberal V., Scott M. L., Benezra R., Sorger P. K. (2000). Chromosome missegregation and apoptosis in mice lacking the mitotic checkpoint protein Mad2. *Cell* **101**(6): 635-645.
- Emre D., Terracol R., Poncet A., Rahmani Z., Karess R. E. (2011). A mitotic role for Mad1 beyond the spindle checkpoint. *Journal of Cell Science* **124**(10): 1664.
- Foltz D. R., Jansen L. E. T., Black B. E., Bailey A. O., Yates J. R., Cleveland D. W. (2006). The human CENP-A centromeric nucleosome-associated complex. *Nature Cell Biology* **8**(5): 458-469.
- Gascoigne K. E., Takeuchi K., Suzuki A., Hori T., Fukagawa T., Cheeseman I. M. (2011). Induced ectopic kinetochore assembly bypasses the requirement for CENP-A nucleosomes. *Cell* **145**(3): 410-422.
- Gassmann R., Essex A., Hu J. S., Maddox P. S., Motegi F., Sugimoto A., O'Rourke S. M., Bowerman B., McLeod I., Yates III J. R. (2008). A new mechanism controlling kinetochore-microtubule interactions revealed by comparison of two dynein-targeting components: SPDL-1 and the Rod/Zwilch/Zw10 complex. *Genes & Development* **22**(17): 2385-2399.
- Griffis E. R., Stuurman N., Vale R. D. (2007). Spindly, a novel protein essential for silencing the spindle assembly checkpoint, recruits dynein to the kinetochore. *The Journal of Cell Biology* **177**(6): 1005-1015.
- Guimaraes G. J., Dong Y., McEwen B. F., DeLuca J. G. (2008). Kinetochore-microtubule attachment relies on the disordered N-terminal tail domain of Hec1. *Current Biology* **18**(22): 1778-1784.
- Hauf S., Cole R. W., LaTerra S., Zimmer C., Schnapp G., Walter R., Heckel A., Van Meel J., Rieder C. L., Peters J. M. (2003). The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. *The Journal of Cell Biology* **161**(2): 281-294.
- Hayashi T., Fujita Y., Iwasaki O., Adachi Y., Takahashi K., Yanagida M. (2004). Mis16 and Mis18 are required for CENP-A loading and histone deacetylation at centromeres. *Cell* **118**(6): 715-729.
- Hori T., Amano M., Suzuki A., Backer C., Welburn J., Dong Y., McEwen B., Shang W., Suzuki E., Okawa K. (2008). CCAN makes multiple contacts with centromeric DNA to provide distinct pathways to the outer kinetochore. *Cell* **135**(6): 1039-1052.
- Howell B., Hoffman D., Fang G., Murray A., Salmon E. (2000). Visualization of Mad2 dynamics at kinetochores, along spindle fibers, and at spindle poles in living cells. *Journal of Cell Biology* **150**(6): 1233-1250.
- Howell B., McEwen B., Canman J., Hoffman D., Farrar E., Rieder C., Salmon E. (2001). Cytoplasmic dynein/dynactin drives kinetochore protein transport to the spindle poles and has a role in mitotic spindle checkpoint inactivation. *The Journal of Cell Biology* **155**(7): 1159-1172.
- Howell B. J., Moree B., Farrar E. M., Stewart S., Fang G., Salmon E. (2004). Spindle checkpoint protein dynamics at kinetochores in living cells. *Current Biology* **14**(11): 953-964.
- Johnson V. L., Scott M. I. F., Holt S. V., Hussein D., Taylor S. S. (2004). Bub1 is required for kinetochore localization of BubR1, Cenp-E, Cenp-F and Mad2, and chromosome congression. *Journal of Cell Science* **117**(8): 1577.
- Kim H., Lee M., Lee S., Park B., Koh W., Lee D. J., Lim D. S. (2009). Cancer-upregulated gene 2 (CUG2), a new component of centromere complex, is required for kinetochore function. *Molecules and Cells* **27**(6): 697-701.
- Kline S. L., Cheeseman I. M., Hori T., Fukagawa T., Desai A. (2006). The human Mis12 complex is required for kinetochore assembly and proper chromosome segregation. *The Journal of Cell Biology* **173**(1): 9.
- Knowlton A. L., Lan W., Stukenberg P. T. (2006). Aurora B is enriched at merotelic attachment sites, where it regulates MCAK. *Current Biology* **16**(17): 1705-1710.
- Lampson M. A., Renduchitala K., Khodjakov A., Kapoor T. M. (2004). Correcting improper chromosome-spindle attachments during cell division. *Nature Cell Biology* **6**(3): 232-237.
- Lee S., Gang J., Jeon S. B., Choo S. H., Lee B., Kim Y. G., Lee Y. S., Jung J., Song S. Y., Koh S. S. (2007). Molecular cloning and functional analysis of a novel oncogene, cancer-upregulated gene 2 (CUG2). *Biochem. Biophys. Res. Commun.* **360**(3): 633-639.
- Liu S., Hittle J., Jablonski S., Campbell M., Yoda K., Yen T. (2003). Human CENP-I specifies localization of CENP-F, Mad1 and Mad2 to kinetochores and is essential for mitosis. *Nature Cell Biology* **5**: 341-345.

- Liu S., Rattner J., Jablonski S., Yen T. (2006). Mapping the assembly pathways that specify formation of the trilaminar kinetochore plates in human cells. *Journal of Cell Biology* **175**(1): 41-53.
- Luo X., Tang Z., Rizo J., Yu H. (2002). The Mad2 spindle checkpoint protein undergoes similar major conformational changes upon binding to either Mad1 or Cdc20. *Molecular Cell* **9**(1): 59-71.
- Maresca T. J., Salmon E. (2010). Welcome to a new kind of tension: translating kinetochore mechanics into a wait-anaphase signal. *Journal of Cell Science* **123**(6): 825-835.
- Maresca T. J., Salmon E. D. (2009). Intrakinetochore stretch is associated with changes in kinetochore phosphorylation and spindle assembly checkpoint activity. *The Journal of Cell Biology* **184**(3): 373-381.
- Martin-Lluesma S., Stucke V. M., Nigg E. A. (2002). Role of Hec1 in spindle checkpoint signaling and kinetochore recruitment of Mad1/Mad2. *Science* **297**(5590): 2267.
- Mattiuzzo M., Vargiu G., Totta P., Fiore M., Ciferri C., Musacchio A., Degraffi F. (2011). Abnormal kinetochore-generated pulling forces from expressing a N-terminally modified Hec1. *PLoS One* **6**(1): e16307.
- McClelland M. L., Gardner R. D., Kallio M. J., Daum J. R., Gorbsky G. J., Burke D. J., Stukenberg P. T. (2003). The highly conserved Ndc80 complex is required for kinetochore assembly, chromosome congression, and spindle checkpoint activity. *Genes & Development* **17**(1): 101.
- McEwen B. F., Heagle A. B., Cassels G. O., Buttle K. F., Rieder C. L. (1997). Kinetochore fiber maturation in PtK1 cells and its implications for the mechanisms of chromosome congression and anaphase onset. *The Journal of Cell Biology* **137**(7): 1567-1580.
- Meraldi P., Draviam V. M., Sorger P. K. (2004). Timing and checkpoints in the regulation of mitotic progression. *Developmental Cell* **7**(1): 45-60.
- Musacchio A., Hardwick K. G. (2002). The spindle checkpoint: structural insights into dynamic signalling. *Nature Reviews Molecular Cell Biology* **3**(10): 731-741.
- Musacchio A., Salmon E. (2007). The spindle-assembly checkpoint in space and time. *Nature Reviews Molecular Cell Biology* **8**: 379-393.
- Okada M., Cheeseman I. M., Hori T., Okawa K., McLeod I., Yates III J. R., Desai A., Fukagawa T. (2006). The CENP-H-I complex is required for the efficient incorporation of newly synthesized CENP-A into centromeres. *Nature Cell Biology* **8**: 446-457.
- Perpelescu M., Fukagawa T. (2011). The ABCs of CENPs. *Chromosoma*: 1-22.
- Pinsky B. A., Kung C., Shokat K. M., Biggins S. (2005). The Ipl1-Aurora protein kinase activates the spindle checkpoint by creating unattached kinetochores. *Nature Cell Biology* **8**(1): 78-83.
- Prendergast L., van Vuuren C., Kaczmarczyk A., Doering V., Hellwig D., Quinn N., Hoischen C., Diekmann S., Sullivan K. F. (2011). Premitotic Assembly of Human CENPs-T and -W Switches Centromeric Chromatin to a Mitotic State. *PLoS Biology* **9**(6): e1001082.
- Rattner J., Rao A., Fritzler M., Valencia D., Yen T. (1993). CENP-F is a ca 400 kDa kinetochore protein that exhibits a cell-cycle dependent localization. *Cell Motil Cytoskeleton* **26**(3): 214-226.
- Regnier V., Vagnarelli P., Fukagawa T., Zerjal T., Burns E., Trouche D., Earnshaw W., Brown W. (2005). CENP-A is required for accurate chromosome segregation and sustained kinetochore association of BubR1. *Molecular and Cellular Biology* **25**(10): 3967.
- Rieder C. L., Maiato H. (2004). Stuck in Division or Passing through:: What Happens When Cells Cannot Satisfy the Spindle Assembly Checkpoint. *Developmental Cell* **7**(5): 637-651.
- Ruchaud S., Carmena M., Earnshaw W. C. (2007). Chromosomal passengers: conducting cell division. *Nature Reviews Molecular Cell Biology* **8**(10): 798-812.
- Santaguida S., Musacchio A. (2009). The life and miracles of kinetochores. *The EMBO Journal* **28**(17): 2511-2531.
- Schaar B., Chan G., Maddox P., Salmon E., Yen T. (1997). CENP-E function at kinetochores is essential for chromosome alignment. *Journal of Cell Biology* **139**(6): 1373-1382.
- Shannon K. B., Canman J. C., Salmon E. D. (2002). Mad2 and BubR1 function in a single checkpoint pathway that responds to a loss of tension. *Molecular Biology of the Cell* **13**(10): 3706-3719.
- Shelby R. D., Monier K., Sullivan K. F. (2000). Chromatin assembly at kinetochores is uncoupled from DNA replication. *The Journal of Cell Biology* **151**(5): 1113-1118.
- Sironi L., Mapelli M., Knapp S., De Antoni A., Jeang K. T., Musacchio A. (2002). Crystal structure of the tetrameric Mad1-Mad2 core complex: implications of a 'safety belt' binding mechanism for the spindle checkpoint. *The EMBO Journal* **21**(10): 2496-2506.

- Starr D. A., Saffery R., Li Z., Simpson A. E., Choo K., Yen T. J., Goldberg M. L. (2000). HZWint-1, a novel human kinetochore component that interacts with HZW10. *Journal of Cell Science* **113**(11): 1939.
- Sullivan K. F. (2001). A solid foundation: functional specialization of centromeric chromatin. *Current Opinion in Genetics & Development* **11**(2): 182-188.
- Suzuki A., Hori T., Nishino T., Usukura J., Miyagi A., Morikawa K., Fukagawa T. (2011). Spindle microtubules generate tension-dependent changes in the distribution of inner kinetochore proteins. *The Journal of Cell Biology* **193**(1): 125-140.
- Tadeu A. M. B., Ribeiro S., Johnston J., Goldberg I., Gerloff D., Earnshaw W. C. (2008). CENP-V is required for centromere organization, chromosome alignment and cytokinesis. *The EMBO Journal* **27**(19): 2510-2522.
- Tomkiel J., Cooke C. A., Saitoh H., Bernat R. L., Earnshaw W. C. (1994). CENP-C is required for maintaining proper kinetochore size and for a timely transition to anaphase. *The Journal of Cell Biology* **125**(3): 531.
- Vallee R. (1990). Mitosis: dynein and the kinetochore. *Nature* **345**(6272): 206.
- Van Hooser A. A., Ouspenski I. I., Gregson H. C., Starr D. A., Yen T. J., Goldberg M. L., Yokomori K., Earnshaw W. C., Sullivan K. F., Brinkley B. (2001). Specification of kinetochore-forming chromatin by the histone H3 variant CENP-A. *Journal of Cell Science* **114**(19): 3529.
- Waters J. C., Chen R. H., Murray A. W., Salmon E. (1998). Localization of Mad2 to kinetochores depends on microtubule attachment, not tension. *The Journal of Cell Biology* **141**(5): 1181-1191.
- Welburn J. P. I., Vleugel M., Liu D., Yates J. R., Lampson M. A., Fukagawa T., Cheeseman I. M. (2010). Aurora B phosphorylates spatially distinct targets to differentially regulate the kinetochore-microtubule interface. *Molecular Cell* **38**(3): 383-392.
- Wood K. W., Sakowicz R., Goldstein L. S. B., Cleveland D. W. (1997). CENP-E is a plus end-directed kinetochore motor required for metaphase chromosome alignment. *Cell* **91**(3): 357-366.
- Yamamoto T. G., Watanabe S., Essex A., Kitagawa R. (2008). SPDL-1 functions as a kinetochore receptor for MDF-1 in *Caenorhabditis elegans*. *The Journal of Cell Biology* **183**(2): 187-194.
- Yen T. J., Compton D., Wise D., Zinkowski R., Brinkley B., Earnshaw W., Cleveland D. (1991). CENP-E, a novel human centromere-associated protein required for progression from metaphase to anaphase. *The EMBO Journal* **10**(5): 1245.
- Yen T. J., Li G., Schaar B., Szilak I., Cleveland D. W. (1992). CENP-E is a putative kinetochore motor that accumulates just before mitosis. *Nature* **359**: 536-539.

CHAPTER 4

Examining the mechanism of multipolar spindle formation following CENP-W depletion

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Key words: centrosome, multipolarity, CENP-W/T complex, motor proteins, TPX2

4.1. SUMMARY

The CENP-W/T complex was previously reported to be required for mitosis. HeLa cells depleted of CENP-W displayed profound mitotic defects, with mitotic timing delay, disorganized prometaphases and multipolar spindles as major phenotypic consequences. In this study, we examined the mechanism of multipolar spindle formation induced by CENP-W depletion. Using time lapse live cell microscopy with histone H2B and tubulin fluorescently-tagged cells, we observed frequent pole fragmentation events following CENP-W depletion. Examination of centriolar abnormalities in these cells revealed two things. First, we do not observe promiscuous centriole overduplication. Secondly, we do observe numerous abnormal centriole splitting events in mitotic cells. In addition, a large proportion of the examined centrosomes lacked centrioles, but were positively stained with different centrosomal markers. These observations suggested that perturbation in spindle force distribution caused by defective kinetochores could contribute to a mechanical mechanism for spindle pole disruption. Application of the two drugs, monastrol and nocodazole, allowed investigation of this hypothesis. Inhibition of centrosome separation by monastrol reduced the incidence of spindle pole fragmentation, indicating that normal bipolar spindle force dynamics are required for spindle pole disruption. Surprisingly, an increased frequency of bipolar spindles was observed in cells treated with monastrol under CENP-W depletion conditions. ‘Spindle free’ nocodazole arrested cells did not exhibit pole fragmentation after CENP-W depletion, indicating that microtubules per se are required for the observed fragmentation. These observations support a mechanical origin for spindle pole fragmentation mediated by inappropriate distribution of force within the spindle. To test this idea, we attempted to mechanically stabilize spindle poles by overexpression of the human microtubule cross-linking protein, TPX2. TPX2 expression did suppress spindle pole fragmentation. Consistent with a known function of the CENP-W/T complex in mitosis, Hec1 was depleted from the kinetochores in the affected cells. We propose that CENP-W, by influencing proper kinetochore assembly, particularly microtubule docking sites, can confer spindle pole resistance to traction forces exerted by motor proteins during chromosome congression. Taken together, our findings are consistent with a model in which centrosome integrity is controlled by the pathways regulating kinetochore-microtubule attachment stability.

4.2. INTRODUCTION

Cell division is controlled and regulated by the two mechanisms: one is the replication of chromosomal DNA called cell cycle, and the other is duplication of the centrosome or the equivalent organelle that will function as the spindle pole (Winey 1999; Zachariae and Nasmyth 1999). The process requires an intricate coordination, since uncontrolled divisions can lead to tumors (Holland and Cleveland 2009). Therefore, cells have regulatory proteins that control the cell cycle at various stages (checkpoints). If cell cycle control is activated, it inhibits cell division. Thus, the cell has time to perform repair work in the DNA or, if a damage is irreparable, to induce cell death (Morgan 2007). At the same time, centrosome number in a cell must be tightly coordinated within the cell cycle, since any deregulation in centrosome duplication can lead to the formation of multiple spindle poles during mitosis, resulting in the unequal distribution of chromosomes and the production of aneuploid progeny. This in consequence can lead to genetic instability and contribute to tumorigenesis (Fukasawa 2005; Weaver and Cleveland 2006). To help accurately segregate their DNA during mitosis cells had developed two intricate structures, that are assembled and disassembled every cell cycle: one is a microtubule-based organization, called the spindle, and the other is a complex protein assembly that links the chromosomes to their mitotic spindle, called kinetochore (Bakhoun *et al.* 2008; Holland and Cleveland 2009).

The kinetochores are assembled during G2 phase of the cell cycle upon specific site of centromeric chromatin, called centromere, and these proteinaceous structures serve as the attachment point for microtubules on each sister chromatid (Cheeseman and Desai 2008). The core of the centromere chromatin consists of the histone H3 variant containing CENP-A nucleosomes and the constitutive centromere-associated network (CCAN), a group of over 15 tightly associated chromatin proteins that forms the foundation on which the kinetochore and spindle assembly checkpoint (SAC) assemble (Sullivan 2001). The SAC ensures proper chromosome segregation by sensing chromosome attachments and arresting the cell cycle at the metaphase-anaphase transition until correct bipolar attachment is achieved (Musacchio and Salmon 2007). Therefore kinetochores are necessary go-between for centromeric chromatin and microtubule dependent movement of chromosomes during mitosis (Cheeseman and Desai 2008). This poleward chromosome movement is driven on one side by forces derived from kinetochore-associated microtubule motor proteins, such as kinesin-related proteins CENP-E and MCAK as well as cytoplasmic dynein; and on the other side by the continuous poleward flux of tubulin subunits within the spindle lattice (Sharp *et al.* 2000; Gordon *et al.* 2001). The

mitotic spindle is a fusiform microtubule-based, very flexible superstructure, self-assembling from microtubules and organizing chromosomes movements in a process called bi-orientation (Rieder and Salmon 1994; Wittmann *et al.* 2001).

