The role of ZW10 and its binding partners in chromosome segregation

by

Irina Michailovna Shapiro

M.S. Biophysics
St.Petersburg State Technical University, St.Petersburg, Russia, 2000

SUBMITTED TO THE DEPARTMENT OF BIOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BIOLOGY
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

SEPTEMBER 2007

Signature of Author: _____

Department of Biology
August 31, 2007

Certified by: _____

Peter K. Sorger
Professor of Systems Biology - HMS
Professor of Biological Engineering - MIT
Thesis Supervisor

Accepted by: _____

Stephen P. Bell
Professor of Biology
Chairman, Biology Graduate Committee

The role of ZW10 and its binding partners in chromosome segregation

by

Irina M. Shapiro

Submitted to the Department of Biology on August 31, 2007
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Biology

Abstract

Kinetochores are proteinacious structures that assemble on centromeric DNA and fulfill several important functions during chromosome segregation. They attach chromosomes to microtubules of the mitotic spindle in a bipolar fashion and monitor the state of the kinetochore-microtubule attachment. In case errors are present, a monitoring mechanism, the spindle assembly checkpoint, inhibits metaphase to anaphase transition until proper bipolar attachments are formed. The mechanics of the attachment process is poorly understood and functions of many players involved are largely unknown. The relationship between the formation of kinetochore-microtubule attachment and its monitoring by the checkpoint remains unclear.

I combined an RNAi technology with the high resolution microscopy to analyze the function of six microtubule-binding proteins and a checkpoint protein complex ROD/ZW10/Zwilch (RZZ) in chromosome segregation and spindle checkpoint signaling. I discovered a non-redundant role for CLIP-170, dynein/dynactin, LIS1 and TOG1 in chromosome congression to the metaphase plate. Selective depletion of dynein/dynactin and CLIP-170 from mitotic kinetochores by RNAi of ZW10, uncovered a novel role for these proteins in the initial kinetochore-microtubule encounter. Surprisingly, my results also demonstrate that ZW10 functions in the spindle checkpoint signaling independently of previously proposed downstream player, Mad2. In addition, I identified a ZW10's interaction with BubR1 that may suggest a possible role for ZW10 in the checkpoint signaling pathway. Thus RZZ complex acts at the interphase of attachment and signaling at kinetochores suggesting a close link between structural establishment of kinetochore-microtubule binding and its monitoring by the spindle checkpoint, in contrary to classical thinking.

Thesis supervisor: Peter K. Sorger
Title: Professor of Systems Biology – HMS, Professor of Biological Engineering - MIT

Acknowledgements

I would like to thank Peter for giving me the opportunity to join his lab. I am grateful to you for creating a wonderful research environment in which no experimental approach is unthinkable. I have learned a great deal from your way of looking at problems from systems point of view and from your desire to embrace new technology.

I would like to thank Frank Solomon for his help as a member of my Thesis committee from the beginning to the end and for his support during the frustrations of my graduate career. Thank you for listening to my complaints, concerns and fears and for your always objective advice. Knowing that you are there to turn to made my problems more bearable.

I would like to thank Frank Gertler for his help as a member of my Thesis committee from the beginning to the end and for his helpful input and encouragement. I am grateful to you for giving me the opportunity to join your lab and for your support during the writing of this thesis. You invaluable advice, sense of humor and enthusiasm made the whole experience much more pleasant.

I would like to thank Steve Bell and Jagesh Shah for joining my defense committee.

I would like to thank Viji Draviam for everything she had done for me over these years. You taught me a great deal of what I know now about science, experimental research and scientific writing. I am also forever grateful to you for teaching me how to think about scientific questions and how to approach scientific problems. You also taught me to accept nothing less than the best science. Your enthusiasm, sense of humor and an everyday smile made my day to day life happy and something to look forward to. You are one of the best scientists I know, and I am grateful for having had an opportunity to work with you and learn from you.

I would also like to thank all past and present members of Sorger lab who assisted with my experiments and made an environment in the lab so exiting and pleasant to come to everyday. I am grateful to Patrick Meraldi and Andrew McAinsh for valuable scientific discussions, experimental advice and a constant source of enthusiasm and humor. I am grateful to Suzanne Gaudet for her encouragement and a truly broad knowledge of science that she shares with everyone. I would like to thank Peter De Wulf for teaching me velocity sedimentation techniques. Finally, I must thank Heather Hess, Ying Yue and Kate Leitermann for their help along the way.

I would like to thank graduate students in the Sorger lab: Dan Rines, Jessica Tytell, Emily Gillett, Chris Espelin, Rob Hagan, Stephanie Xie, John Albeck and Bree Aldridge. You were wonderful at welcoming me in the lab, lending a hand and answering tonnes of questions I had. Working with you and learning from you was a pleasure.

Finally, I would like to thank my family for their love and support over the years. Many thanks to my parents for encouraging me to follow my dreams and pointing me back to the right direction when things were not going great and I was about to change tracks. I would like to thank Roma and Julia for making everything in life more wonderful. Roma, I am grateful to you for your patience and support through the ups and downs, thank you for believing in me even when I did not believe in myself anymore. Julia, thank you for keeping me focused on what really matters. Knowing that no matter how things are in the lab, I will always be a wife and a mother, keeps things in prospective.

Table of contents

Chapter 1

Kinetochore-microtubule attachment and spindle checkpoint signaling.

Outline

1. Introduction: chromosome segregation, genomic instability and the spindle checkpoint.

Genomic integrity is crucial for successful propagation of genetic material through generations. In every division a cell has to ensure that both of its progenies receive exactly one copy of each chromosome. Chromosomes attach to microtubules (MTs) of the mitotic spindle in a bipolar fashion and position themselves at the spindle equator so that upon dissolution of sister chromatid cohesion each daughter cell obtains one chromosome of each kind. Kinetochore- MT attachment is an error prone process since MTs exhibit dynamic instability and capture kinetochores in a stochastic fashion. The spindle assembly checkpoint monitors the state of kinetochore- MT attachment and delays cells at the metaphase to anaphase transition until all chromosomes become correctly attached to spindle MTs. Despite the fact that cell division has been studied for over a century, structural details of the kinetochore-MT interface and biochemical functions of the spindle checkpoint machinery remain unclear.

Chromosomal instability (CIN), a phenotype in which cell division is accompanied by an abnormally high rate of chromosome loss and gain(Lengauer et al., 1997), contributes to genomic instability that is thought to be critical for tumorogenesis (Hanahan and Weinberg, 2000). An outstanding question in the field remains whether aneuploidy is a cause for cellular transformation or whether it is a consequence of cancer cells having an abnormal cell cycle. Chromosome missegregation caused by errors in spindle assembly checkpoint is thought to be one cause of CIN, although evidence for this connection remains weak (Draviam et al., 2004). It is crucial for our understanding of human cancers to gain an extensive knowledge of chromosome segregation events in normal cells. This chapter examines currently explored mechanistic details of kinetochore- MT attachment and its error-sensing and correction by spindle assembly checkpoint.

1.1 Chromosome segregation and kinetochore-MT attachment.

Chromosome segregation in eukaryotic cells relies on a self-assembling array of microtubule fibers that emanate from organizing centers called centrosomes in mammals or spindle pole bodies in yeast. During mitosis, MTs form attachments with sister chromatids, the cell cortex and anti-parallel MTs emanating from the opposite pole. The kinetochore, a multi-protein complex that assembles on centromeric DNA, serves as a link between the chromosome and MTs. Kinetochore proteins perform multiple functions during mitosis: capture astral MTs, form mature attachments, orchestrate chromosome movements necessary for congression, monitor and correct improper attachments. Since MTs undergo periods of rapid shrinkage and growth allowing them to randomly scan the volume of the cell (Mitchison and Kirschner, 1984; Mitchison and Kirschner, 1985), kinetochore binding to MTs is a difficult task involving activities of many proteins that requires a tight control and a correction system. In recent years, additional mechanisms that help establish kinetochore-MT attachment have become apparent. It is now thought that attachment is achieved by a combination of random search and kinetochore-mediated MT growth pathways. In the following paragraphs, I will present an overview of the spindle structure, kinetochore architecture and a current understanding of the mechanism of kinetochore-MT attachment.

1.1.1 Mitotic spindle.

Although a bipolar mitotic spindle of a constant shape is assembled in every cell division and provides the mechanical network for chromosome segregation, it is made of highly dynamic microtubules. MTs are linear polarized polymers of tubulin heterodimers. α- and β- tubulin molecules form 100kDa dimers that then interact to form protofilaments that further assemble

into hollow MTs, 25 nm in diameter. Cytoplasmic and spindle MTs typically have 12-15 protofilaments (Amos and Klug, 1974; Mandelkow et al., 1986). The head–to–tail arrangement of the dimers along protofilaments gives MTs an intrinsic structural polarity (Mandelkow et al., 1986). α- tubulin is exposed at more stable "minus" ends of MTs while β-tubulin is exposed at more dynamic "plus" ends. Polymerized tubulin at the MT ends rapidly exchanges with the pool of unassembled tubulin dimers (Margolis and Wilson, 1978). Half life of tubulin turnover in unattached MTs is 20-60s (Salmon et al., 1984; Saxton et al., 1984), while the kinetochore bound MTs are more stable, with half lives of several minutes (Mitchison et al., 1986).

MT ends have the ability to persistently grow and rapidly shrink – a phenomenon termed "dynamic instability" (Mitchison and Kirschner, 1984). The abrupt transition between growth and shrinkage is called catastrophe, while the switch from shrinkage to growth is called rescue (Walker et al., 1988). The hydrolysis of GTP-bound tubulin provides the fundamental basis for dynamic instability. Both α- and β-tubulin can bind GTP, but only β-tubulin can hydrolyze and exchange GTP to GDP (Spiegelman et al., 1977). MT growth is controlled by the presence of GTP or GDP at the MT end. Incorporation of GTP-tubulin dimers is followed by the hydrolysis of GTP into GDP, thus producing a lattice of GDP-bound protofilaments capped at the end with the newly associated GTP-bound tubulin. Loss of the tubulin- GTP cap leads to catastrophe, when GTP-tubulin association is inhibited and rapid GDP-tubulin dissociation proceeds. Stabilization of the MT end by a new GTP-cap is thought to rescue the MT shrinking phase (Stewart et al., 1990). Changes in MT dynamics upon entry into mitosis mainly depend on changes in catastrophe and rescue rates (Gliksman et al., 1993). MT dynamics provides some of the force required for unattached sister chromatids movements in prometaphase and for anaphase chromosome movements (Dogterom and Yurke, 1997). Motor proteins and MT-associated

proteins (MAPs) also contribute to force generation both directly by altering MT growth rates and indirectly by crosslinking MTs and regulating their dynamics (Cottingham et al., 1999; Desai et al., 1999; Huyett et al., 1998; Rogers et al., 2004).

Mitotic spindle assembly was always thought to be dependent on centrosomes. Centrosomes (microtubule-organizing centers) are found in the polar regions of the mitotic spindle and define the number of spindle poles in the cell (Brinkley, 2001). It has also been postulated that spindle assembly is driven by centrosome nucleated astral MTs that probe the space "searching" for chromosomes (Kirschner and Mitchison, 1986). Upon a kinetochore encounter, MTs are captured and stabilized, and an array of astral MTs is getting transformed into the spindle-like shape. This "Search-and capture" hypothesis offered an explanation for the higher dynamicity of MTs during mitosis and for cell type specific spindle assembly times, based on stochastic nature of MT capture by kinetochores (Kirschner and Mitchison, 1986). Furthermore, this phenomenon was directly observed in newt lung cells (Rieder and Alexander, 1990). However, the search and capture mechanism did not explain the spindle assembly in cells lacking centrosomes, like plant cells, oocytes, etc. In addition, modeling studies show that random search and capture alone might not be sufficient to build metaphase plate in a short period of time (Wollman et al., 2005).

Recent data have revealed that spindle assembly might form by centrosome-independent mechanisms that contribute to spindle formation not only in cells lacking centrosomes but in centrosome containing cells as well. Chromatin has been shown to nucleate spindle assembly in Xenopus cells and extracts by a mechanism dependent on Ran-GTP (Carazo-Salas et al., 1999; Heald et al., 1996; Karsenti et al., 1984; Ohba et al., 1999; Wilde and Zheng, 1999). In mammalian cells, it has been demonstrated that changes of MT dynamics that occur upon entry

into mitosis lead to the loss of some individual MTs and to formation of MT bundles that that get incorporated into the spindle in dynein- dependent manner (Belmont et al., 1990; Rusan et al., 2001; Rusan et al., 2002). Thus the current understanding of mitotic spindle assembly involves kinetochores, centrosomes and MTs that establish connections between spindle components and integrate them into a common structure. The details of non-centrosomal pathway for formation of the kinetochore- MT attachment are discussed below in paragraph 1.4.

1.1.2 Kinetochores and centromeric heterochromatin.

Kinetochores are multiprotein complexes that perform several functions that are crucial for accurate chromosome segregation during mitosis. They are responsible for chromosome attachment to the spindle, for control of MT dynamics, for generation of a force necessary for chromosome movements and for the function of the spindle assembly checkpoint. Kinetochores begin to assemble on centromeric DNA in S phase. Although most centromeres comprise long stretches of short tandem repetitive "satellite" DNA sequences, *S.cerevisiae* centromeres span just 125bp that are necessary and sufficient for accurate chromosome segregation in mitosis and meiosis (Clarke and Carbon, 1980; Cottarel et al., 1989). In *S.cerevisiae*, specialized centromere-specific histone H3 variant Cse4p is targeted to CDEIII conserved DNA segment by centromere- binding protein CBF3 (Hyman et al., 1992; Meluh et al., 1998). Thus DNA sequence alone is sufficient to define the correct localization of budding yeast centromeres. Even the simplest of known kinetochores, a budding yeast kinetochore, consists of more than 65 proteins. These proteins form at least 17 discrete subcomplexes that assemble on centromeric DNA in a hierarchical order (De Wulf et al., 2003).

S.pombe and metazoan centromeres are far more complex then those of *S.cerevisiae*. *S.pombe* centromeres span 40-100kb and are surrounded by inverted 'inner' repeats that in turn are surrounded by 'outer' repeats (Baum et al., 1994; Takahashi et al., 1992). Human centromeres comprise 0.3-5 Mb and contain 1,500-30,000 copies of AT-rich 171bp α-satellite repeats (McDermid et al., 1986). Centromeric identity in these organisms is defined by the substitution of histone H3 by homologous centromere –specific protein, CENP-A, that is packaged into chromatin (Palmer et al., 1991). Interestingly, despite specificity between centromere-binding proteins and centromeric DNA, no sequence determinants have been identified for any complex centromere. Ablation or deletion of the centromere results in the formation of a neocentromere on the same chromososme that is capable of transmitting genetic information (Alonso et al., 2003; Lo et al., 2001a; Lo et al., 2001b). The function of centromeres is conserved in evolution, but their DNA sequences are not. Recent studies suggest that Mis18 protein complex and histone deacetylase RbAp46/48 mark centromeric heterochromatin and prepare it for recruitment of CENP-A by regulating acetylation of the centromeric histones (Fujita et al., 2007). CENP-A also provides an epigenetic mark to specify centromere location by generating a more rigid nucleosome than its counterparts assembled with Histone H3 (Black et al., 2007). This structural alteration depends on the CENP-A centromere targeting domain that has been demonstrated to be required for maintaining centromeric identity (Black et al., 2007).

Although most of the kinetochore associated proteins are conserved from yeast to humans, vertebrates developed a more elaborate kinetochore structure, probably to accommodate the complexity of kinetochore-MT attachment process and an increased number of kinetochore-MT binding sites. Although *S.cerevisiae* kinetochore attaches to a single MT, a mammalian kinetochore binds 20-40 MTs (Brinkley and Cartwright, 1971; Winey et al., 1995). Currently

more than 100 proteins have been reported to associate with the centromere-kinetochore complex in human cells (Cheeseman et al., 2004; Foltz et al., 2006; Izuta et al., 2006). Vertebrate kinetochores always have been modeled in the context of their trilaminar structure that was elucidated by conventional electron microscopy experiments (Brinkley and Stubblefield, 1966; Jokelainen, 1967). Inner chromosome associated layer has been observed to be separated from the MT-binding outer layer by an electron-lucent zone. Vertebrate kinetochores have also been suggested to contain repeated subunits of common structure that would act as kinetochore-MT docking sites, each subunit being functionally equivalent to *S.cerevisiae* MT binding site (Zinkowski et al., 1991); (Joglekar et al., 2006). The use of high-pressure freezing and freeze substitution, which minimizes structural changes, has demonstrated that mammalian kinetochore appears as a ball of fibrous material that is connected directly to a denser surface of the centromeric heterochromatin (McEwen et al., 1998). Recent electron tomography studies have also shown that the kinetochore outer plate is a meshwork of crosslinked fibers with several fibers extending from the outer plate to bind MT walls, thus supporting a network model of the kinetochore-MT attachment (Dong et al., 2007).

1.1.3 Kinetochore assembly.

Kinetochores assemble on centromeric DNA in a cell cycle- dependent manner. Although some proteins are constitutively associated with centromeres throughout the cell cycle, others are transiently detected at this subcellular location from late G2 to telophase of mitosis. The most dynamic group of proteins localizes to kinetochores only in mitosis. Despite the temporal order of recruitment of kinetochore proteins, a linear assembly pathway can not be isolated. Kinetochore assembly in human cells, Drosophila, *C.elegans* and *Xenopus* appears to be

complex (Figure 1.1) (Blower and Karpen, 2001; Cheeseman et al., 2004; Liu et al., 2006; Vigneron et al., 2004).

CENP-A, a specialized isoform of histone H3 (Palmer et al., 1991), is the first protein that binds to the centromeric DNA and provides the base for kinetochore assembly. CENP-A is suggested to be required for the recruitment of inner kinetochore proteins CENP-C, CENP-H, CENP-I and a centromere protein Aurora B, although the dependence of Aurora B/ MCAK recruitment on CENP-A is controversial (Howman et al., 2000; Liu et al., 2006; Oegema et al., 2001; Van Hooser et al., 2001). Recruitment of inner kinetochore proteins by CENP-A directs three major assembly pathways that contain multiple branches and intersect to form a network that defines the spatial and temporal relationships between kinetochore localizing proteins. CENP-I, a constitutive component of the inner plate, is responsible for kinetochore recruitment of Ndc80 protein complex and CENP-F, stable outer kinetochore elements that associate with kinetochores starting in G2 (Liao et al., 1995; McCleland et al., 2003). Ndc80 complex consists of four proteins that are conserved from yeast to humans: NDC80p/Hec1, Nuf2p, Spc24p and Spc25p (DeLuca et al., 2002; Howe et al., 2001; Wigge and Kilmartin, 2001). Several studies have shown that Ndc80 complex is crucial for correct chromosome segregation, kinetochore-MT attachment and spindle checkpoint signaling; the latter is possibly due to its function in recruitment of Mad1/Mad2 and Zwint-1/ZW10/ROD to kinetochores (DeLuca et al., 2005; He et al., 2001; Lin et al., 2006; Meraldi et al., 2004).

CENP-C seems to also be required for kinetochore recruitment of Ndc80 complex, Mis12, KNL-1 and a number of dynamic outer kinetochore components, including the Bub checkpoint proteins, ZW10/ROD/Zwilch (RZZ) complex and some MT-associated proteins (MAPs) and motors (Cheeseman et al., 2004; Desai et al., 2003; Liu et al., 2006). Position of

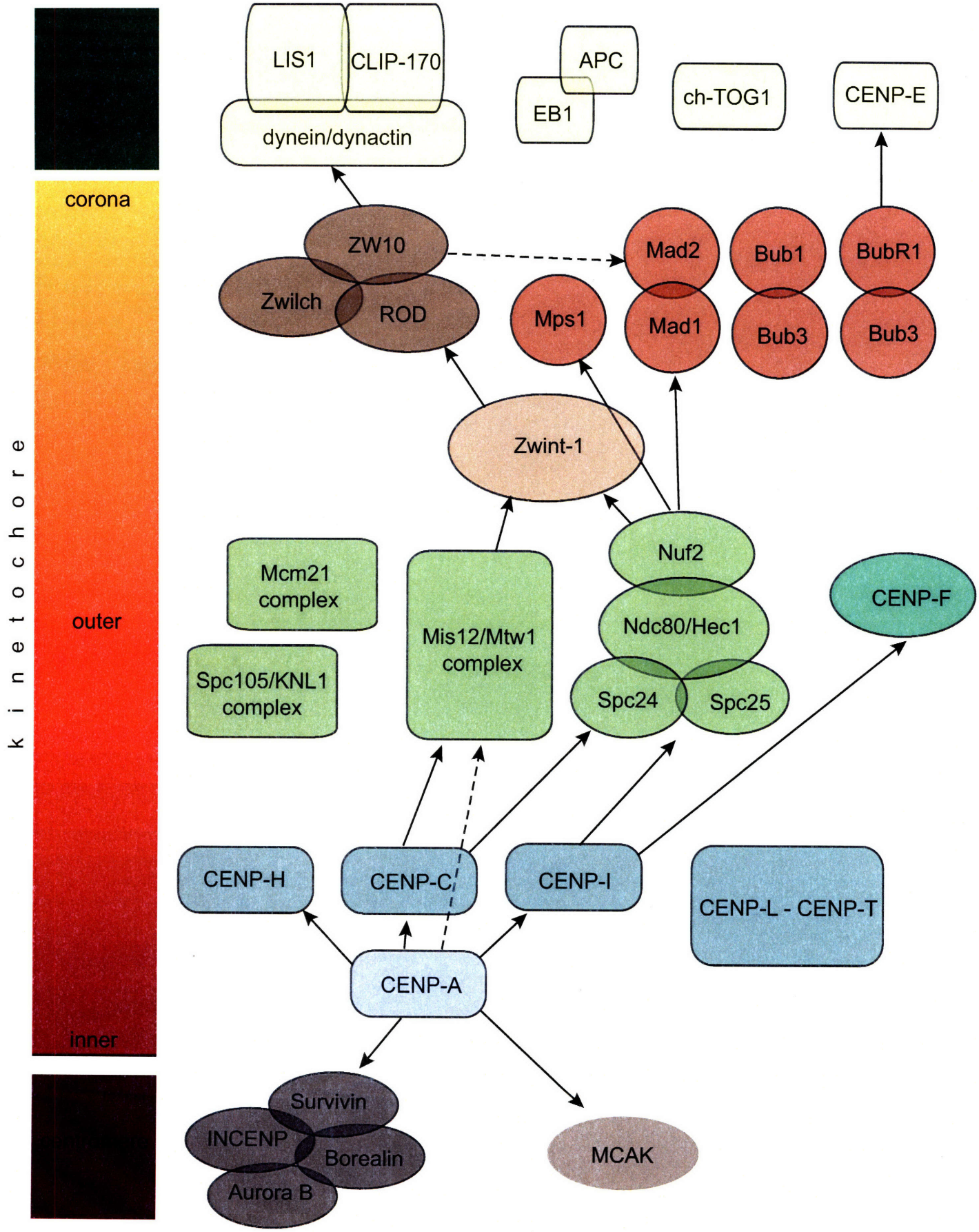

kinetochore
corona
outer
inner
LIS1
CLIP-170
dynein/dynactin
APC
EB1
ch-TOG1
CENP-E
ZW10
Zwilch
ROD
Mps1
Mad2
Mad1
Bub1
Bub3
BubR1
Bub3
Zwint-1
Mcm21 complex
Spc105/KNL1 complex
Mis12/Mtw1 complex
Nuf2
Ndc80/Hec1
Spc24
Spc25
CENP-F
CENP-H
CENP-C
CENP-I
CENP-L - CENP-T
CENP-A
Survivin
INCENP
Borealin
Aurora B
MCAK

Figure 1.1. A network of intersecting pathways that contributes to kinetochore assembly. Centromere-localizing proteins are positioned at the bottom. Microtubule-binding proteins that localize to kinetochores are positioned at the top. Solid arrows indicate recruitment dependencies. Dashed arrows indicate potential interactions.

Mis12 in the assembly pathway is controversial. Mis12/Mtw1 binds CENP-A/Cse4 in *S.cerevisiae* and is required for chromosome biorientation and generation of centromeric tension (Pinsky et al., 2003). Although localization of Mis12 in *S.pombe* was reported to be independent of CENP-A (Takahashi et al., 2000), in *C.elegans* Mis12 has been placed downstream of CENP-A (Cheeseman et al., 2004). Recent study in HeLa cells utilizing an RNAi depletion approach confirms this finding (Liu et al., 2006). Aurora B and MCAK localization to inner centromere from late G2 until metaphase also depends on CENP-A and is not affected when CENP-H and CENP-I are depleted from cells (Andrews et al., 2004; Liu et al., 2006). Thus CENP-A specifies a third assembly branch allowing Aurora B and MCAK to localize to kinetochores.

Kinetochore components that remain stable and constant in level from prometaphase till anaphase, like CENP-A,-C,-H, -I, Ndc80 complex, CENP-F (DeLuca et al., 2002) and KNLs (Cheeseman et al., 2004), provide a base for the assembly of the dynamic outer kinetochore proteins. Dynamic components that change concentrations during mitosis include spindle checkpoint proteins, Mads, Bubs, Cdc20 and RZZ, and MAPs and motors. Many of these proteins are highly recruited to unattached kinetochores in prometaphase and their levels decrease upon kinetochore-MT attachment (Hoffman et al., 2001; Howell et al., 2004; King et al., 2000; Shah et al., 2004). On the contrary, there are other components that associate with kinetochores in a MT-dependent manner. These include MAPs, like EB1 and APC, and Ran pathway proteins, including RanGap1(Joseph et al., 2002; Kaplan et al., 2001; Tirnauer et al., 2002). MAPs, components of the kinetochore outer layer, are of special interest since they mediate kinetochore-MT attachment by providing structural means of association between MT polymer and the multiprotein kinetochore complex and can directly regulate MT dynamics.

1.1.4 Microtubule–associated proteins (MAPs) and motors.

Plus ends of MTs are important for MT destiny since they exhibit dynamic instability and allow MTs to explore cellular space in an effort to capture chromosomes and attach to cell cortex. A complex protein machinery is associated with the MT plus ends in all eukaryotic organisms, which regulates their dynamics and interaction with various cellular structures. MT end binding proteins are divided into two classes: MT depolymerases, including KinI kinesins, and MT plus-end-tracking proteins (+TIPs), which support polymerization.

KinI kinesins, which possess an internal motor domain, utilize ATP hydrolysis to bend MT protofilaments in an inside-out manner thus promoting depolymerization (Desai et al., 1999; Hunter et al., 2003). MCAK is a major vertebrate KinI depolymerase (Wordeman and Mitchison, 1995). Disruption of MCAK function in mammalian cells leads to alignment defects and severe missegregation of chromosomes. Alignment defects are likely caused by the defective kinetochore-MT binding with prevailing merotelic and syntelic attachments, thus suggesting a role for MCAK in the correction of improper attachments (Kline-Smith et al., 2004). MCAK activity is regulated by Aurora B kinase, which recruits MCAK to kinetochores and inhibits its depolymerising activity by phosphorylation (Andrews et al., 2004; Lan et al., 2004).

(+) TIPs include MT-dependent motors, dynein and Centromere-associated protein-E (CENP-E), and the non-motor proteins, Cytoplasmic linker protein-170 (CLIP-170), Clip-associated proteins (CLASPs), lissencephaly protein (LIS1), end-binding protein 1 (EB1), Adenomatous polyposis coli (APC), colonic and hepatic tumor over-expressed gene 1 (ch-TOG1). APC and EB1 are binding partners that localize to kinetochores in a MT-dependent manner and play a role in chromosome segregation (Green and Kaplan, 2003; Kaplan et al., 2001; Tirnauer et al., 2002). Depletion of APC and EB1 causes subtle segregation errors in

anaphase that escape spindle checkpoint control and might contribute to tumorogenesis (Draviam et al., 2006; Green et al., 2005). CLIP-170 and CLASPs are (+) TIPs that localize to kinetochores in the absence of MTs(Dujardin et al., 1998; Maiato et al., 2003). CLIP-170 binds unattached kinetochores in prometaphase and leaves kinetochores upon attachment (Dujardin et al., 1998). Accumulation of CLIP-170 at the kinetochore requires dynein and LIS1 (Tai et al., 2002). CLIP-170 is implicated in MT stabilization and plays a role in chromosome segregation by facilitating the formation of kinetochore-MT attachments (Carvalho et al., 2004; Komarova et al., 2002; Tanenbaum et al., 2006). CLASPs are required for chromosome congression and maintenance of the mitotic spindle (Maiato et al., 2003; Maiato et al., 2002; Pereira et al., 2006). CLASPs bind to EB1 and stabilize MTs by promoting the pause state (Mimori-Kiyosue et al., 2006; Sousa et al., 2007). Although most of the (+) TIPs are MT polymerases, surprisingly one of them (XMAP215/Stu2p/ch-TOG1) has been reported to exhibit both MT stabilizing and MT destabilizing activities (Gard and Kirschner, 1987); (Shirasu-Hiza et al., 2003; van Breugel et al., 2003). This phenomenon might be explained by the ability of Stu2p fragments and its vertebrate homologues to bind free tubulin heterodimers (Al-Bassam et al., 2006). The high affinity of Stu2p/XMAP215 for tubulin could lead to either removal or addition of tubulin subunits at microtubule ends, depending on free tubulin concentration and on many other intracellular factors (Al-Bassam et al., 2007). This hypothesis is supported by the observed discrete growth of MTs in the presence of XMAP215 (Kerssemakers et al., 2006). How cells regulate activity of TOG1 to achieve two diverse functions is unknown.

MT-dependent motors, dynein and CENP-E, are important for chromosome movements in mitosis that are essential for correct segregation. CENP-E is a kinesin-like motor that associates with the fibrous corona of the kinetochore and has been implicated in kinetochore-MT

attachment (Cooke et al., 1997; McEwen et al., 2001; Wood et al., 1997; Yao et al., 1997) Cells depleted of CENP-E exhibit alignment defects with a few mono-oriented chromosomes residing in the vicinity of spindle poles (Schaar et al., 1997). Cytoplasmic dynein is a (-) end directed MT motor implicated in many aspects of intracellular movement. Dynein localizes to the cell cortex, spindle poles and kinetochores, where it functions bound to dynactin, a multisubunit activating complex (McGrail et al., 1995; McGrail and Hays, 1997; Robinson et al., 1999). At the cell cortex, dynein associates with astral microtubules and provides an outward force to separate spindle poles during spindle assembly and anaphase B (Vaisberg et al., 1993). Dynein also plays key roles in the assembly and function of the mitotic spindle. In Xenopus and mammals, dynein focuses the poles of the spindle by cross-linking and sliding microtubule minus ends together (Gaglio et al., 1997; Heald et al., 1997). Dynein activity at kinetochores is modulated by additional proteins, like LIS1, CLIP-170, NudE and Nudel (Tai et al., 2002; Yan et al., 2003). Dynein is highly concentrated at unattached kinetochores in prometaphase. Its levels decrease upon kinetochore- MT attachment followed by relocalization of dynein to the mitotic spindle (Hoffman et al., 2001; King et al., 2000). Dynein is also implicated in poleward movement of chromosomes in prometaphase and anaphase (Savoian et al., 2000; Sharp et al., 2000). Inactivation of the spindle checkpoint has been suggested to depend on dynein activity that is involved in shedding and transport of the checkpoint proteins away from kinetochores following MT capture (Howell et al., 2001; Wojcik et al., 2001).

Despite the increasing number of MAPs that have been discovered in recent years and comprehensive analysis of their function by many laboratories, our understanding of MAPs network at kinetochores is far from complete. There is no integrated map of molecular players that would explain an orchestrated control of MT dynamics in mitosis. Since mammalian

kinetochores are normally associated with at least 20 MTs, it remains unclear how coordination between polymerization states of all MTs is achieved simultaneously. The checkpoint mechanism that monitors functions of MAPs at kinetochores is also poorly understood.

1.1.5 Kinetochore- MT attachment.

Bipolar attachment is crucial for faithful segregation of sister chromatids into two daughter cells. Chromosomes achieve bipolar attachment, when one kinetochore of a pair binds MTs emanating from one spindle pole while the other becomes attached and oriented towards the opposing pole. Kinetochore- MT attachment and spindle formation has been studied for over a century and a unifying model is emerging that reconciles previously competing hypothesis. It is thought now that kinetochore-MT formation happens by two mechanisms that act simultaneously during prometaphase and both involve MT capture by kinetochores. One mechanism is centrosome-based; the other is kinetochore-based (Figure 1.2).

In the centrosome-based mechanism, plus-ends of MTs emanating from two opposite spindle poles exhibit dynamic instability and probe the cytoplasm of the cell in a "search-and-capture" mechanism (Kirschner and Mitchison, 1986). The kinetochore that is closer to the spindle pole during nuclear envelope breakdown (NBD) laterally attaches to the MT and the chromosome moves poleward, thus orienting the kinetochore towards the pole. While a mono-oriented chromosome is moving, its kinetochore binds more MTs that bundle into a kinetochore-fiber whose plus end is imbedded in the kinetochore outer plate (Rieder and Alexander, 1990). The speed of poleward chromosome movement suggests that this motion might be mediated by dynein (Pfarr et al., 1990; Rieder and Alexander, 1990). A MT growing from the opposite pole contacts the sister kinetochore of the mono-oriented chromosome, matures into a kinetochore-

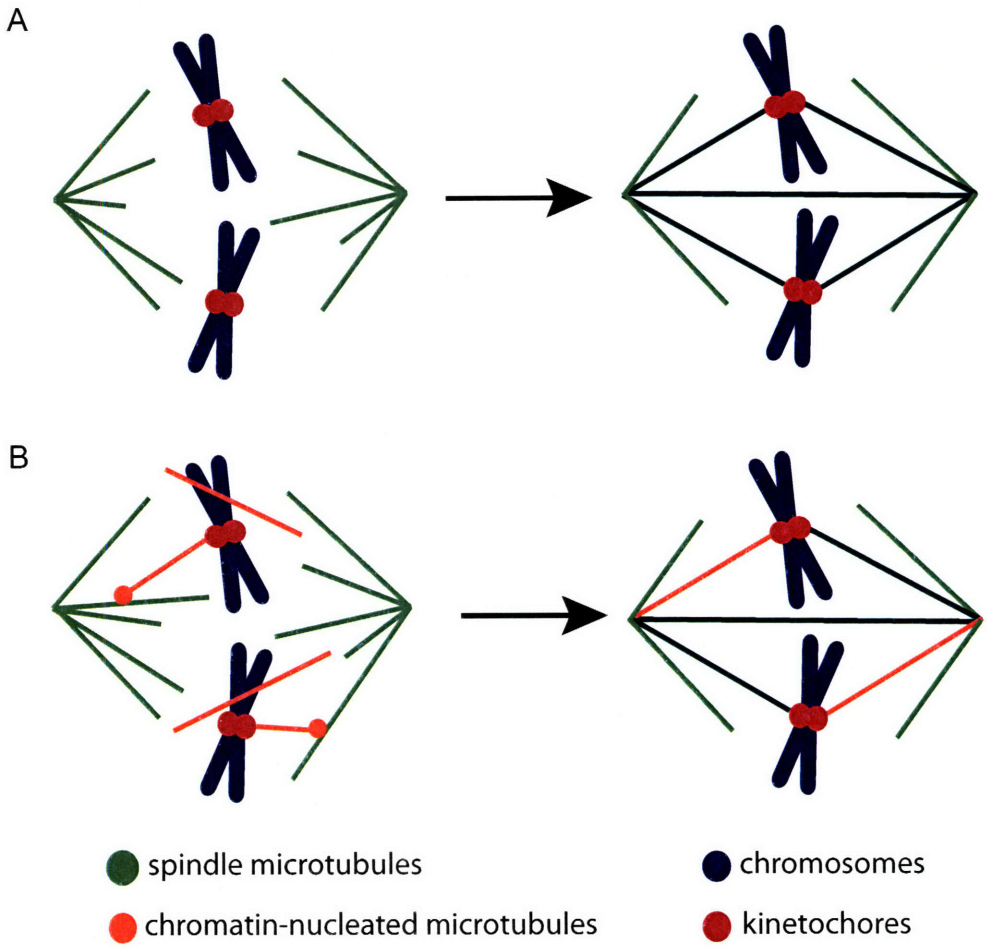


Figure 1.2. Pathways of kinetochore-microtubules formation.
(A) 'Search-and-capture' pathway. Centrosome-nucleated MTs (green) scan the volume of the cell to capture kinetochores (red). (B) Integrated pathway combines 'search-and-capture' and kinetochore-based pathways. Chromatin-nucleated microtubules (orange) are captured by centrosome-generated astral microtubules (green). Following capture, chromatin-nucleated MTs are transported along astral MTs and are incorporated into the forming spindle.The resulting spindle contains both centrosomal and chromatin - nucleated MTs.

fiber and biorients the chromosome. Biorientation is followed by congression of the chromosome to the spindle equator (Rieder and Alexander, 1990). Interestingly, a recent study that combined a live-cell light microscopy and correlative electron microscopy has demonstrated that mono-oriented chromosomes in mammalian cells can congress to the metaphase plate, in contrary to classical thinking that biorientation is a prerequisite for congression. In this CENP-E – dependent process, a mono-oriented chromosome glides along a kinetochore-MT of already congressed chromosome towards the metaphase plate (Kapoor et al., 2006). Although a 'search-and-capture' mechanism explains attachment of MTs to an isolated kinetochore, it is a random low efficiency process. It is predicted that a single kinetochore capture would take only several minutes, but it would consume hours to capture all 46 kinetochore pairs in mammalian cells (Hill and Kirschner, 1982; Wollman et al., 2005). Also, the search-and-capture mechanism does not explain kinetochore-MT attachment in cells lacking centrosomes, including plants and animal oocytes (Heald et al., 1997).

In the kinetochore-based mechanism, MTs are nucleated by chromatin in a process dependent on the small GTPase Ran that forms a concentration gradient around chromosomes (Heald et al., 1996; Kalab et al., 2002). The activity of chromosome-associated Ran-GTP exchange factor RCC1 is involved in gradient formation (Carazo-Salas et al., 1999). Nucleation of MTs by kinetochores might present a problem, since the plus ends of MTs would move away from kinetochores as MTs polymerize, the opposite to MT polarity in the mitotic spindle. Electron microscopy studies resolved this paradox, revealing that kinetochores nucleate short MTs that form not on kinetochores but immediately adjacent to kinetochores (Witt et al., 1980). Attachment of MT plus ends to kinetochore possibly happens after MT elongation through the activity of kinetochore associated plus-end motors (Witt et al., 1980). After kinetochore-

nucleated MTs have been formed, astral MTs capture kinetochore-bound MTs, their minus ends are transported poleward by dynein motors and kinetochore -MTs get incorporated into mitotic spindle (Khodjakov et al., 2003). The nucleation of MTs by kinetochores has been observed as a part of normal formation of mitotic spindle in centrosomal cells (Maiato et al., 2004).

The two mechanisms of formation of kinetochore-MTs appear to contribute simultaneously to spindle assembly. It is possible that those chromosomes that are positioned in the vicinity of centrosomes at NEB capture astral MTs and attach by centrosomal-based mechanism (Maiato et al., 2004). Chromosomes that reside far from centrosomes and have a lesser chance to encounter an astral MT start to nucleate MTs at their kinetochores. These kinetochore-MTs elongate and thus obtain a higher possibility to capture an astral MT and get incorporated into the spindle (Khodjakov et al., 2003). A combination of multiple mechanisms expedites the process of kinetochore-MT attachment and spindle assembly in the cell.

1.1.6 The role of MAPs and motors in the MT capture by the kinetochore.

Most of the kinetochore-localized MAPs and motors have been implicated in chromosome congression and some were suggested to play a role in kinetochore-MT attachment (Dujardin et al., 1998; Echeverri et al., 1996; Kaplan et al., 2001; Kapoor et al., 2006; Kline-Smith et al., 2004; McEwen et al., 2001; Pereira et al., 2006; Schaar et al., 1997; Shirasu-Hiza et al., 2003; van Breugel et al., 2003). It is clear now that disruption of the function of a particular MAP or motor destabilizes kinetochore binding to MTs only to a certain extent. Since attachment is a multi-stage process that is orchestrated by the activities of many MAPs and motors, it is still unclear which protein is required at which stage and when its function is redundant. There is some information available regarding the later step of attachment - correction of improper

configurations. MCAK dependent depolymerising activity regulated by Aurora B is involved at this stage (Andrews et al., 2004; Kline-Smith et al., 2004; Lan et al., 2004). Congression of mono-oriented chromosomes to the metaphase plate has been shown to depend on CENP-E (Kapoor et al., 2006). An earlier step, maturation of attachment and MT bundling, has been proposed to depend on the activity of Bub1 checkpoint kinase (Meraldi and Sorger, 2005). Until recently it remained unclear which proteins are involved in the initial kinetochore-MT encounter.

It has been observed in newt lung cells that chromosomes start moving poleward soon after one of its unattached kinetochores contacts a growing astral MT (Hayden et al., 1990; Rieder and Alexander, 1990). The chromosome velocity of 25-55 μm min^{-1} is very similar to the velocity of cytoplasmic dynein observed *in vitro* (Paschal et al., 1987). Moreover, the speed of poleward chromosome movement in prometaphase and anaphase is reduced in drosophila embryos after anti-dynein antibody injection (Sharp et al., 2000). Inhibition of dynactin function using overexpression of p50 subunit of dynactin caused congression errors in COS-7 cells (Echeverri et al., 1996). Based on these indirect observations it has been proposed that dynein is involved in the initial MT capture at kinetochores. On the contrary, inhibition of dynein/dynactin activity by microinjection of purified p50 dynamitin protein or concentrated 70.1 anti-dynein antibody did not perturb chromosome congression in prometaphase or chromosome segregation in anaphase (Howell et al., 2001). Thus the role of dynein in kinetochore-MT attachment remains controversial and molecular players involved in the initial kinetochore-MT encounter remain unknown.

1.1.7 Conclusion.

Kinetochores are multiprotein structures that play several roles during cell division. They attach replicated chromosomes to the opposite poles of the mitotic spindle, facilitate chromosome movement to the spindle equator and inhibit anaphase onset until all chromosomes are properly attached and positioned. Achievement of bipolar attachment is crucial for errorless partitioning of the genetic material. Although ~100 kinetochore proteins have been identified today, regulation of attachment formation and correction of improper attachments are poorly understood. Recent studies shed the light on the composition of the core MT-binding site at the kinetochore. The Ndc80 complex has been shown to facilitate core MT attachment and is regulated by Aurora B phosphorylation (DeLuca et al., 2006). In addition, a kinetochore-MT network composed of Ndc80, Mis12 and KNL-1 complexes was reconstituted *in vitro* and has been demonstrated to bind MTs directly (Cheeseman et al., 2006). Aurora B kinase is the only protein identified to date that appears to detect and destabilize defective attachments (Lampson et al., 2004; Pinsky et al., 2006). Although kinetochore components involved in these processes are known, their regulation remains unclear.

To gain a complete understanding of the structure and function of the attachment site, additional components of the core MT binding machinery have to be identified. Those will likely include MAPs and motors, one candidate being dynein which is implicated in the initial kinetochore-MT encounter. Additional correction mechanisms have to be elucidated, since all chromosomes in the cell achieve bipolar attachment at different times, Aurora B has to be regulated by individual kinetochores which would turn off Aurora B activity upon attachment. It remains to be determined how MTs attach to kinetochores in a way that MTs retain their

dynamic properties without kinetochores 'falling off 'and without interfering with motor-based transport along MTs.

1.2 Spindle checkpoint overview.

Checkpoints are regulated transition points in the cell cycle where progression to the next cell cycle phase is delayed until upstream events are completed successfully. The canonical checkpoint was defined by Weinert and Hartwell in their study of the *S.cerevisiae* mutants deficient for the response to DNA damage (Weinert and Hartwell, 1988). Whereas wild-type yeast arrest in G2 in response to X-ray irradiation, *rad9* mutant cells fail to arrest, do not enter mitosis and exhibit low viability. However, simultaneous treatment with X-rays and nocodazole, a microtubule depolymerising drug that delays cells in mitosis allows sufficient time for DNA repair, thus increasing viability. Furthermore, *rad9* cells grow normally and are viable in the absence of DNA damaging agents (Weinert and Hartwell, 1988). On the basis of these results it was proposed that Rad9p performs a control function in the cell cycle. Rad9p does not participate in the DNA repair *per se* but delays cells in G2 until DNA repair has occurred. Thus the model was developed that defined checkpoints as pathways that monitor underlying cell cycle events but do not participate in them.

The spindle assembly checkpoint (SAC) monitors kinetochore-MT attachment and delays dissolution of the sister chromatid cohesion and progression through the metaphase-to-anaphase transition until all kinetochores are attached to MTs in a bipolar fashion. Abrogation of the checkpoint machinery leads to the premature anaphase onset and genomic instability that might cause tumorogenesis (Michel et al., 2001). SAC is an essential pathway in metazoans that is activated at the beginning of mitosis (Basu et al., 1999; Dobles et al., 2000). Interestingly, in

S.cerevisiae, SAC is non-essential and becomes essential only in response to damage of the kinetochore-MT attachment (Hardwick and Murray, 1995). The explanation of the seeming controversy comes from the fact that in multicellular organisms mitotic lesions occur in nearly every cell cycle, in contrast to yeast, where fidelity of mitotic chromosome transmission is high because spindles are formed earlier in the cell cycle (Hartwell and Smith, 1985). Thus checkpoint activity is required frequently in higher eukaryotes thus making SAC an essential pathway.

A minimal checkpoint is thought to consist of a sensor, which detects underlying errors, a transducer, which transmits and possibly amplifies the sensed signal, and an effector, which stops cell cycle progression until the defect has been repaired. The details of SAC activation and silencing are incomplete but it is generally believed that kinetochores control SAC. Critical discoveries that support this hypothesis are that impairment of the kinetochore components or mutations in centromeric DNA activate SAC in *S.cerevisiae* (Pangilinan and Spencer, 1996; Spencer and Hieter, 1992). Strikingly, a single unattached kinetochore is sufficient to delay cells at the metaphase to anaphase transition (Rieder et al., 1995). It is thought that SAC monitors attachment of kinetochores to the spindle MTs and tension imposed on a kinetochore pair by stretching of the centromeric chromatin upon biorientation (Nicklas et al., 1995; Rieder et al., 1995; Rieder et al., 1994; Stern and Murray, 2001). The nature of biochemical signal that holds SAC active in response to kinetochore-MT binding defects remains unclear. Checkpoint signaling is transmitted by spindle checkpoint kinases, including Bub1, BubR1 and Mps1, as well as by other checkpoint proteins that localize to unattached kinetochores during mitosis (Hoffman et al., 2001; Meraldi et al., 2004; Taylor et al., 1998). The details of checkpoint protein localization and function are discussed in the following paragraphs.

The final target of SAC is the anaphase promoting complex/cyclosome (APC/C) – a multisubunit E3 ubiquitin ligase that polyubiquitinates its key substrates, cyclin B and Pds1p/securin, targeting them for degradation by 26S proteasome (Cohen-Fix et al., 1996; Evans et al., 1983). CDC20 is a co-activator of APC/C that is negatively regulated by SAC (Visintin et al., 1997). Sequestration of CDC20 by Mad2 binding inhibits its function and prevents APC/C activation, thus leading to inhibition of the metaphase to anaphase transition (Fang et al., 1998). APC/C activity is critical for destruction of two proteins, Pds1 and Cyclin B. Pds1p/securin is an inhibitor of Esp1p/separase, a protease required to cleave the cohesin complex that holds sister chromatids together (Ciosk et al., 1998). The cohesin protein complex consists of Smc1, Smc3, Scc1 and Scc3 which localize to centromeres and along chromosome arms following DNA replication (Michaelis et al., 1997). The cleavage of Scc1 cohesin subunit by Esp1p/separase is a prerequisite for sister chromatid separation (Uhlmann et al., 1999). In addition, proteolysis of cyclin B inactivates mitotic kinase CDK1, which allows anaphase initiation and concomitant exit from mitosis (Murray and Kirschner, 1989).

The checkpoint protein complex consisting of Mad2, BubR1 and Bub3 acts as a SAC effector. It binds to CDC20, thus keeping it from activating APC/C until all chromosomes achieve bipolar attachment (Sudakin et al., 2001). Upon bi-orientation of all chromosomes the checkpoint is released and cells proceed to anaphase. To ensure completion of MT attachment before onset of separation, APC/C activity is tightly regulated spatially and temporally. In the following paragraphs I will describe the functions of particular checkpoint proteins in the spindle checkpoint signaling and the details of the mechanism of APC/C regulation.

1.2.1 Checkpoint inputs: attachment vs. tension.

Although it is clear that SAC inhibits APC/C to arrest cells in metaphase, the primary defect sensed by spindle checkpoint remains controversial. The simplest hypothesis is that checkpoint monitors the state of kinetochore-MT interactions that include two aspects: kinetochore-MT attachment (occupancy) and tension exerted by spindle MTs on centromeres. However, it remains unclear whether these signals are separable or interdependent with a lot of contradictory evidence supporting each hypothesis. Evidence for lack of attachment being a trigger for checkpoint activation is based on the analysis of chromosome behavior in mitotic rat PtK cells (Rieder et al., 1995). Rieder *et al* have demonstrated that a single unattached kinetochore is sufficient to induce a metaphase arrest. Ablation of unattached kinetochore by laser microsurgery relieves the arrest supporting the hypothesis that the unattached kinetochore is the source of the checkpoint activation signal. At the same time the other kinetochore of a pair keeps monotelic attachement to spindle MTs but being under no tension it does not trigger mitotic arrest, indicating that the lack of tension is not sufficient to activate the checkpoint (Rieder et al., 1995). The change in kinetochore localization of checkpoint proteins depending on the attachment status further supports the notion that SAC monitors kinetochore- MT binding (Howell et al., 2001); (Hoffman et al., 2001; Waters et al., 1998).

In case SAC senses only the lack of attachment, syntelic attachments, a binding of both kinetochores in a pair to MTs emanating from the same centrosome, would fail to engage the checkpoint and would lead to aneuploidy. Thus the mechanism has to exist for the SAC to respond to the lack of tension across centromeres. The tension sensing hypothesis is supported by micromanipulation of chromosomes in praying mantid spermatocytes (Li and Nicklas, 1995). These cells arrest for many hours in meiosis I when there is an unpaired chromosome X.

However when tension is applied to unpaired X chromosome by pulling it with the microneedle, the checkpoint is satisfied and cells enter anaphase. The tension sensing hypothesis was also supported by studies in budding yeast. Using *cdc6* mutants that enter mitosis with unreplicated chromosomes (Piatti et al., 1995), it was observed that without sister chromatids, monotelic attachment of kinetochores to MTs caused a metaphase delay (Stern and Murray, 2001). Important caveats exist in experiments on which tension model is based. Tension-micromanipulation experiments have only been performed on meiotic insect cells, implying that results might be system specific. It is not clear whether unreplicated *cdc6* kinetochores are functionally equivalent to the replicated ones. And most importantly, the interdependence of tension and attachment has been demonstrated using the grasshopper spermatocyte system in which kinetochores with 'weak' attachments have been created using micromanipulation (Nicklas et al., 2001). Kinetochores with just few attached MTs do not accumulate high amounts of checkpoint proteins despite the lack of tension, however the checkpoint is silenced only after kinetochores obtain a full occupancy and come under tension (Nicklas et al., 2001). These experiments indicate that tension stabilizes attachment and *vice versa.* Another layer of complication in distinguishing between attachment and tension triggering the checkpoint is caused by techniques used to mimic loss of attachment and tension phenotypes. To generate unattached kinetochores cells are usually exposed to nocodazole, while the absence of tension is achieved by exposure to taxol. Taxol treated kinetochores are positioned closer together indicating the lack of tension. The average number of kinetochore-bound MTs is unperturbed, although it exhibits much broader distribution, suggesting that taxol treatment alters MT occupancy (McEwen et al., 1997). Thus taxol treatment might trigger the checkpoint because it generates unoccupied MT binding sites, and not because SAC monitors lack of tension at

kinetochores. The separation of attachment and tension signals is based on the evidence from different organisms and combines data from studies of meiosis and mitosis. Given the difficulty of experimental dissection of these two signals, it is reasonable to suggest that they might be manifestations of the same phenomena. Differences between attachment and tension triggering the checkpoint might reflect differences between organisms and cell types.

1.2.2 Spindle checkpoint proteins and their functions.

The canonical spindle checkpoint genes were first isolated in 1991 when two independent screens in *S.cerevisiae* identified mutations resistant to inhibition of the cell-cycle progression by spindle poisons. The screen for yeast mutants that did not form colonies after growth on low-dose benomyl plates revealed mitotic arrest-deficient genes named *MAD1, MAD2* and *MAD3* (Li and Murray, 1991). A second screen for mutants that fail to arrest in mitosis in response to the complete loss of MTs yielded genes named budding uninhibited by benzimidazole, including *BUB1, BUB2* and *BUB3* (Hoyt et al., 1991). Canonical *BUB* and *MAD* genes are non-essential in yeast, although *bub1Δ* and *bub3Δ* have slow growth phenotypes and *bub* and *mad* mutants exhibit high rate of spontaneous chromosome loss when grown at normal conditions (Hoyt et al., 1991; Li and Murray, 1991; Roberts et al., 1994; Warren et al., 2002). Out of six initially discovered canonical checkpoint genes, MAD1-3, BUB1 and BUB3 are the players of the spindle assembly checkpoint while BUB2 is a part of the checkpoint that coordinates mitotic exit with spindle positioning (Farr and Hoyt, 1998; Hardwick and Murray, 1995; Wang et al., 2000).

Following the discovery of the six canonical checkpoint genes, the number of proteins found to play a role in SAC continues to expand. MPS1 was found to be an essential checkpoint kinase identified initially in a screen for mutants defective for spindle pole body duplication

(Weiss and Winey, 1996). Checkpoint proteins are highly conserved throughout evolution; homologs of Mad1p, Mad2p, Bub1p, Bub3p, Mps1p were discovered in higher organisms (Sharp-Baker and Chen, 2001); (Abrieu et al., 2001; Campbell et al., 2001; Li and Benezra, 1996; Taylor et al., 1998). Mad3p does not have a metazoan homolog, but a closely related protein BubR1 (Bub - related 1) has been identified in metazoans composed of Mad3p-like N-terminal domain and Bub1p- like C-terminal kinase domain (Taylor et al., 1998). Additional genes have been discovered to play a role in the spindle checkpoint signaling including the member of the passenger complex, Aurora B/Ipl1, implicated in the sensing of inter-kinetochore tension and correction of syntelic attachments (Biggins and Murray, 2001; Lampson et al., 2004). CENP-E, a plus-end directed kinesin-like motor, has been suggested to play a role in the checkpoint signaling by affecting BubR1 kinase activity (Mao et al., 2003; Weaver et al., 2003). Members of the RZZ checkpoint complex, ROD, ZW10 and Zwilch, have been found only in metazoans and may assist in recruiting Mad1/ Mad2 to mitotic kinetochores (Buffin et al., 2005; Kops et al., 2005; Starr et al., 1997). Despite uncertainty about molecular details of the relationship between various checkpoint components, it is clear that functions of all of the checkpoint proteins are required for the successful performance of SAC to ensure correct chromosome segregation in mitosis.

A common feature of all checkpoint proteins is their kinetochore localization in mitosis. First vertebrate homologs of the checkpoint proteins Mads and Bubs, and then their yeast counterparts, were shown to concentrate at unattached kinetochores in prometaphase and become depleted from kinetochores upon bipolar kinetochore-MT attachment (Chen et al., 1998; Gillett et al., 2004; Li and Benezra, 1996; Taylor et al., 1998). Despite similarity in localization, checkpoint protein dynamics at kinetochores varies greatly. FRAP analysis revealed that Mad1

and Bub1 are stable components of the kinetochore, whereas Mad2, BubR1 and Mps1 dynamically exchange with replenishment half-lives of 10-25 sec (Howell et al., 2004; Shah et al., 2004). Localization of Mads and Bubs to kinetochores in prometaphase can be further enhanced by addition of nocodazole to induce MT depolymerization and prevent formation of kinetochore-MT attachments (Chen et al., 1998; Jablonski et al., 1998; Waters et al., 1998). The amount of Mad2 at kinetochores becomes reduced beyond detection limit as kinetochore occupancy increases in metaphase. Because Mad2 is recruited to unattached kinetochores and delocalizes to the spindle and centrosomes upon attachment, its kinetochore localization is interpreted as a marker for the lack of attachment (Howell et al., 2001; Waters et al., 1998). In contrast, levels of Bub1 and BubR1 at kinetochores decrease in metaphase but still remain noticeable (Hoffman et al., 2001). Delocalization of checkpoint proteins after achievement of bipolar attachment is proposed to silence the SAC (Howell et al., 2001). However, checkpoint proteins may remain active in the cytosol after shedding from kinetochores or, alternatively, silencing of the checkpoint by some active mechanism may lead to removal of checkpoint proteins from kinetochores.

1.2.3 Additional roles of checkpoint proteins in chromosome segregation.

Accurate segregation of chromosomes is achieved by restraining APC activity and generating a temporal gap between nuclear envelope breakdown and anaphase onset. This temporal gap is maintained until the correct bipolar attachment of all chromosomes is complete. Although spindle checkpoint proteins were identified a long time ago, precise biochemical functions of many of them are unclear. Recent studies reveal additional roles for checkpoint proteins in mitosis apart from their function in the checkpoint signaling (discussed in detail

below) (Lampson and Kapoor, 2005; Maia et al., 2007; Meraldi and Sorger, 2005; Pinsky et al., 2006). In support for multiple roles checkpoint proteins play in mitosis, analysis of Mad2 function in *Drosophila* SAC have shown that cells lacking Mad2 divide correctly because of rapid chromosome attachment and robust checkpoint. This finding suggests that although many checkpoint protein mutations in the *Drosophila* have been reported lethal, their effect may result not only from elimination of the checkpoint function of these proteins but also from elimination of additional functions that checkpoint proteins play in mitosis (Buffin et al., 2007). Almost all checkpoint proteins known today seem to have multiple roles in regulation of chromosome segregation.

Checkpoint proteins Mad2 and BubR1 are thought to monitor fidelity of kinetochore-MT attachment due to their accumulation on unattached kinetochores (Li and Benezra, 1996; Taylor et al., 2001). Recently an additional role for Mad2 and BubR1 was discovered in the control of the mitotic timing (Buffin et al., 2007; Meraldi et al., 2004). A detailed analysis of mitotic progression in mammalian cells revealed that depletion of Mad2 or BubR1 affects the overall timing of mitosis in contrast to depletion of Mad1, Bub1 and Bub3 that results in the checkpoint deficiency without altering the duration of mitosis. Furthermore, this role of Mad2 and BubR1 is kinetochore independent. Thus the timing of anaphase onset and the spindle checkpoint are controlled by separate mechanisms (Meraldi et al., 2004).

Aurora B/Ipl1 kinase is implicated in the checkpoint response to the lack of tension as well as in the correction of improper attachments. Mutations in *IPL1* gene lead to massive chromosome missegregation, specifically when caused by formation of syntelic attachments (Biggins et al., 1999; Chan and Botstein, 1993). It has been proposed that Aurora B/Ipl1 promotes the turnover of kinetochore microtubule interactions that do not generate tension

(Tanaka et al., 2002). Evidence from studying AuroraB/Ipl1 function in *mtw1* budding yeast mutants and using small molecule inhibitors in mammalian cells indicates that Aurora B selectively disassembles kinetochore microtubules that are syntelically attached (Lampson et al., 2004; Pinsky et al., 2003). Together, these experiments indicate that Aurora B/Ipl1 might be responsible for the instability of attachments at kinetochores that lack tension. Checkpoint function of Aurora B/Ipl1 is required to delay a metaphase to anaphase transition in response to the lack of tension but not attachment (Biggins and Murray, 2001; Hauf et al., 2003). The mechanism by which Aurora B/Ipl1 detects tensionless attachments and signals to the checkpoint is becoming apparent. Coupling mutations in genes required for correct kinetochore-MT attachment with the deletion of Ipl1p resulted in restoration of attachments and checkpoint shut off (Pinsky et al., 2006). Thus Ipl1 activates the checkpoint in response to tension defects by creating unattached kinetochores (Pinsky et al., 2006). Therefore tension defects have to be converted into attachment errors to engage the checkpoint, further emphasizing interdependence of attachment and tension at kinetochores.

Several other proteins originally defined as "checkpoint proteins" have additional functions in establishing and possibly monitoring attachment including mitotic kinases Bub1 and BubR1. Depletion of Bub1 leads to abrogation of the spindle checkpoint and chromosome missegregation caused by improper kinetochore-MT attachments (Meraldi and Sorger, 2005). Since depletion of Bub1 displaces MCAK, a MT depolymerase, from mitotic kinetochores, it is possible that errors in attachment caused by Bub1 depletion result from lack of MCAK activity (Huang et al., 2007). In the case of BubR1, although its role in the checkpoint signaling is relatively well studied, its additional function in formation of stable kinetochore-MT attachments is not yet understood (Lampson and Kapoor, 2005). Several studies have shown that BubR1

interacts with a kinesin-like motor protein CENP-E (Chan et al., 1999; Yao et al., 2000). CENP-E modulates BubR1 kinase activity in *in vitro* kinase assays and in *Xenopus* egg extracts (Mao et al., 2003). BubR1 in turn is proposed to monitor function of CENP-E at the attachment site as judged by in vitro kinase assays performed in the presence of MTs (Mao et al., 2005). However, recent findings indicate that BubR1 function in the formation of correct kinetochore-MT binding sites can not be attributed to its interaction with CENP-E. Analysis of kinetochore-MT interactions in *Drosophila* S2 cells depleted of BubR1 or CENP-meta (*Drosophila* homologue of CENP-E) by siRNA treatment reveals antagonistic effects of BubR1 and CENP-E on the stability of attachments (Maia et al., 2007). Moreover, simultaneous depletion of both proteins appears to partially rescue the chromosome detachment phenotype associated with BubR1 depletion (Maia et al., 2007).Thus attachment defects observed in the absence of BubR1 might result from impaired function of yet to be discovered MAPs or motors that require BubR1 for their kinetochore localization; alternatively, BubR1 kinase might alter the function of known MT-binding proteins by phosphorylation resulting in their inactivation.

1.2.4 Classical definition of checkpoint proteins.

Regulation of metaphase-to anaphase transition is a complex process where most of the players have multiple functions. In the light of recent discoveries, current terminology that refers to all proteins whose absence overcomes an arrest imposed by MT poison as 'checkpoint proteins' seems to be outdated. Removal of some of the kinetochore structural proteins allow cells to overcome a mitotic arrest caused, for example, by nocodazole treatment. These proteins do not participate in checkpoint signaling *per se* but form the platform for kinetochore recruitment of 'real' checkpoint proteins, so that calling structural kinetochore members

'checkpoint proteins' is incorrect although their function fits conventional definition. In the past, the role of checkpoint proteins in the inhibition of metaphase to anaphase transition was studied in isolation, meaning that often temporal and spatial interactions with other kinetochore proteins were not taken into consideration. Abundance of data on multiple functions of checkpoint players suggests that the checkpoint signaling network has to be analyzed as a whole including the regulation of cytoplasmic timers, spindle assembly components and kinetochore assembly regulators. To draw out their influences is crucial to understand the interplay of multiple events that mark the sudden onset of anaphase which occurs only after the attachment of the last chromosome is complete.