Centrosomes, as the primary microtubule organizing centres in animal cells (MTOC) are the core of the spindle poles and therefore their numbers need to be strongly controlled, duplicating exactly once per cell cycle, around the time of the DNA replication, and not exceeding two at the entry to mitosis (Winey 1999; Nigg 2006). Centrosomes are usually positioned near the nucleus of the cell and are composed of a core structure consisting of a pair of centrioles, called a mother and a daughter centriole. During interphase, mother and daughter centrioles are usually found closely associated, held together by a dynamic flexible linker allowing partial movements of the daughter centriole around the mother. During late G₂, centrosomes separate in a two-step process and this is thought to generate the first cue for the formation of a bipolar spindle. The first step involves disassembling of the fibrous linker connecting the two centriole pairs, and the second engages microtubule-based motor proteins, such as Eg5 kinesin and cytoplasmic dynein to be recruited to the centrosomes. It has been proposed that minus-end directed dynein, when anchored to either nuclear envelope or cell cortex, pulls on the microtubules that are nucleated by the two daughter centrosomes (Gonczy *et al.* 1999). However, centrosomes are not required for bipolarity. It has been shown in many studies, on different organisms, that cells without centrosomes can form bipolar spindles in a centrosome-independent pathway (Khodjakov *et al.* 2000). Moreover, the study on the metaphase arrested *Xenopus* egg extracts, which do not contain either centrosomes or kinetochores, revealed the importance of microtubule motor proteins in spindle assembly (Heald *et al.* 1996).

The establishment and maintenance of bipolar spindle is thought to be due to the concerted action of variety of dynamic processes occurring in the spindle, including: microtubules undergoing dynamic instability (coming from growing and shrinking of the tubulin subunits), poleward microtubule flux, chromosome movements, motor-driven antiparallel microtubule sliding, dynein-dynactin dependent, minus-end directed microtubule transport and orientation movement of the spindle poles (Wittmann *et al.* 2001). Within the mitotic spindle, microtubule dynamics seem to be regulated mainly by microtubule-associated proteins (MAPs), that are required for focusing of the minus-end of the microtubules to form spindle poles (Dionne *et al.* 1999; Wittmann *et al.* 2000). The main MAPs represent nuclear protein of the mitotic apparatus (NuMa) and targeting protein for Xklp2 (TPX2), that bind and stabilize microtubules. Conversely, the plus-ends of microtubules are embedded at kinetochores, where various of

microtubule plus-end associated proteins control the polymerization state of the kinetochore microtubules (Cheeseman and Desai 2008). These include proteins promoting microtubule polymerization, such as MAP215, EB1 or CLASP family proteins; as well as microtubule depolymerases, such as kinesin-13 family proteins (MCAK) and kinesin-8 family members (Howard and Hyman 2007; Varga *et al.* 2009). In addition, it has been shown, that in mammalian cells the kinetochore activity itself influences mechanisms of spindle pole organization, by TPX2 microtubule cross-linking activity, which was found to be sufficient to maintain focused poles even in cells deficient in Nuf2, NuMa and HSET activities (Manning and Compton 2007). Beside the motor proteins and MAPs, it has been demonstrated as well, that CCAN components regulate the turnover of kinetochore-MT plus-ends, by controlling dynamic instability (Amaro *et al.* 2010).

Within the complicated array of centromere and kinetochore proteins, many of their functions in recruiting ‘sensors’, transmitting a signal from unattached chromosomes and capturing the spindle-MTs, are still unknown. In order to extend our knowledge of the role of CENP-W/T complex of the centromere in the global formation of the efficient spindles, we performed functional studies on CENP-W depleted GFP-CENP-W cells.

4.3. MATERIALS AND METHODS

4.3.1. Cell culture, RNAi and Drug treatment

HeLa cells were cultured as previously described (Chapter 2, section 2.3.1). For cells stably expressing either GFP-CENP-W (a gift from Dr. Hori) or mCherry-tubulin (obtained as previously described, Chapter 3, section 3.4.3), a medium with selective antibiotics (blasticidin or G418, respectively) was used.

In siRNA experiments, synthetic double stranded ON-TARGET plus SMARTpool sequences (all from Dharmacon) for CENP-W (L-032901-01), CENP-T (L-014577-01) and non-targeting control pool (D-001810-10) were used. Targeted duplexes were transfected at 100 nM concentration into cells using DharmaFECT 1 reagent (Dharmacon), according to the manufacturer's instruction. Cells were analyzed 24h and/or 48h post-transfection.

To inhibit microtubule dynamics cells were incubated with nocodazole at 20nM and 1 μ M. To analyze spindle pole fragmentation, cells were incubated with 100 μ M monastrol (all from Sigma). All of the treatments were performed for the last 4h before harvesting.

4.3.2. Immunofluorescence

Cells immunostaining and fluorescent microscopy were carried out as previously described (Chapter 3, section 3.3.2). Cells were fixed in either with 4% PFA for 20 min or for 10 min with ice-cold methanol (for γ -tubulin and centrin antibodies). Primary antibody dilutions were as follows: mouse anti- α -tubulin (DM1 α , Sigma, 1:1000), rabbit anti- γ -tubulin (Sigma, 1:1000), rabbit anti-pericentrin (Bethyl, 1:300), rabbit anti-NuMa (Bethyl, 1:200), mouse anti-centrin-3 (Abnova, 1:500), rabbit anti-TPX2 (Bethyl, 1:100), mouse anti-Hec1 (GeneTex, 1:100). Secondary antibodies conjugated to FITC, TRITC or Cy-5 (all from Jackson Laboratories) were chosen as appropriate and used as recommended by the supplier. DNA was counterstained with DAPI.

4.3.3. Western blotting

Protein samples were processed as previously described (Chapter 2, section 2.3.4.). The membranes were incubated with primary antibodies: mouse anti-GFP (Roche, 1:1000), mouse anti-tubulin (DM1 α , Sigma, 1:5000), rabbit anti-CENP-W (Abcam, 1:1000) and rabbit anti-TPX2 (Bethyl, 1:1000). Goat anti-mouse or goat anti-rabbit HRP antibodies (Jackson Laboratories) were successively applied. Antigens on the membrane were revealed by enhanced chemiluminescence (ECL, Milipore). Densitometric analysis of immunoblots were determined using the Gene Snap software from Syngene.

4.3.4. DNA plasmid preparation and transfection

Mini plasmid DNA preparations of photoactivatable (PA)-GFP and pYFPC3-TPX2 (a kind gift from Dr. Isabelle Vernos) constructs were generated using Qiagen Mini Prep kit following the manufacturer's instructions. Transfection of GFP-CENP-W HeLa cells with plasmid DNAs was carried out by electroporation using a Nucleofector™ II device (Lonza Biologics, Slough, United Kingdom). Briefly, HeLa cells were trypsinised and 1×10^6 cells for each DNA plasmid were spun in a centrifuge at 1200 r.p.m for 5min. The supernatant was removed from cell pellet, 4 µg of plasmid DNA was pipetted directly on top of the cell pellet followed by 100µl of Ingenio electroporation solution (Mirus supplied through Cambridge Bioscience, Cambridge, United Kingdom). The cell pellet was resuspended in this solution and transferred to an Ingenio cuvette (Cambridge Bioscience, Cambridge United Kingdom). The Amaxa program I-013 was selected and cells were transfected. Following nucleofection, cells were placed on a glass coverslips in 12-well dishes with pre-warmed medium and placed back in 37°C incubator, allowing the cells to grow and adhere before siRNA transfection. The siRNA administration was carried out 24h later and transiently transfected cells were analyzed 24h or 48h later by fluorescence microscopy or western blotting.

4.4. RESULTS

4.4.1. Spindle pole fragmentation in CENP-W depleted cells

As previously demonstrated (Prendergast *et al.* 2011), following CENP-W depletion, cells demonstrated severe mitotic defects, with most cells exhibiting disorganized prometaphases, often with multipolar spindles. In order to investigate the spindle microtubule behavior in cells depleted of CENP-W protein, HeLa H2B-GFP cells were transfected with mCherry- α -tubulin DNA and a stable line was generated. Consistent with previous findings, live cell imaging on tubulin labeled H2B-GFP cells confirmed that cells depleted of CENP-W experienced a significant delay in mitosis, with a prolonged congression periods and whole spindle rolling motions (Prendergast *et al.* 2011).

High frequency of a multipolar spindle phenotype of CENP-W depleted cells suggested difficulties in formation or maintaining a bipolar spindle pole integrity. Such a problem could be due to abnormal centrosome behavior, and could be caused by two mechanisms: 1) entering mitosis with more than two centrosomes, or 2) fragmentation of poles within mitosis. We further examined spindle dynamics of CENP-W depleted cells using live cell imaging of mCherry- α -tubulin cells. Visualization of spindle microtubules demonstrated that multipolar spindles arise from bipolar spindles during prometaphase (Figure 4.1A). In this example a mitotic cell showed a bipolar spindle at the beginning of the observation (0-36 min). While the chromosomes initially dispersed within a bipolar spindle, a new supernumerary spindle pole appeared (36 min), and the spindle re-organized to a tri-polar structure with chromosomes adopting an appropriate configuration. Additionally, quantitative results of observed mitoses in mCherry- α -tubulin cells demonstrated that over 40% of mitotic cells within the CENP-W depleted population increased the number of their poles, going from bipolar to tri-, tetra- and multipolar cells within the duration of the 12h imaging (Figure 4.1B; data not shown). It is noticeable that pole fragmentation occurred also in the rolling cells suggesting that rolling itself contributes to mechanical pole disruption. As compared to 1.3% of the spontaneously occurring pole fragmentation of control cells, these findings support the hypothesis that in CENP-W depleted cells the integrity of the spindle pole is weakened, leading to frequent pole fragmentation events and thus multipolarity.

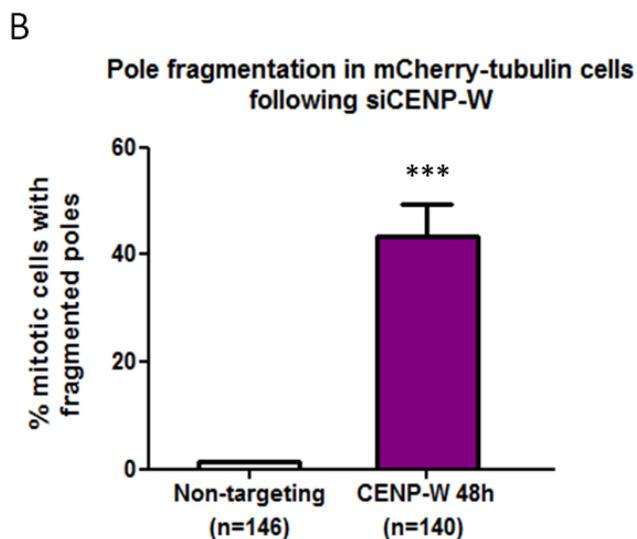
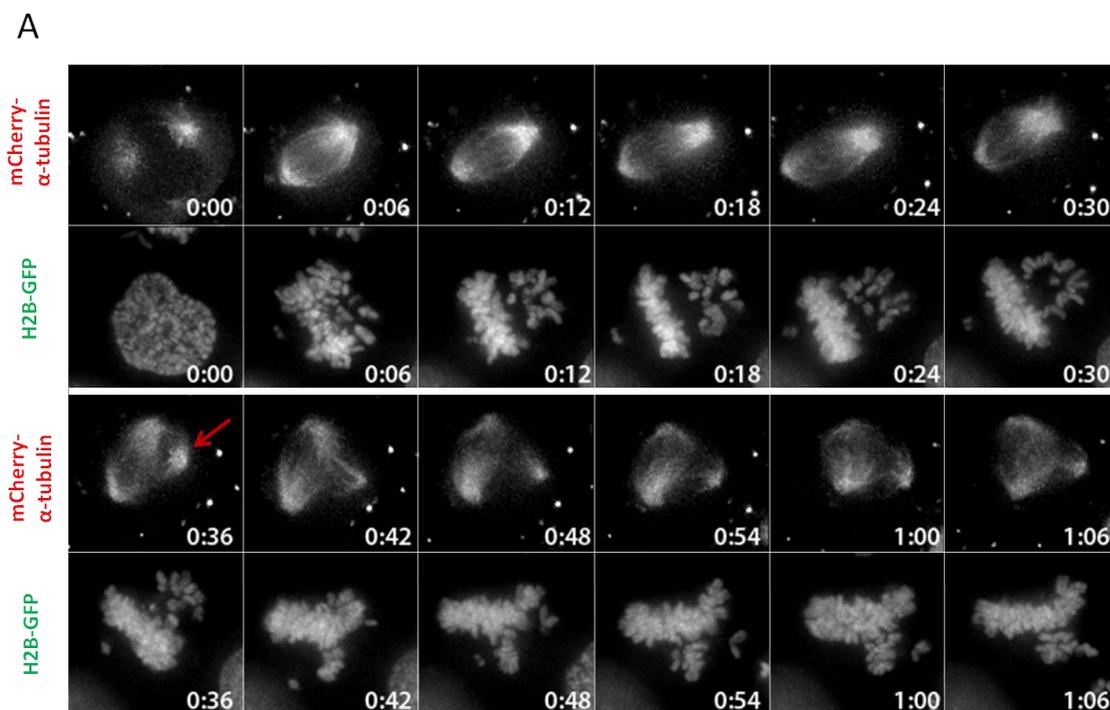


Figure 4.1. Tubulin labeling confirms spindle pole fragmentation in CENP-W depleted cells. (A) HeLa H2B-GFP cells were transfected with mCherry- α -tubulin to directly visualize spindle MTs following transfection with pooled siCENP-W for 48h. It is clear that the cell initially possessed bipolar spindle (time 0-36min) but then supernumerary pole appeared under mCherry-tubulin fluorescence (36, red arrow). Spindle poles moved apart and the chromatin re-organized within the multipolar spindle. Time = hr:min. **(B)** Graph represents mean percentage (+SEM) of the pole fragmentation events occurring in mitotic cells following CENP-W depletion. *** $p < 0.0001$ (one-way ANOVA followed by Dunnett's multiple comparison).

4.4.2. Centrosomal abnormalities following CENP-W depletion

The findings from live cell studies, documenting the occurrence of the pole fragmentation events support the hypothesis of destabilized pole integrity as a consequence of CENP-W depletion. However, since most of the pole fragmentation events were observed 48h following transfection with siRNA, we could not rule out the possibility of amplified centrosomes from the first cell cycle (24h post-transfection) being ‘carried over’ to the next one, due to cytokinesis failure and therefore giving rise to the multipolar spindles in the following mitosis (48h post-transfection). Alternatively, the pole fragmentation could arise from centriole overduplication and/or premature centriole disengagement occurring in the preceding interphase (Loncarek *et al.* ; Inanc *et al.* 2010) following CENP-W depletion.

To directly address the question of whether centriole number is altered following CENP-W depletion, we used HeLa cells stably expressing GFP-tagged CENP-W, which would allow us to observe centriole number specifically within the depleted population using fluorescence microscopy. Firstly, the ability to deplete over-expressed CENP-W protein in these cells was evaluated, both by western blotting and immunofluorescence (Figure 4.2). Using a 48h siRNA transfection protocol, we successfully knocked down both tagged and endogenous CENP-W to 18% and 22% of control levels, respectively.

With an efficient knockdown method, the experimental strategy for investigating the aberrations of centrosome and centriole numbers in CENP-W depleted cells was applied (as illustrated in Figure 4.S1A). Briefly, HeLa GFP-CENP-W cells were treated with siRNA and then fixed at the times which allowed to assess the centrosomal alterations in the first and the second mitosis following siRNA depletion (24h and 48h later, respectively). The cells were processed by immunofluorescence for centriole detection (with centrin-3 antibody) and different centrosomal markers, such as pericentrin, NuMa and γ -tubulin (Figure 4.3A, Figure S4.1B and Figure S4.2). Consistent with live cell studies a major increase of supernumerary centrosomal foci was observed following CENP-W depletion. Moreover, a significant increase was observed rapidly, with almost 30% of cells showing supernumerary centrosomal foci 24h post-transfection, (Figure 4.3B). These findings strongly suggest that, spindle multipolarity following CENP-W siRNA treatment is due to fragmentation of spindle poles rather than abnormal amplification of centrosome number due to cytokinesis failure or centrosome overduplication.

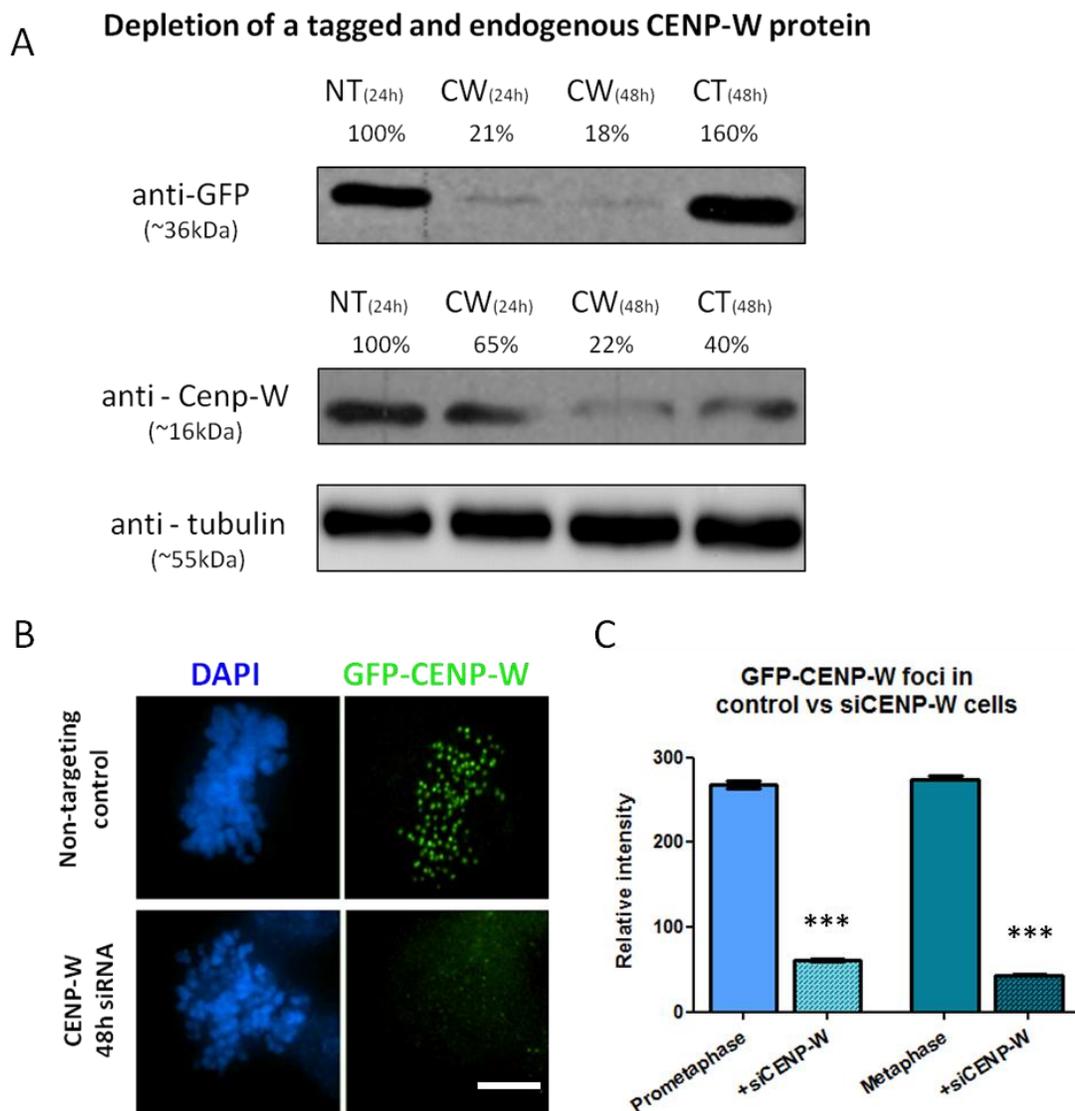


Figure 4.2. Depletion of CENP-W protein in GFP-CENP-W HeLa cells. (A) HeLa cells expressing GFP-CENP-W were transfected with pooled siRNA for CENP-W (CW), CENP-T (CT) and non-targeting (NT) control. Samples harvested at indicated times were blotted against GFP and CENP-W for detection of a tagged and endogenous protein, respectively. Numbers represent relative protein abundance, measured by quantification of chemiluminescence signal and normalized to tubulin. (B) Representative image of GFP-CENP-W (green) cells, depleted with CENP-W and stained with DAPI (blue). Scale bar 5 μ m. (C) Graph represents quantitative measurements of the mean (+SEM) of relative GFP fluorescence signal intensity of the mitotic prometaphases and metaphases cells, following siRNA transfection. Values plotted were background corrected. *** $p < 0.0001$ (one-way ANOVA followed by Dunnett's multiple comparison).

Subsequently, we looked at the composition of centrosomes in the affected cells. First, we examined the centrin-3 foci appearance to inspect the possible overduplication or other abnormalities of centrioles. Overall, centriole numbers were not seen to increase in mitotic cells depleted of CENP-W, with 95% of cells containing 4 centrioles per cell (Figure 4.3B). However, a different perturbation was observed – an abnormal centriole splitting. Typically pair centrioles are closely apposed, within about a diameter of each other. Centrioles that were positioned alone or with greater than about one diameter separation were scored as abnormally split. In a normal mitosis (non-targeting control treated cells) each spindle pole possessed one pair of centrioles, visible as two adjacent centrin-3 spots (Figure 4.3A). In CENP-W depleted prometaphases, 30% of mitotic cells exhibited abnormal separation of the paired centrioles in one or more centrosomes, leading to spindle poles containing one single centrin dot, because paired centrioles had split (Figure 4.3A and C).

In addition, clear non-centriole associated centrosomal foci were observed, consistent with a spindle pole fragmentation process occurring in mitosis, after CENP-W depletion (see red arrows in Figure 4.3A). Taken together, these findings demonstrated that spindle multipolarity in CENP-W depleted cells did not originate from centrosome overduplication occurring in the previous cell cycle, but is generated during mitosis, associated with abnormal centriole splitting.

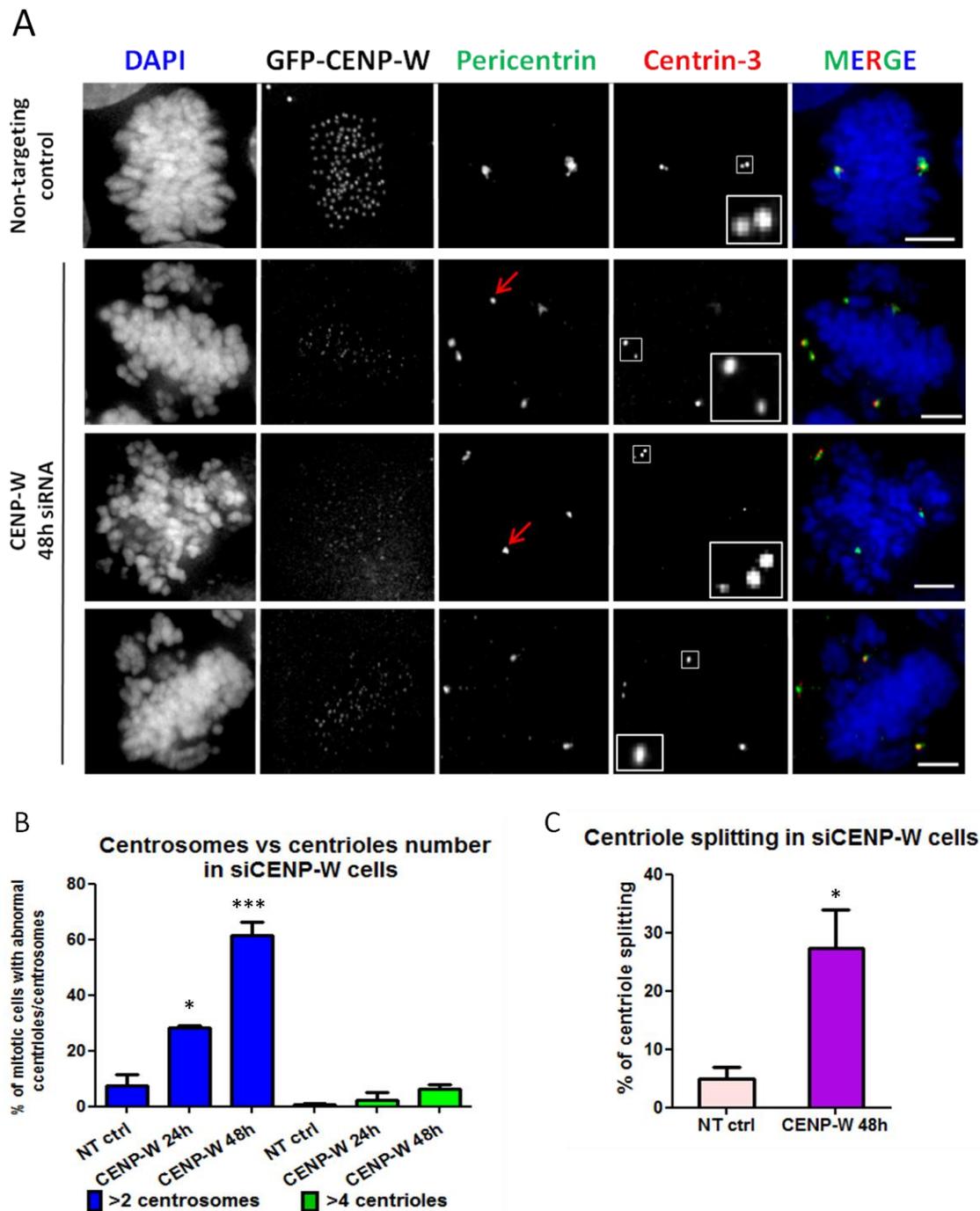


Figure 4.3. Abnormal centriole splitting in CENP-W depleted cells. (A) Representative panel of GFP-CENP-W cells transfected with siCENP-W for 48h, stained for pericentrin (for centrosome detection, green) and centrin-3 (for centriole detection, red). siCENP-W disorganized prometaphases exhibited a multipolar spindle (red arrows indicate non-centriolar associated poles). The boxed enlargements show centriole pair in non-targeting control (NT ctrl) metaphase cell and different centriole abnormalities observed upon siCENP-W treatment, scale bar 5 μ m. (B) Graph represents mean percentage (+SEM) of mitotic cells with >2 pericentrin or >4 centrin foci (at least 100 cells were scored, in triplicate assays). (C) Graph represents mean percentage (+SEM) of mitotic cells with split centrioles. * $p < 0.01$, *** $p < 0.0001$ (one-way ANOVA followed by Dunnett's multiple comparison).

4.4.3. Kinetochores associated molecular dysfunction of CENP-W depleted cells

Considering that in human cells there are no known direct interactions between CENP-W and any of the centrosomal components, as well as no reports of CENP-W being a part of the centrosome complex (Hori *et al.* 2008), we presumed that there must be some additional kinetochores associated dysfunction. Given the fundamental role of Hec1 in stable microtubule-kinetochores attachments, we decided to examine the binding capacity of the Hec1 in cells depleted of CENP-W, that would explain the observed phenotype. The choice of this protein was additionally supported by the literature review, documenting the phenotype of the Hec1-depleted cells displaying, surprisingly similar to CENP-W depleted cells, mitotic defects, with major being prometaphase-like chromosome configurations, mitotic arrest and disorganized spindles (Martin-Lluesma *et al.* 2002; DeLuca *et al.* 2005). Furthermore, the recently published work on a modified Hec1 derivative, reporting the abnormal kinetochores-generated pulling forces from expressing an EGFP-Hec1 (Mattiuzzo *et al.* 2011), shed a new light on our findings. Indeed, consistently with our suspicions, immunofluorescence staining for Hec1, revealed a severe kinetochores binding problem, following CENP-W depletion. In control cells, Hec1 was present on kinetochores throughout mitosis, with a major fluorescent intensity decline only following anaphase onset (Figure 4.4 A and B). However, following CENP-W depletion, a significant reduction of the fluorescent intensity was observed, associated with the impaired binding of this outer kinetochores protein in cells depleted of CENP-W, with some residual occupancy of the protein during the earlier stages of mitosis (Figure 4.4 A and C).

The weakened Hec1 binding following CENP-W depletion is consistent with the observed phenotype and in accordance with the kinetochores-microtubules attachments being impaired in these cells. In addition, it provides explanation for our previous findings of reduced Mad1/Mad2 binding to the kinetochores depleted of CENP-W/T complex (Chapter 3), since Hec1 was shown to be required, together with Mps1 kinase, for recruitment of the Mad1/Mad2 checkpoint complex to kinetochores (Martin-Lluesma *et al.* 2002). Furthermore, Hec1-depleted cells were reported to display persistent spindle checkpoint activity, although they lacked significant amount of Mad1 and Mad2 at kinetochores, similarly as CENP-W and -T depleted mitotic HeLa cells. Therefore we concluded, that impaired Hec1 binding to the kinetochores and subsequent kinetochores-MT attachment problems were respectively, the primary consequence of CENP-W loss and the possible cause of multipolar spindle formation and centrosomal abnormalities, reported in depleted cells.

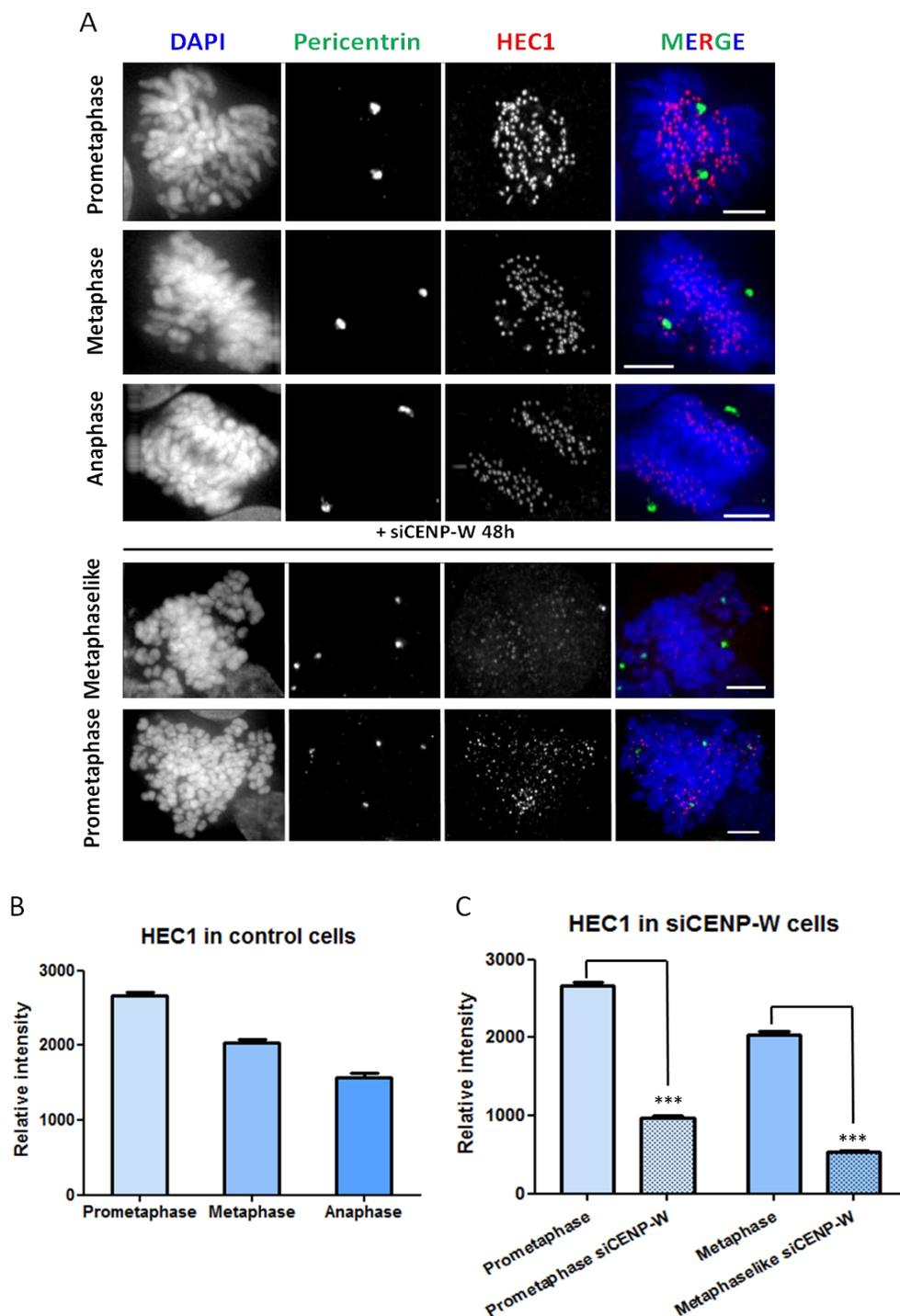


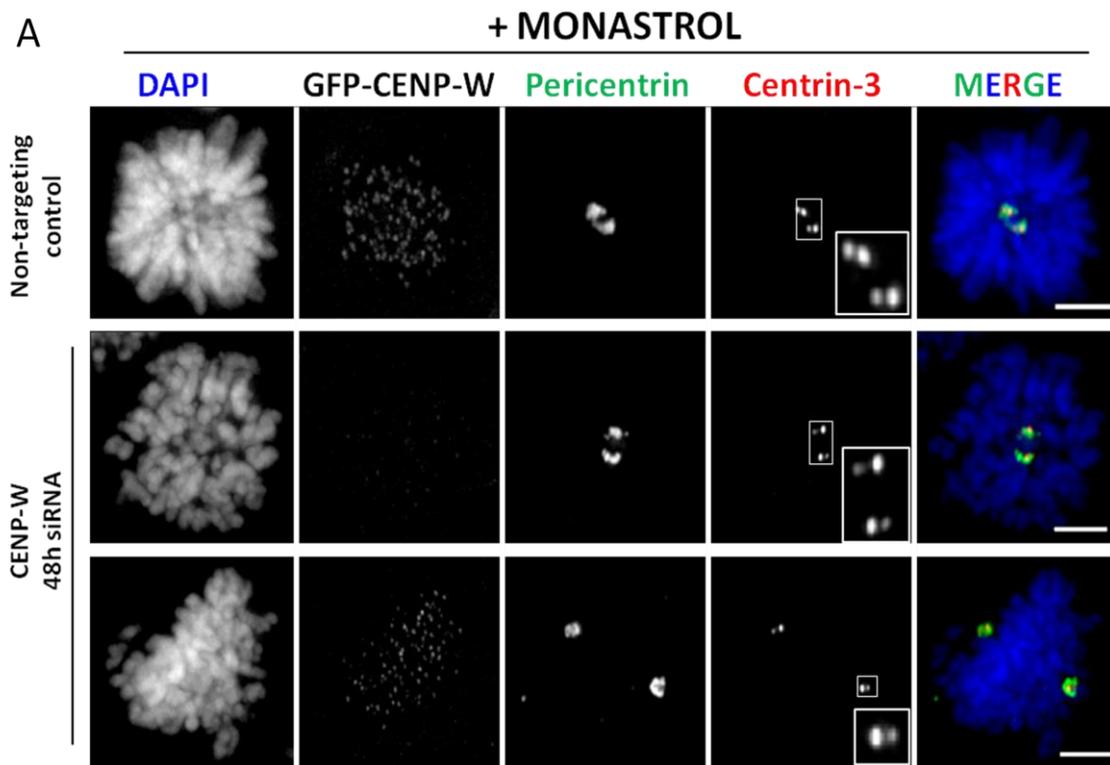
Figure 4.4. Impaired kinetochore binding of Hec1 protein following CENP-W depletion (A) Representative panel of GFP-CENP-W cells transfected with siCENP-W for 48h, and stained for pericentrin (green) and Hec1 foci (red) across different mitotic stages, scale bar 5 μ m. **(B)** Relative Hec1 fluorescence signal intensity was quantified and plotted as the mean \pm SEM versus mitotic stage. **(C)** Graph represents quantitative measurements of the mean (+SEM) of relative Hec1 fluorescence signal intensity of the mitotic prometaphases and metaphases cells, following siRNA transfection. Values plotted were background corrected (at least 1000 kinetochores were scored in duplicate assays). *** $p < 0.0001$ (one-way ANOVA followed by Dunnett's multiple comparison).

4.4.4. CENP-W phenotype suppression following drug treatment

4.4.4.1. The effect of monastrol treatment

The quantitative analysis of centrosomal alterations following CENP-W depletion revealed frequent abnormal centriole splitting events in these cells, suggesting that there might be a cause-effect relationship between the splitting and centrosome fragmentation. However, the appearance of non-centriolar associated centrosomal foci indicates that pole fragmentation can be independent of centriole splitting. Interestingly, following live cell studies, all of the observed events of spindle pole fragmentation seemed to originate from a bipolar array (Figure 4.1A). To further investigate the question of whether in CENP-W depleted cells pole fragmentation requires the centrosome separation and bipolar structure formation, we applied monastrol (MON). This widely used drug acts at the entry into mitosis, preventing the separation of centrosomes through Eg5 kinesin inhibition and arresting the cells in mitosis with monopolar spindles (Kapoor *et al.* 2000).

HeLa GFP-CENP-W cells were depleted of CENP-W and next exposed to a 4h drug treatment, 44h post-transfection with siRNA. More than 90% of control MON-treated cells displayed two pairs of adjacent centrioles at the centre of the monopolar spindle (Figure 4.5A, Figure 4.6, Figure 4.7). Simultaneously, monastrol treatment on CENP-W depleted cells suppressed the abnormal centriole splitting, giving two distinctive phenotypes: either 4 centrioles in a close distance within a monopolar spindle, or two pairs of adjacent centrioles organized in a bipolar manner (Figure 4.5A). Nevertheless, following monastrol treatment, a significant decrease of abnormally split centrioles in CENP-W depleted cells was observed (Figure 4.5B), suggesting that centriole splitting takes place on bipolar spindles after centrosome separation at prophase.