1.2.5 Checkpoint outputs: biochemical pathways controlling APC/C.

The nature of the diffusible APC/C inhibitory signals emitted by unattached kinetochores has not been established. Recent studies have revealed an attractive candidate called mitotic checkpoint complex (MCC) that contains checkpoint proteins Mad2, BubR1, Bub3 and Cdc20. MCC binds to APC/C and inhibits its ubiquitin-ligase activity for securin and cyclin B (Fang et al., 1998; Sudakin et al., 2001; Wassmann and Benezra, 1998; Wu et al., 2000). Mad2 and BubR1 alone can inhibit APC/C with low efficiency, but since BubR1 has a distinct binding site on Cdc20 from Mad2, Mad2 and BubR1 have a synergistic effect on APC/C inhibition (Fang, 2002; Tang et al., 2001). Currently it is unclear when and where in the duration of mitosis Mad2, BubR1, Bub3 and Cdc20 interact to form MCC. Binding of Mad2 to Cdc20 is required for BubR1 binding to Cdc20 thus suggesting that Mad2-Cdc20 subcomplex might act as a seed to promote MCC formation (Davenport et al., 2006). How the MCC inhibits APC/C activity is

poorly understood. Although there is a lot of evidence suggesting that kinetochores are necessary to sustain checkpoint signaling, their contribution to the production of MCC is controversial.

The kinetochore- independent theory of MCC formation is based on the following set of experiments. In budding yeast MCC is detectable in the *ndc10* mutants that have defective checkpoint as well as in metaphase arrested cells where SAC is inactive, suggesting that MCC formation in *S.cerevisiae* does not require checkpoint activation (Fraschini et al., 2001; Poddar et al., 2005). Depletion of Mad2 or BubR1 affects the overall timing of mitosis in HeLa cells in a kinetochore-independent manner (Meraldi et al., 2004). Therefore checkpoint control in mitosis might act in two phases. The first phase includes control over the average duration of mitosis by cytosolic timer consisting of Mad2 and BubR1. During the second phase, unattached kinetochores generate a 'wait anaphase' signal that prolongs prometaphase by keeping the checkpoint active in case cells still have unattached kinetochores after extinction of the timer. It is possible that Mad2 and BubR1 work together as part of a cytosolic multiprotein complex. This complex may correspond to the MCC whose biochemical properties are consistent with those of a kinetochore-independent regulator (Sudakin et al., 2001). MCC has been isolated from mitotic as well as from interphase cell extracts, although only mitotic MCC is capable of inhibiting APC/C (Sudakin et al., 2001). Therefore unattached prometaphase kinetochores instead of being involved in MCC formation might sensitize APC/C to inhibition by MCC (Doncic et al., 2006; Sear and Howard, 2006; Sudakin et al., 2001).

Kinetochore localization of checkpoint proteins strongly suggests that kinetochores contribute to MCC formation. All members of MCC cycle dynamically on and off kinetochores, including Mad2 whose kinetochore recruitment follows biphasic kinetics (Howell et al., 2001; Howell et al., 2004; Shah et al., 2004). FRAP studies indicate that Mad2 at kinetochores exist in

two pools: a stably-bound pool and a high turnover pool. Mad1, a stable kinetochore component during prometaphase, forms a complex with Mad2 and is required for Mad2 kinetochore recruitment (De Antoni et al., 2005; Howell et al., 2004; Shah et al., 2004). C-terminal part of Mad2 contains two antiparallel β-sheets separated from the rest of the molecule by the linker loop that shuttles between two conformations: an 'open' state (O-Mad2) capable of binding Mad1 or Cdc20, and a 'closed' state (C-Mad2) in which Mad1 or Cdc20 are topologically trapped by Mad2 (De Antoni et al., 2005). Mad1-C-Mad2 contributes to a stable pool of Mad2 at kinetochores and serves as a receptor for a rapidly cycling form of Mad2, O-Mad2 (De Antoni et al., 2005; Luo et al., 2002; Sironi et al., 2002). A Mad2 mutant that does not form C-Mad2-O-Mad2 heterodimers, was not recruited to kinetochores, suggesting that the fast-exchanging pool of Mad2 is recruited to the kinetochores through a C-Mad2-O-Mad2 heterodimerization event (De Antoni et al., 2005). Since Mad1 and Cdc20 bind to the similar region of Mad2, Cdc20 triggers the same conformational change in Mad2 as Mad1 does. Therefore O-Mad2 changes its conformation to C-Mad2 after binding to Cdc20 (Luo et al., 2002; Sironi et al., 2002). Based on these findings a model was proposed, referred to as the Mad2 template model, to explain the regulation of Mad2 by Mad1 (Figure 1.3A). The C–Mad2 molecule tightly bound to Mad1 recruits another O–Mad2 molecule to the kinetochores through a C-Mad2-O-Mad2 interaction. The relatively loosely bound O–Mad2 molecule is passed on to Cdc20. The Cdc20-bound O-Mad2 adopts the C-Mad2 conformation and can presumably recruit another O–Mad2 through O-Mad2-C-Mad2 heterodimerization. This way, the C–Mad2–Cdc20 complex can amplify itself by self-propagation away from the kinetochores and might provide a molecular mechanism to explain the observation that a single unattached kinetochore is sufficient to activate the checkpoint (Rieder et al., 1995). While Mad2 oligomerisation model explains signal

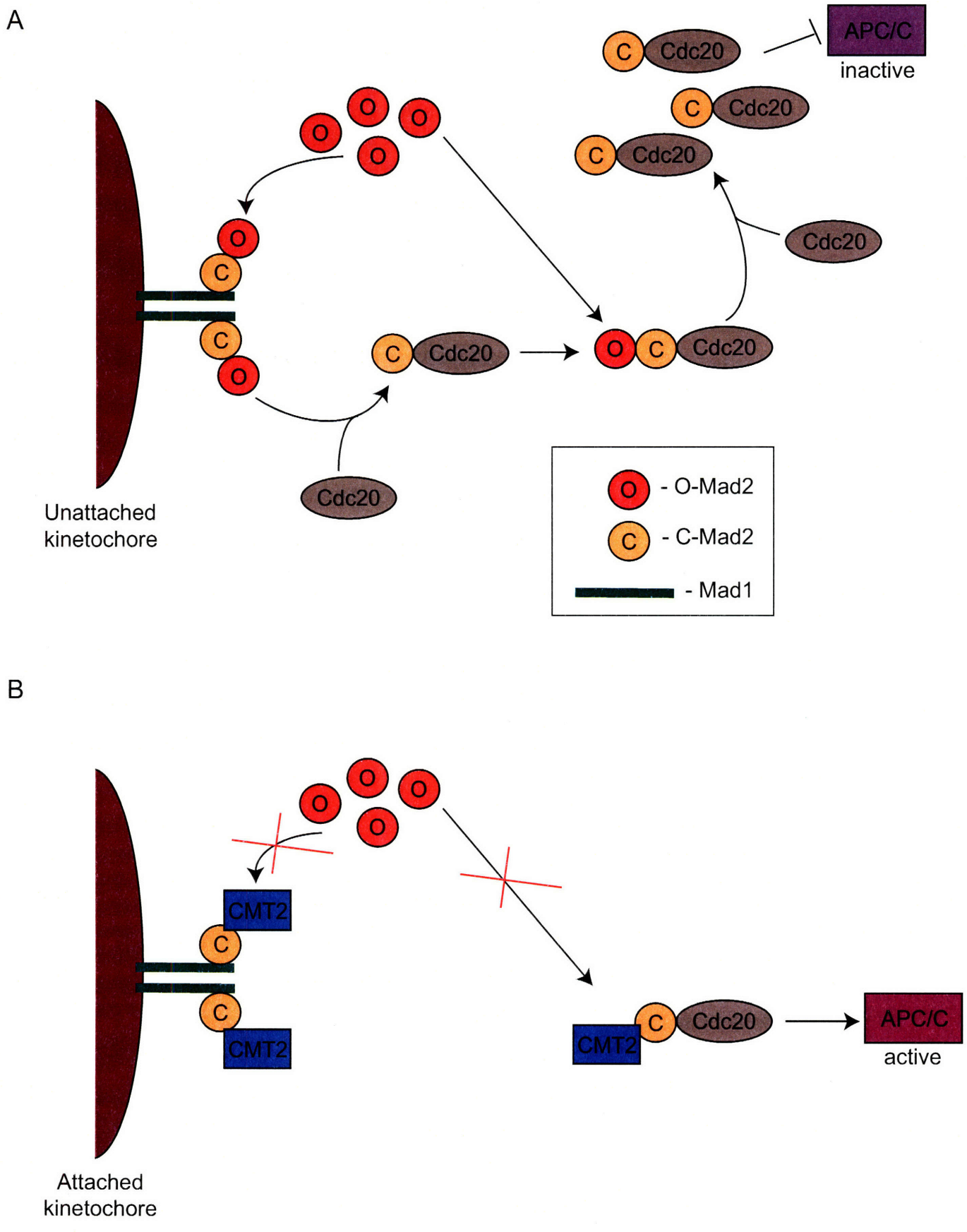

A
C
Cdc20
APC/C
inactive
C
Cdc20
C
Cdc20
O
O
O
O
O
C
C
O
Cdc20
C
Cdc20
O
C
Cdc20
Cdc20
Unattached
kinetochore
O - O-Mad2
C - C-Mad2
- Mad1
B
O
O
O
CMT2
C
C
CMT2
C
Cdc20
CMT2
APC/C
active
Attached
kinetochore

Figure 1.3. Mad2 template model and schematic of the checkpoint silencing by CMT2. (A) Mad2 template model. Unattached kinetochores bind Mad1–C-Mad2 (closed Mad2). Mad1–C-Mad2 recruits O-Mad2 (open Mad2) to kinetochores and facilitates its structural conversion. O-Mad2 binds Cdc20 and turns into C-Mad2-Cdc20 thus preventing Cdc20 from activation of APC/C. C-Mad2–Cdc20 might be involved in a cytosolic auto-amplification reaction based on the same interaction. **(B)** CMT2 inhibits Mad2 by preventing dimerization and releasing inhibition of C-Mad2-Cdc20. Upon checkpoint silencing, CMT2 binds C-Mad2-Madl at the kinetochore and C-Mad2-Cdc20 in the cytosol. Kinetochore CMT2 inhibits Mad2 dimerization and prevents replenishment of the cytosolic pool. CMT2 binding to C-Mad2-Cdc20 renders the complex competent for APC/C activation.

amplification from unattached kinetochore, the role of Bub checkpoint proteins in regulating CDC20 and Mad2 function remains unclear. Moreover, how kinetochore-MT attachment regulates Mad2-mediated checkpoint signaling is also poorly understood.

1.2.6 Checkpoint silencing.

Several mechanisms contribute to inactivation of the spindle checkpoint after bipolar attachment of kinetochores to spindle MTs. Checkpoint silencing can happen by biochemical modification of checkpoint components that keep checkpoint active or by removal of checkpoint proteins from kinetochores, where they contribute to checkpoint activation. The pathway of biochemical modification is based on CMT2, a negative regulator of the checkpoint signaling, and on the balance between ubiquitination and deubquitination activities that dynamically control APC/C (Reddy et al., 2007; Stegmeier et al., 2007). Removal of checkpoint proteins from kinetochores depends on the function of dynein motor (Howell et al., 2001; Wojcik et al., 2001). Checkpoint inactivation likely depends on the concerted action of all of these mechanisms.

1.2.7 Silencing by biochemical modification.

One of the mechanisms is based on CMT2 ($p31^{Comet}$), a Mad2 binding protein that negatively regulates the checkpoint (Habu et al., 2002; Xia et al., 2004). The Mad2 template model predicts that kinetochore bound, but not cytosolic, Mad1-C-Mad2 complexes should trigger a conformational change in free O-Mad2 and that C-Mad2-Cdc20 should not propagate itself through binding to O-Mad2 without a kinetochore-generated signal (Figure 1.3A). Thus C-Mad2 catalytic function at kinetochores has to be under tight control. CMT2 may fulfill this role since it competes with O-Mad2 for binding to C-Mad2 and therefore prevents C-Mad2-O-Mad2

dimerization (Mapelli et al., 2006; Vink et al., 2006; Xia et al., 2004). Kinetochores might inhibit CMT2 ability to bind to C-Mad2 at unattached kinetochores, possibly by phosphorylation (Hagan R., manuscript in preparation), thus allowing C-Mad2-O-Mad2 interaction and accumulation of C-Mad2-Cdc20, resulting in the checkpoint activation. Upon achievement of bipolar attachement, CMT2 may be activated by phosphatases that remove inhibitory phosphorylation and will trigger SAC inactivation. Since there are no known orthologs of CMT2 in lower eukaryotes, there are additional mechanisms that contribute to checkpoint inactivation.

Recent findings suggest one of these mechanisms. A screen for novel regulators of the cell cycle progression and spindle checkpoint revealed a novel deubiquitinating enzyme USP44 that controls APC/C activity in mitosis (Stegmeier et al., 2007). USP44 inhibits disassembly of Mad2-Cdc20 complexes by antagonizing APC-dependent ubiqutination of Cdc20, thus preventing premature inactivation of APC/C (Stegmeier et al., 2007). On the other hand, APC/C itself can drive the disassembly of checkpoint complexes thus causing checkpoint inactivation. APC/C ubiquitinates Cdc20, a member of MCC, leading to release of Mad2. Free Mad2 may again bind Cd20 when unattached kinetochores are present. However, when all kinetochores achieve bipolar attachment, disassembly of MCC might predominate (Reddy et al., 2007). Thus dynamic regulation of APC/C seems to involve a fine-tuned balance of ubiquitination and deubiquitination activities. How this ubiquitination-deubiquitination switch is regulated to promote a rapid transition in the state of APC/C activity once the last chromosome achieves a bipolar attachment remains to be investigated.

1.2.8 Silencing by checkpoint proteins removal.

Many checkpoint proteins, including Mad2, Mad2, RZZ complex, MPS1 and CENP-F, are redistributed to the spindle MTs and centrosomes following MT capture (Hoffman et al., 2001; Howell et al., 2000; Liao et al., 1995; Williams et al., 1996). Cytoplasmic dynein has been implicated in the shedding and transport of checkpoint proteins away from kinetochores (Howell et al., 2001; Wojcik et al., 2001). Cells depleted of dynein motor are arrested in metaphase and retain high levels of Mad2 and Rod on their kinetochores (Draviam et al., 2006; Howell et al., 2001; Wojcik et al., 2001). These observations led to the proposal that dynein-mediated shedding contributes to SAC inactivation by removing checkpoint proteins from correctly attached kinetochores (Howell et al., 2001; Wojcik et al., 2001). The caveat of this model lays in the assumption that depletion of dynein by antibody injection or mutation does not generate spindle problems such as misaligned kinetochores (Howell et al., 2001; Wojcik et al., 2001). On the contrary, other studies have shown that dynein depletion leads to congression errors in metaphase caused by improper kinetochore-MT attachements (Draviam et al., 2006; Echeverri et al., 1996; Yang et al., 2007). If this is the case, high levels of checkpoint proteins at kinetochores observed in the absence of dynein may be attributed to the presence of incorrect attachments that activate the checkpoint and not to dynein shedding functions at kinetochores.

The spindle checkpoint is a very complex system of molecular interactions and signaling that controls progression through mitosis and ensures correct separation of the genetic material in to two daughter cells. Recently a lot of progress has been made in identification of the critical parameters of SAC as well as the *in vitro* reconstitution of their function. Still, many questions remain unresolved and new molecular players are yet to be discovered. The precise biochemical nature of the 'wait anaphase' signal is still unclear and the rules that govern formation of MCC,

its role in the inhibition of APC/C and its dissociation in to individual components are not known. The connection between establishment of kinetochore-MT attachment by MT binding proteins and monitoring of attachment by checkpoint proteins is unclear. The missing components of the checkpoint signaling cascade are kinase regulatory pathways, which govern most signal transduction networks. Although many mitotic kinases have been known for a long time their substrates remain unclear.

1.3 RZZ: Rod/ZW10/Zwilch checkpoint complex.

Although most checkpoint proteins have clear homologs in higher eukaryotes, several other proteins have been identified in metazoans that are also essential for the spindle checkpoint. Rough deal (Rod), Zeste-white 10 (ZW10) and Zwilch form a complex referred to as RZZ (Scaerou et al., 2001; Williams et al., 2003). RZZ members do not have obvious homologs in yeast and contain no recognizable protein motifs that may provide a hint to their function (Starr et al., 1997). They are essential for checkpoint function and mutations in ZW10, ROD and Zwilch have been found in a panel of human colorectal tumors, implying that RZZ complex might be a cause of chromosomal instability and cancer (Wang et al., 2004b). There is no data available on the exact mechanistic role of RZZ members in the spindle checkpoint signaling and chromosome segregation. In the following sections I discuss the details of RZZ discovery and the current understanding of its function.

1.3.1 Identification of RZZ complex.

Zeste-white 10 (zw10) and *rough deal (rod)* genes were originally identified in *Drosophila* in screens for mutations that affect fidelity of mitotic chromosome behavior (Karess and Glover,

1989; Smith et al., 1985). The phenotypes caused by disruption of either gene's function are similar in Drosophila and in C. elegans. Depletion of ZW10 or Rod leads to lagging chromatids and DNA bridges in anaphase and greatly increases missegregation of sister chromatids during mitosis (Scaerou et al., 1999; Williams et al., 1992). No additive effects are observed in *zw10/rod* double null mutants (Scaerou et al., 2001). Both proteins are required for the spindle checkpoint function in *Drosophila, Xenopus* and human cells (Basto et al., 2000; Chan et al., 2000; Kops et al., 2005). Immunofluorescence analysis revealed identical localization pattern for ZW10 and Rod (Scaerou et al., 2001; Williams et al., 1992). Both proteins require each other for kinetochore binding and form a complex of about 800kDa that has been isolated by gel exclusion chromatography (Scaerou et al., 2001). A third member of the complex, Zwilch, was identified by the immunoaffinity chromatography with anti-ZW10 antibody. *Zwilch* mutations result in phenotypes identical to those of *zw10* and *rod* (Williams et al., 2003). The same core complex has been identified in human cells and no additional stable components have been found (Kops et al., 2005). Since the combined molecular weight Rod, ZW10 and Zwilch (240kDa, 85kDa and 75kDa, respectively) is about half of the complex mass, it is likely that RZZ contains two copies of each protein or the complex acts as a dimer. Currently, there is no information available on the biochemistry and assembly of the complex.

1.3.2 Intracellular localization of RZZ.

During interphase Rod, ZW10 and Zwilch are observed in the cytoplasm but it is not clear whether they already associate in a complex (Basto et al., 2004; Williams et al., 1992). Right after nuclear envelope breakdown RZZ members enter the nucleus and accumulate on unattached kinetochores (Basto et al., 2004; Williams et al., 1992; Williams et al., 2003). Upon kinetochore-

MT attachment Rod and ZW10 get redistributed along spindle MTs towards the poles and their levels at kinetochores decline as determined using immunofluorecence analysis in *Drosophila* embryos (Scaerou et al., 1999; Williams et al., 1992). After anaphase onset RZZ delocalizes back to kinetochores where it remains until the end of anaphase, while its MT association disappears (Chan et al., 2000; Scaerou et al., 2001). In telophase and cytokinesis a small amount of ZW10 is observed at the central spindle and midzone, while Rod is seen at the spindle poles (Scaerou et al., 2001; Williams et al., 1996). Spatial separation of ZW10 and Rod at the end of mitosis indicates that these proteins might have separate functions apart from their role in RZZ.

Redistribution of RZZ from kinetochores to the spindle in metaphase is an example of kinetochore 'shedding' observed by live-cell imaging in Drosophila embryos and neuroblasts (Basto et al., 2004; Buffin et al., 2005; Wojcik et al., 2001). Removal of the checkpoint proteins from kinetochores and their transport along kinetochore-MTs towards centrosomes is a dynein dependent process (Howell et al., 2001). The speed of migration of Rod particles along MTs is consistent with dynein mediated transport (Basto et al., 2004). Moreover, in neuroblasts with hypomorphic dynein mutation that blocks dynein movement along MTs, Rod accumulates on metaphase kinetochores and its redistribution to kinetochore MTs is inhibited (Wojcik et al., 2001). Thus RZZ removal from kinetochores after kinetochore-MT attachment is complete seems to depend on the function of dynein motor. On the other hand, RZZ complex is required for the recruitment of dynein/dynactin to mitotic kinetochores. p50 dynamitin subunit has been identified as a binding partner for ZW10 in the two-hybrid screen (Starr et al., 1998). Mutations in both *rod* and *zw10* deplete dynein from kinetochores indicating that the whole RZZ is required for dynein kinetochore localization (Starr et al., 1998; Williams et al., 1996). Therefore, by recruiting dynein to kinetochores, RZZ is thought to set a platform for its own removal in

metaphase, as well as removal of other checkpoint proteins from kinetochores, including Mad2, and checkpoint inactivation.

Although the kinetochore recruitment of many checkpoint proteins is interdependent, several studies in Drosophila and human cells have demonstrated that RZZ kinetochore localization does not dependent of BubR1, Bub3, Mad2, Mps1, CENP-E and CENP-F (Basu et al., 1998; Chan et al., 2000; Liu et al., 2003a; Liu et al., 2003b). A novel kinetochore protein Zwint-1 has been identified in a two-hybrid screen for ZW10 interacting proteins (Starr et al., 2000). Zwint-1 is recruited to kinetochores in early prophase, before ZW10 localizes there, and remains kinetochore bound till mid-anaphase, which makes it a good candidate for ZW10 kinetochore recruiting protein (Wang et al., 2004a). Indeed, recent data have shown that siRNA depletion of Zwint-1 inhibits kinetochore localization of ZW10. Abrogation of Zwint-1 function causes the same mitotic defects as depletion of ZW10 or Rod, including defective checkpoint and anaphase segregation errors (Wang et al., 2004a). By affinity tag chromatography, Zwint-1 was found in a complex with Knl-1 and Mis12, constitutive inner kinetochore components, and in the association with Ndc80, an outer kinetochore plate component (Cheeseman et al., 2004; Obuse et al., 2004). Immunofluorescence analysis in human cells revealed that Hec1 sequentially recruits Zwint-1 and ZW10 to mitotic kinetochores (Lin et al., 2006). Thus, Zwint-1 seems to be a component of the kinetochore outer plate targeted to kinetochore by the inner plate components, which, in turn, recruits RZZ, a member of the fibrous corona.

1.3.3 Functions of RZZ.

Since RZZ is required for kinetochore localization of dynein/dynactin complex (Lin et al., 2006; Starr et al., 1998; Williams et al., 2003), chromosome segregation defects observed in

zw10 and *rod* mutants might be due to the lack of dynein function at kinetochores. Perturbation of dynein activity has been shown to slow down anaphase migration (Savoian et al., 2000; Sharp et al., 2000). This might explain lagging anaphase chromatids caused by depletion of ZW10 or Rod. As has been discussed in the paragraph 1.1.6, the role of dynein at kinetochores in prometaphase is controversial. Although some reports claim that dynein plays a role in chromosome congression at the metaphase plate, others did not observe any alignment defects in dynein depleted cells (Echeverri et al., 1996; Howell et al., 2001). Depletion of ZW10 or Rod in *Drosophila* did not result in alignment defects and caused only missegregated chromosomes and DNA bridges in anaphase (Scaerou et al., 1999; Williams et al., 1992). Thus it remains unclear whether RZZ plays role in chromosome congression and whether it is important for monitoring dynein function at mitotic kinetochores.

How does RZZ contribute to a functional checkpoint? Two studies have shown that RZZ depletion in *Drosophila*, human and *Xenopus* cells blocks recruitment of Mad1 and Mad2 to unattached kinetochores (Buffin et al., 2005; Kops et al., 2005). Thus RZZ complex has been suggested to play a structural role at kinetochores and to participate in the checkpoint by ensuring kinetochore binding of Mad1 and Mad2. Localization of Mad2-GFP has been observed in *rod* or zw10 mutated *Drosohpila* neuroblasts. However, the extent of reduction of kinetochore-associated Mad2 was variable. In 20% of cells authors did not detect any Mad2 at kinetochores in *rod* mutants, while in 80% of cells some Mad2 signal was detectable at prometaphase kinetochores (Buffin et al., 2005). Therefore, the role of Rod/Zw10 may be to enhance the affinity of Mad1/Mad2 for its binding site, increasing its stability on kinetochores prior to MT capture. Moreover, depletion of Zwint-1 leads to a partial checkpoint inactivation and a partial reduction of Mad2 at kinetochores (Lin et al., 2006). This suggests that both ZW10

and Mad2 are required for a full activation of the spindle checkpoint. Altogether, this data leaves a poor understanding of the role of RZZ complex in the spindle checkpoint signaling.

ZW10 seems to have some cellular functions independent of Rod. *zw10* mutant spermatocytes exhibit defects in cytokinesis that correlates with the localization of ZW10 to the midbody of the dividing cells (Scaerou et al., 2001; Williams et al., 1996). During interphase, ZW10 has been shown to form a complex with syntaxin-18 and several other proteins involved in vesicle transport between Golgi and endoplasmic reticulum (Hirose et al., 2004), suggesting a role for ZW10 in membrane trafficking. Since membrane vesicle dynamics is important for cytokinesis, this data supports a cytokinetic role for ZW10. Depletion of ZW10 by RNAi treatment has been observed to cause the dispersal of Golgi, endosomes and lysosomes. Golgi membrane-associated dynein is decreased in ZW10 depleted cells, suggesting a role for ZW10 in dynein cargo binding during interphase. Furthermore, it demonstrates that ZW10 serves a role during interphase related to that at the kinetochore, acting as an anchor for dynein and dynactin.

Despite the significant progress that has been made in recent years in understanding the intracellular role of RZZ, many important questions remain unanswered regarding the biochemistry and function of RZZ complex. First of all, it is unclear how RZZ promotes recruitment and maintenance of Mad1/Mad2 at kinetochores. Affinity chromatography experiments and two-hybrid screens performed on members of RZZ complex have not yet identified a link to Mad1 or Mad2 (Kops et al., 2005; Starr et al., 2000; Williams et al., 2003). Thus RZZ may associate with Mad1 and Mad2 indirectly through unknown protein interactions or, alternatively, the kinetochore may serve as a platform for their interaction that can not occur in solution. The nature of the signal that leads to dynein-mediated shedding of Rod and Mad2 off kinetochores is unknown. Moreover, since Mad2 disappears from kinetochores in metaphase,

while Rod continues to be recruited and transported poleward along kinetochore-MTs, it is important to understand determinants of the kinetochore recruitment of Rod and Mad2. Lastly, there is no information available on the biochemistry of RZZ complex. The temporal and spatial mode of RZZ assembly and how it is regulated needs to be determined. In conclusion, RZZ appears to play an important role in mitosis by recruiting an activator of the checkpoint, Mad2, and the proposed silencer of the checkpoint, dynein/dynactin, to kinetochores.

1.4. Conclusion

Accurate segregation of sister chromatids in mitosis critically depends on successful establishment of physical links between kinetochores and MTs. Proper monitoring of the fidelity of kinetochore-MT binding and its coupling to the cell cycle machinery is equally important. An enormous amount of work has to be accomplished by each cell in a very short period of time to ensure precise, rapid and faithful chromosome segregation in every cell division. Organisms evolved a complex structure of proteins that accomplish tasks described above and a complex network of signaling interactions to monitor functions of these proteins. A combination of yeast genetic analysis with the power of live cell imaging observations in mammalian cells has lead to the discovery of many proteins involved in kinetochore functions on different levels. Still, broad questions remain unanswered regarding the mechanistic steps involved in chromosome segregation.

First, although correct formation of stable but dynamic links between chromosomes and spindle MTs is essential for accurate chromosome segregation, the molecular mechanisms by which kinetochores bind MTs remain poorly understood. Many of the molecules involved have been identified but have not been assigned a clear function in the mechanisms that control MT

capture at kinetochores and chromosome behavior in mitosis. In chapter 2 of this thesis I analyze the functions of six kinetochore-bound MT-associated proteins (kMAPs) using RNAi, live-cell microscopy and quantitative image analysis and address the role of these proteins in mitosis.

Second, little is known regarding the biochemical and structural ways by which spindle checkpoint proteins localize to kinetochores, monitor kinetochore-MT binding and signal to the checkpoint to ensure an adequate response to the state of MT attachment. RZZ is one of the poorly studied protein complexes involved in the spindle checkpoint, since it has been found only in metazoans and most of the pioneering work that lead to the discovery of the canonical checkpoint components has been done in budding yeast. In chapter 3 of this thesis I analyze the role of RZZ in chromosome segregation and checkpoint signaling and propose a model for RZZ function at kinetochores.

Third, the structure and composition of RZZ complex that plays a crucial role in metazoan checkpoint is poorly understood. The lack of structure-function information on the members of RZZ makes the biochemical analysis of the RZZ complex difficult and identification of individual functions of RZZ members virtually impossible. In chapter 4 of this thesis I investigate the dynamic localization of ZW10 in mitosis and describe a mutational analysis of ZW10.

Checkpoint signaling for a long time has been considered a separate entity performed by a secluded set of proteins while the establishment of bipolar attachment is accomplished by a different group of proteins that make physical links between MTs and kinetochores. In chapter 5 of this thesis I discuss an intrinsic connection between establishment of attachment and its monitoring by the spindle checkpoint and propose future avenues of research.

1.5 References

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

Al-Bassam, J., N.A. Larsen, A.A. Hyman, and S.C. Harrison. 2007. Crystal structure of a TOG domain: conserved features of XMAP215/Dis1-family TOG domains and implications for tubulin binding. *Structure.* 15:355-62.

Al-Bassam, J., M. van Breugel, S.C. Harrison, and A. Hyman. 2006. Stu2p binds tubulin and undergoes an open-to-closed conformational change. *J Cell Biol.* 172:1009-22.

Alonso, A., R. Mahmood, S. Li, F. Cheung, K. Yoda, and P.E. Warburton. 2003. Genomic microarray analysis reveals distinct locations for the CENP-A binding domains in three human chromosome 13q32 neocentromeres. *Hum Mol Genet.* 12:2711-21.

Amos, L., and A. Klug. 1974. Arrangement of subunits in flagellar microtubules. *J Cell Sci.* 14:523-49.

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

Basto, R., R. Gomes, and R.E. Karess. 2000. Rough deal and Zw10 are required for the metaphase checkpoint in Drosophila. *Nat Cell Biol.* 2:939-43.

Basto, R., F. Scaerou, S. Mische, E. Wojcik, C. Lefebvre, R. Gomes, T. Hays, and R. Karess. 2004. In vivo dynamics of the rough deal checkpoint protein during Drosophila mitosis. *Curr Biol.* 14:56-61.

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

Basu, J., E. Logarinho, S. Herrmann, H. Bousbaa, Z. Li, G.K. Chan, T.J. Yen, C.E. Sunkel, and M.L. Goldberg. 1998. Localization of the Drosophila checkpoint control protein Bub3 to the kinetochore requires Bub1 but not Zw10 or Rod. *Chromosoma.* 107:376-85.

Baum, M., V.K. Ngan, and L. Clarke. 1994. The centromeric K-type repeat and the central core are together sufficient to establish a functional Schizosaccharomyces pombe centromere. *Mol Biol Cell.* 5:747-61.

Belmont, L.D., A.A. Hyman, K.E. Sawin, and T.J. Mitchison. 1990. Real-time visualization of cell cycle-dependent changes in microtubule dynamics in cytoplasmic extracts. *Cell.* 62:579-89.

Biggins, S., and A.W. Murray. 2001. The budding yeast protein kinase Ipl1/Aurora allows the absence of tension to activate the spindle checkpoint. *Genes Dev.* 15:3118-29.

Biggins, S., F.F. Severin, N. Bhalla, I. Sassoon, A.A. Hyman, and A.W. Murray. 1999. The conserved protein kinase Ipl1 regulates microtubule binding to kinetochores in budding yeast. *Genes Dev.* 13:532-44.

Black, B.E., M.A. Brock, S. Bedard, V.L. Woods, Jr., and D.W. Cleveland. 2007. An epigenetic mark generated by the incorporation of CENP-A into centromeric nucleosomes. *Proc Natl Acad Sci U S A.* 104:5008-13.

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

Brinkley, B.R. 2001. Managing the centrosome numbers game: from chaos to stability in cancer cell division. *Trends Cell Biol.* 11:18-21.

Brinkley, B.R., and J. Cartwright, Jr. 1971. Ultrastructural analysis of mitotic spindle elongation in mammalian cells in vitro. Direct microtubule counts. *J Cell Biol.* 50:416-31.

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

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

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

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

Carazo-Salas, R.E., G. Guarguaglini, O.J. Gruss, A. Segref, E. Karsenti, and I.W. Mattaj. 1999. Generation of GTP-bound Ran by RCC1 is required for chromatin-induced mitotic spindle formation. *Nature.* 400:178-81.

Carvalho, P., M.L. Gupta, Jr., M.A. Hoyt, and D. Pellman. 2004. Cell cycle control of kinesin-mediated transport of Bik1 (CLIP-170) regulates microtubule stability and dynein activation. *Dev Cell.* 6:815-29.

Chan, C.S., and D. Botstein. 1993. Isolation and characterization of chromosome-gain and increase-in-ploidy mutants in yeast. *Genetics.* 135:677-91.

Chan, G.K., S.A. Jablonski, D.A. Starr, M.L. Goldberg, and T.J. Yen. 2000. Human Zw10 and ROD are mitotic checkpoint proteins that bind to kinetochores. *Nat Cell Biol.* 2:944-7.

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

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

Cheeseman, I.M., S. Niessen, S. Anderson, F. Hyndman, J.R. Yates, 3rd, K. Oegema, and A. Desai. 2004. A conserved protein network controls assembly of the outer kinetochore and its ability to sustain tension. *Genes Dev.* 18:2255-68.

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

Ciosk, R., W. Zachariae, C. Michaelis, A. Shevchenko, M. Mann, and K. Nasmyth. 1998. An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. *Cell.* 93:1067-76.

Clarke, L., and J. Carbon. 1980. Isolation of a yeast centromere and construction of functional small circular chromosomes. *Nature.* 287:504-9.

Cohen-Fix, O., J.M. Peters, M.W. Kirschner, and D. Koshland. 1996. Anaphase initiation in Saccharomyces cerevisiae is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. *Genes Dev.* 10:3081-93.

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

Cottarel, G., J.H. Shero, P. Hieter, and J.H. Hegemann. 1989. A 125-base-pair CEN6 DNA fragment is sufficient for complete meiotic and mitotic centromere functions in Saccharomyces cerevisiae. *Mol Cell Biol.* 9:3342-9.

Cottingham, F.R., L. Gheber, D.L. Miller, and M.A. Hoyt. 1999. Novel roles for saccharomyces cerevisiae mitotic spindle motors. *J Cell Biol.* 147:335-50.

Davenport, J., L.D. Harris, and R. Goorha. 2006. Spindle checkpoint function requires Mad2-dependent Cdc20 binding to the Mad3 homology domain of BubR1. *Exp Cell Res.* 312:1831-42.

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

De Wulf, P., A.D. McAinsh, and P.K. Sorger. 2003. Hierarchical assembly of the budding yeast kinetochore from multiple subcomplexes. *Genes Dev.* 17:2902-21.

DeLuca, J.G., Y. Dong, P. Hergert, J. Strauss, J.M. Hickey, E.D. Salmon, and B.F. McEwen. 2005. Hec1 and nuf2 are core components of the kinetochore outer plate essential for organizing microtubule attachment sites. *Mol Biol Cell.* 16:519-31.

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

DeLuca, J.G., B. Moree, J.M. Hickey, J.V. Kilmartin, and E.D. Salmon. 2002. hNuf2 inhibition blocks stable kinetochore-microtubule attachment and induces mitotic cell death in HeLa cells. *J Cell Biol.* 159:549-55.

Desai, A., S. Rybina, T. Muller-Reichert, A. Shevchenko, A. Shevchenko, A. Hyman, and K. Oegema. 2003. KNL-1 directs assembly of the microtubule-binding interface of the kinetochore in C. elegans. *Genes Dev.* 17:2421-35.

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

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

Dogterom, M., and B. Yurke. 1997. Measurement of the force-velocity relation for growing microtubules. *Science.* 278:856-60.

Doncic, A., E. Ben-Jacob, and N. Barkai. 2006. Noise resistance in the spindle assembly checkpoint. *Mol Syst Biol.* 2:2006 0027.

Dong, Y., K.J. Vanden Beldt, X. Meng, A. Khodjakov, and B.F. McEwen. 2007. The outer plate in vertebrate kinetochores is a flexible network with multiple microtubule interactions. *Nat Cell Biol.* 9:516-22.

Draviam, V.M., I. Shapiro, B. Aldridge, and P.K. Sorger. 2006. Misorientation and reduced stretching of aligned sister kinetochores promote chromosome missegregation in EB1- or APC-depleted cells. *Embo J.* 25:2814-27.

Draviam, V.M., S. Xie, and P.K. Sorger. 2004. Chromosome segregation and genomic stability. *Curr Opin Genet Dev.* 14:120-5.

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

Echeverri, C.J., B.M. Paschal, K.T. Vaughan, and R.B. Vallee. 1996. Molecular characterization of the 50-kD subunit of dynactin reveals function for the complex in chromosome alignment and spindle organization during mitosis. *J Cell Biol.* 132:617-33.

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

Fang, G. 2002. Checkpoint protein BubR1 acts synergistically with Mad2 to inhibit anaphase-promoting complex. *Mol Biol Cell.* 13:755-66.

Fang, G., H. Yu, and M.W. Kirschner. 1998. The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. *Genes Dev.* 12:1871-83.

Farr, K.A., and M.A. Hoyt. 1998. Bub1p kinase activates the Saccharomyces cerevisiae spindle assembly checkpoint. *Mol Cell Biol.* 18:2738-47.

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

Fraschini, R., A. Beretta, G. Lucchini, and S. Piatti. 2001. Role of the kinetochore protein Ndc10 in mitotic checkpoint activation in Saccharomyces cerevisiae. *Mol Genet Genomics.* 266:115-25.

Fujita, Y., T. Hayashi, T. Kiyomitsu, Y. Toyoda, A. Kokubu, C. Obuse, and M. Yanagida. 2007. Priming of centromere for CENP-A recruitment by human hMis18alpha, hMis18beta, and M18BP1. *Dev Cell.* 12:17-30.

Gaglio, T., M.A. Dionne, and D.A. Compton. 1997. Mitotic spindle poles are organized by structural and motor proteins in addition to centrosomes. *J Cell Biol.* 138:1055-66.

Gard, D.L., and M.W. Kirschner. 1987. A microtubule-associated protein from Xenopus eggs that specifically promotes assembly at the plus-end. *J Cell Biol.* 105:2203-15.

Gillett, E.S., C.W. Espelin, and P.K. Sorger. 2004. Spindle checkpoint proteins and chromosome-microtubule attachment in budding yeast. *J Cell Biol.* 164:535-46.

Gliksman, N.R., R.V. Skibbens, and E.D. Salmon. 1993. How the transition frequencies of microtubule dynamic instability (nucleation, catastrophe, and rescue) regulate microtubule dynamics in interphase and mitosis: analysis using a Monte Carlo computer simulation. *Mol Biol Cell.* 4:1035-50.

Green, R.A., and K.B. Kaplan. 2003. Chromosome instability in colorectal tumor cells is associated with defects in microtubule plus-end attachments caused by a dominant mutation in APC. *J Cell Biol.* 163:949-61.

Green, R.A., R. Wollman, and K.B. Kaplan. 2005. APC and EB1 function together in mitosis to regulate spindle dynamics and chromosome alignment. *Mol Biol Cell.* 16:4609-22.

Habu, T., S.H. Kim, J. Weinstein, and T. Matsumoto. 2002. Identification of a MAD2-binding protein, CMT2, and its role in mitosis. *Embo J.* 21:6419-28.

Hanahan, D., and R.A. Weinberg. 2000. The hallmarks of cancer. *Cell.* 100:57-70.

Hardwick, K.G., and A.W. Murray. 1995. Mad1p, a phosphoprotein component of the spindle assembly checkpoint in budding yeast. *J Cell Biol.* 131:709-20.

Hartwell, L.H., and D. Smith. 1985. Altered fidelity of mitotic chromosome transmission in cell cycle mutants of S. cerevisiae. *Genetics.* 110:381-95.

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

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

Hayden, J.H., S.S. Bowser, and C.L. Rieder. 1990. Kinetochores capture astral microtubules during chromosome attachment to the mitotic spindle: direct visualization in live newt lung cells. *J Cell Biol.* 111:1039-45.

He, X., D.R. Rines, C.W. Espelin, and P.K. Sorger. 2001. Molecular analysis of kinetochore-microtubule attachment in budding yeast. *Cell.* 106:195-206.

Heald, R., R. Tournebize, T. Blank, R. Sandaltzopoulos, P. Becker, A. Hyman, and E. Karsenti. 1996. Self-organization of microtubules into bipolar spindles around artificial chromosomes in Xenopus egg extracts. *Nature.* 382:420-5.

Heald, R., R. Tournebize, A. Habermann, E. Karsenti, and A. Hyman. 1997. Spindle assembly in Xenopus egg extracts: respective roles of centrosomes and microtubule self-organization. *J Cell Biol.* 138:615-28.

Hill, T.L., and M.W. Kirschner. 1982. Subunit treadmilling of microtubules or actin in the presence of cellular barriers: possible conversion of chemical free energy into mechanical work. *Proc Natl Acad Sci U S A.* 79:490-4.

Hirose, H., K. Arasaki, N. Dohmae, K. Takio, K. Hatsuzawa, M. Nagahama, K. Tani, A. Yamamoto, M. Tohyama, and M. Tagaya. 2004. Implication of ZW10 in membrane trafficking between the endoplasmic reticulum and Golgi. *Embo J.* 23:1267-78.

Hoffman, D.B., C.G. Pearson, T.J. Yen, B.J. Howell, and E.D. Salmon. 2001. Microtubule-dependent changes in assembly of microtubule motor proteins and mitotic spindle checkpoint proteins at PtK1 kinetochores. *Mol Biol Cell.* 12:1995-2009.

Howe, M., K.L. McDonald, D.G. Albertson, and B.J. Meyer. 2001. HIM-10 is required for kinetochore structure and function on Caenorhabditis elegans holocentric chromosomes. *J Cell Biol.* 153:1227-38.

Howell, B.J., D.B. Hoffman, G. Fang, A.W. Murray, and E.D. Salmon. 2000. Visualization of Mad2 dynamics at kinetochores, along spindle fibers, and at spindle poles in living cells. *J Cell Biol.* 150:1233-50.

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

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

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

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

Huang, H., J. Feng, J. Famulski, J.B. Rattner, S.T. Liu, G.D. Kao, R. Muschel, G.K. Chan, and T.J. Yen. 2007. Tripin/hSgo2 recruits MCAK to the inner centromere to correct defective kinetochore attachments. *J Cell Biol.* 177:413-24.

Hunter, A.W., M. Caplow, D.L. Coy, W.O. Hancock, S. Diez, L. Wordeman, and J. Howard. 2003. The kinesin-related protein MCAK is a microtubule depolymerase that forms an ATP-hydrolyzing complex at microtubule ends. *Mol Cell.* 11:445-57.

Huyett, A., J. Kahana, P. Silver, X. Zeng, and W.S. Saunders. 1998. The Kar3p and Kip2p motors function antagonistically at the spindle poles to influence cytoplasmic microtubule numbers. *J Cell Sci.* 111 (Pt 3):295-301.

Hyman, A.A., K. Middleton, M. Centola, T.J. Mitchison, and J. Carbon. 1992. Microtubule-motor activity of a yeast centromere-binding protein complex. *Nature.* 359:533-6.

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

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

Joglekar, A.P., D.C. Bouck, J.N. Molk, K.S. Bloom, and E.D. Salmon. 2006. Molecular architecture of a kinetochore-microtubule attachment site. *Nat Cell Biol.* 8:581-5.

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

Joseph, J., S.H. Tan, T.S. Karpova, J.G. McNally, and M. Dasso. 2002. SUMO-1 targets RanGAP1 to kinetochores and mitotic spindles. *J Cell Biol.* 156:595-602.

Kalab, P., K. Weis, and R. Heald. 2002. Visualization of a Ran-GTP gradient in interphase and mitotic Xenopus egg extracts. *Science.* 295:2452-6.

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

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

Karess, R.E., and D.M. Glover. 1989. rough deal: a gene required for proper mitotic segregation in Drosophila. *J Cell Biol.* 109:2951-61.

Karsenti, E., S. Kobayashi, T. Mitchison, and M. Kirschner. 1984. Role of the centrosome in organizing the interphase microtubule array: properties of cytoplasts containing or lacking centrosomes. *J Cell Biol.* 98:1763-76.

Kerssemakers, J.W., E.L. Munteanu, L. Laan, T.L. Noetzel, M.E. Janson, and M. Dogterom. 2006. Assembly dynamics of microtubules at molecular resolution. *Nature.* 442:709-12.

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

King, J.M., T.S. Hays, and R.B. Nicklas. 2000. Dynein is a transient kinetochore component whose binding is regulated by microtubule attachment, not tension. *J Cell Biol.* 151:739-48.

Kirschner, M.W., and T. Mitchison. 1986. Microtubule dynamics. *Nature.* 324:621.

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

Komarova, Y.A., A.S. Akhmanova, S. Kojima, N. Galjart, and G.G. Borisy. 2002. Cytoplasmic linker proteins promote microtubule rescue in vivo. *J Cell Biol.* 159:589-99.

Kops, G.J., Y. Kim, B.A. Weaver, Y. Mao, I. McLeod, J.R. Yates, 3rd, M. Tagaya, and D.W. Cleveland. 2005. ZW10 links mitotic checkpoint signaling to the structural kinetochore. *J Cell Biol.* 169:49-60.

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

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

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

Lengauer, C., K.W. Kinzler, and B. Vogelstein. 1997. Genetic instability in colorectal cancers. *Nature.* 386:623-7.

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

Li, X., and R.B. Nicklas. 1995. Mitotic forces control a cell-cycle checkpoint. *Nature.* 373:630-2.

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

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

Lin, Y.T., Y. Chen, G. Wu, and W.H. Lee. 2006. Hec1 sequentially recruits Zwint-1 and ZW10 to kinetochores for faithful chromosome segregation and spindle checkpoint control. *Oncogene.* 25:6901-14.

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

Liu, S.T., J.C. Hittle, S.A. Jablonski, M.S. Campbell, K. Yoda, and T.J. Yen. 2003b. Human CENP-I specifies localization of CENP-F, MAD1 and MAD2 to kinetochores and is essential for mitosis. *Nat Cell Biol.* 5:341-5.

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

Lo, A.W., J.M. Craig, R. Saffery, P. Kalitsis, D.V. Irvine, E. Earle, D.J. Magliano, and K.H. Choo. 2001a. A 330 kb CENP-A binding domain and altered replication timing at a human neocentromere. *Embo J.* 20:2087-96.

Lo, A.W., D.J. Magliano, M.C. Sibson, P. Kalitsis, J.M. Craig, and K.H. Choo. 2001b. A novel chromatin immunoprecipitation and array (CIA) analysis identifies a 460-kb CENP-A-binding neocentromere DNA. *Genome Res.* 11:448-57.

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

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

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

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

Maiato, H., P. Sampaio, C.L. Lemos, J. Findlay, M. Carmena, W.C. Earnshaw, and C.E. Sunkel. 2002. MAST/Orbit has a role in microtubule-kinetochore attachment and is essential for chromosome alignment and maintenance of spindle bipolarity. *J Cell Biol.* 157:749-60.

Mandelkow, E.M., R. Rapp, and E. Mandelkow. 1986. Microtubule structure studied by quick freezing: cryo-electron microscopy and freeze fracture. *J Microsc.* 141:361-73.

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

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

Mapelli, M., F.V. Filipp, G. Rancati, L. Massimiliano, L. Nezi, G. Stier, R.S. Hagan, S. Confalonieri, S. Piatti, M. Sattler, and A. Musacchio. 2006. Determinants of conformational dimerization of Mad2 and its inhibition by p31comet. *Embo J.* 25:1273-84.

Margolis, R.L., and L. Wilson. 1978. Opposite end assembly and disassembly of microtubules at steady state in vitro. *Cell.* 13:1-8.

McCleland, M.L., R.D. Gardner, M.J. Kallio, J.R. Daum, G.J. Gorbsky, D.J. Burke, and P.T. Stukenberg. 2003. The highly conserved Ndc80 complex is required for kinetochore assembly, chromosome congression, and spindle checkpoint activity. *Genes Dev.* 17:101-14.

McDermid, H.E., A.M. Duncan, M.J. Higgins, J.L. Hamerton, E. Rector, K.R. Brasch, and B.N. White. 1986. Isolation and characterization of an alpha-satellite repeated sequence from human chromosome 22. *Chromosoma.* 94:228-34.

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

McEwen, B.F., A.B. Heagle, G.O. Cassels, K.F. Buttle, and C.L. Rieder. 1997. Kinetochore fiber maturation in PtK1 cells and its implications for the mechanisms of chromosome congression and anaphase onset. *J Cell Biol.* 137:1567-80.

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

McGrail, M., J. Gepner, A. Silvanovich, S. Ludmann, M. Serr, and T.S. Hays. 1995. Regulation of cytoplasmic dynein function in vivo by the Drosophila Glued complex. *J Cell Biol.* 131:411-25.

McGrail, M., and T.S. Hays. 1997. The microtubule motor cytoplasmic dynein is required for spindle orientation during germline cell divisions and oocyte differentiation in Drosophila. *Development.* 124:2409-19.

Meluh, P.B., P. Yang, L. Glowczewski, D. Koshland, and M.M. Smith. 1998. Cse4p is a component of the core centromere of Saccharomyces cerevisiae. *Cell.* 94:607-13.

Meraldi, P., V.M. Draviam, and P.K. Sorger. 2004. Timing and checkpoints in the regulation of mitotic progression. *Dev Cell.* 7:45-60.

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

Michaelis, C., R. Ciosk, and K. Nasmyth. 1997. Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. *Cell.* 91:35-45.

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

Mimori-Kiyosue, Y., I. Grigoriev, H. Sasaki, C. Matsui, A. Akhmanova, S. Tsukita, and I. Vorobjev. 2006. Mammalian CLASPs are required for mitotic spindle organization and kinetochore alignment. *Genes Cells.* 11:845-57.

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

Mitchison, T., and M. Kirschner. 1984. Dynamic instability of microtubule growth. *Nature.* 312:237-42.

Mitchison, T.J., and M.W. Kirschner. 1985. Properties of the kinetochore in vitro. II. Microtubule capture and ATP-dependent translocation. *J Cell Biol.* 101:766-77.

Murray, A.W., and M.W. Kirschner. 1989. Cyclin synthesis drives the early embryonic cell cycle. *Nature.* 339:275-80.

Nicklas, R.B., S.C. Ward, and G.J. Gorbsky. 1995. Kinetochore chemistry is sensitive to tension and may link mitotic forces to a cell cycle checkpoint. *J Cell Biol.* 130:929-39.

Nicklas, R.B., J.C. Waters, E.D. Salmon, and S.C. Ward. 2001. Checkpoint signals in grasshopper meiosis are sensitive to microtubule attachment, but tension is still essential. *J Cell Sci.* 114:4173-83.

Obuse, C., O. Iwasaki, T. Kiyomitsu, G. Goshima, Y. Toyoda, and M. Yanagida. 2004. A conserved Mis12 centromere complex is linked to heterochromatic HP1 and outer kinetochore protein Zwint-1. *Nat Cell Biol.* 6:1135-41.

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

Ohba, T., M. Nakamura, H. Nishitani, and T. Nishimoto. 1999. Self-organization of microtubule asters induced in Xenopus egg extracts by GTP-bound Ran. *Science.* 284:1356-8.

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

Pangilinan, F., and F. Spencer. 1996. Abnormal kinetochore structure activates the spindle assembly checkpoint in budding yeast. *Mol Biol Cell.* 7:1195-208.

Paschal, B.M., S.M. King, A.G. Moss, C.A. Collins, R.B. Vallee, and G.B. Witman. 1987. Isolated flagellar outer arm dynein translocates brain microtubules in vitro. *Nature.* 330:672-4.

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

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

Piatti, S., C. Lengauer, and K. Nasmyth. 1995. Cdc6 is an unstable protein whose de novo synthesis in G1 is important for the onset of S phase and for preventing a 'reductional' anaphase in the budding yeast Saccharomyces cerevisiae. *Embo J.* 14:3788-99.

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

Pinsky, B.A., S.Y. Tatsutani, K.A. Collins, and S. Biggins. 2003. An Mtw1 complex promotes kinetochore biorientation that is monitored by the Ipl1/Aurora protein kinase. *Dev Cell.* 5:735-45.

Poddar, A., P.T. Stukenberg, and D.J. Burke. 2005. Two complexes of spindle checkpoint proteins containing Cdc20 and Mad2 assemble during mitosis independently of the kinetochore in Saccharomyces cerevisiae. *Eukaryot Cell.* 4:867-78.

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

Rieder, C.L., and S.P. Alexander. 1990. Kinetochores are transported poleward along a single astral microtubule during chromosome attachment to the spindle in newt lung cells. *J Cell Biol.* 110:81-95.

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

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

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

Robinson, J.T., E.J. Wojcik, M.A. Sanders, M. McGrail, and T.S. Hays. 1999. Cytoplasmic dynein is required for the nuclear attachment and migration of centrosomes during mitosis in Drosophila. *J Cell Biol.* 146:597-608.

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

Rusan, N.M., C.J. Fagerstrom, A.M. Yvon, and P. Wadsworth. 2001. Cell cycle-dependent changes in microtubule dynamics in living cells expressing green fluorescent protein-alpha tubulin. *Mol Biol Cell.* 12:971-80.

Rusan, N.M., U.S. Tulu, C. Fagerstrom, and P. Wadsworth. 2002. Reorganization of the microtubule array in prophase/prometaphase requires cytoplasmic dynein-dependent microtubule transport. *J Cell Biol.* 158:997-1003.

Salmon, E.D., R.J. Leslie, W.M. Saxton, M.L. Karow, and J.R. McIntosh. 1984. Spindle microtubule dynamics in sea urchin embryos: analysis using a fluorescein-labeled tubulin and measurements of fluorescence redistribution after laser photobleaching. *J Cell Biol.* 99:2165-74.

Savoian, M.S., M.L. Goldberg, and C.L. Rieder. 2000. The rate of poleward chromosome motion is attenuated in Drosophila zw10 and rod mutants. *Nat Cell Biol.* 2:948-52.

Saxton, W.M., D.L. Stemple, R.J. Leslie, E.D. Salmon, M. Zavortink, and J.R. McIntosh. 1984. Tubulin dynamics in cultured mammalian cells. *J Cell Biol.* 99:2175-86.

Scaerou, F., I. Aguilera, R. Saunders, N. Kane, L. Blottiere, and R. Karess. 1999. The rough deal protein is a new kinetochore component required for accurate chromosome segregation in Drosophila. *J Cell Sci.* 112 (Pt 21):3757-68.

Scaerou, F., D.A. Starr, F. Piano, O. Papoulas, R.E. Karess, and M.L. Goldberg. 2001. The ZW10 and Rough Deal checkpoint proteins function together in a large, evolutionarily conserved complex targeted to the kinetochore. *J Cell Sci.* 114:3103-14.

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

Sear, R.P., and M. Howard. 2006. Modeling dual pathways for the metazoan spindle assembly checkpoint. *Proc Natl Acad Sci U S A.* 103:16758-63.

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

Sharp-Baker, H., and R.H. Chen. 2001. Spindle checkpoint protein Bub1 is required for kinetochore localization of Mad1, Mad2, Bub3, and CENP-E, independently of its kinase activity. *J Cell Biol.* 153:1239-50.

Sharp, D.J., G.C. Rogers, and J.M. Scholey. 2000. Cytoplasmic dynein is required for poleward chromosome movement during mitosis in Drosophila embryos. *Nat Cell Biol.* 2:922-30.

Shirasu-Hiza, M., P. Coughlin, and T. Mitchison. 2003. Identification of XMAP215 as a microtubule-destabilizing factor in Xenopus egg extract by biochemical purification. *J Cell Biol.* 161:349-58.

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

Smith, D.A., B.S. Baker, and M. Gatti. 1985. Mutations in genes encoding essential mitotic functions in Drosophila melanogaster. *Genetics.* 110:647-70.

Sousa, A., R. Reis, P. Sampaio, and C.E. Sunkel. 2007. The Drosophila CLASP homologue, Mast/Orbit regulates the dynamic behaviour of interphase microtubules by promoting the pause state. *Cell Motil Cytoskeleton.* 64:605-620.

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

Spiegelman, B.M., S.M. Penningroth, and M.W. Kirschner. 1977. Turnover of tubulin and the N site GTP in Chinese hamster ovary cells. *Cell.* 12:587-600.

Starr, D.A., R. Saffery, Z. Li, A.E. Simpson, K.H. Choo, T.J. Yen, and M.L. Goldberg. 2000. HZwint-1, a novel human kinetochore component that interacts with HZW10. *J Cell Sci.* 113 (Pt 11):1939-50.

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

Starr, D.A., B.C. Williams, Z. Li, B. Etemad-Moghadam, R.K. Dawe, and M.L. Goldberg. 1997. Conservation of the centromere/kinetochore protein ZW10. *J Cell Biol.* 138:1289-301.

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

Stern, B.M., and A.W. Murray. 2001. Lack of tension at kinetochores activates the spindle checkpoint in budding yeast. *Curr Biol.* 11:1462-7.

Stewart, R.J., K.W. Farrell, and L. Wilson. 1990. Role of GTP hydrolysis in microtubule polymerization: evidence for a coupled hydrolysis mechanism. *Biochemistry*. 29:6489-98.

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

Tai, C.Y., D.L. Dujardin, N.E. Faulkner, and R.B. Vallee. 2002. Role of dynein, dynactin, and CLIP-170 interactions in LIS1 kinetochore function. *J Cell Biol.* 156:959-68.

Takahashi, K., E.S. Chen, and M. Yanagida. 2000. Requirement of Mis6 centromere connector for localizing a CENP-A-like protein in fission yeast. *Science*. 288:2215-9.

Takahashi, K., S. Murakami, Y. Chikashige, H. Funabiki, O. Niwa, and M. Yanagida. 1992. A low copy number central sequence with strict symmetry and unusual chromatin structure in fission yeast centromere. *Mol Biol Cell*. 3:819-35.

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

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

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

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

Taylor, S.S., D. Hussein, Y. Wang, S. Elderkin, and C.J. Morrow. 2001. Kinetochore localisation and phosphorylation of the mitotic checkpoint components Bub1 and BubR1 are differentially regulated by spindle events in human cells. *J Cell Sci.* 114:4385-95.

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

Uhlmann, F., F. Lottspeich, and K. Nasmyth. 1999. Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. *Nature*. 400:37-42.

Vaisberg, E.A., M.P. Koonce, and J.R. McIntosh. 1993. Cytoplasmic dynein plays a role in mammalian mitotic spindle formation. *J Cell Biol.* 123:849-58.

van Breugel, M., D. Drechsel, and A. Hyman. 2003. Stu2p, the budding yeast member of the conserved Dis1/XMAP215 family of microtubule-associated proteins is a plus end-binding microtubule destabilizer. *J Cell Biol.* 161:359-69.

Van Hooser, A.A., Ouspenski, II, H.C. Gregson, D.A. Starr, T.J. Yen, M.L. Goldberg, K. Yokomori, W.C. Earnshaw, K.F. Sullivan, and B.R. Brinkley. 2001. Specification of kinetochore-forming chromatin by the histone H3 variant CENP-A. *J Cell Sci.* 114:3529-42.

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

Vink, M., M. Simonetta, P. Transidico, K. Ferrari, M. Mapelli, A. De Antoni, L. Massimiliano, A. Ciliberto, M. Faretta, E.D. Salmon, and A. Musacchio. 2006. In vitro FRAP identifies the minimal requirements for Mad2 kinetochore dynamics. *Curr Biol*. 16:755-66.

Visintin, R., S. Prinz, and A. Amon. 1997. CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis. *Science.* 278:460-3.

Walker, R.A., E.T. O'Brien, N.K. Pryer, M.F. Soboeiro, W.A. Voter, H.P. Erickson, and E.D. Salmon. 1988. Dynamic instability of individual microtubules analyzed by video light microscopy: rate constants and transition frequencies. *J Cell Biol.* 107:1437-48.

Wang, H., X. Hu, X. Ding, Z. Dou, Z. Yang, A.W. Shaw, M. Teng, D.W. Cleveland, M.L. Goldberg, L. Niu, and X. Yao. 2004a. Human Zwint-1 specifies localization of Zeste White 10 to kinetochores and is essential for mitotic checkpoint signaling. *J Biol Chem.* 279:54590-8.

Wang, Y., F. Hu, and S.J. Elledge. 2000. The Bfa1/Bub2 GAP complex comprises a universal checkpoint required to prevent mitotic exit. *Curr Biol.* 10:1379-82.

Wang, Z., J.M. Cummins, D. Shen, D.P. Cahill, P.V. Jallepalli, T.L. Wang, D.W. Parsons, G. Traverso, M. Awad, N. Silliman, J. Ptak, S. Szabo, J.K. Willson, S.D. Markowitz, M.L. Goldberg, R. Karess, K.W. Kinzler, B. Vogelstein, V.E. Velculescu, and C. Lengauer. 2004b. Three classes of genes mutated in colorectal cancers with chromosomal instability. *Cancer Res.* 64:2998-3001.

Warren, C.D., D.M. Brady, R.C. Johnston, J.S. Hanna, K.G. Hardwick, and F.A. Spencer. 2002. Distinct chromosome segregation roles for spindle checkpoint proteins. *Mol Biol Cell.* 13:3029-41.

Wassmann, K., and R. Benezra. 1998. Mad2 transiently associates with an APC/p55Cdc complex during mitosis. *Proc Natl Acad Sci U S A.* 95:11193-8.

Waters, J.C., R.H. Chen, A.W. Murray, and E.D. Salmon. 1998. Localization of Mad2 to kinetochores depends on microtubule attachment, not tension. *J Cell Biol.* 141:1181-91.

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

Weinert, T.A., and L.H. Hartwell. 1988. The RAD9 gene controls the cell cycle response to DNA damage in Saccharomyces cerevisiae. *Science.* 241:317-22.

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

Wigge, P.A., and J.V. Kilmartin. 2001. The Ndc80p complex from Saccharomyces cerevisiae contains conserved centromere components and has a function in chromosome segregation. *J Cell Biol.* 152:349-60.