B

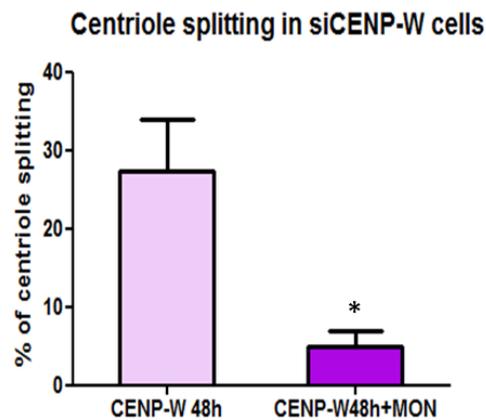


Figure 4.5. Monastrol treatment rescues abnormal centriole splitting. (A) Representative panel of GFP-CENP-W cells transfected with siCENP-W for 48h, incubated with monastrol (MON) for the last 4h of transfection and stained for pericentrin (centrosome, green) and centrin-3 (centriole, red). The boxed enlargements show centriole pairs of mitotic cells in control and siCENP-W treated cells following monastrol incubation, scale bar 5 μ m. (B) Graph represents mean percentage (+SEM) of the mitotic cells with split centrioles following monastrol and/or siCENP-W treatment (at least 100 cells were scored in duplicate assays). * $p < 0.05$ (one-way ANOVA followed by Dunnetts multiple comparison).

In addition to the rescue of the centriole splitting phenotype by monastrol, we observed another striking phenotype in which CENP-W depletion inhibited monopolar spindle formation in monastrol. As expected, following monastrol, nearly 90% of mitotic cells of non-targeting siRNA treated cells possessed single centrosome foci and monopolar spindle, as visualized by immunostaining with γ -tubulin (or pericentrin) and α -tubulin, respectively (Figure 4.6 and 4.7). However, in CENP-W depleted cells, over 50% of mitotic spindles observed had a normal bipolar structure with relatively well organized chromosomes within metaphase plate and spindles (Figure 4.6). These findings were confirmed using two different centrosomal markers, in a two independent experiments.

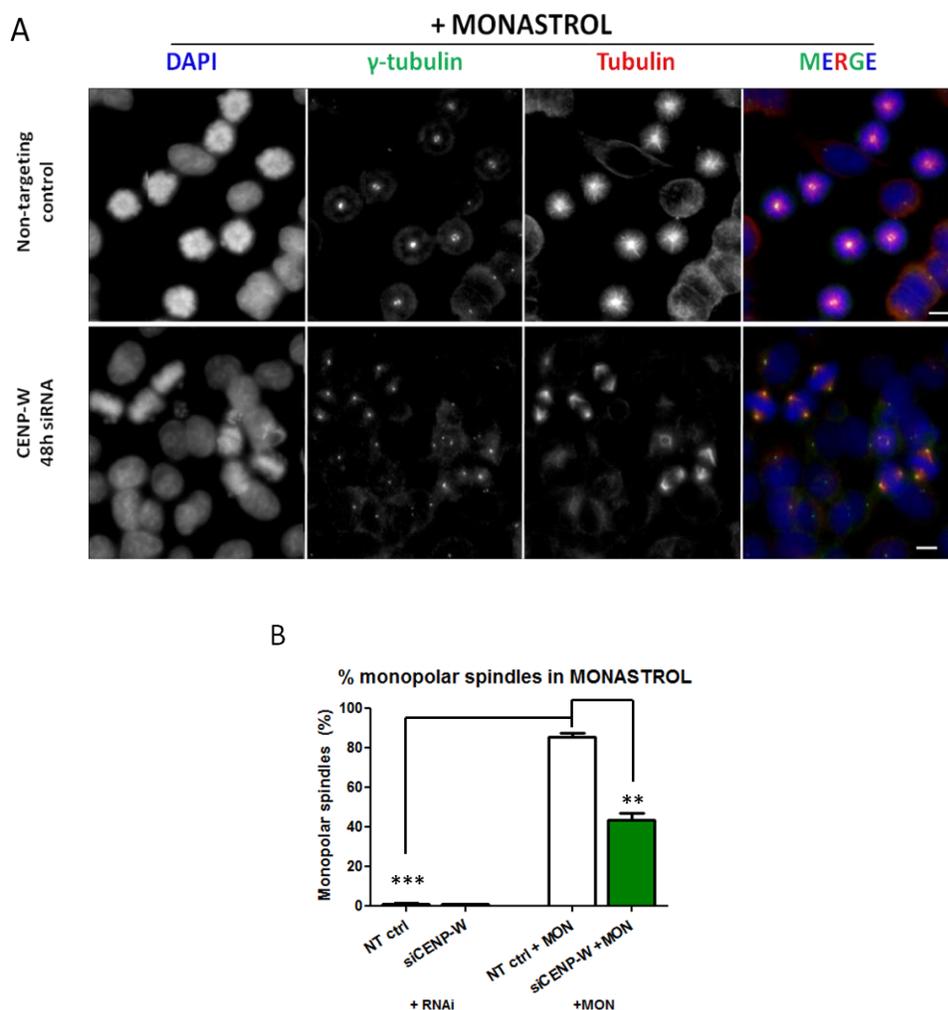


Figure 4.6. CENP-W depletion counteracts monopolarity in monastrol treated cells. (A) Representative panel of GFP-CENP-W cells transfected with siCENP-W for 48h, incubated with monastrol (MON) for the last 4h of transfection and stained for γ -tubulin (green) and α -tubulin (red), scale bar 10 μ m. **(B)** Graph represents mean percentage (+SEM) of the mitotic cells with monopolar spindles following monastrol and/or siRNA treatment (at least 100 cells were scored, in duplicate assays). ** $p < 0.001$, *** $p < 0.0001$ (one-way ANOVA followed by Dunnett's multiple comparison).

Moreover, the quantitative analysis of centrosome foci appearance (both of pericentrin and γ -tubulin staining), revealed a significant decrease of supernumerary centrosomal markers in CENP-W depleted cells, following monastrol treatment, comparable to the untreated control (Figure 4.6B). Based on our previous 24h post-transfection results (Figure 4.3B), indicating the ability of pole fragmentation even in a first mitosis, it is unlikely that in a drug experiment the entire population of multipolar CENP-W depleted cells arose during the last 4h before harvesting, in a non-MON treated cells. Therefore, we concluded that monastrol treatment following CENP-W depletion rescues abnormal centriole splitting and consequently suppresses its downstream effect of the pole fragmentation. Moreover, it does so, not only in cells that initiated mitosis in the presence of a drug, but also counteracts both monopolarity and multipolarity in a CENP-W depleted population, which makes Eg5 kinesin a candidate for mediating CENP-W multipolar effects.

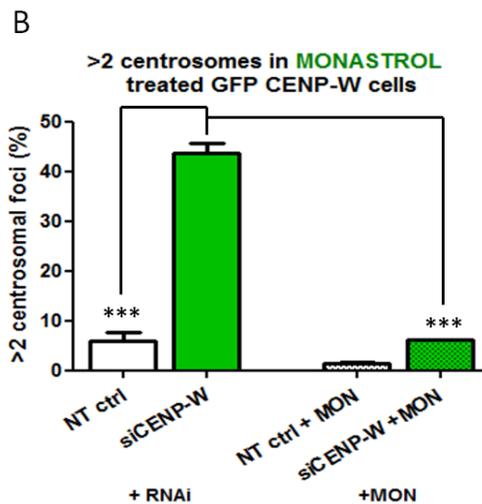
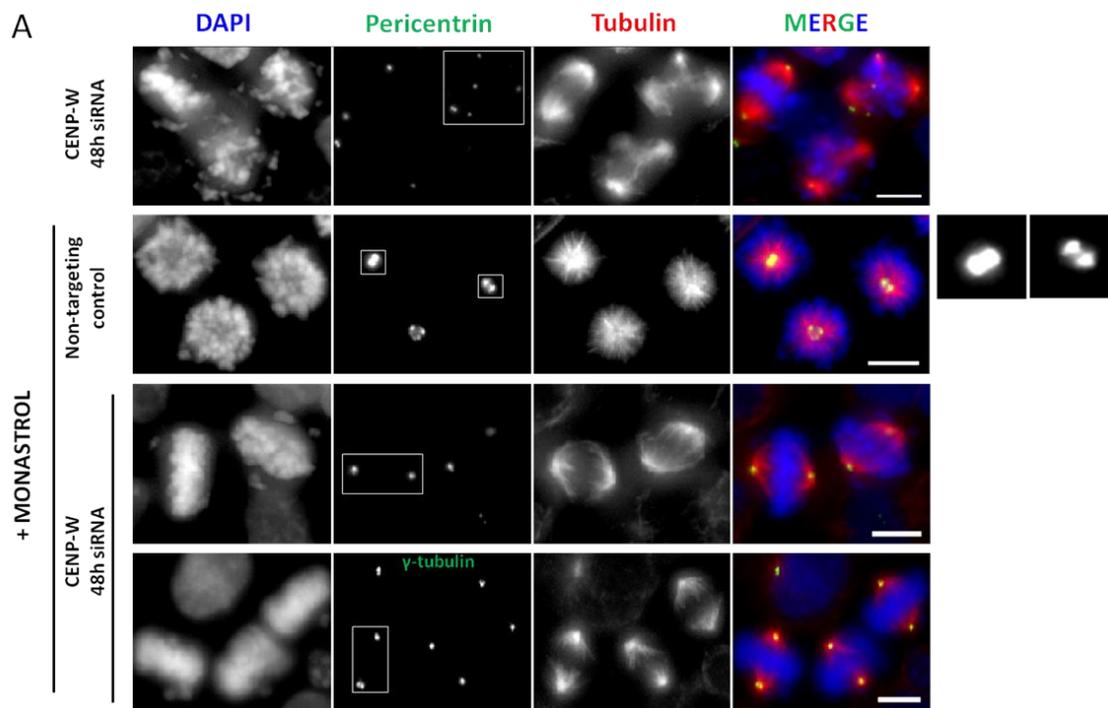


Figure 4.7. Monastrol treatment suppresses centrosome fragmentation in CENP-W depleted cells. (A) Representative panel of GFP-CENP-W cells processed as previously described (Figure 4.6A). Insets show pericentrin foci before and after monastrol. The boxed enlargement shows single pericentrin foci characteristic for monastrol treatment, scale bar 10 μ m. (B) Graph represents mean percentage (+SEM) of the mitotic cells with supernumerary centrosomes (based on pericentrin and γ -tubulin foci) following monastrol and/or siRNA treatment (at least 100 cells were scored, in duplicate assays). *** $p < 0.0001$ (one-way ANOVA followed by Dunnett's multiple comparison).

4.4.4.2. The effect of nocodazole treatment

In a normal mitosis, the formation and maintenance of the bipolar spindle is achieved by microtubule polymerization/depolymerization dynamics combined with finely tuned balance of forces, exerted on different locations of the mitotic spindle apparatus: kinetochores and centrosomes, chromosome arms and interdigitating microtubules (Dumont and Mitchison 2009). The above forces are generated by minus-end directed dyneins or plus-end directed kinesins or can come from microtubule dynamics directly (Sharp *et al.* 2000). Once established that in HeLa cells CENP-W depletion induces centrosome fragmentation, associated with centriole splitting and abnormal spindle dynamics, we wished to identify the cause of this impairing multipolar effect. We hypothesize that the abnormal force generation, presumably excessive pulling on centrosomes caused by CENP-W depletion, produced unbalance of the forces distribution and was the origin of spindle pole fragmentation in these cells. If centrosome fragmentation is indeed a force dependent process, then it should exhibit MT-dependence and therefore simple removal of the spindles should abolish the observed phenotype of pole fragmentation.

To investigate this hypothesis, we used nocodazole (NOC) treatment at concentrations sufficient to prevent spindle formation, resulting in a characteristic prometaphase-like arrest state (Figure 4.8). It has been reported that these high concentration of nocodazole ($\geq 1\mu\text{M}$) induce nearly complete depolymerization of microtubules, resulting in punctuate aggregates of tubulin scattered throughout the cytoplasm of the mitotic cells, with only interphase cells containing short microtubule fragments (Jordan *et al.* 1992). Therefore, by incubating GFP-CENP-W cells with $1\mu\text{M}$ nocodazole we abolished the spindle MTs in these cells and fixed specimen processed for immunofluorescence with centrosome markers (pericentrin and γ -tubulin), to monitor pole fragmentation. Consistent with our hypothesis, following nocodazole treatment the centrosomes were intact, near the periphery of the cells and the frequency of aberrant centrosome numbers observed in CENP-W depleted cells was significantly reduced (Figure 4.8 and S4.2). We can conclude that spindle pole fragmentation is dependent on microtubule function in these cells.

Subsequently, we performed a nocodazole titration assay, to search for the concentration of a drug that would provide a reasonably highly arrested population of mitotic cells (around 50% of mitotic index), with a relatively normal bipolar spindles (Figure S4.3). The goal of this experiment was to obtain “kinetically suppressed” spindles with normal bipolar structure but inhibited MT dynamics (Jordan *et al.* 1992). Exposure of cells to the low dose of nocodazole (20nM) for 4h, did not result in a major change in centrosome number after CENP-W depletion, although it slightly suppressed the multipolarity in these cells ($p \leq 0.01$). However, the same

treatment in the control cells resulted in an increased population of mitotic cells with multipolar spindles, that was not significantly different than the multipolar population of CENP-W depletion alone, without the drug treatment (Figure S4.4), as described previously (Jordan *et al.* 1992). Thus, we conclude that modulating only slightly the force production on the spindle, by manipulating microtubule dynamics with nocodazole, can result in multipolarity. We therefore propose that CENP-W depletion mimics low dose nocodazole effect under our observation conditions.

Taken together the combined results of the two drug treatment experiments demonstrated that spindle pole fragmentation following CENP-W depletion is a microtubule-dependent process and requires Eg5 kinesin function. The data suggested that centrosome disruption and associated events of centriole splitting are most likely due to unbalance of the forces generated within the spindle and therefore exerting abnormal pulling forces at centrosomes, tearing the latter apart and producing supernumerary centrosomes. The *de novo* emerged centrosomes are capable of developing asters associated with MT bundles, thus triggering multipolarity in CENP-W depleted cell.

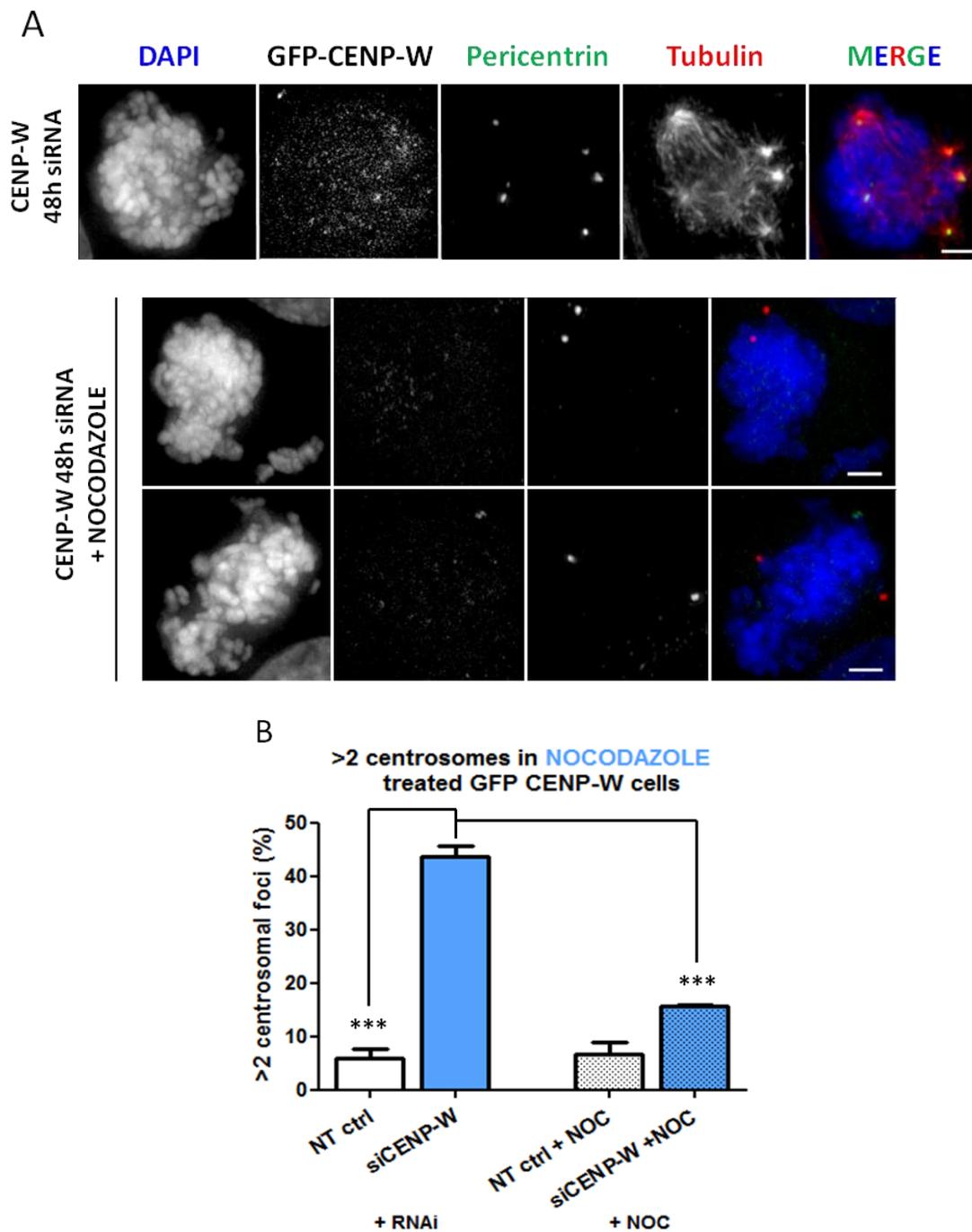


Figure 4.8. Suppression of siCENP-W-dependent spindle pole fragmentation in nocodazole cells. (A) Representative panel of GFP-CENP-W cells transfected with siCENP-W for 48h, incubated with 1 μ M nocodazole (NOC) for the last 4h of transfection and stained for pericentrin (green) and α -tubulin (red). In the nocodazole panel the α -tubulin channel is not shown, scale bar 5 μ m. **(B)** Graph represents mean percentage (+SEM) of the mitotic cells with supernumerary centrosomes (based on pericentrin and γ -tubulin foci) following nocodazole and/or siRNA treatment (at least 100 cells were scored in duplicate assays). *** $p < 0.0001$ (one-way ANOVA followed by Dunnett's multiple comparison).

4.4.5. The effect of overexpression of the human TPX2 (hTPX2) on spindle pole behavior

The mitotic spindle is an extremely dynamic and a very flexible structure, self-assembling from microtubules and associated structural and regulatory proteins, and is required for chromosome segregation during cell division (Wittmann *et al.* 2001). Among many other microtubule associated proteins, the targeting protein for Xklp2 (TPX2) displays multiple functions in spindle assembly. TPX2 is a 98 kDa Ran-regulated, cross-linking microtubule-associated protein, that nucleates, binds and bundles microtubules *in vitro*. It has been shown that its function is required for spindle pole organization, Aurora A activation and for microtubule formation at kinetochores (Gruss and Vernos 2004; Ma *et al.* 2010).