Wilde, A., and Y. Zheng. 1999. Stimulation of microtubule aster formation and spindle assembly by the small GTPase Ran. *Science.* 284:1359-62.

Williams, B.C., M. Gatti, and M.L. Goldberg. 1996. Bipolar spindle attachments affect redistributions of ZW10, a Drosophila centromere/kinetochore component required for accurate chromosome segregation. *J Cell Biol.* 134:1127-40.

Williams, B.C., T.L. Karr, J.M. Montgomery, and M.L. Goldberg. 1992. The Drosophila l(1)zw10 gene product, required for accurate mitotic chromosome segregation, is redistributed at anaphase onset. *J Cell Biol.* 118:759-73.

Williams, B.C., Z. Li, S. Liu, E.V. Williams, G. Leung, T.J. Yen, and M.L. Goldberg. 2003. Zwilch, a new component of the ZW10/ROD complex required for kinetochore functions. *Mol Biol Cell.* 14:1379-91.

Winey, M., C.L. Mamay, E.T. O'Toole, D.N. Mastronarde, T.H. Giddings, Jr., K.L. McDonald, and J.R. McIntosh. 1995. Three-dimensional ultrastructural analysis of the Saccharomyces cerevisiae mitotic spindle. *J Cell Biol.* 129:1601-15.

Witt, P.L., H. Ris, and G.G. Borisy. 1980. Origin of kinetochore microtubules in Chinese hamster ovary cells. *Chromosoma.* 81:483-505.

Wojcik, E., R. Basto, M. Serr, F. Scaerou, R. Karess, and T. Hays. 2001. Kinetochore dynein: its dynamics and role in the transport of the Rough deal checkpoint protein. *Nat Cell Biol.* 3:1001-7.

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

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

Wordeman, L., and T.J. Mitchison. 1995. Identification and partial characterization of mitotic centromere-associated kinesin, a kinesin-related protein that associates with centromeres during mitosis. *J Cell Biol.* 128:95-104.

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

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

Yan, X., F. Li, Y. Liang, Y. Shen, X. Zhao, Q. Huang, and X. Zhu. 2003. Human Nudel and NudE as regulators of cytoplasmic dynein in poleward protein transport along the mitotic spindle. *Mol Cell Biol.* 23:1239-50.

Yang, Z., U.S. Tulu, P. Wadsworth, and C.L. Rieder. 2007. Kinetochore dynein is required for chromosome motion and congression independent of the spindle checkpoint. *Curr Biol.* 17:973-80.

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

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

Zinkowski, R.P., J. Meyne, and B.R. Brinkley. 1991. The centromere-kinetochore complex: a repeat subunit model. *J Cell Biol.* 113:1091-110.

Chapter 2

Role of microtubule associated proteins and motors in chromosome segregation.

Note:

This chapter is adapted with permission from Draviam *et al* 2006.

V.M. Draviam, I.Shapiro, B Aldridge, and P.K. Sorger. "Misorientation and reduced stretching of aligned sister kinetochores promote chromosome missegregation in EB1- or APC- depleted cells" EMBO Journal (2006) 25, pp 2814-2827

Experiments in figures where the function of CLIP-170, DHC and LIS1 has been investigated are the author's own work. All other experiments described in this chapter are the work of Viji Draviam.

Outline

2.1 Abstract

The correct formation of stable yet dynamic links between chromosomes and spindle microtubules (MTs) is essential for accurate chromosome segregation. However, the molecular mechanisms by which kinetochores bind MTs and checkpoints monitor this binding remain poorly understood. Here we analyze the functions of six kinetochore-bound microtubule-associated proteins (kMAPs) using RNAi, live-cell microscopy and quantitative image analysis. We find that RNAi-mediated depletion of four kMAPs, CLIP170, LIS1, DHC and TOG1 causes failure in chromosome congression and activation of the spindle checkpoint. In contrast, the Adenomatous Polyposis Coli protein (APC) and its binding partner, EB1 do not perturb kinetochore-MT attachment, but APC or EB1 depletion generates lesions that are poorly detected by the checkpoint and may cause missegregation. This study shows that depletion of CLIP-170, LIS1, DHC or TOG1 generates severe errors in congression, indicating a non-redundant role for these proteins in mitosis and chromosome congression.

2.2 Introduction

Kinetochores are multi-protein structures that assemble on centromeric DNA and mediate the attachment of chromosomes to microtubules (MTs). During metaphase, pairs of sister kinetochores undergo a complex series of movements in which they capture the (+) ends of MTs emanating from centrosomes and also nucleate the formation of MTs that are guided toward the poles, so as to form stable bipolar attachments to the mitotic spindle (Rieder, 2005). Following the completion of bipolar attachment by all chromatid pairs, and the consequent silencing of the Mad and Bub-dependent spindle checkpoint,

sister chromatids disjoin and move towards poles, around which daughter cells form (Musacchio and Hardwick, 2002). In many MT-based processes, motors and MT associated proteins (MAPs) work together to regulate MT dynamics in a spatially controlled fashion and to generate forces necessary for directed movement (Howard and Hyman, 2003). Despite an extensive amount of data characterizing the role of MAPs and motors in regulation of MT dynamics in interphase, their function in chromosome segregation is poorly understood.

Previous work in a variety of eukaryotes has identified many MAPs that localize to kinetochores (Akhmanova and Hoogenraad, 2005). The dynein/dynactin motor complex and the +end binding proteins CLIP170 and LIS1 accumulate on kinetochores that are unattached to MTs and at +ends of growing MTs (Dujardin et al., 1998; Faulkner et al., 2000). Dynein/dynactin acts as a mediator between CLIP-170, LIS1 and kinetrochores (Coquelle et al., 2002; Tai et al., 2002). In contrast, (+)end binding protein EB1 is recruited selectively to kinetochores that are associated with growing MTs (Tirnauer et al., 2002). Adenomatous Polyposis Coli protein (APC), an EB1 binding partner, exhibits similar localization pattern (Kaplan et al., 2001). Finally, CLASP1 localizes to kinetochores regardless of MT-attachment status (Maiato et al., 2003) where it controls MT dynamics at attached kinetochores (Maiato et al., 2003; Maiato et al., 2005).

CLIP-170, Lis1 and dynein interact with each other at kinetochores and other subcellular locations (Lansbergen et al., 2004; Maiato et al., 2005; Tai et al., 2002). CLIP-170 is implicated in MT stabilization acting as an anti-catastrophe or rescue factor (Brunner and Nurse, 2000; Komarova et al., 2002). In addition, CLIP-170 is suggested to

promote MT capture at cortical sites through interaction with dynein/dynactin (Goodson et al., 2003; Lansbergen et al., 2004; Sheeman et al., 2003). The role of CLIP-170 in chromosome segregation has been unclear at the time when I started this work. LIS1, is reported to suppress microtubule dynamics in vitro by reducing catastrophes (Han et al., 2001). In the case of dynein/dynactin or LIS1, published data are contradictory with respect to function in mitosis and MT binding (Echeverri et al., 1996; Faulkner et al., 2000; Green et al., 2005; Howell et al., 2001). While overexpression of dynamitin (p50) subunit of dynactin results in unalined chromosomes in metaphase (Echeverri et al., 1996), injection of an anti-dynein antibody 70.1 or a purified p50 into PtK1 cells does not disrupt chromosome congression at the metaphase plate (Howell et al., 2001). Similarly, injection of anti-LIS1 antibody into NRK cells causes unaligned chromosomes that lead to missegregation in anaphase (Faulkner et al., 2000) while depletion of LIS1 in 293T cells evokes a mitotic arrest and causes spindle mispositioning (Green et al., 2005). Therefore, the role of dynein/dynactin/ CLIP-170/LIS1 proteins in chromosome segregation is poorly understood.

Depletion of the EB1 or APC MAPs is reported to cause chromosome misalignment and missegregation (Green et al., 2005; Kaplan et al., 2001), implying a defect in the checkpoint response, but it is unknown why APC and EB1 depleted cells divide rather than arrest when misaligned chromosomes are present. EB1, APC, CLIP-170 and dynein/dynactin/LIS1 are localised to kinetochores of mitotic cells, may possibly function in regulating MT–kinetochore attachment and/or the dynamics of kinetochore fibres (Coquelle et al., 2002; Dujardin et al., 1998; Kaplan et al., 2001; Tirnauer et al., 2002). Since these proteins have been mostly analysed by separate studies, and often in

different experimental systems, it is difficult to predict whether they participate in synergistic, mutually exclusive or redundant pathways, a question that clearly requires further examination.

In this chapter, I describe our investigation of the effects of disrupting proteins at the kinetochore-MT interface on chromosome segregation in mitosis. We use live-cell microscopy and quantitative image analysis to directly compare the fates of mitotic cells that have been depleted of one of six kMAPs by RNAi. We find that depletion of CLIP170, Dynein heavy chain (DHC), LIS1 or TOG1 prevents normal chromosome congression and results in prolonged checkpoint-dependent mitotic arrest, eventually leading to cell death. In contrast, depletion of EB1 or APC does not significantly interfere with congression but leads to sporadic chromosome missegregation at anaphase. Severity of congression defect caused by depletion of CLIP-170, DHC, LIS1 or TOG1 suggests a crucial role these proteins play in kinetochore-MT attachment.

2.3 Results

2.3.1 Effect of MAPs depletion on chromosome congression at the metaphase plate.

To investigate the effect of depleting kMAPs on kinetochore-MT binding, checkpoint activation, and chromosome segregation we combined RNAi, live-cell microscopy and quantitative image analysis. CLIP170, LIS1, DHC, TOG1, APC and EB1 were targeted with 4-5 different siRNA oligonucleotides each and the degree of depletion monitored by immunoblotting and immunofluorescence (Figure 2.1; Supplementary Table and data not shown). For each gene, two oligonucleotides resulting in at least 80-90% protein depletion were chosen for further study (Supplementary Table). RNAi-

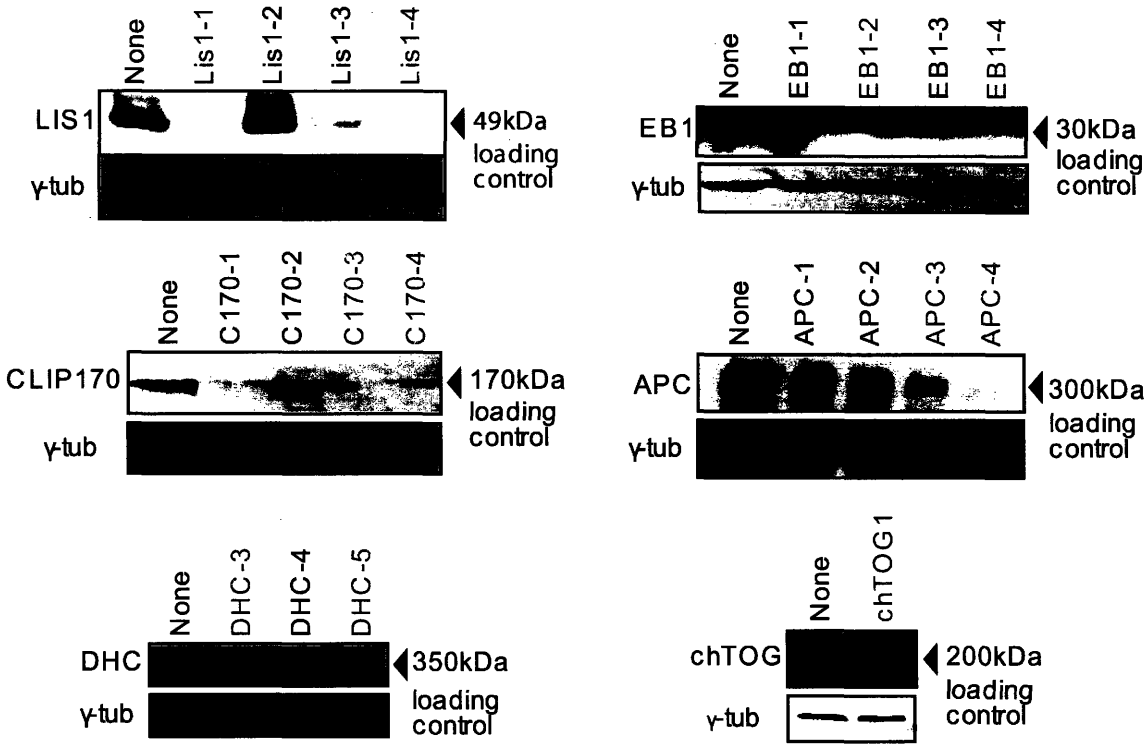


Figure 2.1 Extent of RNAi-mediated protein depletion. Immunoblots of lysates from cells transfected with siRNA against individual kMAPs and probed with antibodies as indicated. γ-tubulin antibody was used as loading control. Oligos (in green) were chosen for time-lapse analysis.

treated HeLa cells (n~100 per gene) expressing Histone2B (H2B) fused to DsRed were imaged every 3min for 4-12 h. In each cell, the time of anaphase onset was determined relative to nuclear breakdown (NBD), which was set as t=0. Chromosomes that had not congressed to the metaphase plate prior to anaphase, or that remained away from the metaphase plate for >45min in arrested cells, were scored as unaligned; those that remained stranded at the spindle equator after anaphase A (chromatid disjunction) were scored as lagging. The fragmentation of chromatin and membrane blebbing (markers of cell death), the orientation of the metaphase plate, elongation of cells in anaphase B and ingression of the furrow during cytokinesis were scored from phase contrast and fluorescence images.

In control cells (n=81), chromosomes were observed to align at the spindle equator by t= 20 ± 1.5min, followed 6-8min later by anaphase (at t= 26 ± 2min; (Meraldi et al., 2004). Fewer than 7% of cells had unaligned chromosomes at the time of anaphase onset and no cell death was observed. In contrast, 70-90% cells (n~100 per gene) depleted of TOG1, DHC, CLIP170 or LIS1 remained arrested in mitosis for >3h without visible cell body elongation or cytokinetic furrow ingression (Figure 2.2 A,B). The severity of the misalignment defect and the precise morphology of the mitotic spindle varied among kMAP depletions, but in all cases, the majority of cells arrested in mitosis had failures of chromosome congression with two or more chromosomes scattered along the spindle axis distant and far from the spindle equator (Figure 2.2 A,C); ~90% of cells that remained arrested for >3hr underwent cell death. Thus chromosome congression to the metaphase plate is impaired in cells depleted of DHC, CLIP-170, TOG1 or LIS1 leading to checkpoint activation and inhibition of metaphase to anaphase transition.

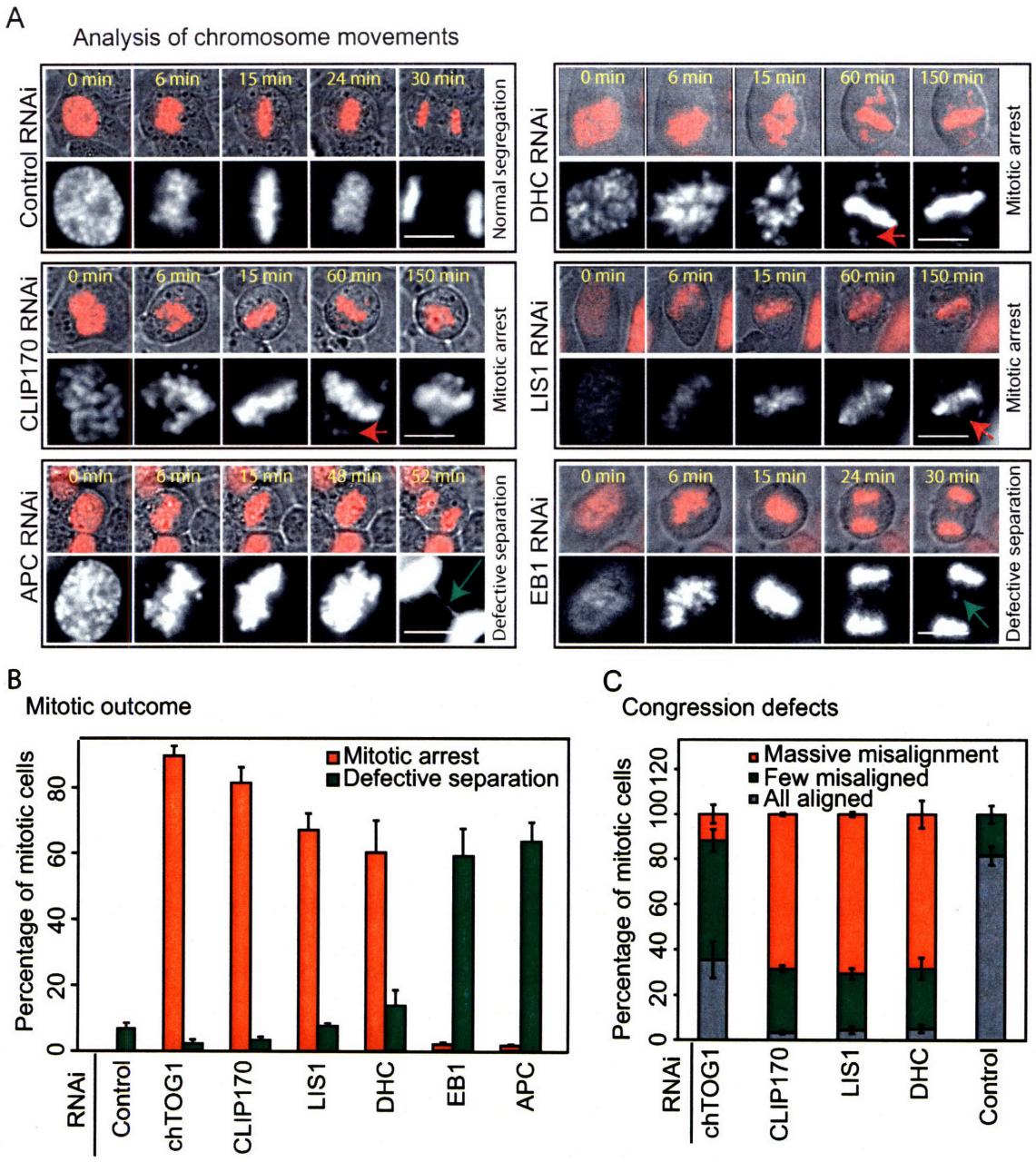


Figure 2.2 Analysis of mitotic outcome in kMAP-depleted cells **(A)** Overlay of phase-contrast and fluorescent images of H2B-DsRed expressing HeLa cells treated with siRNA as indicated. Grey-scale panels are 2X magnified fluorescent images of top panels Nuclear breakdown is set as t=0. Red and green arrows mark uncongressed and lagging chromosomes respectively. Bar: 10μm **(B)** Mitotic outcome in control-, or kMAP-depleted cells. Cells were scored as arrested prior to anaphase (orange) or having undergone defective anaphase in the presence of lagging or unaligned chromosomes (green). Error bars show SD based on at least three experiments. **(C)** Congression defects in control-, or kMAP-depleted cells. Misalignment was visually scored as >5 (massive), 1-5 (few), or no chromosome away from the metaphase plate

2.3.2 Centromere stretching in cells depleted of MAPs.

Although only a few chromosomes remained unaligned in TOG1, DHC, CLIP-170 or LIS1- depleted cells, the majority of chromosomes congressed to the metaphase plate. To determine whether aligned chromosomes were correctly attached to spindle MTs we measured inter-centromeric distances in cells depleted of DHC, as a measure of microtubule-mediated pull associated with inter-kinetochore tension. During metaphase, sister kinetochores are subjected to MT-pulling forces and their extent of separation oscillates between 1.0 and 2.0μm (Figure 2.3; (Shelby et al., 1996)). Cells were treated with MG132, a proteasome inhibitor, to block metaphase to anaphase transition and allow cells more time to build up an inter-kinetochore tension. RNAi-treated HeLa cells (n~ 10) expressing CENPB fused to GFP (CENPB-GFP) were imaged every 3min for 40 min. As expected, when control cells treated with MG132 were filmed between t = 0 and 33min after NBD, transient separation was observed and mean inter-kinetochore distances increased over time up to 1.6μm (Figure 2.3). In DHC - depleted cells, brief periods of transient separation were detected but mean inter-kinetochore distances were only around 0.4 - 0.8μm. Even after addition of MG132, DHC-depleted kinetochores did not exhibit a higher degree of separation (Figure 2.3). Uniformly low level of tension across congressed sisters in DHC-depleted cells suggests possible defects in kinetochore-MT attachment that do not interfere with alignment at the metaphase plate, but may contribute to the failure to progress through metaphase to anaphase transition.

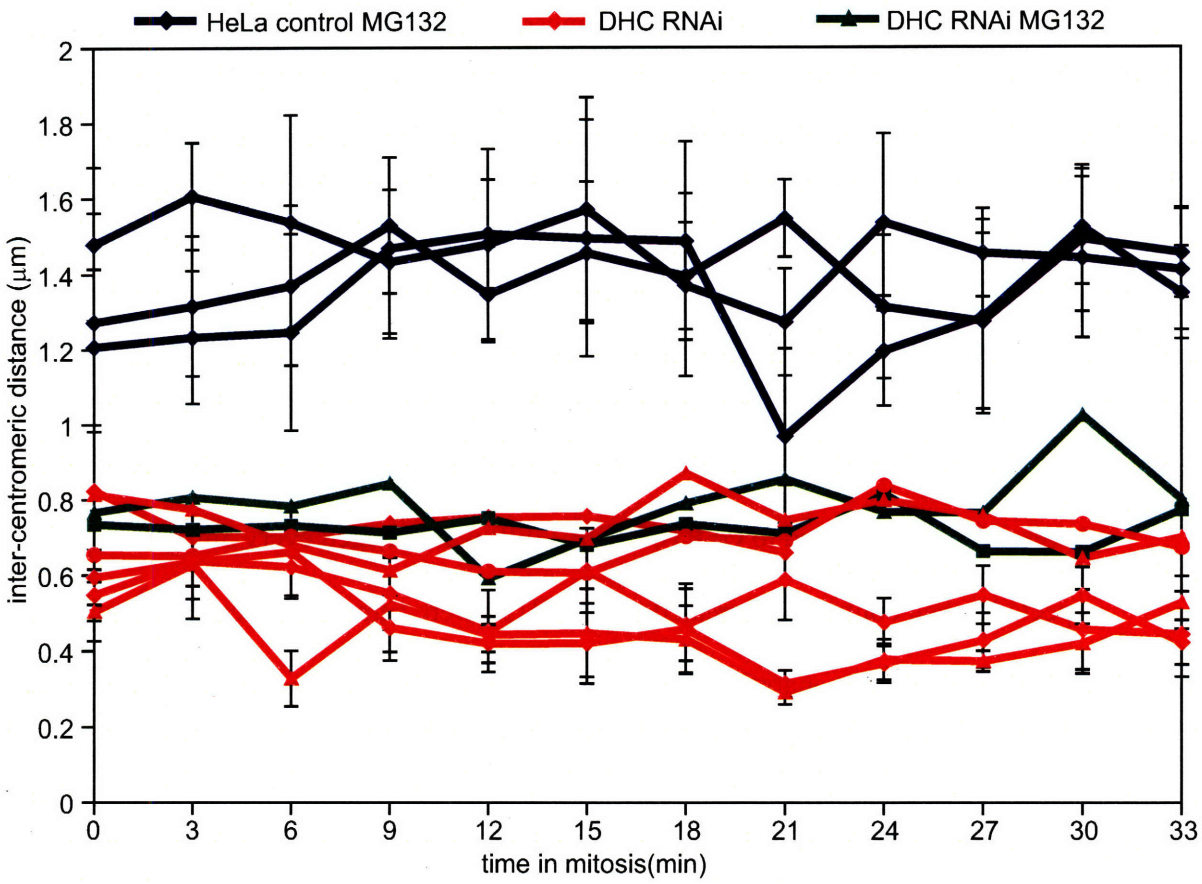


Figure 2.3. DHC depletion reduces centromeric stretching.
Inter-kinetochore distances in CENPB-GFP expressing HeLa cells, treated with siRNA against control or DHC, calculated from 3D images taken every 3min for 33min period. Cells were treated with 1 μM, as indicated.

2.3.3 Checkpoint activation in cells depleted of MAPs.

Recruitment of checkpoint proteins Mads and Bubs to unattached kinetochores has been correlated with activation of the spindle checkpoint and mitotic arrest. Therefore I assessed the kinetochore recruitment of Mad2 and Bub1 in cells depleted of various MAPs. In TOG1, DHC, CLIP170 or LIS1-depleted cells, high-levels of Mad2 and Bub1 were present on misaligned kinetochores, whereas in control cells the levels of kinetochore-bound Mad2 and Bub1 decreased as prometaphase proceeded and kinetochores aligned (Figure 2.4A). To confirm that mitotic arrest provoked by kMAP depletion was checkpoint dependent, cells were codepleted of Mad2. In all cases, >98% of codepleted cells bypassed the checkpoint as evidenced by early anaphase onset (Figure 2.4B) followed by anaphase B elongation and cleavage furrow ingression. Taken together, these data show that, in the absence of TOG1, DHC, CLIP170 or LIS1, chromosome congression to the metaphase plate is impaired, congressed chromosomes exhibit reduced centromere stretching, a sustained checkpoint signal is generated and cell death ensues.

2.3.4 Mitotic phenotypes of EB1 or APC depletion

The phenotypes associated with EB1 or APC depletion were strikingly different from those of other kMAP depletions. Chromosomes congressed correctly to the spindle equator in most cells and anaphase ensued with little or no delay (Figure 2.2A and 2.5A). During anaphase, however, 60-70% of EB1- or APC-depleted cells (n>200 cells) had one or more chromosomes or chromatin strands stranded at spindle equator (Figure 2.2A and 2.5B). The appearance of these lagging strands suggested that kinetochores were not

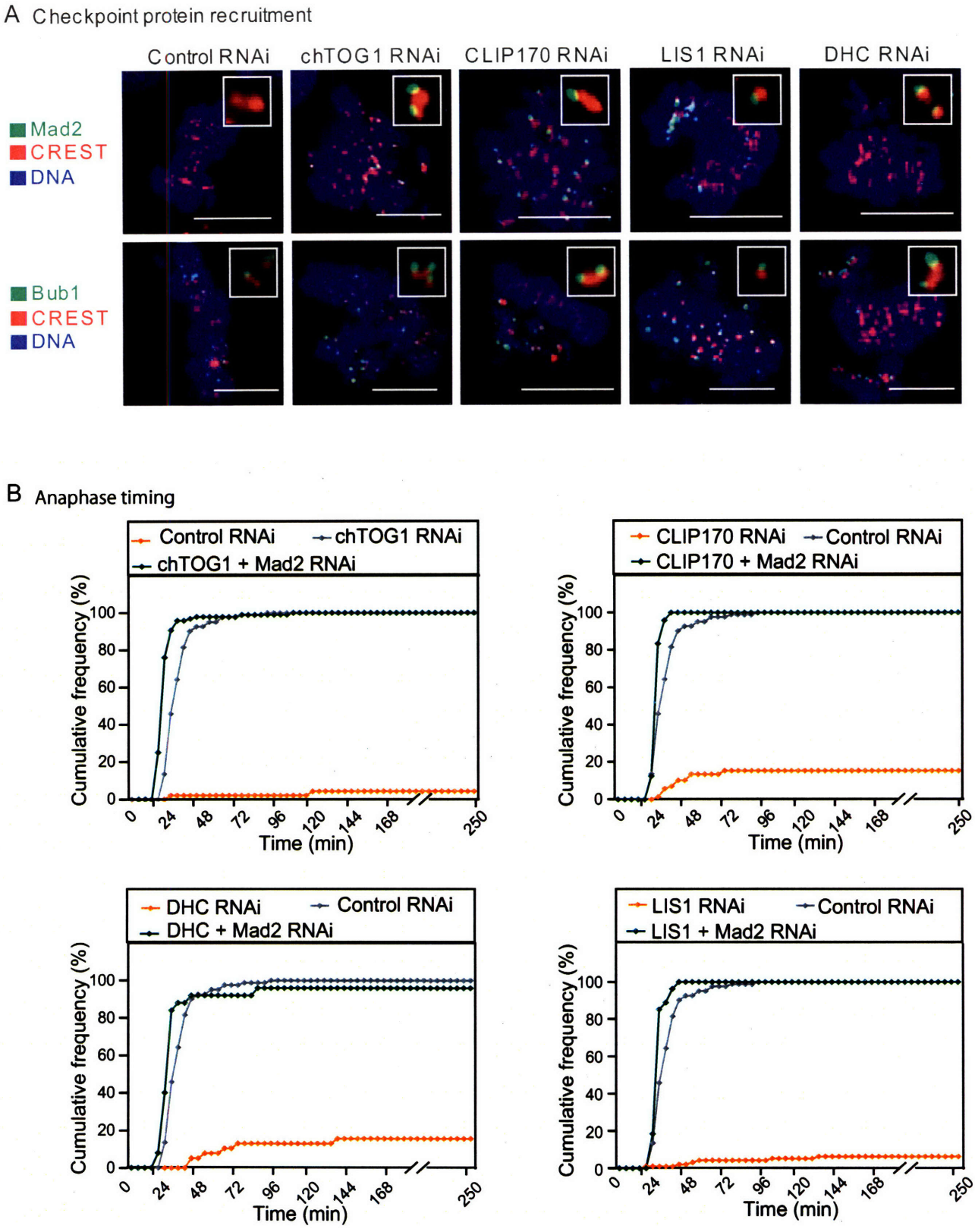


Figure 2.4. Kinetochore recruitment of checkpoint proteins.
(A) Images of metaphase cells treated with siRNA against MAPs, as indicated, and stained with anti-Bub1 or anti-Mad2 antibody (red) and CREST sera to visualize kinetochores. Inset: 0.25×0.25mμ. Bar: 10.mμ(green). **(B)** Cumulative frequency plots of anaphase onset times in siRNA-treated cells as indicated.

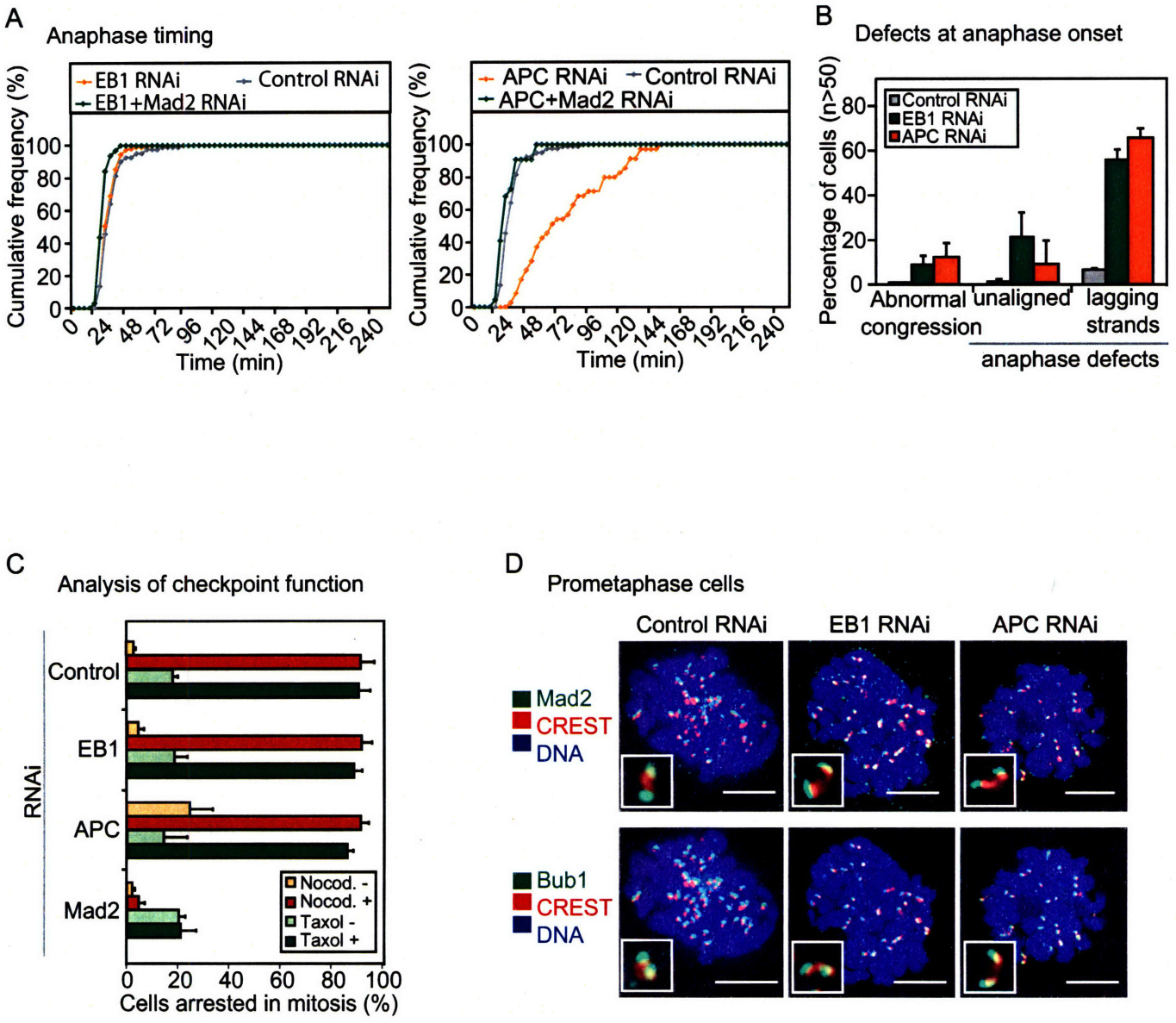


Figure 2.5. Depletion of EB1 or APC does not abrogate spindle checkpoint response. **(A)** Cumulative frequency plots of anaphase times in siRNA-treated cells as indicated. **(B)** Segregation defects in control-, EB1-, or APC-depleted cells. Abnormal congression denotes the presence of unaligned chromosomes 3min prior to anaphase onset. Cells with anaphase defects were scored as having unaligned chromosomes during anaphase separation or containing lagging chromosomes. Error bars show SD based on at least three different experiments **(C)** Percentage of control-, EB1-, APC-, or Mad2-depleted cells arrested in mitosis. In the presence or absence of nocodazole, cells in mitosis for >50min after NBD were scored as arrested. In the presence or absence of taxol, mitotic arrest was scored by counting DAPI stained condensed DNA. **(D)** Images of prometaphase cells treated with siRNA as indicated, and stained with CREST sera to visualize kinetochores (red), and with Mad2 or Bub1 (green) antibodies.

bound to MTs in a manner compatible with normal disjunction. One possibility for missegregation was that EB1 or APC depletion interfered with checkpoint function. To test this, EB1- and APC-depleted cells were treated with nocodazole, an MT-depolymerizing drug, or taxol, an MT-stabilizing drug, and the extent of mitotic arrest evaluated by live and fixed cell imaging (see Figure 2.5C legend for details). Whereas Mad2 depletion (a positive control) abrogated checkpoint arrest, arrest was efficient and sustained in drug-treated EB1- or APC-depleted cells (Figure 2.5C). Moreover, immunostaining revealed high levels of kinetochore-bound Mad1, Mad2, Mps1, Bub1 and BubR1 checkpoint proteins in control, EB1- and APC-depleted prometaphase cells, consistent with an intact checkpoint (Figure 2.5D; data not shown). Thus, EB1 or APC depletion did not interfere with the operation of the spindle checkpoint.

2.4 Discussion

By combining RNAi-mediated protein depletion, live-cell imaging and quantitative analysis, the roles of kMAPs in chromosome-MT binding and spindle checkpoint function were investigated. The six kMAPs of this study share the property that they localize to kinetochores and spindle MTs, but fall into two classes with respect to mitotic depletion phenotypes. Depletion of CLIP170, DHC, LIS1 or TOG1 perturbs chromosome congression and generates unattached kinetochores that provoke a checkpoint-dependent mitotic arrest. The arrest is sustained and associated with increased cell death. Depletion of EB1 or APC causes chromosomes to congress to the spindle equator and cells proceed with little or no delay but occasionally missegeregate

chromosomes. Thus, CLIP-170, DHC, LIS1 or TOG1 play a crucial non-redundant role in kinetochore-MT attachment.

The role of dynein motor at kinetochores has been controversial (Echeverri et al., 1996; Howell et al., 2001). Although the function of dynein in the poleward chromosome movement in prometaphase has been suggested previously based on measurements of the velocity of chromosome movements (Rieder and Alexander, 1990), dynein antibody injection studies eliminated a role for dynein in chromosome congression (Howell et al., 2001). Our analysis indicates that in cells depleted of DHC, chromosomes do not congress to the metaphase plate and cells arrest in mitosis for a prolonged period of time and commit to cell death. This data suggests a possible role for dynein in kinetochore-MT attachment, as has been proposed earlier (Echeverri et al., 1996; Rieder and Alexander, 1990). Moreover, a recent report from Rieder lab confirms dynein function in poleward chromosome movement, although initial kinetochore-MT encounter is not abrogated in anti-dynein antibody injected cells (Yang et al., 2007). Further attempts to investigate the role of dynein/dynactin complex at kinetochores by depletion were unsuccessful. Detailed analysis of dynein/dynactin function in MT binding is obstructed by massive cell death resulted from the depletion of these proteins. A likely explanation being that dynein/dynactin complex functions at multiple locations in the cell, including spindle, cortical sites and vesicular trafficking (Carminati and Stearns, 1997; Fath et al., 1997). Therefore, to address the function of dynein/dynactin I chose to selectively deplete this complex from kinetochores by interfering with the function of ZW10. ZW10 is a member of the RZZ checkpoint complex that has been demonstrated to recruit dynein/dynactin to

kinetochores (Starr et al., 1998). Detailed analysis of ZW10 depletion and its effect on kinetochore-bound dynein function are presented in Chapter 3 of this thesis.

CLIP-170 has been demonstrated to depend on dynein/dynactin for kinetochore localization (Coquelle et al., 2002). Our data indicates the role of CLIP-170 in chromosome congression that is in agreement with the recent observations suggesting that CLIP-170 plays a role in kinetochore-MT attachment (Tanenbaum et al., 2006). Using RNAi and mutational analysis of CLIP-170 it has been demonstrated that CLIP-170 does not affect dynamics of kinetochore-MTs or their stability but plays a role in the formation of kinetochore- MT binding site (Tanenbaum et al., 2006). LIS1 interacts with CLIP-170 and dynein/dynactin at kinetochores (Coquelle et al., 2002; Tai et al., 2002). Its function in nuclear positioning in different organisms is relatively well understood (Dujardin et al., 2003; Eshel et al., 1993; Han et al., 2001; Liu et al., 1999), while the function of LIS1 in chromosome segregation is controversial (Faulkner et al., 2000; Green et al., 2005). Our data indicates the role for LIS1 in chromosome congression, in agreement with the study that also employed an RNAi mediated approach of LIS1 depletion (Green et al., 2005). TOG1/Stu2/XMAP215 has been demonstrated to exhibit both MT-stabilizing and MT-destabilizing activities (Holmfeldt et al., 2004; Shirasu-Hiza et al., 2003; van Breugel et al., 2003) (Brittle and Ohkura, 2005). TOG1 is also important for the formation of bipolar spindle and centrosomal integrity (Cassimeris and Morabito, 2004).Our findings demonstrate that TOG1 is also critical for chromosome segregation.

Another class of MAPs analyzed in our study included EB1 and APC. Their depletion caused occasional chromosome missegregation despite a complete alignment of chromosomes at the metaphase plate and functional checkpoint. Further analysis

indicated that depletion of EB1 and APC leads to misorientation of centromeric pairs at the metaphase plate, caused by insufficient pulling forces imposed on kinetochore pairs by spindle MTs. Lesions generated by the depletion of EB1 and APC are invisible to the spindle checkpoint and thus contribute to aneuploidy (Draviam et al., 2006).

Altogether our data demonstrates that there are many kinetochore localizing MAPs that have non-redundant functions in chromosome segregation, since depletion of any of them interferes with chromosome congression and possibly with kinetochore-MT attachment. A detailed analysis of individual functions of kMAPs will be necessary to understand the structure of the MT-binding site at kinetochores and how it is regulated. It will be also important to integrate functions of all MAPs and motors regulating MT dynamics at kinetochores in a network of interactions. First attempts have been made to reconstitute MAPs interacting network in vitro using Xenopus egg extracts and a visual immunoprecipitation analysis (Niethammer et al., 2007). Counteracting activities of mitotic microtubule polymerases and depolymerases have been revealed and their action in concert to regulate MT dynamics during chromosome separation are beginning to be understood (Niethammer et al., 2007). Further efforts will be required to reconstitute kinetochore-MT binding site and generate a protein interaction network that controls binding and polymer dynamics in mitosis.

2.5 Materials and methods

Live-cell imaging

Cells were imaged using 20X NA0.75 objective as described in (Meraldi et al., 2004). To image centromere dynamics, 100X NA1.4 objective equipped with a heater was used.

Cell culture

Vectors encoding H2B-DsRed or CENPB-GFP cDNA were transfected using *Fugene6* and cells were sorted using *FACS*. Cells were synchronized using thymidine (20mM; 18h) and nocodazole (40ng/ml) was added 10min prior to imaging. Taxol (40nM) was added 10h after thymidine wash and mitotic cells were scored 6h later.

RNAi

siRNA oligonucleotides (Dharmacon Research, Inc.) targeting DHC, CLIP-170, LIS1, TOG, APC and EB1 are indicated in Supplementary table. RNAi oligos were transfected into HeLa cells as described (Elbashir et al., 2001) and analyzed 48 hrs after transfection. Lamin A siRNA was used as a control (Elbashir et al., 2001).

Antibodies

Cells were fixed, permeabilized and blocked as described (Meraldi et al., 2004). Antibodies used were as follows: affinity-purified goat anti-Bub1 (a gift of S. Taylor, University of Manchester, Manchester, UK), human anti-CREST (Meraldi et al., 2004), mouse anti-EB1 (BD Transduction Labs), rabbit anti-CLIP-170 (Santa-Cruz).

Immunoblotting

Whole cell extracts were prepared by cell lysis in SDS sample buffer with 15% mercaptoethanol, resolved by SDS-PAGE, transferred to PVDF (Immobilon) membranes by semi-dry blotting (Hoefer). Membranes were incubated in blocking buffer (3% low-fat dried milk, PBS, 0.1% Tween-20) and probed with antibodies, as indicated (Supplementary Table), in blocking buffer. Anti-mouse and anti-sheep HRP-conjugated secondary antibodies (Amersham Pharmacia) were applied in blocking buffer and blots

were developed by enhanced chemiluminescence (Supersignal West Femto Maximum kit; Pierce).

Depleted protein	siRNA oligos*	Mitotic phenotype	Post-transfection time when phenotype was analyzed (in hrs.)	Antibodies (Dilutions; and Source)
chTOG1	AAG AGC AGU CGC AAA UGA AGC* #	Mitotic arrest; One primary metaphase plate with multiple smaller plates	36	aXMAP215 (1:200) Gift of Hyman lab
CLIP170	1. AUA AGC UCU UUG CAA GAA A 2. AAA UGG AAG ACA CGU UAA A 3. AAG CUA AUG GCC UGC AGA CAA* 4. UUU CUU GCA AAG AGC UUA UTT*	Mitotic arrest; single metaphase plate with few unattached chromosomes	56	H300 (1:400) SantaCruz
LIS1	1. AAG UCC AGU CAC UCG AGU CAU* 2. AAG AGG CUA GUG GUA UAU UUA 3. AAG GCC GUA UAU UAA CUA GAA 4. GUA UGG UAC GGC CAA AUCA*	Mitotic arrest; single metaphase plate with few unattached chromosomes	72	H300 (1:200) SantaCruz
DHC	1. GCA CUG UUA UGU CAA CUUA 2. AAG GAA GCA CUG CGU GAA CAU 3. AAG GAU CAA ACA UGA CGG AAU* 4. AAG GCG CUG GAA UUG ACA GAU* 5. GGC CAA GGA GGC GCT GGA A	Mitotic arrest; single metaphase plate with few unattached chromosomes	48	DHC (1:800) BD biosciences
APC	1. AAC CAA GGU GGA AAU GGU GUA 2. AAG GUG CCA UGG AAC AGG CUG 3. AAG CGG CAG AAU GAA GGU CAA* 4. AAG UGG CAG GAA GCU CAU GAA*	Defective segregation (lagging strands and occasional unaligned chromosome); Single metaphase plate	76	c-APC28.9 (1:500) Abcam
EB1	1. AAU CCU GUA GAG GCA CGA CAA 2. AAG GUG CAG CUA AAU CAA GUC* 3. AAA UUC GUU CAG UGG UUC AAG 4. AAU UGC CUU GAA GAA AGU GAA*	Defective segregation (lagging strands); Single metaphase plate	48	EB1 (1:1000) BD biosciences

*Oligos used to analyse mitotic phenotype by time-lapse microscopy

Gergely et al., 2003

2.6 Acknowledgements

I thank Viji Draviam for assistance with RNAi depletion experiments, live-cell imaging and imunofluorescence. I also thank her for helpful discussions and critical reading of this chapter. We thank S Taylor, W Earnshaw, Y Mimori-Kiyosue, T Schroer, T Hyman, and K Sullivan for reagents and members of the Sorger lab for helpful discussion.

2.7 References

Akhmanova, A., and C.C. Hoogenraad. 2005. Microtubule plus-end-tracking proteins: mechanisms and functions. *Curr Opin Cell Biol.* 17:47-54.

Brittle, A.L., and H. Ohkura. 2005. Mini spindles, the XMAP215 homologue, suppresses pausing of interphase microtubules in Drosophila. *Embo J.* 24:1387-96.

Brunner, D., and P. Nurse. 2000. CLIP170-like tip1p spatially organizes microtubular dynamics in fission yeast. *Cell.* 102:695-704.

Carminati, J.L., and T. Stearns. 1997. Microtubules orient the mitotic spindle in yeast through dynein-dependent interactions with the cell cortex. *J Cell Biol.* 138:629-41.

Cassimeris, L., and J. Morabito. 2004. TOGp, the human homolog of XMAP215/Dis1, is required for centrosome integrity, spindle pole organization, and bipolar spindle assembly. *Mol Biol Cell.* 15:1580-90.

Coquelle, F.M., M. Caspi, F.P. Cordelieres, J.P. Dompierre, D.L. Dujardin, C. Koifman, P. Martin, C.C. Hoogenraad, A. Akhmanova, N. Galjart, J.R. De Mey, and O. Reiner. 2002. LIS1, CLIP-170's key to the dynein/dynactin pathway. *Mol Cell Biol.* 22:3089-102.

Draviam, V.M., I. Shapiro, B. Aldridge, and P.K. Sorger. 2006. Misorientation and reduced stretching of aligned sister kinetochores promote chromosome missegregation in EB1- or APC-depleted cells. *Embo J.* 25:2814-27.

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

Dujardin, D.L., L.E. Barnhart, S.A. Stehman, E.R. Gomes, G.G. Gundersen, and R.B. Vallee. 2003. A role for cytoplasmic dynein and LIS1 in directed cell movement. *J Cell Biol.* 163:1205-11.

Echeverri, C.J., B.M. Paschal, K.T. Vaughan, and R.B. Vallee. 1996. Molecular characterization of the 50-kD subunit of dynactin reveals function for the complex in chromosome alignment and spindle organization during mitosis. *J Cell Biol.* 132:617-33.

Elbashir, S.M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature.* 411:494-8.

Eshel, D., L.A. Urrestarazu, S. Vissers, J.C. Jauniaux, J.C. van Vliet-Reedijk, R.J. Planta, and I.R. Gibbons. 1993. Cytoplasmic dynein is required for normal nuclear segregation in yeast. *Proc Natl Acad Sci U S A.* 90:11172-6.

Fath, K.R., G.M. Trimbur, and D.R. Burgess. 1997. Molecular motors and a spectrin matrix associate with Golgi membranes in vitro. *J Cell Biol.* 139:1169-81.

Faulkner, N.E., D.L. Dujardin, C.Y. Tai, K.T. Vaughan, C.B. O'Connell, Y. Wang, and R.B. Vallee. 2000. A role for the lissencephaly gene LIS1 in mitosis and cytoplasmic dynein function. *Nat Cell Biol.* 2:784-91.

Goodson, H.V., S.B. Skube, R. Stalder, C. Valetti, T.E. Kreis, E.E. Morrison, and T.A. Schroer. 2003. CLIP-170 interacts with dynactin complex and the APC-binding protein EB1 by different mechanisms. *Cell Motil Cytoskeleton.* 55:156-73.

Green, R.A., R. Wollman, and K.B. Kaplan. 2005. APC and EB1 function together in mitosis to regulate spindle dynamics and chromosome alignment. *Mol Biol Cell.* 16:4609-22.

Han, G., B. Liu, J. Zhang, W. Zuo, N.R. Morris, and X. Xiang. 2001. The Aspergillus cytoplasmic dynein heavy chain and NUDF localize to microtubule ends and affect microtubule dynamics. *Curr Biol.* 11:719-24.

Holmfeldt, P., S. Stenmark, and M. Gullberg. 2004. Differential functional interplay of TOGp/XMAP215 and the KinI kinesin MCAK during interphase and mitosis. *Embo J.* 23:627-37.

Howard, J., and A.A. Hyman. 2003. Dynamics and mechanics of the microtubule plus end. *Nature.* 422:753-8.

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

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

Komarova, Y.A., A.S. Akhmanova, S. Kojima, N. Galjart, and G.G. Borisy. 2002. Cytoplasmic linker proteins promote microtubule rescue in vivo. *J Cell Biol.* 159:589-99.

Lansbergen, G., Y. Komarova, M. Modesti, C. Wyman, C.C. Hoogenraad, H.V. Goodson, R.P. Lemaitre, D.N. Drechsel, E. van Munster, T.W. Gadella, Jr., F. Grosveld, N. Galjart, G.G. Borisy, and A. Akhmanova. 2004. Conformational changes in CLIP-170 regulate its binding to microtubules and dynactin localization. *J Cell Biol.* 166:1003-14.

Liu, Z., T. Xie, and R. Steward. 1999. Lis1, the Drosophila homolog of a human lissencephaly disease gene, is required for germline cell division and oocyte differentiation. *Development.* 126:4477-88.

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

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

Meraldi, P., V.M. Draviam, and P.K. Sorger. 2004. Timing and checkpoints in the regulation of mitotic progression. *Dev Cell.* 7:45-60.

Musacchio, A., and K.G. Hardwick. 2002. The spindle checkpoint: structural insights into dynamic signalling. *Nat Rev Mol Cell Biol.* 3:731-41.

Niethammer, P., I. Kronja, S. Kandels-Lewis, S. Rybina, P. Bastiaens, and E. Karsenti. 2007. Discrete states of a protein interaction network govern interphase and mitotic microtubule dynamics. *PLoS Biol.* 5:e29.

Rieder, C.L. 2005. Kinetochore fiber formation in animal somatic cells: dueling mechanisms come to a draw. *Chromosoma.* 114:310-8.

Rieder, C.L., and S.P. Alexander. 1990. Kinetochores are transported poleward along a single astral microtubule during chromosome attachment to the spindle in newt lung cells. *J Cell Biol.* 110:81-95.

Sheeman, B., P. Carvalho, I. Sagot, J. Geiser, D. Kho, M.A. Hoyt, and D. Pellman. 2003. Determinants of S. cerevisiae dynein localization and activation: implications for the mechanism of spindle positioning. *Curr Biol.* 13:364-72.

Shelby, R.D., K.M. Hahn, and K.F. Sullivan. 1996. Dynamic elastic behavior of alpha-satellite DNA domains visualized in situ in living human cells. *J Cell Biol.* 135:545-57.

Shirasu-Hiza, M., P. Coughlin, and T. Mitchison. 2003. Identification of XMAP215 as a microtubule-destabilizing factor in Xenopus egg extract by biochemical purification. *J Cell Biol.* 161:349-58.

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

Tai, C.Y., D.L. Dujardin, N.E. Faulkner, and R.B. Vallee. 2002. Role of dynein, dynactin, and CLIP-170 interactions in LIS1 kinetochore function. *J Cell Biol.* 156:959-68.

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

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

van Breugel, M., D. Drechsel, and A. Hyman. 2003. Stu2p, the budding yeast member of the conserved Dis1/XMAP215 family of microtubule-associated proteins is a plus end-binding microtubule destabilizer. *J Cell Biol.* 161:359-69.

Yang, Z., U.S. Tulu, P. Wadsworth, and C.L. Rieder. 2007. Kinetochore dynein is required for chromosome motion and congression independent of the spindle checkpoint. *Curr Biol.* 17:973-80.

Chapter 3

A role for ZW10 in kinetochore -microtubule capture and spindle checkpoint signaling.

Note:

All experiments presented in this chapter were designed and executed by the author.

Outline

3.1 Abstract

Correct kinetochore-microtubule attachment is essential for proper chromosome segregation. Errors in attachment, if left uncorrected, can lead to aneuploidy and possibly cancer. Establishment of bipolar attachment is a multistep process initiated as a stochastic capture of microtubules (MTs) by kinetochores followed by correction of improper attachments. Here I demonstrate that in human cells depleted of ZW10 many chromosomes do not align at the spindle equator prior to separation, indicating a novel role for ZW10 in chromosome congression. I investigate the state of kinetochore-MT attachment in ZW10 depleted cells and conclude that many kinetochores fail the early step of achieving a bipolar attachment – the microtubule encounter. Comparison of ZW10 with other checkpoint proteins involved in attachment, like Aurora B or Bub1, reveals differential recruitment pattern of various MAPs and motors to mitotic kinetochores that may potentially explain differences in attachment phenotypes caused by depletion of these checkpoint proteins. Moreover, ZW10 depleted cells fail to delay anaphase onset in the presence of elevated levels of Mad2 at unaligned chromosomes in anaphase suggesting a Mad2 independent role for ZW10 in the spindle checkpoint. These findings demonstrate that ZW10 is unique in that it integrates an early MT capture function with checkpoint signaling at kinetochores.

3.2 Introduction

Zeste-white 10(zw10) and rough deal(rod) genes were originally identified in Drosophila and are restricted to metazoans (Ref.). Unlike the Mads, Bubs, Mps1 and AuroraB/Ipl1 genes, close homologues of ZW10 or its binding partners ROD and Zwilch have not been found in fungi (Starr et al., 1997). ZW10 and ROD are considered checkpoint proteins, since abrogation of their function in *Drosophila*, human cells and in *Xenopus* mitotic extracts leads to precautious

onset of anaphase in the presence of MT inhibitors (Basto et al., 2000; Chan et al., 2000; Kops et al., 2005). Depletion of ZW10, ROD or Zwilch produces identical mitotic phenotypes, causing lagging chromosomes and DNA bridges in anaphase that in turn lead to missegregation and aneuploidy in both mitotic and meiotic cells (Scaerou et al., 2001; Starr et al., 1997). ZW10 , ROD and Zwilch require each other for kinetochore localization (Scaerou et al., 2001; Williams et al., 2003) and form a complex (RZZ) recruited to kinetochores via Zwint-1 (Wang et al., 2004), which in turn depends on Hec1 for its kinetochore localization (Lin et al., 2006). RZZ is itself required for kinetochore recruitment of the dynein-dynactin and Mad1-Mad2 complexes (Buffin et al., 2005; Kops et al., 2005; Starr et al., 1998). RZZ complex has been proposed to play a role in checkpoint inactivation since its components as well as Mad2 are transported towards the minus ends of MTs and away from kinetochores in metaphase by dynein/dynactin (so-called "kinetochore shedding")(Karess, 2005).

Apart from its proposed function in checkpoint inactivation, dynein has been implicated in the initial kinetochore-MT attachment. Dynein is necessary for poleward transport of chromosomes and kinetochore-nucleated MTs in prometaphase(Khodjakov et al., 2003; Maiato et al., 2004; Rieder and Alexander, 1990; Savoian et al., 2000; Sharp et al., 2000). However, anti-dynactin (p50) antibody injection studies excluded requirement for dynein in congression and chromosome alignment at the metaphase plate (Howell et al., 2001). Inhibition or depletion of dynein abrogates its function at multiple subcellular regions making it difficult to dissect the role of dynein in mitosis. Thus the role of kinetochore-bound dynein remains unclear.

Since kinetochore-MT binding is an error-prone process, spindle checkpoint monitors the state of kinetochore-MT attachment and delays cells in mitosis if at least one improperly attached kinetochore is present (Rieder et al., 1995). When all pairs of sister kinetochores have achieved bipolar attachment, the spindle assembly checkpoint is silenced and cells proceed into anaphase.

Mads and Bubs, canonical checkpoint proteins, were originally discovered in budding yeast in screens for genes required to sustain a mitotic arrest in response to gross spindle damage(Hoyt et al., 1991; Li and Murray, 1991). Subsequent studies have revealed that the functional checkpoint in metazoans requires many additional components, including ROD/ZW10/Zwilch, Zwint-1, CENP-E and CENP-I (Karess, 2005). Some kinetochore proteins, like CENP-A, play a structural role and are involved in the checkpoint by providing a platform for spatial organization of downstream checkpoint components. Others, like AuroraB, Bub1 and Ndc80 protein complex, are required for correct kinetochore-MT binding in addition to their checkpoint function (DeLuca et al., 2006; Meraldi and Sorger, 2005; Pinsky et al., 2006). Depletion or inhibition of checkpoint kinases AuroraB or Bub1 gives rise to distinct phenotypes and increases the frequency of syntelic attachments, binding by both kinetochores in a pair of sisters to MTs emanating from a single pole, or lateral attachments, binding of kinetochores to the walls rather than the ends of MTs (Lampson et al., 2004; Meraldi et al., 2004). Aurora B is implicated in correction of syntelic malorientations and Bub1 might interfere with the formation of end-on attachments (Lampson et al., 2004; Meraldi et al., 2004), suggesting the role for these proteins in later steps of achievement of bipolar attachment. This leaves formation and regulation of early attachment steps poorly understood.

Here I investigate the effect of disrupting the function of RZZ complex on progression of mitosis. Live-cell imaging is used to uncover a new role for RZZ and its binding partner, dynein/dynactin, in chromosome segregation. I find that depletion of RZZ member ZW10 interferes with chromosome congression at the metaphase plate. Moreover, I demonstrate that efficient chromosome-MT capture early in mitosis critically depends on RZZ, an unexpected function for a complex known to regulate exit from mitosis. Additional evidence is also presented for a possible Mad2 independent role for ZW10 in the spindle checkpoint control. I

speculate that the role for RZZ at kinetochores is to coordinate early MT capture at kinetochores and its monitoring by the checkpoint.

3.3 Results

3.3.1 Depletion of ZW10 leads to congression and segregation errors

Because all three components of the RZZ complex are interdependent for kinetochore localization in human cells (Chan et al., 2000; Williams et al., 2003), the kinetochore functions of RZZ as a whole can be probed by depleting any single protein. Moreover, mutations in different components of *Drosophila* RZZ give rise to nearly identical mitotic phenotypes(Scaerou et al., 1999; Williams et al., 2003) implying that kinetochores are the primary sites of RZZ's mitotic function. To evaluate the extent of RZZ protein depletion by siRNA transfection, we used 3D immunofluorescence of individual kinetochores, with CREST staining as a quantitation control (deconvolution imaging was performed in nocodazole-treated cells, as described previously (Meraldi and Sorger, 2005). In human cells, efficient depletion of RZZ subunits proved difficult and preliminary experiments established ZW10 to be the best siRNA target. Two siRNA oligos were identified that effectively depleted ZW10. Oligo1 showed ≥8-fold depletion as judged by quantitative immunoblotting of cell extracts and by immunofluorescence of individual kinetochores, whereas oligo2 showed ≥15 fold depletion (Figure 3.1 A-C).

To investigate the consequences of ZW10 depletion on mitosis, chromosome movements were followed in ~100 individual HeLa cells expressing Histone-2B fused to GFP (H2B-GFP) by acquiring images every 3 min for 6 hrs (Figure 3.1D). Cells depleted of ZW10 proceeded through mitosis in the presence of nocodazole (data not shown) confirming previous reports that ZW10 is necessary for mitotic arrest in response to gross spindle damage (Chan et al., 2000) and

establishing the efficacy of RNAi-mediated protein depletion. Moreover, in ~60% of ZW10-depleted but only 2% of control cells, chromosomes failed to reach the spindle equator and align correctly prior to anaphase onset (Figure 3.1, D and E). As a consequence of these alignment errors and the absence of a functional checkpoint, >45% of ZW10-depleted cells contained DNA bridges and paired chromatids that lagged at the spindle equator in anaphase (Figure 1F). Therefore I conclude that ZW10 is required for chromosome congression at the metaphase plate.