As discussed above, spindle pole fragmentation in CENP-W depleted cells is proposed to be a consequence of unbalanced forces generated within CENP-W depleted spindles. A role for microtubules was directly assessed in the drug-based assays discussed above. Here, we hypothesized that strengthening the spindle poles should result in a similar suppression of multipolarity. To address this biochemical-based rescue approach we used overexpression of fluorescently-tagged TPX2, hypothesizing that its microtubule cross-linking activity could increase the mechanical stability and integrity of spindle poles.

To characterize the impact of the over-expressed recombinant TPX2 protein on spindle pole formation, firstly we determined its subcellular localization in mitotic HeLa cells. For that purpose, we used immunofluorescence microscopy with an affinity-purified polyclonal antibody against hTPX2 (Figure 4.9A). As previously reported (Gruss *et al.* 2002), during prophase, hTPX2 assembled at the centre of the microtubule asters, decorating growing microtubules between two poles. As cells progressed to metaphase, hTPX2 increased in intensity at the spindle poles and expanded along spindle MTs. The protein remained concentrated at the spindle poles at the onset of anaphase, and next re-localized to the spindle midzone during telophase (data not shown). Following the exit from mitosis hTPX2 was no longer visible in the nucleus, indicating its degradation in the subsequent G1.

To confirm that localization model for a recombinant protein, we expressed a fusion of yellow fluorescent protein and hTPX2 (TPX2-YFP) in HeLa GFP-CENP-W cells. As expected, TPX2-YFP recombinant protein displayed a very similar to the endogenous protein pattern of localization (Figure 4.9B). Next, we evaluated the efficiency and persistence of the over-expressed fusion protein over time, by western blotting and fluorescent microscopy (Figure S4.5). The tagged version of the protein remained detectable up to 60h post-transfection, as

depicted on the western blot and fluorescent images (Figure S4.5A and B). We did not observe any major mitotic defects following TPX2-YFP overexpression, in contrast to a previous report for a different fluorescent tags of TPX2 (Garrett *et al.* 2002; Gruss *et al.* 2002). Thus, exogenously expressed TPX2-YFP is efficiently incorporated into mitotic spindles, presumably augmenting the endogenous protein.

To examine the functional activity of the microtubule cross-linking, correlated with spindle pole organization of the human TPX2, we tested the ability of a recombinant TPX2 protein (TPX2-YFP) to rescue centrosome fragmentation in cells depleted of CENP-W. To address that question the experimental strategy illustrated in Figure 4.10A, was applied. Briefly, HeLa GFP-CENP-W cells were transfected for overexpression of TPX2, with TPX2-YFP plasmid DNA and plated onto a glass coverslips. The cells were treated with siRNA 24h later and then fixed at the indicated times, which would allow to assess the centrosomal abnormalities in the first and the second cell cycle following siRNA depletion (24h and 48h later, respectively). Control cells were transfected with a plasmid expressing photactivatable green fluorescent protein (PA-GFP), as an additional experimental control to rule out a possibility of the transfection condition impacting on centrosome alterations. The cells were then processed with immunofluorescence for centrosomal marker, using pericentrin antibody. The efficiency of TPX2-YFP expression in the entire populations of transfected cells was confirmed with western blotting detection for endogenous and a tagged version of the protein (Figure S4.5C).

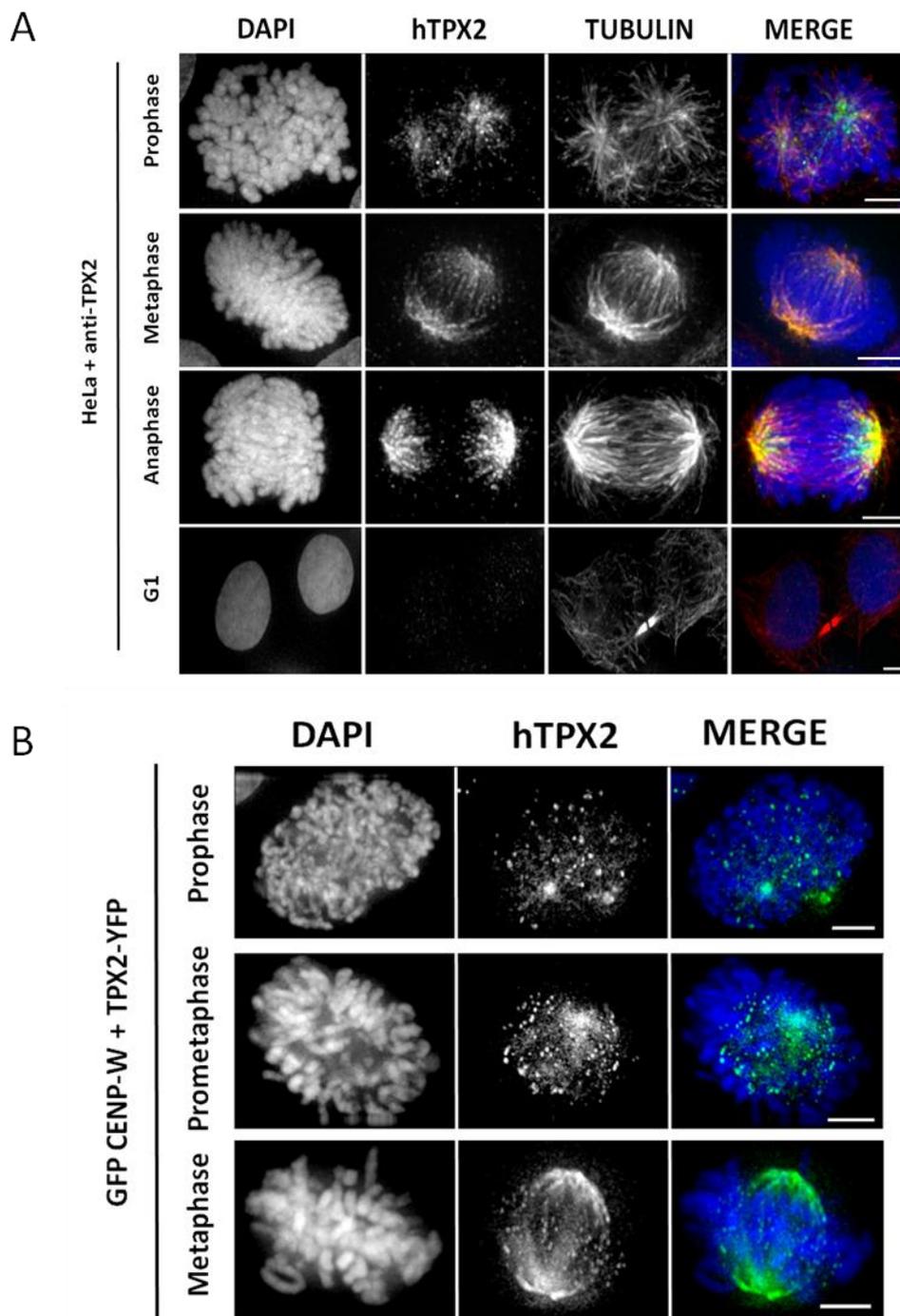
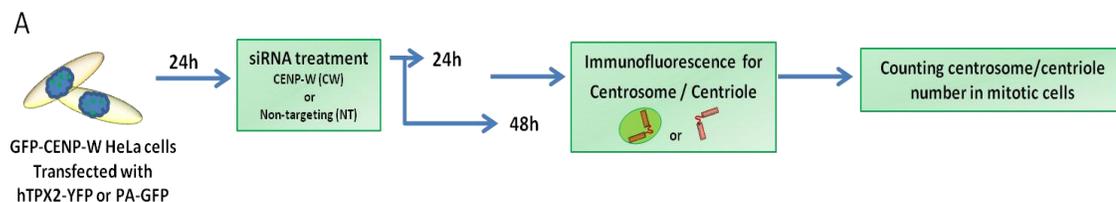


Figure 4.9. Localization and overexpression of hTPX2 in HeLa cells. (A) Exponentially growing HeLa cells were fixed with 4% PFA and processed for immunofluorescence with antibody against hTPX2 (green) and α -tubulin (red). Co-localization across the different stages of mitosis of the spindles with hTPX2 is shown in yellow, in the overlay. Scale bar 5 μ m. (B) Representative panel of GFP-CENP-W HeLa cells overexpressing TPX2-YFP protein, scale bar 5 μ m.

Following TPX2 overexpression, in both siRNA treated populations (non-targeting control and CENP-W), two distinctive phenotypes were observed, the one of a low and one of a high levels of the recombinant TPX2 protein overexpression (Figure 4.10B). Consistently with our hypothesis, cells displaying high levels of the over-expressed TPX2 protein possessed two compact pericentrin foci, following CENP-W depletion. In contrast, those of the low expression level of the recombinant protein, were found to possess three or more centrosomes during mitosis (see red arrow in Figure 4.10B), indicating the ongoing pole fragmentation in these cells. Occasionally, the multipolar cells with high levels of the TPX2 overexpression were also found in the population of CENP-W depleted cells (data not shown). Nevertheless, the quantitative results of the pericentrin foci appearance, following CENP-W depletion in cells with over-expressed TPX2, revealed a major significant decrease in the mitotic cells with supernumerary centrosomes, in both populations of a 24h and 48h post-transfection with siRNA (Figure 4.10C). Concurrent data obtained from the control PA-GFP transfected cells illustrated no significant difference in the appearance of the supernumerary pericentrin foci following CENP-W depletion in these cells (Figure 4.10D), providing additional support, that in our experimental design, the observed suppression of the multipolarity of CENP-W depleted cells was due to TPX2 protein overexpression.

These findings demonstrated that, in mitotic cells depleted of CENP-W, the overexpression of one of the major proteins involved in spindle pole organization and formation of the kinetochore-MTs can enhance the ability of spindle poles to resist fragmentation. The combined biochemical and chemical rescue approaches showed that in CENP-W depleted cells, the phenotype of pole fragmentation can be rescued by altering the force distribution exerted on the mitotic apparatus, responsible for maintaining bipolar spindle microtubules.



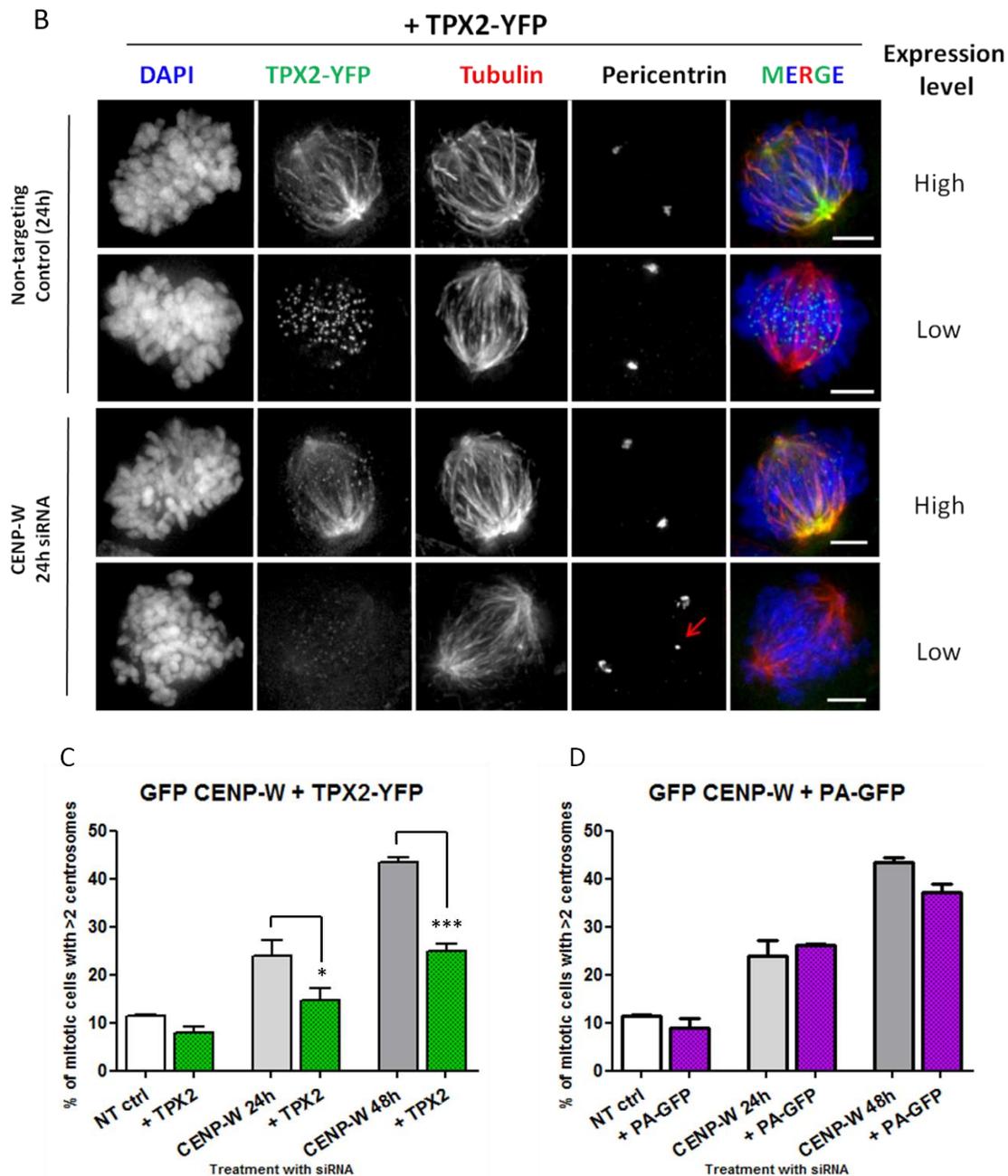


Figure 4.10. Overexpression of hTPX2 suppresses multipolarity in CENP-W depleted cells. (A) Schematic experimental strategy used in TPX2 experiment. (B) Representative panel of GFP-CENP-W cells transfected with TPX2-YFP (green), followed by siRNA depletion of CENP-W and stained for α -tubulin (red) and pericentrin. In the overlay, yellow color indicates co-localization of red microtubules and green TPX2-YFP overexpression. Red arrow indicates supernumerary pericentrin foci in siCENP-W cell with low levels of TPX2 overexpression. Scale bar 5 μ m. (C-D) Graphs represent mean percentage (+SEM) of the mitotic cells with supernumerary pericentrin foci following depletion of CENP-W in TPX2-YFP or PA-GFP transfected cells (n=150 mitotic cells, in triplicate assays). * p<0.01, *** p<0.0001 (one-way ANOVA followed by Dunnetts multiple comparison).

4.5 DISCUSSION

The aim of this study was to investigate the mechanism of multipolar spindle formation following CENP-W depletion. As reported in previous studies, CENP-W/T complex of the CCAN plays an essential role in mitosis (Hori *et al.* 2008; Prendergast *et al.* 2011). Depletion of CENP-W protein induces severe mitotic aberrations in human cells, namely defective chromosome congression leading to prolonged mitosis with disorganized prometaphase-like chromosomes scattering near the spindle poles and formation of multipolar spindles, associated with centriole splitting.

Multipolarity is a hallmark of tumour cells and may arise from supernumerary centrosomes content in a cell as a consequence of centrosome overduplication or cytokinesis failure (Borel *et al.* 2002; Habedanck *et al.* 2005; Inanc *et al.* 2010). Loss of spindle pole integrity during mitosis is an alternative way leading to multipolarity independent of centrosome amplification, due to, for example, premature centriole disengagement or loss or alterations of centrosomal components (Gergely *et al.* 2003; Cassimeris and Morabito 2004; Thein *et al.* 2007; Logarinho *et al.* 2012). Our quantitative analysis of centrosome versus centriole counts in CENP-W depleted cells, excluded the first possibility. Since the massive increase in centrosomal foci appearance initiated already in a short-term experiment, and did not correlate with a similar increase of the centriole number, we concluded that multipolar spindle formation did not originate from centrosome amplification, but rather from pole fragmentation due to loss of its integrity and therefore premature centriole splitting. Similarly, a recent work reported that modification of kinetochore composition due to GFP-Hec1 overexpression in HeLa cells leads to severe mitotic defects and abnormal kinetochore generated forces terminating in a multipolar spindles, originated from centriole splitting (Mattiuzzo *et al.* 2011). We hypothesize that similar unbalance of the forces distribution, mediated by the mechanochemical motors associated with a plus-end microtubules at kinetochores is the primary cause of pole fragmentation in CENP-W depleted cells.

Alternatively, the two recent reports documenting that extensive metaphase delay causes cohesion fatigue and induces premature chromatid separation, followed by centriole disengagement provides another possible explanation for vast spindle multipolarity (Daum *et al.* 2011; Stevens *et al.* 2011). However, our combined results argue that cohesion fatigue is unlikely to be the primary reason for the observed phenotype of CENP-W depletion. Although we cannot exclude that cumulative events of CENP-W loss result in some cohesion fatigue,

particularly due to the similarity of phenotypes of the extensive chromosome scattering occurring asynchronously and often accompanied by continuous rotation of the entire spindle following prolonged metaphase arrest (Daum *et al.* 2011); still, we will further discuss lines of evidence that argue against the cohesion fatigue based explanation.

Firstly, the live cell observations of mCherry- α -tubulin expressing cells depleted of CENP-W illustrated that the initial spindle pole fragmentation events occurred almost immediately following bipolar spindle establishment, and at least several hours before centriole disengagement reported in cells exposed to prolonged metaphase arrest induced by five independent perturbations (Stevens *et al.* 2011). Moreover, following CENP-W depletion, only ~25% of cells with supernumerary centrosomal foci demonstrated an abnormal centriole split, a consequent multipolar phenotype normally associated with cohesion fatigue (Daum *et al.* 2011; Stevens *et al.* 2011). The remaining 30-35% of cells had increased number of poles that were acentriolar and derived from fragmentation of the pre-existing poles.

Secondly, using drug-based approach, we managed to efficiently rescued spindle pole fragmentation by affecting microtubule dynamics or antiparallel MT sliding, respectively by nocodazole and monastrol application. Although initial chromosome scattering near the spindle poles in cells depleted of CENP-W protein could indicate loss of sister chromatid cohesion, this phenotype was not persisting following drug treatments, which excludes the irreversible mechanism of cohesion fatigue and premature sister chromatids separation as being the main mechanism driving spindle pole fragmentation and multipolarity in our system. Additionally, we observed a significant decrease of monopolarity in CENP-W depleted cells following Eg5 inhibition. The latter could indicate that in our experimental design, 4h drug treatment would not prevent from centrosome separation in the mitotic cells that had already been arrested in mitosis, before the exposure to monastrol. However, this scenario seems unlikely, since in the mitotic population of CENP-W depleted MON-treated cells we would also observed cells with multipolar spindles that had been arrested for the first 44h, before drug treatment, and would have managed to fragment their poles. Therefore, we concluded that multipolar spindles in CENP-W depleted cells arose after the establishment of a bipolar spindle and Eg5-mediated pushing forces are required for their maintenance. Moreover, we speculate that CENP-W exhibits additional influence on spindle dynamics, since its depletion counteracted Eg5-inhibition mediated monopolar spindle formation in our experimental system. Perhaps, CENP-W directly or indirectly plays a role in kinetochore-dynein recruitment or function, which was suggested earlier based on the premature dynein-dependent depletion of Mad1-Mad2 complex

from CENP-W/T depleted kinetochores. Consistently, it has been shown that simultaneous inhibition of Eg5 and dynein depletion similarly rescues monopolarity in human cells (Tanenbaum *et al.* 2008), which provides additional support that spindles of CENP-W-depleted cells experience a profound unbalance of the force generation between plus-end directed pushing forces of Eg5 kinesin and minus-end directed dynein.

Another argument arises from the TPX2 overexpression experiment, which demonstrated that additional reinforcing and stabilization of spindle poles mediated by this protein, prevented from centrosome fragmentation. Importantly, we found that endogenous TPX2 was localized and maintained normally in CENP-W depleted cells alone (data not shown). TPX2 exhibits many functions during the mitotic spindle assembly, such as binding and cross-linking microtubules and focusing spindle poles. Moreover, it has been found to influence spindle assembly and maintaining focused spindle poles even in the absence of other stabilizing factors, such as NuMa or HSET (Manning and Compton 2007). However, to our knowledge, TPX2 protein has never been implicated in chromatid cohesion establishment or its removal. Therefore, we concluded that the suppressed centrosome fragmentation observed in the CENP-W depleted cells following TPX2 overexpression was due to enhanced ability to resist fragmentation conferred by additional TPX2 activities, which disagrees with a cohesion fatigue model.

Cumulative results, of two drugs and TPX2 overexpression experiments, indicated that multipolarity associated with the depletion of CENP-W protein, was indeed the consequence of the unbalance of the forces distribution in the spindle MTs, rather than the cohesion fatigue following extended mitotic arrest. To our knowledge, no other CCAN protein possesses such a strong potency to affect spindle polarity, with the exception of CENP-L and CENP-O, which depletion induces monopolar spindles, but the detailed mechanism to explain these phenotypes is still unclear (McAinsh *et al.* 2006; McClelland *et al.* 2007). The latter monopolarity, however, can be suppressed by the loss of kinetochore-MT attachments, achieved by co-depletion of both CENP-O and Nuf2R proteins, which substantially rescued bipolar spindle assembly (McAinsh *et al.* 2006). Contrary, our findings argue that model, since depletion of inner centromere component CENP-W affected Hec1 recruitment to the outer kinetochore-MT binding sites and resulted in multipolarity. Our data demonstrated, for the first time, how the loss of one of the centromere protein from the CCAN, can impact on a very delicate but finely tuned balance of the forces and alter the equilibrium towards pulling the excessive strength on centrosomes, resulting in their fragmentation. Therefore, based on our experimental data, and findings from other groups that recently have enlarged our understanding of the complex microtubule

dynamism (Mattiuzzo *et al.* 2011; Logarinho *et al.* 2012), we propose a model for sequence of events that may explain the observed phenotype of CENP-W depletion.

In a normal metaphase cell all the forces are balanced within bi-oriented spindles (Sharp *et al.* 2000; Mattiuzzo *et al.* 2011). The remaining unattached kinetochores exhibit CENP-E motor protein which facilitates microtubule binding mediated by Ndc80/Hec1 complex. The forces exerted by the two plus-end directed kinesins CENP-E and Eg5 are compensated by minus-end directed dynein/dynactin forces (Tanenbaum *et al.* 2008), maintaining the balance within the spindle until all kinetochores achieve bi-orientation. Perturbation of CENP-W function at kinetochores depletes the latter of Ndc80/Hec1 mediated binding. Hec1 depleted cells retain high levels of the kinetochore corona component CENP-E (DeLuca *et al.* 2005). This plus-end directed kinesin has been shown to be a key player mediating perturbed kinetochore traction forces during congression of mono-oriented or laterally-microtubule attached chromosomes, since co-depletion of CENP-E re-balanced the force generation within the spindle leading to decreased multipolarity (Mattiuzzo *et al.* 2011; Logarinho *et al.* 2012). CENP-E plus-end directed forces are thought to overcome the dynein-mediated minus-end forces, and such an unbalance can produce spindle pole fragmentation associated with centriole splitting, independent of centrosome amplification. In many cases of CENP-W depleted cells, such spindle conformation is not transient and cells are either permanently arrested due to Aurora B dependent activity or can enter anaphase, thus leading to aneuploidy. On the other hand, since multipolar anaphase often results in lethality of daughter cells in several cancer cell lines (Ganem *et al.* 2009), CENP-W protein may be considered as potential chemotherapeutic target in human cancers.

4. 6. SUPPLEMENTAL MATERIALS

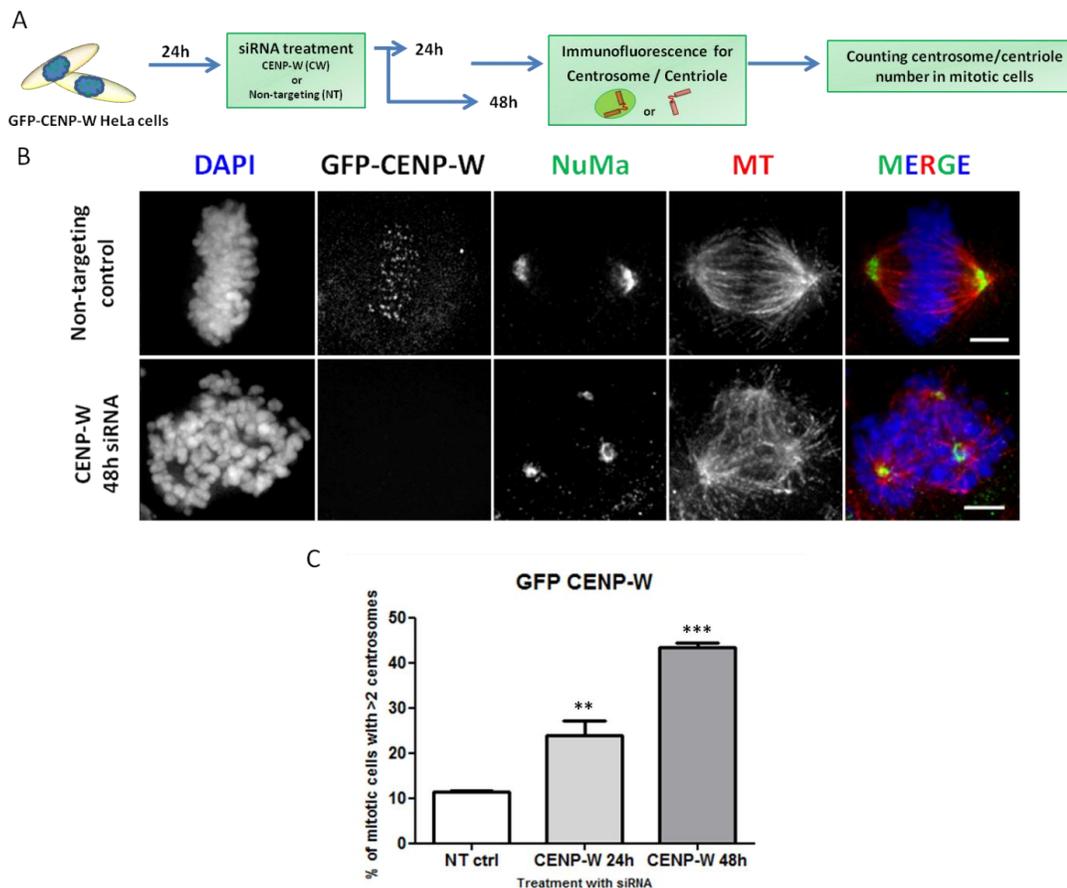


Figure S4.1. Fragmentation of centrosomal material in CENP-W depleted cells. (A) Schematic experimental strategy used in pole fragmentation experiment. (B) Representative panel of GFP-CENP-W cells transfected with siCENP-W for 48h and stained for NuMa (green) and α -tubulin (red), scale bar 5 μ m. (C) Graph represents mean percentage (+SEM) of the mitotic cells with supernumerary pericentriolar foci following depletion of CENP-W in transfected cells (n=100 mitotic cells, in triplicate assays). ** p<0.001, *** p<0.0001 (one-way ANOVA followed by Dunnett's multiple comparison).

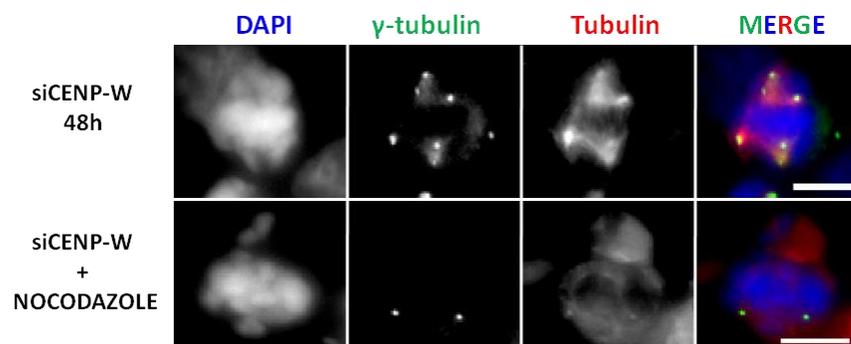


Figure S4.2. Suppression of supernumerary γ -tubulin foci in nocodazole siCENP-W treated cells. Representative panel of GFP-CENP-W cells transfected with siCENP-W for 48h, incubated with 1 μ M nocodazole (NOC) for the last 4h of transfection and stained for γ -tubulin (green) and α -tubulin (red). Scale bar 10 μ m.

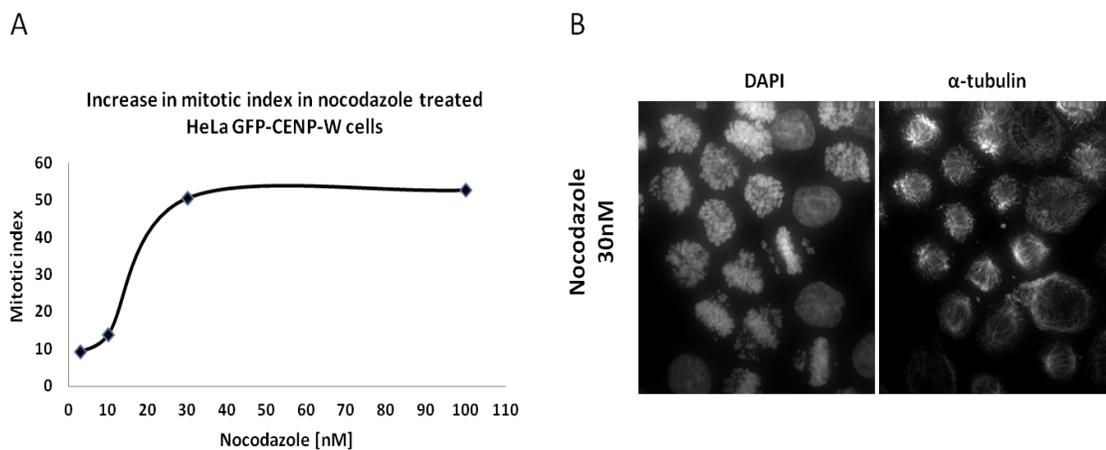


Figure S4.3. Titration of nocodazole (A) Graph represents change in mitotic index following an increasing concentrations of nocodazole (0-100nM). (B) Representative image of GFP-CENP-W cells treated with 30nM nocodazole and stained for spindle microtubules and chromosome visualization with α -tubulin and DAPI, respectively.

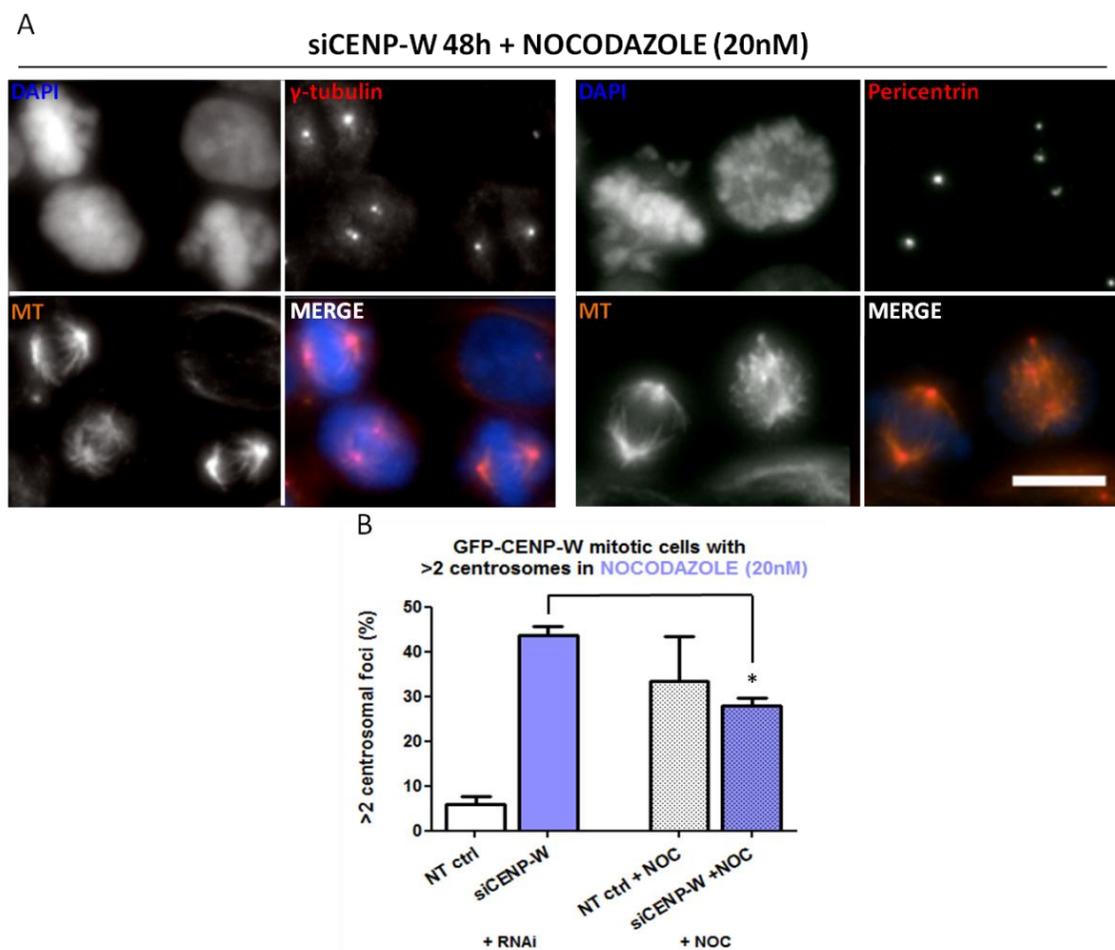


Figure S4.4. Unbalance of the forces in siCENP-W cells followed by spindle disruption with low dose of nocodazole. (A) Representative panel of GFP-CENP-W cells transfected with siCENP-W for 48h, incubated with 20nM nocodazole (NOC) for the last 4h of transfection and stained for pericentrin/ γ -tubulin (red) and α -tubulin (orange), scale bar 10 μ m. (B) Graph represents mean percentage of the mitotic cells with supernumerary centrosomes (based on pericentrin and γ -tubulin foci) following nocodazole and/or siRNA treatment (at least 100 cells were scored for each centrosomal marker). * p<0.01 (one-way ANOVA followed by Dunnetts multiple comparison).

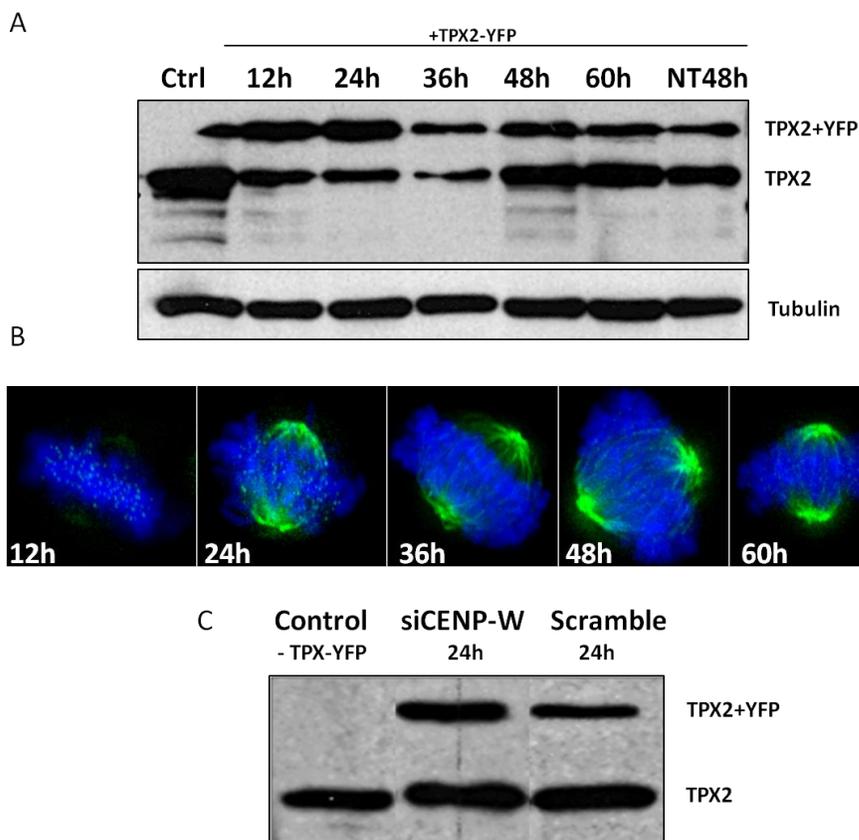


Figure S4.5. Evaluation of the overexpression of hTPX2 in GFP-CENP-W HeLa cells over time. (A) HeLa cells expressing GFP-CENP-W were transiently transfected with TPX2-YFP and/or non-targeting (NT) control siRNA. Samples harvested at indicated times were blotted and probed against TPX2 for detection of a tagged and endogenous protein. Tubulin was used as the protein loading control. (B) Representative image of GFP-CENP-W cells, fixed at indicated times, overexpressing TPX2-YFP protein (green) and stained with DAPI (blue). (C) HeLa GFP-CENP-W, co-transfected with TPX2-YFP and indicated siRNAs, do overexpress TPX2-YFP protein.