3.3.2 Congression defects arise from structural perturbations at the kinetochore

Is chromosome mis-segregation in ZW10-depleted cells simply a consequence of accelerated passage through mitosis? This question has been previously addressed in the context of Mad2 and Bub1 inactivation. In checkpoint defective Mad2-depleted cells, missegregation arises because the mean time before anaphase onset of ~12 min is simply insufficient, relative to ~24 min in unperturbed cells, to complete congression (Meraldi et al., 2004). In contrast, in Bub1-depleted cells, anaphase takes place at approximately the same time as in unperturbed cells and extensive missegregation reflects a direct role for Bub1 in promoting end-on MT binding (Meraldi and Sorger, 2005). To assay mitotic progression in the absence of ZW10, individual siRNA-transfected cells expressing H2B-GFP were imaged and the times between nuclear envelope breakdown (NBD; set as t=0) and anaphase A were determined. In ZW10 depleted cells (n=134) the modal time of anaphase onset (t= 30 ± 10 min) was no shorter, and possibly slightly longer, than in control cells (n=111; t = 25 ± 3 min) (Figure 3.1G). Thus, ZW10 depletion does not accelerate passage through mitosis. As further evidence that insufficient mitotic timing does account for missegregation, ZW10 siRNA-treated cells were synchronized by aphidicholin block-and-release, and the proteasome inhibitor MG132 was then added as cells entered mitosis. MG132 blocks several steps of cell cycle, but in our experiments with

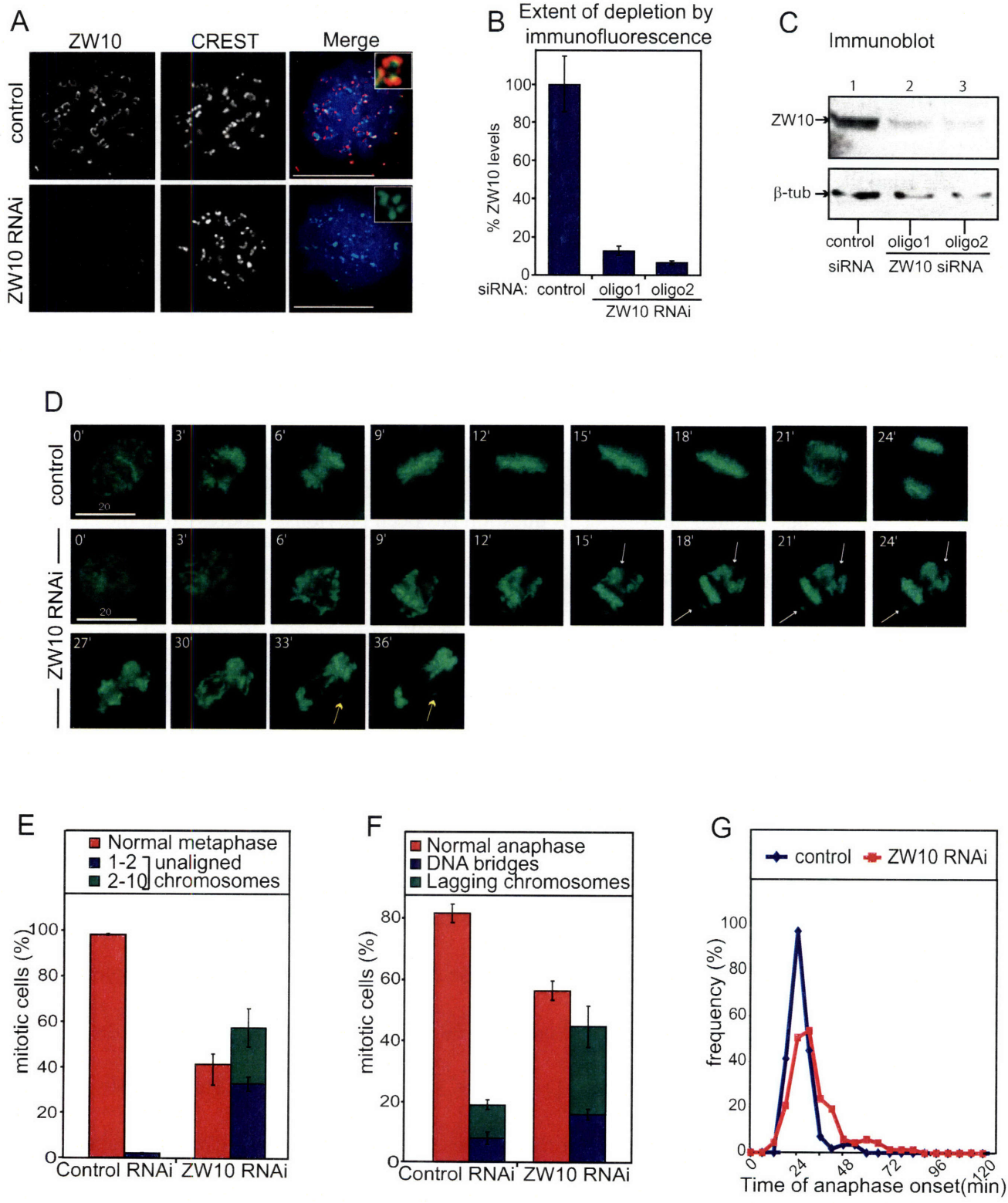

A
ZW10
CREST
Merge
control
ZW10 RNAi
B
Extent of depletion by immunofluorescence
% ZW10 levels
100
80
60
40
20
0
siRNA: control oligo1 oligo2
ZW10 RNAi
C
Immunoblot
1 2 3
ZW10
β-tub
control oligo1 oligo2
siRNA ZW10 siRNA
D
control
ZW10 RNAi
0' 3' 6' 9' 12' 15' 18' 21' 24'
27' 30' 33' 36'
20
E
Normal metaphase
1-2 unaligned
2-10 chromosomes
mitotic cells (%)
100
80
60
40
20
0
Control RNAi ZW10 RNAi
F
Normal anaphase
DNA bridges
Lagging chromosomes
mitotic cells (%)
80
60
40
20
0
Control RNAi ZW10 RNAi
G
control
ZW10 RNAi
frequency (%)
100
80
60
40
20
0
0 24 48 72 96 120
Time of anaphase onset(min)

Figure 3.1. Depletion of ZW10 leads to metaphase and anaphase errors. (A) Representative immunofluorescence images of HeLa cells following transfection with either control or ZW10 siRNA and co- stained with ZW10 antibody, CREST anti-sera and DAPI for DNA. Cells were treated with nocodazole for 2 hrs prior to fixation. Insets are 3D-rendered and 8X-magnified. Scale bar: 10 μm. **(B)** Immunofluorescence intensity of ZW10 on individual kinetochores in cells transfected with siRNAs as indicated. Error bars show SD from 50 kinetochores in 5 cells. ZW10 intensity levels were determined from deconvolved 3D reconstructions relative to a CREST reference and corrected for background noise (see Materials and methods for details). **(C)** Immunoblots of lysates from cells transfected with control siRNA (LaminA)(Elbashir et al., 2001) or either of two siRNA oligos against ZW10 and then probed with anti-ZW10 antibody. Endogenous β-tubulin level is shown as loading control. **(D)** Representative still images from live-cell movies of H2B-GFP expressing HeLa cells transfected with siRNAs as indicated. Scale bar: 20 μm. White and yellow arrows indicate unaligned and lagging chromosomes, respectively. **(E)** Congression defects in control or ZW10 depleted cells. Misalignment was visually scored as 1-2 (few) or 2-10 (many) unaligned chromosomes. Error bars represent SD from three independent experiments. **(F)** Anaphase defects in cells transfected with control or ZW10 siRNA. Error bars represent SD from three independent experiments. **(G)** Frequency distribution of anaphase times in H2B-GFP expressing HeLa cells transfected with siRNAs, as indicated, with NBD set as T=0, as determined from live-cell movies.

synchronized cells, it causes a specific arrest at the metaphase to anaphase transition. One hour after MG132 addition, 48% of ZW10 depleted cells, but only 4% of control cells contained unaligned chromosomes (Figure 3.2 A,B). Mad2 depleted cells, in contrast, contained only 9% unaligned chromosomes under the same conditions (Figure 3.2B). Thus depletion of ZW10 generates long-lasting problems in kinetochore-MT attachment and chromosome alignment that are independent of changes in mitotic timing and checkpoint inactivation.

3.3.3 Segregation defects can be rescued by expression of exogenous ZW10

To establish that segregation errors caused by RNAi of ZW10 were not consequences of off-target effects, I attempted to complement depletion phenotypes by transfecting cells with cDNA encoding ZW10-GFP that carries silent mutations in sequences complementary to our siRNA oligos. Immunoblotting of extracts from these cells showed ZW10-GFP levels to be comparable to those of endogenous ZW10 in control cells (Figure 3.3A). Live-cell imaging revealed the presence of bright, fluorescent, kinetochore foci, thereby establishing that ZW10-GFP localized correctly (Figure 3.3B). When siRNA/ZW10-GFP transfected cells were treated with nocodazole for 16 hr, the mitotic index was nearly as great as in control cells (65-75%) and significantly higher than in Mad2 depleted cells, or ZW10 depleted cells lacking a ZW10-GFP expressing plasmid (8-10%)(Figure 3.3C). Thus, the loss of checkpoint control following ZW10 depletion was efficiently complemented by ZW10-GFP. Importantly, errors in chromosome alignment and attachment were also complemented: imaging of ~100 live cells showed that 80% initiated anaphase normally with all chromosomes aligned at the spindle equator; as compared to 95% of control cells (Figure 3.3 D,G). Some siRNA/ZW10-GFP transfected cells (20%) still had unaligned chromosomes at anaphase onset (Figure 3.3G), probably as a consequence of heterogeneity arising from the use of transient transfection. Nonetheless, I conclude that

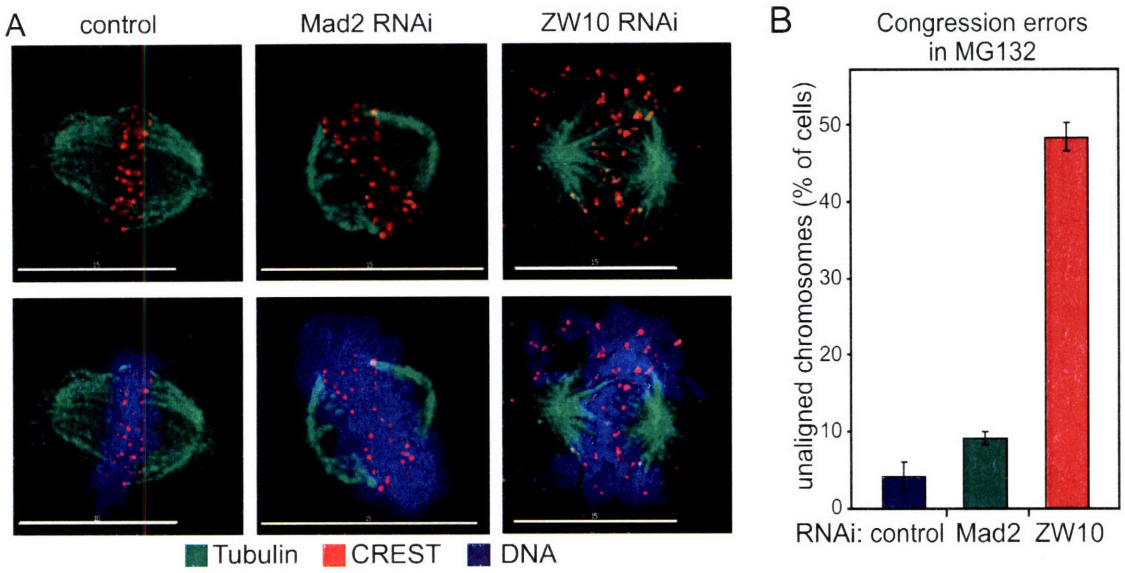


Figure 3.2. Congression errors in ZW10 depleted cells do not result from insufficient time for completion of mitosis. (A) Immunofluorescence images of HeLa cells transfected with siRNAs, as indicated, and treated with MG132 for 1 hour prior to immunostaining with antibodies against β-tubulin, CREST antisera and DAPI. Scale bar: 15 μm. **(B)** Fraction of cells with unaligned chromosomes after MG132 treatment (See Materials and methods for details). The average number of unaligned chromosomes per cell for ≥90 cells in three independent experiments is shown. Error bars represent SEM.

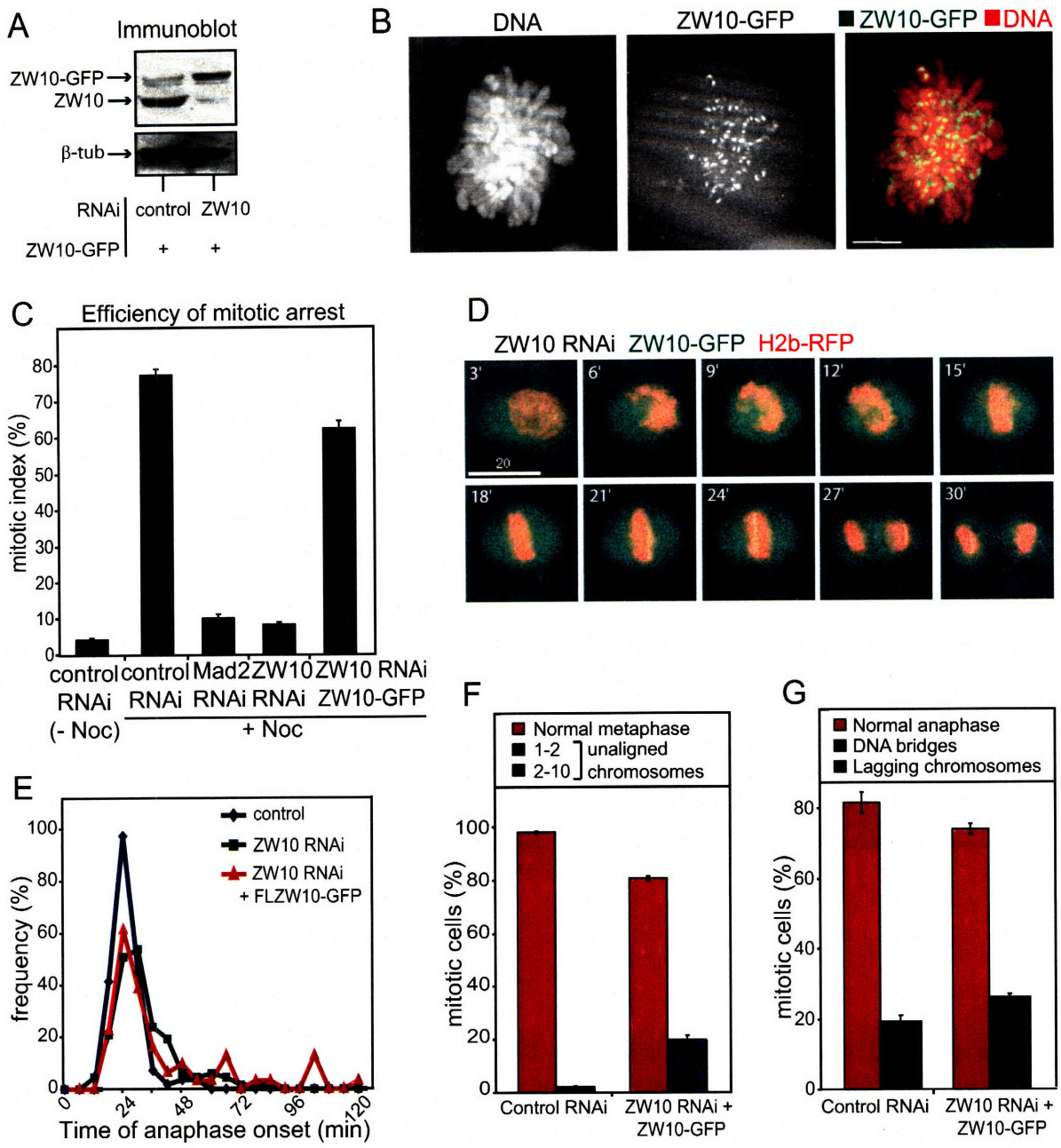


Figure 3.3. Complementation of ZW10 RNAi phenotype.
(A) Immunoblots of lysates from cells transfected with ZW10-GFP and siRNAs as indicated and probed with anti-ZW10 antibody. Endogenous β-tubulin levels are shown as loading controls. **(B)** Representative still image of ZW10 siRNA-treated mitotic cell expressing H2B-DsRed (red) and ZW10-GFP (green). Cells were treated with nocodazole for 30min prior to image acquisition. **(C)** Mitotic index of nocodazole-treated cells transfected with siRNAs, as indicated, and treated with 100 ng/ml nocodazole for 16 h. Cells were fixed and stained with DAPI to visualize nuclei. Mitotic index was measured by counting the number of mitotic cells (n~ 200 cells) in three independent experiments. Error bars represent SEM. **(D)** Representative still images from live-cell movies of H2B-DsRed- expressing HeLa cells treated with ZW10 siRNA and transfected with a plasmid containing RNAi-resistant ZW10-GFP cDNA. Scale bar: 20 μm. **(E)** Frequency plots of anaphase times as determined from live-cell movies of H2B-DsRed- expressing HeLa cells treated with siRNA, as indicated, or transfected with ZW10 siRNA and a plasmid containing RNAi-resistant ZW10-GFP cDNA. NBD was set as T=0. **(F,G)** Congression (F) and anaphase (G) defects in cells transfected with control siRNA alone or with a combination of siRNA and a plasmid containing RNAi-resistant ZW10-GFP cDNA. Error bars represent SEM from three independent experiments.

chromosome segregation errors generated by ZW10-targeting siRNA oligos are likely to arise from depletion of ZW10 and not from off-target effects.

3.3.4 ZW10 plays a crucial role in kinetochor-MT attachment

To determine reasons for chromosome mis-segregation following ZW10-depletion, I examined the overall structure of the mitotic spindle and the status of kinetochore-MT attachments. When cells transfected with control and ZW10 siRNA oligos were fixed, stained with anti-β-tubulin antibody and compared by immunofluorescence microscopy, bipolar spindles of similar size and MT density were observed, suggesting that ZW10 is not necessary for the basic steps of bipolar spindle assembly (Figure 3.4A). Under normal conditions, spindles in human cells contain a dense array of MTs so that kinetochore-MTs are obscured. However, if cells are cold-treated, the majority of spindle MTs depolymerize, leaving behind those bound stably to kinetochores. In cells depleted of ZW10 and cooled to 4°C for 10min prior to fixation, simple inspection revealed many fewer k-MTs than in control cells (Figure 3.4B). In addition, on average, ~50% of chromosomes in ZW10 depleted mitotic cells, but <8% of chromosomes in control cells, were unaligned with kinetochore pairs lacking one or both sets of kinetochore-MTs (Figure 3.4C). The incidence of lateral MT binding was also elevated but syntelic attachments, which are characteristic of Aurora B depletion, were conspicuously absent (Figure 3.4 D,E). Thus ZW10 is essential for establishment of stable kinetochore-MT attachment. Furthermore, the distinctive pattern of unattached and monopolar kinetochore pairs in ZW10 depleted cells suggests an important functional difference between ZW10 and either Bub1 or Aurora B.

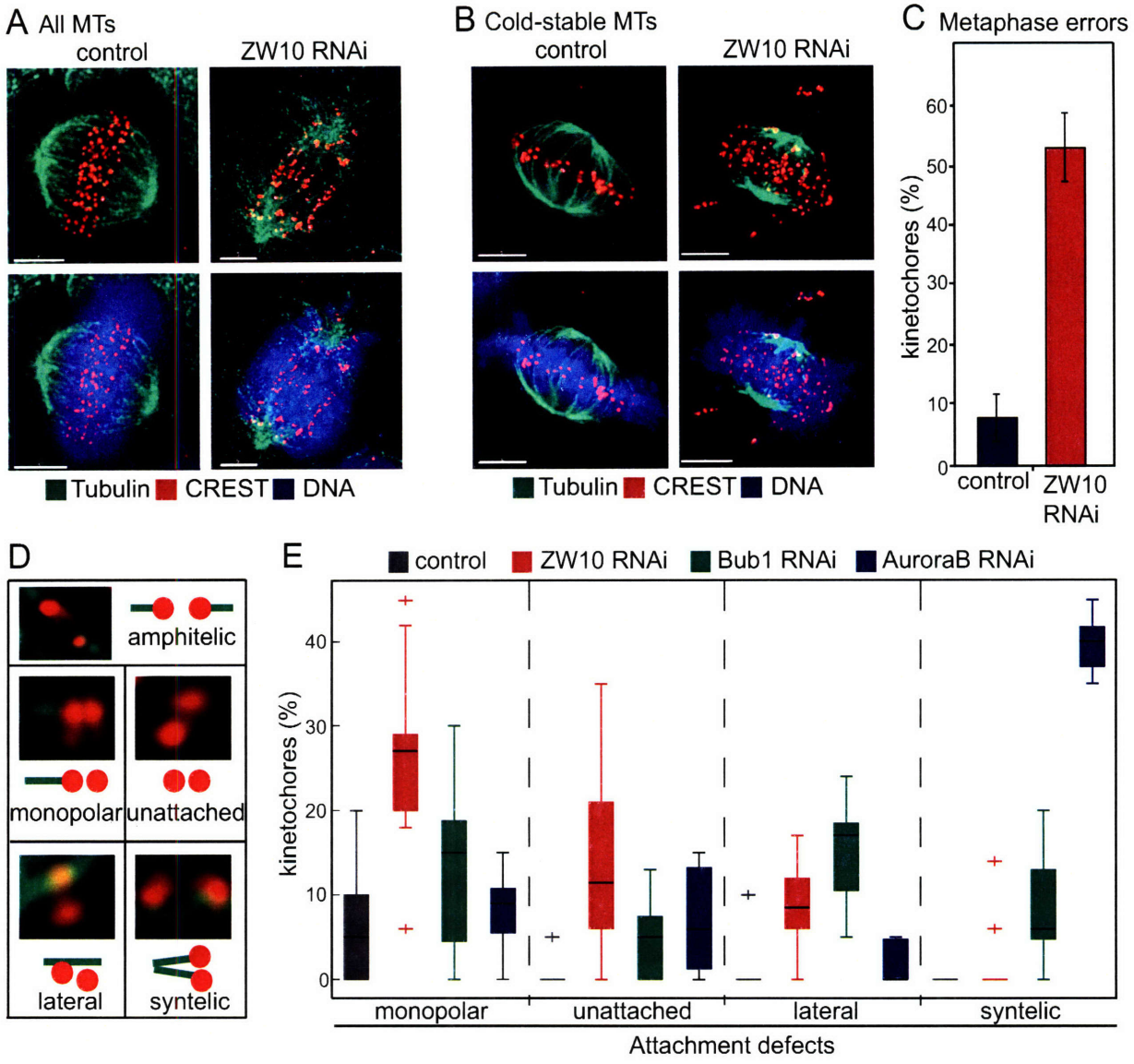


Figure 3.4. Depletion of ZW10 leads to attachment defects distinct from defects observed in Bub1 or Aurora B depletions. **(A,B)** Immunofluorescence images of HeLa cells without (A) and with cold-treatment (B) following control or ZW10 siRNA treatment, and co-stained with β-tubulin antibodies for MTs (green), CREST antisera for kinetochores (red) and DAPI for DNA (blue). Scale bar: 5μm. **(C)** Average number of kinetochore pairs that displayed attachment errors following cold treatment and transfected with control or ZW10 siRNA. Error bars represent SD from 10 cells (≥200 kinetochores). **(D)** Representative images of attachment errors scored in (E). Images are 3D-rendered and 8X-magnified.
(E) Distribution of defective attachments quantified as fraction of kinetochores (n≥20) per cell in cells(n≥8) transfected with siRNA as indicated. Monopolar, unattached, lateral or syntelic attachment defects were quantified. The boxes have lines at the lower quartile, median, and upper quartile values. The whiskers are lines extending from each end of the boxes to show the extent of the rest of the data. Outliers are data with values beyond the ends of the whiskers.

3.3.5 Chromosome tracking reveals ZW10's role in early kinetochore-MT capture

To better visualize the functional consequences of ZW10-depletion for early mitosis I used high resolution deconvolution microscopy to capture 3D image stacks of H2B-GFP expressing HeLa cells (n=24) every 45 sec for a period of 20-30 min. In the earliest time interval during which chromosome movement could be assayed after the morphological manifestation of NBD (0-3 min), ~80% of chromosomes in control cells (n=36) exhibited directed movement towards the spindle equator (Figure 3.5A and 3.5C). Directed movement was interspersed with brief pauses, but all chromosomes made it to the center of the spindle by t~15 min (for trajectory see Figure 5D; Materials and methods for details). This is normal behavior for mitotic chromosomes and reflects the acquisition first of monopolar then bipolar kinetochore-MT attachment, followed by congression to the spindle midzone (Rieder and Salmon, 1998). In ZW10 depleted cells, in contrast, <10% of chromosomes exhibited directed movement right after NBD, and chromosomes remained scattered throughout the spindle (Figure 3.5 B,C,E). The fraction of chromosomes undergoing congression increased with time but did not exceed 40% (Figure 3.5 B,C). Thus, by the time of anaphase initiation, many chromosomes remained distant from the spindle midzone and were missegregated (Figure 3.5E). The absence of normal movement in ZW10 depleted cells immediately after NBD demonstrates that the early steps of kinetochore-MT attachment do not occur with normal efficiency. The subsequent acquisition of directed motility by some chromosomes (Figure 3.5C) suggests either that ZW10 depletion is not complete, with respect to kinetochore-MT binding, or that other MT attachment pathways take over. In either case, the live-cell data presented here demonstrates that early MT attachment at kinetochores is critically dependent on ZW10 function.

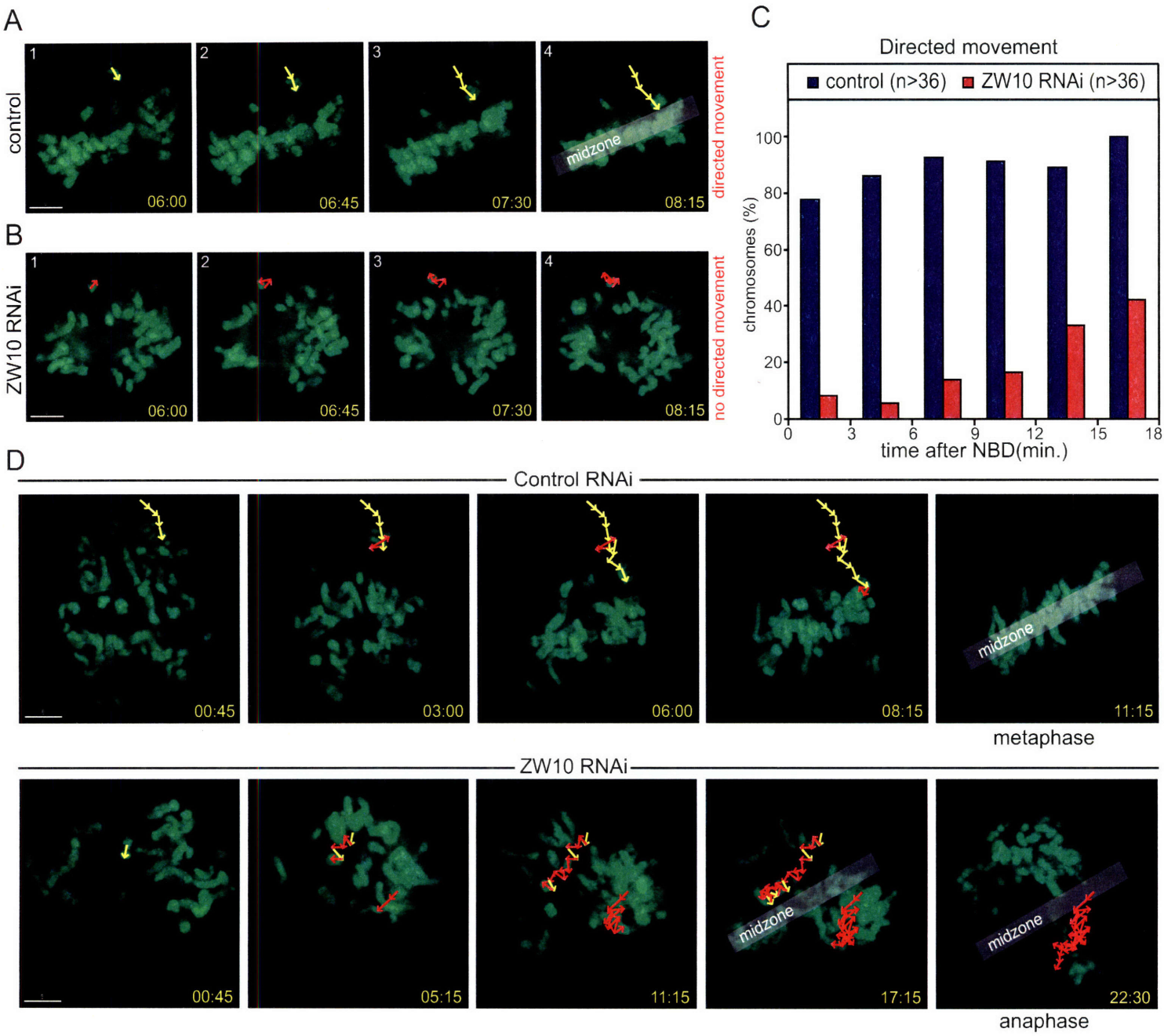


Figure 3.5. ZW10 is necessary for early kinetochore-MT encounter.
(A-B) Representative consecutive still images from live-cell movies of H2B-GFP expressing HeLa cells treated with control (A) or ZW10 siRNA (B). Each image is a maximum intensity projection of 6 Z-stacks acquired every 45sec through 3μm thick section. **(A)** Directed movement of a tracked chromosome (yellow arrows). **(B)** Absence of directed movement in tracked chromosome (red arrows) (for details see Materials and methods). **(C)** Fraction of chromosomes exhibiting directed movement towards the future midzone based on the scoring systems illustrated in (A-B) (n≥36). Directed movement was quantified in control (red bars) or ZW10 siRNA- treated cells (blue bars). NBD set as t=0. (for details see Materials and methods) **(D-E)** Representative images from live-cell movies of cells transfected with control (D) or ZW10 siRNA (E) illustrating the dramatic reduction of directed movement in ZW10-depleted cells compared to control cells. Grey bar indicates future midzone.

3.3.6 Distribution of kinetochore-bound MAPs and motors

Why is the phenotype of ZW10 depletion, in which majority of chromosomes are monopolar or unattached and MT capture is perturbed, different from that of Bub1 or AuroraB depletion, in which lateral and syntelic attachments predominate, respectively? One possibility is that kinetochore recruitment of MAPs and motors is differentially affected. To investigate this possibility, I compared kinetochore binding by six MAPs and motors in ZW10, Aurora B or Bub1 depleted cells: MCAK, a kinesin involved in MT depolymerization (Walczak, 2003); EB1, a plus-end MAP whose kinetochore association is MT-dependent (Tirnauer et al., 2002); CLIP-170, a plus-end MT rescue factor whose kinetochore association is MT-independent (Akhmanova and Hoogenraad, 2005; Dujardin et al., 1998); p50 and p150Glued, components of the dynein/dynactin motor complex and CENP-E, a minus-end directed kinesin involved in congression (Kapoor et al., 2006) (Table1; in some cases the analysis was performed in the presence of nocodazole; see Materials and Methods for further detail). Whereas ZW10-depletion blocked efficient kinetochore binding by p50 and p150Glued (as shown previously (Starr et al., 1998); Supplemental Figures 1 and 3) as well as by CLIP-170, Aurora B or Bub1 depletion affected only MCAK binding: the other five MAPs and motors I assayed were recruited normally (Gorbsky, 2004; Murata-Hori and Wang, 2002) (Liu et al., 2006)(Table 1, Supplemental Figures 1, 2, 3; data not shown). Thus RNAi of ZW10 interferes with kinetochore binding by a different set of MAPs and motors than RNAi of Bub1 or Aurora B, potentially explaining functional differences among these three proteins.

3.3.7 Kinetochore localization of checkpoint proteins in ZW10 depleted cells

Why are attachment lesions generated by depletion of ZW10 not monitored by the spindle checkpoint? Immunofluorescence analysis using antibodies against Mad2, BubR1 and

Table 1. Kinetochore localization of MAPs and motors in cells depleted of ZW10, Bub1 or Aurora B.

Class of MAPs		**Depolymerizing factors**	**Factors promoting polymerization**		**MT-bound motors**		**Kinesin-like proteins**
MAPs used in this study		MCAK	EB1	CLIP-170	p150Glued	p50	CENP-E
Kinetochore recruitment[a,b]		MT-independent[c]	MT-dependent[d]	MT-independent[e]	MT-independent[f]	MT-independent[f]	MT-independent[f]
Results	**ZW10 RNAi**	+	+	-	-[g]	-[g]	+
	Bub1 RNAi	-	+	+	+	+	+
	AuroraB RNAi	-[h]	+	+	+[i]	+[i]	+[i]

(a) For consistency, all dependency experiments shown were performed as part of the current work but some have been reported previously, as indicated

(b) For some MAPs analysis was performed in cells treated with nocodazole (See Materials and methods for details)

(c) Ref. (Moore and Wordeman, 2004)

(d) Ref. (Tirnauer et al., 2002)

(e) Ref. (Dujardin et al., 1998)

(f) Ref. (Hoffman et al., 2001)

(g) Ref. (Starr et al., 1998)

(h) Ref. (Gorbsky, 2004)

(i) Ref. (Murata-Hori and Wang, 2002)

Bub1 showed recruitment of BubR1 and Bub1 to be independent of ZW10, in agreement with previous studies (Chan et al., 2000; Kops et al., 2005) (Supplemental Figure 4). Surprisingly, Mad2 localization at kinetochores varied depending on the RNAi oligo used to deplete ZW10, although attachment defects were generated by both oligos to the same extent. Cyclin B immunostaining was used as a temporal marker for metaphase to anaphase transition. In control cells (n>30), Mad2 levels were high on unaligned kinetochores in prometaphase and were absent in anaphase cells (n=15). (Figure 3.6 A,B). Treatment of cells with RNAi oligo1 (n>30) (Kops et al., 2005) lead to abrogation of Mad2 kinetochore signal, as previously reported (Supplemental Figure 5; (Buffin et al., 2005; Kops et al., 2005). However, depletion of ZW10 by RNAi oligo 2 (n>30) lead to a different result. Although, Mad2 levels were high at unattached kinetochores in prometaphase, as in control cells, surprisingly, Mad2 remained at unaligned kinetochores till anaphase onset in ZW10 depleted cells, leading to ~50% of anaphase cells (n=16) having Mad2 kinetochore signal (Figure 3.6 A,B). Thus ZW10 depleted cells can initiate anaphase in the presence of Mad2 at unaligned kinetochores.