Acknowledgments

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4.7. REFERENCES

- Amaro A. C., Samora C. P., Holtackers R., Wang E., Kingston I. J., Alonso M., Lampson M., McAinsh A. D., Meraldi P. (2010). Molecular control of kinetochore-microtubule dynamics and chromosome oscillations. *Nature Cell Biology* **12**(4): 319-329.
- Bakhoun S. F., Thompson S. L., Manning A. L., Compton D. A. (2008). Genome stability is ensured by temporal control of kinetochore-microtubule dynamics. *Nature Cell Biology* **11**(1): 27-35.
- Borel F., Lohez O. D., Lacroix F. B., Margolis R. L. (2002). Multiple centrosomes arise from tetraploidy checkpoint failure and mitotic centrosome clusters in p53 and RB pocket protein-compromised cells. *Proceedings of the National Academy of Sciences* **99**(15): 9819.
- Cassimeris L., Morabito J. (2004). TOGp, the human homolog of XMAP215/Dis1, is required for centrosome integrity, spindle pole organization, and bipolar spindle assembly. *Molecular Biology of the Cell* **15**(4): 1580-1590.
- Cheeseman I., Desai A. (2008). Molecular architecture of the kinetochore-microtubule interface. *Nature Reviews Molecular Cell Biology* **9**(1): 33-46.
- Daum J., Potapova T., Sivakumar S., Daniel J., Flynn J., Rankin S., Gorbsky G. (2011). Cohesion Fatigue Induces Chromatid Separation in Cells Delayed at Metaphase. *Current Biology: CB* **21**: 1018-1024.
- DeLuca J. G., Dong Y., Hergert P., Strauss J., Hickey J. M., Salmon E., McEwen B. F. (2005). Hec1 and Nuf2 are core components of the kinetochore outer plate essential for organizing microtubule attachment sites. *Molecular Biology of the Cell* **16**(2): 519-531.
- Dionne M. A., Howard L., Compton D. A. (1999). NuMA is a component of an insoluble matrix at mitotic spindle poles. *Cell Motility and the Cytoskeleton* **42**(3): 189-203.
- Dumont S., Mitchison T. J. (2009). Force and length in the mitotic spindle. *Current Biology* **19**(17): R749-R761.
- Fukasawa K. (2005). Centrosome amplification, chromosome instability and cancer development. *Cancer letters* **230**(1): 6-19.
- Ganem N. J., Godinho S. A., Pellman D. (2009). A mechanism linking extra centrosomes to chromosomal instability. *Nature* **460**(7252): 278-282.
- Garrett S., Auer K., Compton D. A., Kapoor T. M. (2002). hTPX2 is required for normal spindle morphology and centrosome integrity during vertebrate cell division. *Current Biology* **12**(23): 2055-2059.
- Gergely F., Draviam V. M., Raff J. W. (2003). The ch-TOG/XMAP215 protein is essential for spindle pole organization in human somatic cells. *Genes & Development* **17**(3): 336-341.
- Gonczy P., Pichler S., Kirkham M., Hyman A. A. (1999). Cytoplasmic dynein is required for distinct aspects of MTOC positioning, including centrosome separation, in the one cell stage *Caenorhabditis elegans* embryo. *The Journal of Cell Biology* **147**(1): 135-150.
- Gordon M. B., Howard L., Compton D. A. (2001). Chromosome movement in mitosis requires microtubule anchorage at spindle poles. *The Journal of Cell Biology* **152**(3): 425.
- Gruss O. J., Vernos I. (2004). The mechanism of spindle assembly: functions of Ran and its target TPX2. *The Journal of Cell Biology* **166**(7): 949-955.
- Gruss O. J., Wittmann M., Yokoyama H., Pepperkok R., Kufer T., Sillje H., Karsenti E., Mattaj I. W., Vernos I. (2002). Chromosome-induced microtubule assembly mediated by TPX2 is required for spindle formation in HeLa cells. *Nature Cell Biology* **4**(11): 871-879.
- Habedanck R., Stierhof Y. D., Wilkinson C. J., Nigg E. A. (2005). The Polo kinase Plk4 functions in centriole duplication. *Nature Cell Biology* **7**(11): 1140-1146.
- Hartwell L. H., Weinert T. A. (1989). Checkpoints: controls that ensure the order of cell cycle events. *Science* **246**(4930): 629.
- Heald R., Tournebise R., Blank T., Sandaltzopoulos R., Becker P., Hyman A., Karsenti E. (1996). Self-organization of microtubules into bipolar spindles around artificial chromosomes in *Xenopus* egg extracts. *Nature* **382**(6590): 420-425.
- Holland A. J., Cleveland D. W. (2009). Boveri revisited: chromosomal instability, aneuploidy and tumorigenesis. *Nature Reviews Molecular Cell Biology* **10**(7): 478-487.
- Hori T., Amano M., Suzuki A., Backer C., Welburn J., Dong Y., McEwen B., Shang W., Suzuki E., Okawa K. (2008). CCAN makes multiple contacts with centromeric DNA to provide distinct pathways to the outer kinetochore. *Cell* **135**(6): 1039-1052.

- Howard J., Hyman A. A. (2007). Microtubule polymerases and depolymerases. *Current Opinion in Cell Biology* **19**(1): 31-35.
- Inanc B., Dodson H., Morrison C. G. (2010). A centrosome-autonomous signal that involves centriole disengagement permits centrosome duplication in G2 phase after DNA damage. *Molecular Biology of the Cell* **21**(22): 3866-3877.
- Jordan M. A., Thrower D., Wilson L. (1992). Effects of vinblastine, podophyllotoxin and nocodazole on mitotic spindles. Implications for the role of microtubule dynamics in mitosis. *Journal of Cell Science* **102**(3): 401.
- Kapoor T. M., Mayer T. U., Coughlin M. L., Mitchison T. J. (2000). Probing spindle assembly mechanisms with monastrol, a small molecule inhibitor of the mitotic kinesin, Eg5. *The Journal of Cell Biology* **150**(5): 975-988.
- Khodjakov A., Cole R. W., Oakley B. R., Rieder C. L. (2000). Centrosome-independent mitotic spindle formation in vertebrates. *Current Biology* **10**(2): 59-67.
- Logarinho E., Maffini S., Barisic M., Marques A., Toso A., Meraldi P., Maiato H. (2012). CLASPs prevent irreversible multipolarity by ensuring spindle-pole resistance to traction forces during chromosome alignment. *Nature Cell Biology* **14**(3): 295-303.
- Loncarek J., Hergert P., Khodjakov A. Centriole reduplication during prolonged interphase requires procentriole maturation governed by Plk1. *Current Biology* **20**(14): 1277-1282.
- Ma N., Tulu U., Ferenz N. P., Fagerstrom C., Wilde A., Wadsworth P. (2010). Poleward transport of TPX2 in the mammalian mitotic spindle requires dynein, Eg5, and microtubule flux. *Molecular Biology of the Cell* **21**(6): 979-988.
- Manning A. L., Compton D. A. (2007). Mechanisms of spindle-pole organization are influenced by kinetochore activity in mammalian cells. *Current Biology* **17**(3): 260-265.
- Martin-Lluesma S., Stucke V. M., Nigg E. A. (2002). Role of Hec1 in spindle checkpoint signaling and kinetochore recruitment of Mad1/Mad2. *Science* **297**(5590): 2267.
- Mattiuzzo M., Vargiu G., Totta P., Fiore M., Ciferri C., Musacchio A., Degrossi F. (2011). Abnormal kinetochore-generated pulling forces from expressing a N-terminally modified Hec1. *PLoS One* **6**(1): e16307.
- McAinsh A. D., Meraldi P., Draviam V. M., Toso A., Sorger P. K. (2006). The human kinetochore proteins Nnf1R and Mcm21R are required for accurate chromosome segregation. *The EMBO Journal* **25**(17): 4033-4049.
- McClelland S. E., Borusu S., Amaro A. C., Winter J. R., Belwal M., McAinsh A. D., Meraldi P. (2007). The CENP-A NAC/CAD kinetochore complex controls chromosome congression and spindle bipolarity. *The EMBO Journal* **26**(24): 5033-5047.
- Morgan D. O. (2007). *The cell cycle: principles of control*, New Science Press.
- Musacchio A., Salmon E. (2007). The spindle-assembly checkpoint in space and time. *Nature Reviews Molecular Cell Biology* **8**: 379-393.
- Nigg E. A. (2006). Cell biology: a licence for duplication. *Nature* **442**(7105): 874.
- Prendergast L., van Vuuren C., Kaczmarczyk A., Doering V., Hellwig D., Quinn N., Hoischen C., Diekmann S., Sullivan K. F. (2011). Premitotic Assembly of Human CENPs-T and-W Switches Centromeric Chromatin to a Mitotic State. *PLoS Biology* **9**(6): e1001082.
- Rieder C. L., Salmon E. (1994). Motile kinetochores and polar ejection forces dictate chromosome position on the vertebrate mitotic spindle. *The Journal of Cell Biology* **124**(3): 223.
- Sharp D., Rogers G., Scholey J. (2000). Microtubule motors in mitosis. *Nature* **407**: 41-47.
- Stevens D., Gassmann R., Oegema K., Desai A. (2011). Uncoordinated loss of chromatid cohesion as a common outcome of extended metaphase arrest. *PLoS One* **6**(8): e22969.
- Sullivan K. F. (2001). A solid foundation: functional specialization of centromeric chromatin. *Current Opinion in Genetics & Development* **11**(2): 182-188.
- Tanenbaum M. E., Mac, uring L. (2008). Dynein, Lis1 and CLIP-170 counteract Eg5-dependent centrosome separation during bipolar spindle assembly. *The EMBO Journal* **27**(24): 3235-3245.
- Thein K. H., Kleylein-Sohn J., Nigg E. A., Gruneberg U. (2007). Astrin is required for the maintenance of sister chromatid cohesion and centrosome integrity. *The Journal of Cell Biology* **178**(3): 345-354.
- Varga V., Leduc C., Bormuth V., Diez S., Howard J. (2009). Kinesin-8 motors act cooperatively to mediate length-dependent microtubule depolymerization. *Cell* **138**(6): 1174-1183.

- Weaver B. A. A., Cleveland D. W. (2006). Does aneuploidy cause cancer? *Current Opinion in Cell Biology* **18**(6): 658-667.
- Winey M. (1999). Cell cycle: driving the centrosome cycle. *Current biology* **9**(12): R449-R452.
- Wittmann T., Hyman A., Desai A. (2001). The spindle: a dynamic assembly of microtubules and motors. *Nature Cell Biology* **3**: E28-E34.
- Wittmann T., Wilm M., Karsenti E., Vernos I. (2000). TPX2, a novel *Xenopus* MAP involved in spindle pole organization. *The Journal of Cell Biology* **149**(7): 1405-1418.
- Zachariae W., Nasmyth K. (1999). Whose end is destruction: cell division and the anaphase-promoting complex. *Genes & Development* **13**(16): 2039.

CHAPTER 5

General discussion

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5.1. CENP-W/T/S/X complex in mitosis and the Checkpoint pathways

Faithful chromosome segregation is a prerequisite for the maintenance of the genome stability and its failure – of tumorigenesis. Consequently, over the course of evolution vertebrate cells have developed molecular machineries and mechanisms to ensure proper genetic material division. The most spectacular and critical is the assembly of two of them: the kinetochores, starting in late G2 phase, and the spindle during mitosis. The main function of the kinetochore is to interact with the centromeric chromatin on one hand and spindle microtubules on the other (Przewloka and Glover 2009). Centromeres are chromosomal loci epigenetically determined by the presence of CENP-A nucleosomes (Allshire and Karpen 2008), which are responsible for the equal segregation of the sister chromatids during mitosis, by directing the assembly of kinetochores. Despite of the considerable progress being made in understanding their composition, the recruitment hierarchy of their components and the principles of their regulation, many of these advances in kinetochore studies are accompanied by a growing number of unanswered question about the function of the individual subunits and of how the structure of different sub-complexes relates to function (Przewloka and Glover 2009; Perpelescu and Fukagawa 2011).

In this thesis, I investigated the requirement for function in kinetochore assembly of the two histone fold domain containing proteins of the CCAN, CENP-W and CENP-T, that form a dimeric complex. Accordingly to the notion that there is a subset of conserved CCAN proteins able to propagate kinetochore identity, we hypothesized that CENP-W/T complex can stimulate a proper kinetochore assembly independently of CENP-A based centromere assembly. Our analysis of the mitotic kinetics revealed that the complex is required for a robust mitosis in each cell cycle. The finding that CENP-W/T complex associates with canonical H3 nucleosomes (Hori *et al.* 2008), coupled with the fact that CENP-T together with CENP-C is sufficient for *de novo* kinetochore assembly in the absence of CENP-A (Gascoigne *et al.* 2011), and further studies of the timing of assembly and heritability of CENP-W/T complex, have lead to a hypothesis that there are two distinct phases in the centromeric chromatin cell cycle, and that CENP-W/T assembly during late S/G2 provides a molecular switch into a mitotic state and a platform upon which kinetochore can assemble (Prendergast *et al.* 2011).

Consistently, further investigation of the cells depleted of CENP-W/T complex revealed severe defects in a bipolar mitotic spindle formation, coupled with an impairment in recruitment to the kinetochores one of the key microtubule-binding component – Hec1. This finding was confirmed by the EM analysis of the DT40 kinetochores depleted of CENP-T, which denotes

that the latter protein interacts directly via its flexible N-terminus tail with the Ndc80 complex of the KMN network (Suzuki *et al.* 2011). We also discovered that kinetochores depleted of the CENP-W/T complex exhibited a profound inability to sustain mitotic checkpoint protein signaling, mediated by Mad1-Mad2 pathway. Consistent with their deficiency (Emre *et al.* 2011), dividing cells frequently displayed lagging chromatids at anaphase, at least some of which were due to persisting merotelic linkages. However, Bub1 and BubR1 checkpoint proteins were still localizing to the unattached prometaphase kinetochores of the depleted cells, which emphasize previously suggested existence of the two independent branches of the SAC signaling, one mediated by Mad-proteins and the other by Bub-proteins (Liu *et al.* 2003). The Bub1 and BubR1 proteins were previously found to depend for their kinetochore localization on KNL-1 protein (Kiyomitsu *et al.* 2007), which in turns is involved in a dimeric complex with another checkpoint protein Zwint-1 (Starr *et al.* 2000), a linker between the KMN and the RZZ complex. Overall, these data suggest that KNL-1 alone might play a checkpoint role.

Our focus on the mitotic checkpoint was originally dictated by the means to identify a possible candidate for a profound mitotic timing delay and correlate that with a subsequent cell fate, since the chronic checkpoint activation has been extensively studied as a potential target in cancer therapy (Kops *et al.* 2005; Weaver and Cleveland 2006; Holland and Cleveland 2009), which will be discussed below. However, in the light of our findings and the recent advancement in the field of HFD CCAN proteins, we propose a model for proper kinetochore-mediated checkpoint signaling. Since CENP-T-W-S-X form a stable heterotetramer (Nishino *et al.* 2012), and CENP-S/X complex was found to be required for KNL-1 kinetochore recruitment (Amano *et al.* 2009), we propose that association of CENP-T/W/S/X with Mis12 subunits is critical, and distal interactions with either KNL-1 (via CENP-S/X) or Ndc80 (via CENP-W/T) specify the two distinct pathways of the SAC signaling. Therefore, we speculate that in the absence of CENP-W/T complex an 'arm' of the CCAN could be in touch with the KNL-1-dependent checkpoint root, via CENP-S/X complex. It would be very informative in the future to study mitotic checkpoint association with the kinetochores simultaneously depleted of the CENP-W/T and CENP-S/X complexes.

5.2. To die or not to die? - a link between mitotic arrest and cell death

The relationship between the function of the spindle assembly checkpoint and cellular responses and sensitivity to antimitotic drugs in mammalian cells is an issue of a great general and clinical interest. Drugs that disrupt microtubules cause cells to accumulate in mitosis due to spindle

checkpoint signaling from unattached kinetochores. Chronic checkpoint activation can result in mitotic slippage, producing aneuploid or polyploid cells. Alternatively, cell death can occur via apoptosis, after failed cytokinesis and entry into G1, or execution of a death pathway directly from mitosis. Prediction of cell responses to antimetabolic drugs has a clinical importance, however little is known about the pathways determining cell fate following treatment with antimetabolic drugs (Yamada and Gorbsky 2006; Shi *et al.* 2008). The variable mitotic checkpoint gene status between different cancers and associated drug resistance, adds up to the complexity of identifying the general link between the SAC-dependent mitotic arrest and cell death. The latter forces scientist to continually search for a new potential targets. Therefore, the long-term goal of this thesis was originally posed by the question: could we find something out about the link between mitotic arrest and cell death that we might then be able to use to screen for genes and drugs that could affect it? Since caspase-3 activity has been associated with a prolonged mitotic arrest, followed by mitotic checkpoint degradation and slippage (Baek *et al.* 2005), we hypothesized that caspase-3 might be required for a mitotic checkpoint function in a normal mitosis.

The direct examination of caspase-3 involvement in a normal mitosis proved a low yield pathway. Our observations were then further confirmed by Lee *et al.* (2011) demonstrating that inhibition of different caspases individually, in combination, or in toto did not affect the duration or fidelity of mitosis in otherwise untreated cells (Lee *et al.* 2011). Furthermore, a recent report attempted to determine the response of individual cells from 15 tumor cell lines to three different types of antimetabolic drugs (Gascoigne and Taylor 2008). Based on these findings, it was suggested that the balance between two competing networks, one involving caspase activation and the other protecting cyclin B1 from degradation, determines cell fate in response to antimetabolic drugs (Gascoigne and Taylor 2008). This implies two outcomes: first, that agents restoring apoptotic pathways may be effective in combination with antimetabolic drugs, especially against drug-resistant tumors (Kim *et al.* 2008). Second, as cancer therapy using antimetabolic drugs exploits the cellular responses to erroneous kinetochore-MT attachment, further investigation of the mechanism and regulation of the chromosome segregation machinery is necessary and might contribute to the discovery of new improved treatments.

Indeed, the data generated here provide an important resource in further uncovering the link between defects in chromosome segregation machinery and cell fate. The examination of mitotic arrest kinetics and spindle structure was focused most directly on understanding functional roles of CENP-W. However, further discoveries gained an interesting scope for the

whole project. The direct examination of caspase activity in cells arrested in mitosis via siRNA-mediated CENP-W depletion resulted in a surprisingly low activity level, contrary to our previous findings of nocodazole arrested cells. One possible explanation for such a tremendous difference of CENP-W dependent arrest from the one caused by spindle disruption, could be due to the fact that survivin, which is a potent caspase-9 inhibitor (Mita *et al.* 2008), inhibits the latter only when bound to spindle microtubules (Li *et al.* 1998). Thus, in the absence of microtubules, nocodazole-arrested cells eventually faster reach the balance of caspase activation and execute apoptosis. Another explanation arises from Bekier *et al.* (2009) work, which shows in a very acute manner that the length of mitotic arrest induced by microtubule-stabilizing (Taxol) drugs determines cell death (Bekier *et al.* 2009). It is suggested that there is a point of no return during prolonged mitotic block and that the mechanism of cell death is linked to gradual changes in cellular biochemistry, by: 1) differential decay of mRNAs encoding proteins that influence apoptotic cell death during mitosis which leads to a gradual inclination towards death, 2) the variability in the fate of cells blocked in mitosis for less than 15h is related to differences in their original levels of proapoptotic and antiapoptotic mRNAs and proteins when they arrive in mitosis (Bekier *et al.* 2009).

Regardless of the explanation a subsequent outcome, that was stimulated by the original question as well as the intermediate observations (no caspase-3 in RNAi arrested cells), was the development of a system in which mitotic arrest leads to very potent cell death. Unexpectedly, we observed that death occurs rapidly after ZM447439-mediated release from siRNA-W mitotic arrest. Since the balance between cell cycle progression and apoptosis is important for both surveillance against genomic defects and responses to drugs that arrest the cell cycle (Yamada and Gorbsky 2006; Lee *et al.* 2011), we propose CENP-W protein as an ideal candidate for a chemotherapy target with a strong potency to change that balance towards cell death. The advantage of such based therapy, apart from potent cell death, is that, in principle it reduces side effects, such as neurotoxicity problem of nondividing cells, as well as overcomes weakened mitotic checkpoint, which is often associated with many cancers (Cahill *et al.* 1998) and contributes to drug resistance.

While preliminary in this thesis, these experiments: a) provide a new and biomedical relevant avenue of research for CENP-W; and b) fulfill the original goal of finding a way to link centromeres, mitosis and cell death. Aurora B inhibition-mediated cell death following CENP-W siRNA is exactly what I had hoped to find and this result can stimulate further investigation to develop screens for the more effective phenotype of CENP-W depletion.

5.3. The spindle – dynamic balance of the forces

For balanced chromosome segregation in mitosis, kinetochores on sister chromatids bind and pull on microtubules emanating from opposite spindle poles. During cell division the mitotic spindle is the second intricate structure assembled in a very coordinated manner to provide means for a bipolar chromosome attachment and segregation. The role of spindle force balance in overall mitotic execution is complicated and requires orchestrated action of the mechanochemical motors, microtubule associated proteins (MAPs) and plus-end associated regulatory proteins, such as microtubule depolymerases. Importantly, the specific movements of the mitotic spindle are not driven by individual mitotic motors acting on their own, but rather multiple complementary and antagonistic motors function simultaneously (Sharp *et al.* 2000). Therefore any change in the balanced equilibrium may result in a bipolar spindle collapse.

The key goal of this work was to find out exactly how CENP-W depletion affects microtubule dynamics and kinetochore interactions and how the latter can influence bipolar spindle formation. In general, mitotic cell shape and polarity are dependent on the precise centrosome duplication, occurring exactly once in a preceding interphase and generating exactly two opposite spindle pole-mediated sites of microtubule nucleation and anchoring, and therefore providing means for chromosome biorientation. Consequently, molecular perturbations that lead to loss of spindle pole integrity are normally associated with misaligned chromosomes and a significant mitotic delay. However, these perturbations are usually related to the loss of centrosomal components and/or a regulatory molecule that can act on both centrosomes and kinetochores, e.g. CLASP (Logarinho *et al.* 2012). To date, only expression of modified Hec1, a non-centrosomal protein was reported to promote spindle pole fragmentations, mediated by abnormal kinetochore generated pulling forces (Mattiuzzo *et al.* 2011). Interestingly, a recent report demonstrated that although an obvious kinetochore component, Hec1 might also be a centrosomal component (Diaz-Rodriguez *et al.* 2008). Whether it functions there or simply gathers there by its diffusion capability along microtubules is not clear. However, it underscores the possibility that kinetochore components play a more direct role in spindle pole structure/function than generally realized.

In this study, I demonstrated, for the first time, how the loss of inner centromere component of the CCAN can alter the balanced forces within the spindle and exerts an excessive pulling forces, presumably mediated by plus-end directed CENP-E, towards centrosomes to promote irreversible multipolarity. Trying to integrate the big picture is very difficult and requires further

investigation of motor candidates being affected or altered by CENP-W depletion. However, it is clear that kinetochore-based mechanisms of the spindle - binding, motoring, fluxing of microtubules – are: a) highly regulated and b) influenced by kinetochore proteins.

5.4. Assessment of aims and future perspectives

I successfully completed the aims of this thesis: **1)** I demonstrated, using two independent approaches (population-based biochemical assays and individual live-cell based evaluation) that neither caspase-3 activity is required nor associated with any mitotic checkpoint protein modulation in a normal mitosis; **2)** I revealed further functional insight into the roles of CENP-W and CENP-T in kinetochore assembly, particularly their requirement in a stable kinetochore-microtubule attachment formation and recruitment for the specific branch of the SAC proteins to localize at the unattached kinetochores; **3)** I discovered that unbalance of the forces generated within the spindle microtubules stands behind the mechanical disruption of the intact spindle poles and results in the formation of multipolar spindles in cells depleted of CENP-W protein.

While informative, the results obtained during this project are not exhaustive. They will however provide a springboard for future research into the development of the further links between both, inner and outer kinetochore components, as well as the influence of the latter on the global establishment and maintenance of the mitotic spindle and associated centrosomes. Furthermore, this work emphasize the need for finding out a system to knock out CENPs proteins in human cells more definitively, e.g. by applying an inducible degron for rapid protein depletion. Overcoming the technical difficulties associated with non-ideal siRNA conditions, by applying fluorescently-tagged expression of the gene of interest and discerning between depleted and non-depleted populations, makes only a minor improvement to the problem. Therefore, this study opens the field for further discoveries. If we had a better system for CENP-W and –T depletion, we could propose a set of experimental strategies to definitively distinguish between the observed differences in phenotypes, which presumably should not be as dramatic. Having the latter established, we could measure, for example, microtubule dynamics and microtubule flux and then begin to think about how spindle architecture and function are actually affected. Finally, this thesis with its preliminary findings regarding the mitotic spindle regulation pathways highlights what an intricate device the spindle is and that fundamental research into its mechanisms continues to provide novel ideas for the fight against cancer.

5.5. REFERENCES

- Allshire R. C., Karpen G. H. (2008). Epigenetic regulation of centromeric chromatin: old dogs, new tricks? *Nature Reviews Genetics* **9**(12): 923-937.
- Amano M., Suzuki A., Hori T., Backer C., Okawa K., Cheeseman I. M., Fukagawa T. (2009). The CENP-S complex is essential for the stable assembly of outer kinetochore structure. *The Journal of Cell Biology* **186**(2): 173.
- Baek K., Shin H., Jeong S., Park J., McKeon F., Lee C., Kim C. (2005). Caspases-dependent cleavage of mitotic checkpoint proteins in response to microtubule inhibitor. *Oncology Research* **15**(3): 161-168.
- Bekier M. E., Fischbach R., Lee J., Taylor W. R. (2009). Length of mitotic arrest induced by microtubule-stabilizing drugs determines cell death after mitotic exit. *Molecular Cancer Therapeutics* **8**(6): 1646-1654.
- Cahill D. P., Lengauer C., Yu J., Riggins G. J., Willson J. K. V., Markowitz S. D., Kinzler K. W., Vogelstein B. (1998). Mutations of mitotic checkpoint genes in human cancers. *Nature* **392**(6673): 300-303.
- Diaz-Rodriguez E., Sotillo R., Schwartzman J. M., Benezra R. (2008). Hec1 overexpression hyperactivates the mitotic checkpoint and induces tumor formation in vivo. *Proceedings of the National Academy of Sciences* **105**(43): 16719.
- Emre D., Terracol R., Poncet A., Rahmani Z., Karess R. E. (2011). A mitotic role for Mad1 beyond the spindle checkpoint. *Journal of Cell Science* **124**(10): 1664.
- Gascoigne K. E., Takeuchi K., Suzuki A., Hori T., Fukagawa T., Cheeseman I. M. (2011). Induced ectopic kinetochore assembly bypasses the requirement for CENP-A nucleosomes. *Cell* **145**(3): 410-422.
- Gascoigne K. E., Taylor S. S. (2008). Cancer cells display profound intra-and interline variation following prolonged exposure to antimetabolic drugs. *Cancer Cell* **14**(2): 111-122.
- Holland A. J., Cleveland D. W. (2009). Boveri revisited: chromosomal instability, aneuploidy and tumorigenesis. *Nature Reviews Molecular Cell Biology* **10**(7): 478-487.
- Hori T., Amano M., Suzuki A., Backer C., Welburn J., Dong Y., McEwen B., Shang W., Suzuki E., Okawa K. (2008). CCAN makes multiple contacts with centromeric DNA to provide distinct pathways to the outer kinetochore. *Cell* **135**(6): 1039-1052.
- Kim M., Liao J., Dowling M. L., Voong K. R., Parker S. E., Wang S., El-Deiry W. S., Kao G. D. (2008). TRAIL inactivates the mitotic checkpoint and potentiates death induced by microtubule-targeting agents in human cancer cells. *Cancer Research* **68**(9): 3440.
- Kiyomitsu T., Obuse C., Yanagida M. (2007). Human Blinkin/AF15q14 is required for chromosome alignment and the mitotic checkpoint through direct interaction with Bub1 and BubR1. *Developmental Cell* **13**(5): 663-676.
- Kops G. J. P. L., Weaver B. A. A., Cleveland D. W. (2005). On the road to cancer: aneuploidy and the mitotic checkpoint. *Nature Reviews Cancer* **5**(10): 773-785.
- Lee K., Kenny A. E., Rieder C. L. (2011). Caspase activity is not required for the mitotic checkpoint or mitotic slippage in human cells. *Molecular Biology of the Cell* **22**(14): 2470-2479.
- Li F., Ambrosini G., Chu E. Y., Plescia J., Tognin S., Marchisio P. C., Altieri D. C. (1998). Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature* **396**(6711): 580-583.
- Liu S., Hittle J., Jablonski S., Campbell M., Yoda K., Yen T. (2003). Human CENP-I specifies localization of CENP-F, Mad1 and Mad2 to kinetochores and is essential for mitosis. *Nature Cell Biology* **5**: 341-345.
- Logarinho E., Maffini S., Barisic M., Marques A., Toso A., Meraldi P., Maiato H. (2012). CLASPs prevent irreversible multipolarity by ensuring spindle-pole resistance to traction forces during chromosome alignment. *Nature Cell Biology* **14**(3): 295-303.
- Mattiuazzo M., Vargiu G., Totta P., Fiore M., Ciferri C., Musacchio A., Degrossi F. (2011). Abnormal kinetochore-generated pulling forces from expressing a N-terminally modified Hec1. *PLoS One* **6**(1): e16307.
- Mita A. C., Mita M. M., Nawrocki S. T., Giles F. J. (2008). Survivin: key regulator of mitosis and apoptosis and novel target for cancer therapeutics. *Clinical Cancer Research* **14**(16): 5000-5005.
- Nishino T., Takeuchi K., Gascoigne K. E., Suzuki A., Hori T., Oyama T., Morikawa K., Cheeseman I. M., Fukagawa T. (2012). CENP-TWSX Forms a Unique Centromeric Chromatin Structure with a Histone-like Fold. *Cell* **148**(3): 487-501.
- Perpelescu M., Fukagawa T. (2011). The ABCs of CENPs. *Chromosoma*: 1-22.

- Prendergast L., van Vuuren C., Kaczmarczyk A., Doering V., Hellwig D., Quinn N., Hoischen C., Diekmann S., Sullivan K. F. (2011). Premitotic Assembly of Human CENPs-T and-W Switches Centromeric Chromatin to a Mitotic State. *PLoS Biology* **9**(6): e1001082.
- Przewloka M. R., Glover D. M. (2009). The kinetochore and the centromere: a working long distance relationship. *Annual Review of Genetics* **43**: 439-465.
- Sharp D., Rogers G., Scholey J. (2000). Microtubule motors in mitosis. *Nature* **407**: 41-47.
- Shi J., Orth J. D., Mitchison T. (2008). Cell type variation in responses to antimitotic drugs that target microtubules and kinesin-5. *Cancer Research* **68**(9): 3269.
- Starr D. A., Saffery R., Li Z., Simpson A. E., Choo K., Yen T. J., Goldberg M. L. (2000). HZWint-1, a novel human kinetochore component that interacts with HZW10. *Journal of Cell Science* **113**(11): 1939.
- Suzuki A., Hori T., Nishino T., Usukura J., Miyagi A., Morikawa K., Fukagawa T. (2011). Spindle microtubules generate tension-dependent changes in the distribution of inner kinetochore proteins. *The Journal of cell biology* **193**(1): 125-140.
- Weaver B. A. A., Cleveland D. W. (2006). Does aneuploidy cause cancer? *Current Opinion in Cell Biology* **18**(6): 658-667.
- Yamada H., Gorbsky G. (2006). Spindle checkpoint function and cellular sensitivity to antimitotic drugs. *Molecular Cancer Therapeutics* **5**(12): 2963-2969.

Appendix A:

Plasmid sequence

Confirmed sequence of destination vector with mCherry tag (red writing), that was gateway adapted with SacII (yellow highlighted), with cloned α -tubulin 1B isoform (blue writing)

TAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCATATATGGAGTTCCGCGT
TACATAACTTACGGTAAATGGCCCGCTGGCTGACCGCCCAACGACCCCGCCCATTTGACGT
CAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCATTGACGTCAATGGGTG
GAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCC
CCCTATTGACGTCAATGACGGTAAATGGCCCGCTGGCATTATGCCCAGTACATGACCTTAT
GGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGT
TTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCAC
CCCATTGACGTCAATGGGAGTTTGTGTTTGGCACCAAAATCAACGGGACTTTCAAAATGTGCG
TAACAACCTCCGCCCATTTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAA
GCAGAGCTGGTTTAGTGAACCGTCAGATCCGCTAGCGCTACCGGTATGGTGAGCAAGGGCG
AGGAGGATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAGGTGCACATGGAGGGCTC
CGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCACC
CAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTC
CCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAGCACCCCGCCGACATCCCCGACTACT
TGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGC
GTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCT
GCGCGGCACCAACTTCCCTCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAG
GCCTCCTCCGAGCGGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGCAGAGGC
TGAAGCTGAAGGACGGCGGCCACTACGACGCTGAGGTCAAGACCACCTACAAGGCCAAGAA
GCCCGTGCAGCTGCCCGGCGCCTACAACGTCAACATCAAGTTGGACATCACCTCCCACAACG
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GGACGAGCTGTACAAGAGATCTCGAGCTCAAGCTTCAAGTTCGAGTTCGACGGTACCGACA
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GAGACGGGCGCTGGCAAGCACGTGCCCGGGCTGTGTTTGTAGACTTGGAAACCCACAGTCAT
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AGGAAGATGCTGCCAATAACTATGCCCGAGGGCACTACACCATTGGCAAGGAGATCATTGA
CCTTGTGTTGGACCGAATTCGCAAGCTGGCTGACCAGTGCACCGGTCTTCAGGGCTTCTTGTT
TTCCACAGCTTTGGTGGGGAAGTGGTTCTGGGTTACCTCCCTGCTCATGGAACGTCTCTCA
GTTGATTATGGCAAGAAGTCCAAGCTGGAGTTCTCCATTTACCCAGCACCCAGGTTTCCAC
AGCTGTAGTTGAGCCCTACAACCTCCATCCTCACCACCCACACCACCCTGGAGCACTCTGATT
GTGCCCTCATGGTAGACAATGAGGCCATCTATGACATCTGTGCTAGAAACCTCGATATCGAG
CGCCCAACCTACACTAACCTTAACCGCTTATTAGCCAGATTGTGTCCTCCATCACTGCTTCC
CTGAGATTTGATGGAGCCCTGAATGTTGACCTGACAGAATTCCAGACCAACCTGGTGCCTTA
CCCCGCATCCACTTCCCTCTGGCCACATATGCCCTGTCATCTCTGCTGAGAAAGCCTACCA
TGAACAGCTTCTGTAGCAGAGATCACCAATGCTTGCTTTGAGCCAGCCAACCAGATGGTGA
AATGTGACCCTCGCCATGGTAAATACATGGCTTGCTGCCTGTTGTACCGTGGTGACGTGGTTC
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TGGTGCCCCACTGGCTTCAAGGTTGGCATCAACTACCAGCCTCCACTGTGGTGCCTGGTGG
AGACCTGGCCAAGGTACAGAGAGCTGTGTGCATGCTGAGCAACACCACAGCCATTGCTGAG
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CAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAATTTACAAATAAAGCATTTTTTT
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CGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCGATTTAGAGCTTGACG
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GGCGTGGCAAGTGTAGCGGTACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGC
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TTTTGCAAAGATCGATCAAGAGACAGGATGAGGATCGTTTCGCATGATTGAACAAGATGGAT
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ACAATCGGCTGCTGATGCCGCCGCTTCCGGCTGTCCGGCTGTGAGCGCAGGGGCCCGCTTTTT
GTCAAGACCGACTGTCCGGTGCCTGAATGAACTGCAAGACGAGGCAGCGCGGCTATCGT
GGCTGGCCACGACGGCGTTCCTTTCGCGAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGG
GACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCC
GAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTG
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CTTGTGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCCG
CAGGCTCAAGGCGAGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCT
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GTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGG
CGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTTCGACGCGCATCGC
CTTCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAA
GCGACGCCAACCTGCCATCACGAGATTTTCGATTCACCCGCCGCTTCTATGAAAGGTTGGG
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AACCCGCGCTATGACGGCAATAAAAAGACAGAATAAAACGCACGGTGTGGGGTCTTTGTTT
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GGCCAATACGCCCGCGTTTTCTTCTTTTCCCACCCACCCCAAGTTCCGGGTGAAGGCCCA
GGGCTCGCAGCCAACGTCGGGGCGGCAGGCCCTGCCATAGCCTCAGGTTACTCATATATACT
TTAGATTGATTTAAACTTCAATTTTAAATTTAAAGGATCTAGGTGAAGATCCTTTTTGATAA
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AGATCAAAGGATCTTCTTGAGATCCTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAA
AACCACCGCTACCAGCGGTGGTTTTGTTGCCGGATCAAGAGCTACCAACTTTTTTCCGAAG
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CCACACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGT
GGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGG
ATAAGGCGCAGCGGTCGGGCTGAACGGGGGTTTCGTGCACACAGCCAGCTTGGAGCGAAC
GACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAAGCGCCACGCTTCCCGAA
GGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGG
GAGCTTCCAGGGGAAACGCCTGGTATCTTTATAGTCTGTGCGGTTTTCCGCCACCTCTGACTT
GAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACG
CGGCCTTTTTACGGTTCCTGGCCTTTTGTGCTGGCCTTTTGTCTCACATGTTCTTCTGCGTTATC
CCCTGATTCTGTGGATAACCGTATTACCGCCATGCAT

Appendix B: Authors Publications

GLYNN, M., **KACZMARCZYK, A.**, PRENDERGAST, L., QUINN, N. & SULLIVAN, K. F.
(2010) Centromeres: assembling and propagating epigenetic function. *Subcell Biochem*,
50, 223-49.

[Appendix B can be found here](#)

Appendix C: Authors Publications

PRENDERGAST, L., VAN VUUREN, C., **KACZMARCZYK, A.**, DOERING, V.,
HELLWIG, D., QUINN, N., HOISCHEN, C., DIEKMANN, S. & SULLIVAN, K. F.
(2011) Premitotic assembly of human CENPs -T and -W switches centromeric
chromatin to a mitotic state. *PLoS Biol*, 9, e1001082.

[Appendix C can be found here](#)