To further investigate an effect of ZW10 depletion on Mad2 kinetochore recruitment pattern, I performed a high-resolution live-cell imaging of Mad2-GFP localization in individual cells. In control cells co-expressing H2b-DsRed and Mad2-GFP, Mad2 localization at unaligned kinetochores was visible in prometaphase. Mad2-GFP kinetochore signal disappeared just before the metaphase to anaphase transition (Figure 3.7A). In contrast, in cells depleted of ZW10, Mad2 remained at unaligned kinetochores till anaphase onset when two chromosome masses separated (Figure 3.7B). Intensity of Mad2 kinetochore signal was quantified and normalized to the background noise. While in control and ZW10 depleted cells (n>10), prometaphase kinetochore signal of Mad2 was about 18 arbitrary units, in anaphase of control cells, Mad2 kinetochore signal was virtually undetectable (Figure 3.7C). On the contrary, in anaphase, kinetochores of

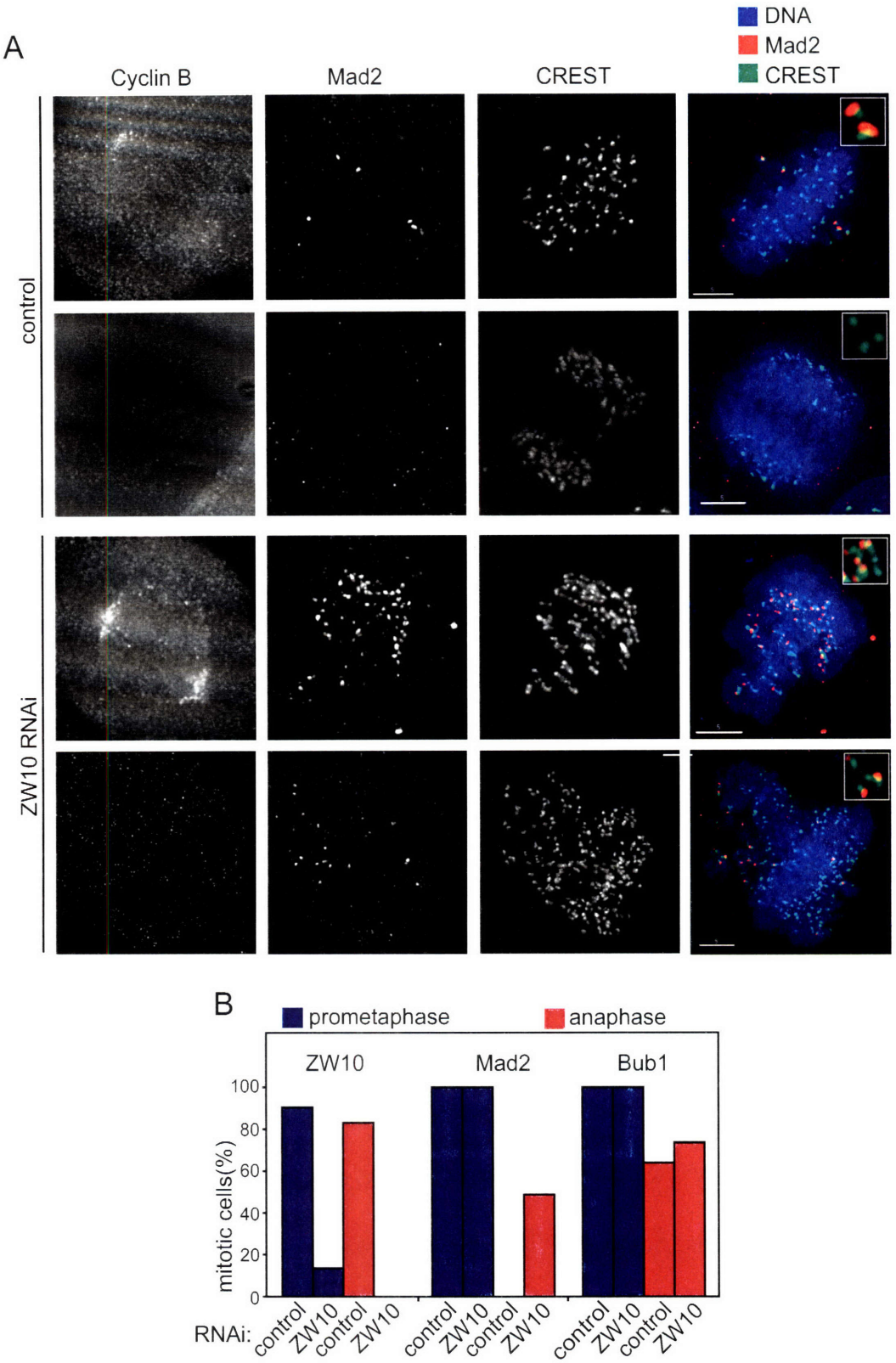


Figure 3.6. Status of Mad2 recruitment in checkpoint deficient cells lacking ZW10. **(A)** Immunofluorescence images of HeLa cells following control or ZW10 siRNA treatment, and co-stained with antibodies against Mad2 (red), CyclinB, CREST antisera for kinetochores (green) and DAPI for DNA (blue). Scale bar: 5μm. **(B)** Percentage of prometaphase and anaphase cells displaying kinetochore localization of ZW10, Mad2 and Bub1 as indicated. Cyclin B status, CREST and DAPI staining were used to define anaphase cells.

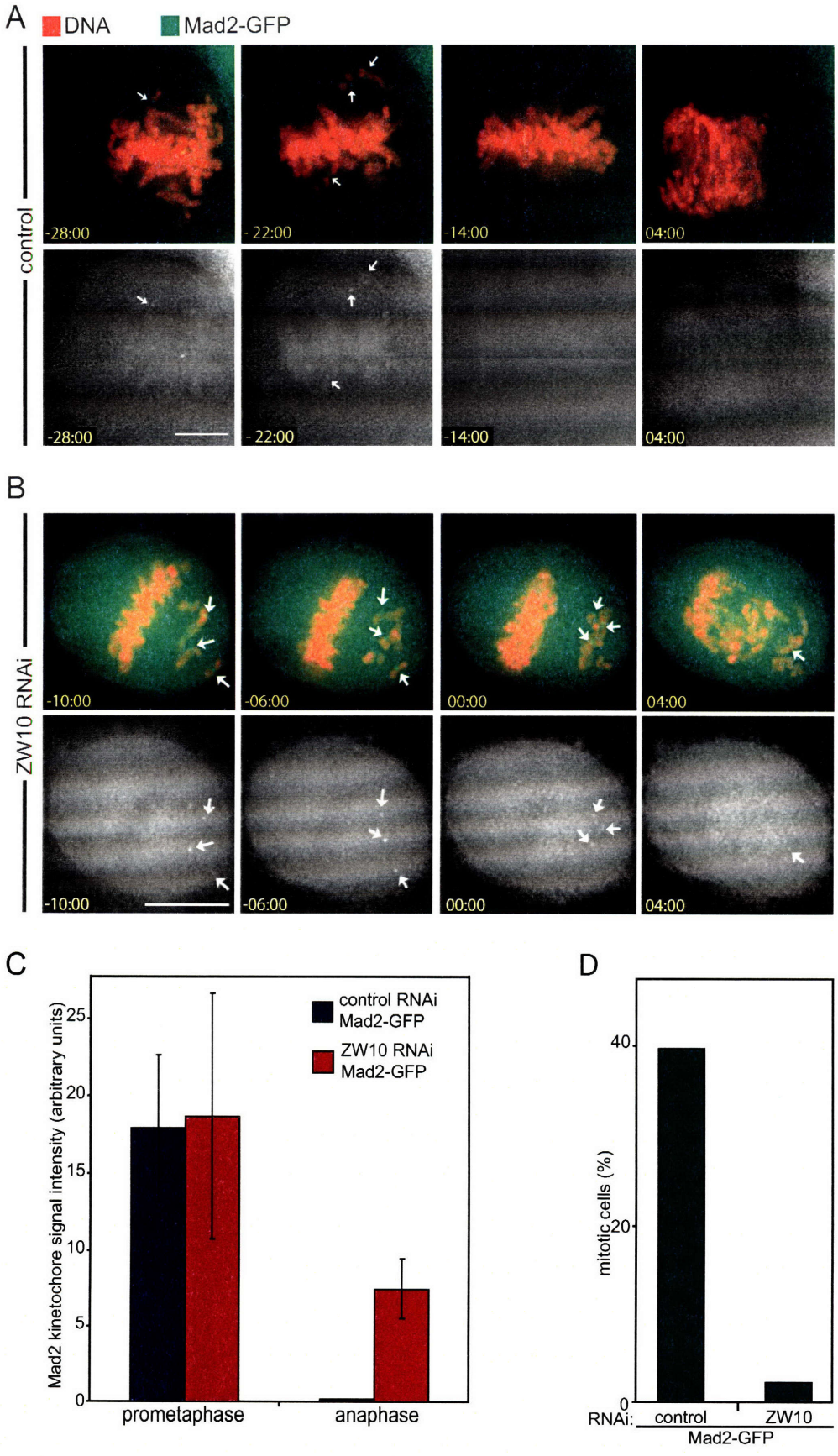

A
DNA
Mad2-GFP
control
-28:00
-22:00
-14:00
04:00
B
ZW10 RNAi
-10:00
-06:00
00:00
04:00
C
Mad2 kinetochore signal intensity (arbitrary units)
control RNAi Mad2-GFP
ZW10 RNAi Mad2-GFP
prometaphase
anaphase
D
mitotic cells (%)
RNAi:
control
ZW10
Mad2-GFP

Figure 3.7. Mad2-GFP localization pattern in HeLa cells depleted of ZW10.
(A-B) Time stills from live cell movies of Mad2-GFP (green) in control cells (A) and cells treated with ZW10 RNAi (B) expressing H2B-RFP (red) to visualize DNA. Overlay of GFP and RFP signals is shown in the upper raw. GFP signal alone is shown in the bottom raw. Anaphase onset is set at t=0. White arrows mark unaligned kinetochores with Mad2-GFP signal. Bar=5μ **(C)** Mad2-GFP signal intensity at kinetochores (arbitrary units) in HeLa cells treated with control (blue bars) or ZW10 (red bars) RNAi and expressing Mad2-GFP and H2B-RFP. Error bars show SD from 50 kinetochores in 10 cells. **(D)** Percentage of mitotic cells in 45min after NBD treated with control or ZW10 RNAi, as indicated, and expressing Mad2-GFP and H2B-RFP.

ZW10 depleted cells retained Mad2 signal whose intensity was measured to about 8 arbitrary units (Figure 3.7C). Thus kinetochore levels of Mad2 in ZW10 depleted cells dropped only about 50% when cells progressed through metaphase to anaphase transition. Since only few molecules of Mad2 are sufficient to arrest cells in mitosis (Howell et al., 2001; Shannon et al., 2002), I conclude that the checkpoint signaling is abrogated in the absence of ZW10 despite Mad2 localization at unaligned kinetochores.

Mad2 overexpression delays cells at the metaphase to anaphase transition (He et al., 1997; Homer et al., 2005). If ZW10 function in the checkpoint is Mad2 independent, we would expect treatment of cells with ZW10 oligo2 to release a mitotic arrest caused by overexpression of Mad2. To test this, we assayed mitotic progression in control and ZW10 depleted cells co-expressing Mad2-GFP and H2bDsRed using live cell imaging. While ~42% of Mad2-GFP overexpressing cells (n>40) remained in mitosis for longer than 45 min after NBD, only 3% of ZW10 siRNA-treated cells (n>40) overexpressing Mad2-GFP were still in mitosis at t=45 min (Figure 3.7D). I conclude that ZW10 RNAi-treatment can override the mitotic arrest induced by Mad2 overexpression, suggesting a Mad2 independent role for ZW10 in the spindle checkpoint signaling.

3.4 Discussion

Here I combine RNAi-mediated protein depletion, live-cell imaging and quantitative analysis to examine the role of ZW10 in chromosome-MT binding and spindle checkpoint. I find that depletion of ZW10 causes defects in chromosome alignment and errors in attachment with prevailing unattached and monotelic chromosomes. Chromosomes in ZW10 depleted cells fail to exhibit directed movement towards the metaphase plate from early mitosis onwards indicating a key role for ZW10 in early kinetochore-MT encounter. A novel role ZW10 plays in the early

steps of formation of kinetochore-MTs distinguishes it from other checkpoint proteins that have been shown to play a role in later steps of attachment, including Aurora B and Bub1. Comparison of MAPs recruited by these proteins to mitotic kinetochores reveals a possible cause for difference in attachment phenotypes observed in each case. While ZW10 is responsible for kinetochore localization of CLIP-170 and dynein/dynactin, AuroraB and Bub1 are required for MCAK recruitment. In addition to ZW10 function in early kinetochore-MT encounter, my work also demonstrates a Mad2 independent role for ZW10 in the spindle checkpoint signaling.

3.4.1 The role of ZW10 in early MT capture at kinetochores

Bipolar binding of paired sister kinetochores to MTs is achieved through a stochastic and dynamic process involving kinetochore capture of MTs emanating from spindle poles, and kinetochore-driven nucleation of MTs that then incorporate into the assembling spindle (Rieder, 2005; Rieder and Salmon, 1998). Initial capture usually involves formation of a transient lateral interaction between a MT and one of a pair of kinetochores followed by poleward motion of the pair. The free kinetochore binds to MTs emanating from the opposite pole (or incorporates kinetochore-nucleated MTs into the spindle). Chromatid pairs that have successfully established bipolar attachment congress to the metaphase plate (Rieder, 2005; Rieder and Salmon, 1998).

In my work, two lines of evidence suggest that ZW10 is required early in mitosis when kinetochore-MT binding initially forms, possibly in initial capture itself. First, ZW10-depleted cells contain large numbers of unattached or partially attached (monopolar) chromatid pairs arrayed more or less randomly around the spindle. Second, live-cell imaging shows that ZW10-depleted chromosomes do not exhibit directed movement towards the metaphase plate that normally starts at the beginning of mitosis. Thus, segregation errors in ZW10-depleted cells appear to arise from failures of initial MT capture rather than subsequent destabilization of

attachment. The role for ZW10 in early MT encounter is particularly surprising because previous studies had explicitly shown that mutants of RZZ members in *Drosophila* displayed chromosome missegregation with lagging chromatids and DNA bridges in anaphase while metaphase congression was unperturbed, implying an anaphase role for RZZ complex (Scaerou et al., 2001; Williams et al., 2003). The role for ZW10 in the early kinetochore-MT capture has been missed in previous studies possibly because in *Drosophila* cells nuclear envelope does not disassemble completely after the NEB (Kiseleva et al., 2001). Since eight *Drosophila* chromosomes are caught in nuclear envelope fragments at the cell equator, it is difficult to distinguish between unaligned chromosomes and chromosomes congressed to the metaphase plate (R.Karess, personal communication). By following cells through mitosis using live cell imaging in HeLa cells we uncovered a prometaphase role for RZZ in MT capture. EM analysis should, in the future, be helpful in revealing more about the structural perturbations at the kinetochore –MT interface that occur in the absence of ZW10.

3.4.2 Comparison of ZW10 to other non-MT binding proteins involved in attachment

Bub1 is implicated in promoting progression of lateral to end-on attachments (Meraldi and Sorger, 2005), while Aurora B is important for correction of syntelic chromosome mal-orientations (Lampson et al., 2004) suggesting a role for these checkpoint proteins in later steps of formation of a bipolar attachment (Figure 3.8A). Differences among the depletion phenotypes of ZW10, Aurora B and Bub1 probably reflect differences in the spectrum of MAPs and motors whose kinetochore association is impaired when these proteins are depleted. While Aurora B and Bub1 are required for recruitment of MCAK, a kinetochore kinesin, ZW10 is required for CLIP-170 and dynein-dynactin recruitment. Since kinetochore localization of MCAK, CENP-E and EB1 does not require ZW10 and only a narrow subgroup of MAPs relies on ZW10 for its

kinetochore recruitment, ZW10 must have a very specific function at the kinetochore-MT interface early on in mitosis.

The work presented here places RZZ complex into a group of checkpoint proteins that have additional functions in regulation of attachment. This finding brings up a question of the general organization of the checkpoint signaling (Figure 3.8B). What is the relationship between sensing and signaling at kinetochores? One possibility might be that multiple MAPs and motors perform essential functions at kinetochores in establishment of attachment and regulation of MT dynamics. Another set of proteins, some of them are considered now checkpoint proteins, performs two functions: first, participate in the checkpoint signaling network as a part of a mechanism that inhibits APC/C in response to incorrect attachment and probably tension. Second, monitors activity of MAPs and motors that require this particular protein for kinetochore recruitment. Then, when malfunction of MT-binding proteins happens, a corresponding checkpoint protein would engage the spindle checkpoint and impose a mitotic arrest. Alternatively malfunction of a MAP or motor may be sensed by all monitoring checkpoint proteins simultaneously.

3.4.3 Early MT capture is mediated by CLIP-170 and dynein/dynactin complex

CLIP-170 has been shown to act as a MT rescue factor (Akhmanova and Hoogenraad, 2005; Xiang, 2006) and has been also suggested to play a role in the formation of kinetocore-MT attachments (Tanenbaum et al., 2006). Based on the measurement of rates of poleward chromosome movement, dynein has been implicated in the (-)-end directed sliding of newly captured kinetochores along MTs (Rieder and Alexander, 1990; Sharp et al., 2000). On the contrary, injection of anti-dynamitin (p50) antibody, known to displace dynein/dynactin complex from mitotic kinetochores (Echeverri et al., 1996), did not cause defects in congression and

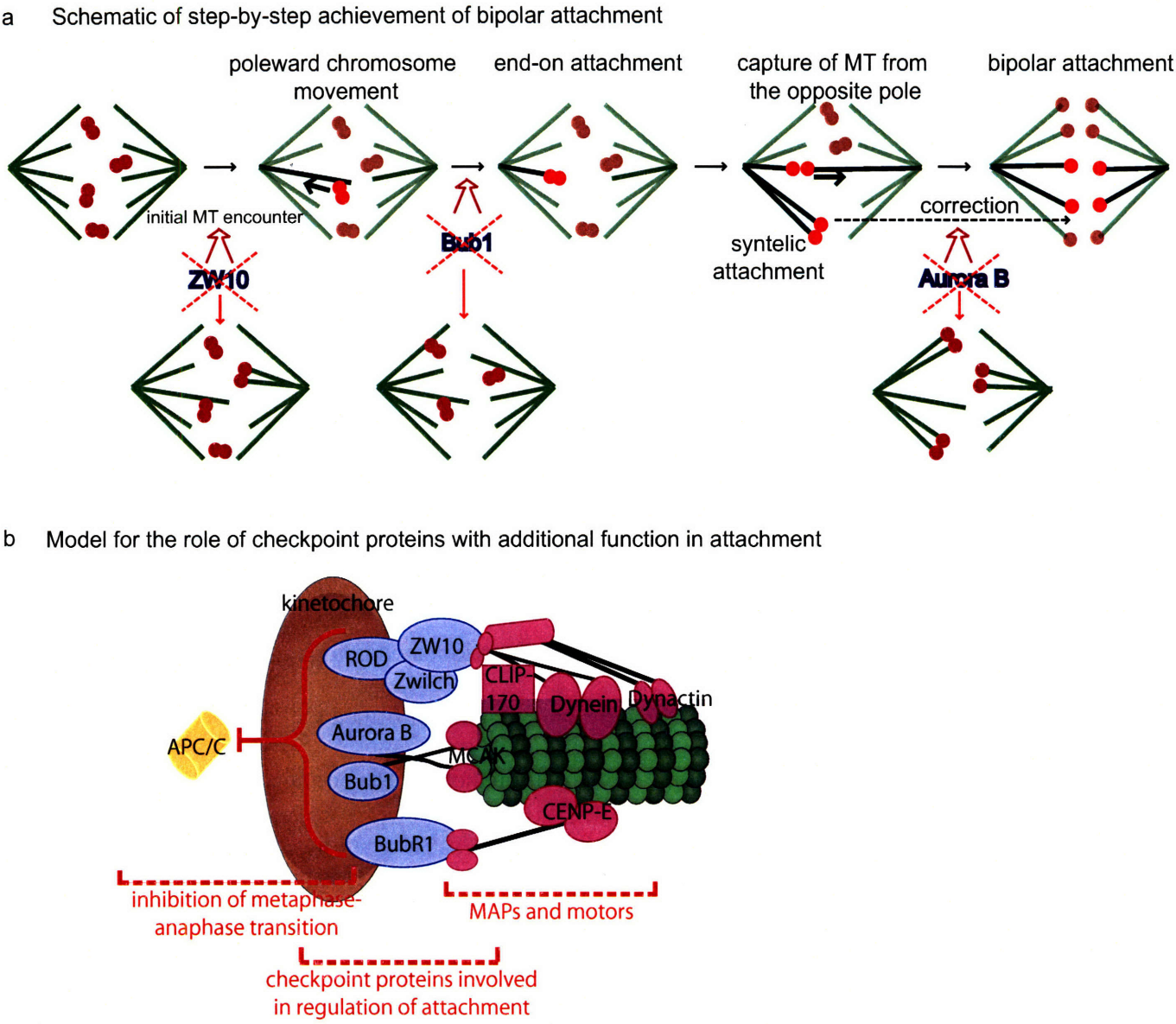


Figure 3.8. Speculative models for the role of ZW10 in kinetochore-MT attachment and for the role of checkpoint proteins, with additional functions in regulation of attachment, in checkpoint signaling. **(A)** Schematic illustration of processes involved in bipolar kinetochore-MT attachment. Disruption of ZW10 function leads to unattached kinetochores suggesting the role for ZW10 in the early step of kinetochore-MT encounter. Abrogation of Bub1 function causes side-on attachments and abrogation of AuroraB function leads to uncorrected syntelic attachments. **(B)** Schematic illustrates the mode of action of checkpoint proteins, ZW10, AuroraB, Bub1 and BubR1. ZW10, a member of RZZ complex, recruits dynein/dynactin to kinetochores thus establishing attachment. AuroraB and Bub1 are required for kinetochore localization of MCAK and play a role in correction of improper attachments. BubR1 recruits CENP-E to kinetochore and monitors its function. Checkpoint signaling and inhibition of APC/C requires the activity of all checkpoint proteins simultaneously.

metaphase chromosome alignment (Howell et al., 2001). These conflicting results can not be reconciled by a simple depletion of CLIP-170 or dynein by RNAi treatment since both proteins localize to multiple subcellular regions and are involved in a variety of mitotic processes (Dujardin and Vallee, 2002; Lansbergen and Akhmanova, 2006).

While this thesis was being written, a report from Rieder lab that assessed functions of dynein and ZW10 at kinetochores was published (Yang et al., 2007). Using live cell analysis and anti-dynein antibody (70.1) injection, that has been previously demonstrated to disrupt the function of dynein (Howell et al., 2001), authors demonstrated that depletion of dynein inhibits poleward motion of kinetochores after initial MT encounter. The authors concluded that ZW10 RNAi enabled the selective depletion of dynein from kinetochores. Tracking of individual kinetochore spots labeled with CENPB-GFP after NBD confirmed the role for ZW10/dynein in prometaphase poleward chromosome motion. Immunofluorescence analysis of cold-stable MTs suggested that ZW10/dynein complex functions in stabilization of kinetochore-MT attachments (Yang et al., 2007).

Since my data indicates that RNAi of ZW10 depletes not only dynein but also CLIP-170 from kinetochores, it is possible that congression and attachment defects associated with ZW10 depletion may be due to cumulative effect of these proteins at kinetochores. Alternatively, because it has been demonstrated previously that dynein acts in a complex with CLIP-170 (Tai et al., 2002), both proteins simultaneously may contribute to formation of kinetochore-MTs. Dynein may be required for chromosome poleward motion after initial encounter, while CLIP-170, that exhibits MT crosslinking functions (Akhmanova et al., 2005; Pierre et al., 1994), might facilitate MT bundling thus contributing to maturation of attachment. An expectation of this model would be a disruption of attachment in cells where the function of CLIP-170 is perturbed. Both CLIP-170 mutant expression and RNAi depletion of CLIP-170 have been demonstrated to

cause attachment defects (Tanenbaum et al., 2006). Thus it is very likely that formation of kinetochore-MTs is not a dynein exclusive role.

3.4.4 ZW10 checkpoint function and Mad2 kinetochore localization.

The difference in Mad2 localization associated with ZW10 depletion by two different RNAi oligos may be explained by comparing the extent of depletion of ZW10 achieved with each oligo. Our results show that depletion of ZW10 attained by RNAi oligo1 (Kops et al., 2005) is more complete and leads to the abrogation of Mad2 kinetochore signal, in agreement with previous studies (Fig.1A; Table 2; (Buffin et al., 2005; Kops et al., 2005). This result implies a structural role for ZW10 at kinetochore as a molecular scaffold for recruiting Mad2 and dynein/dynactin. In contrast, a partial depletion of ZW10 generated using RNAi oligo2 does not displace Mad2 from kinetochores, although it leads to attachment defects as severe as those obtained with RNAi oligo1. More importantly, partially depleted cells fail to delay anaphase onset despite Mad2 localization at unaligned kinetochores suggesting a Mad2 independent role for ZW10 in checkpoint signaling (Table 2). Several cases described in the literature documented a difference in phenotypes observed when kinetochore proteins were depleted by siRNA treatment to a different extent. For example, incomplete or partial depletion of Ndc80/HEC1 or Bub1 can result in a Mad2-dependent mitotic arrest (Johnson et al., 2004; Martin-Lluesma et al., 2002) (DeLuca et al., 2006; DeLuca et al., 2003), while complete depletion of these proteins abrogates the checkpoint despite the presence of unattached kinetochores (Meraldi et al., 2004; Meraldi and Sorger, 2005).

Mad2 is recruited to unattached kinetochores in ZW10 depleted cells to the same extent as in wild type cells, indicating that the 'wait anaphase' signal is correctly generated by improperly attached kinetochores. However, inability of Mad2 to activate the checkpoint in response to

Table 2. Phenotypes associated with differential depletion of ZW10.

RNAi oligo	Extent of ZW10 RNAi-mediated depletion	Congression	Checkpoint	Mad2 recruitment
Oligo1 (Kops et al.)	Partial (87%)	58% defective	abrogated	+
Oligo2	Complete (94%)	56% defective	abrogated	–

attachment errors suggests that checkpoint signaling is perturbed in the absence of ZW10. According to Mad2 'template model' of the checkpoint activation, inactive O-Mad2 gets activated by interacting with Mad1-C-Mad2 heterodimer at kinetochores (De Antoni et al., 2005). This allows now active C-Mad2 to bind to Cdc20 and, together with other checkpoint proteins comprising MCC, sequester Cdc20 thus inhibiting its function as an activator of APC/C (De Antoni et al., 2005). It is possible that RZZ complex is required for the 'activation' step, meaning that, although Mad2 is recruited to unattached kinetochores in the absence of ZW10, it can not achieve C-Mad2 conformation, bind to Cdc20 and inhibit APC/C activation. Alternatively, there are numerous possibilities of how RZZ might be involved in checkpoint signaling downstream of Mad2. RZZ may facilitate the formation of MCC or enhance MCC binding to APC/C or influence APC/C ubiquitination activity.

A similar phenotype was observed in PtK1 cells injected with the fragment of Mad1 called GST-Mad1F10. In these cells, Mad2 localization at unattached kinetochores does not prevent anaphase onset (Canman et al., 2002). GST-Mad1F10 contains the region of Mad1 that includes the Mad1-Mad1 oligomerization domain as well as the Mad2-binding domain and does not localize to kinetochores. As Mad1 competes with Cdc20 for the same binding domain on Mad2, authors proposed that GST-Mad1F10 at high concentrations likely out-competes Cdc20 for Mad2 binding in a dominant-negative fashion. Thus GST-Mad1F10 may disrupt the spindle checkpoint by sequestering Mad2 from either native Mad1 or Cdc20 or from Mad1-binding sites at the kinetochore (Canman et al., 2002). In cells with dysfunctional RZZ, checkpoint signaling may be disrupted in a similar fashion. Although Mad2 kinetochore levels in ZW10 depleted cells drop by about 50% in anaphase compared to prometaphase, remaining amount of Mad2 at anaphase kinetochores should be sufficient to arrest cells in mitosis, since Mad2 is required to sustain checkpoint even after its substantial depletion from kinetochores (Howell et al., 2001;

Shannon et al., 2002). Moreover, the fact that mitotic arrest induced by Mad2 overexpression is rescued by ZW10 depletion, clearly demonstrates that Mad2 and ZW10 act in parallel or ZW10 is downstream of Mad2 in the checkpoint signaling pathway.

My findings are also supported by the recent report that revealed that depletion of Zwint-1 by RNAi treatment in HeLa cells disrupts ZW10 kinetochore localization but Mad2 recruitment is only partially abrogated (Lin et al., 2006). Hec1 depletion perturbs Zwint-1, ZW10 and Mad2 recruitment to kinetochores. Moreover, spindle checkpoint is abrogated in cells depleted of Hec1 or Zwint-1 to different extents. While interference with Hec1 function leads to a complete checkpoint failure, Zwint-1 depletion causes incomplete checkpoint inactivation as judged by mitotic index in nocodazole treated cells. Thus Hec1/Zwint-1/zw10 pathway helps to recruit and maintain Mad2 at kinetochores, while both ZW10 and Mad2 are required for the full activation of the spindle checkpoint (Lin et al., 2006). Future biochemical experiments will uncover the exact role ZW10 plays in the checkpoint control.

3.5 Materials and methods

Cell culture, cDNA

HeLa cells were grown as described(Meraldi et al., 2004). H2B-GFP has been described previously(Meraldi et al., 2004). For transient expression of full length HZW10, ZW10 cDNA was PCR amplified from ZW10-S11/pBS-KS (a gift of B.Williams, Cornell University, Ithaca, NY) and subcloned into pEGFP-C1. RNAi-resistant ZW10 construct was generated using the QuickChange® Mutagenesis kit (Stratagene). mMad2-GFP was used for localization studies. Aminoacid sequence of mmMad2 is 96.6% similar to that of hsMad2.

RNAi, MG132 treatment, complementation assay

ZW10-targetting siRNA oligonucleotides (Dharmacon Research, Inc.): RNAi oligo1 (UGAUCAAUGUGCUGUUCAA)(Kops et al., 2005)and RNAi oligo 2 (GGUACUGCACCAACUAAAG) were transfected into HeLa cells as described (Elbashir et al., 2001) and analyzed 92 hrs after transfection. Lamin A siRNA was used as a control (Elbashir et al., 2001). For MG132 assay, 60hrs post transfection, cells were treated with aphidicolin for 24 hrs and imaged 8 hrs after aphidicolin release. 1 μM MG132 (Sigma) was added and chromosome misalignment was analyzed 1 h after mitotic entry. For complementation assays, cells were treated with siRNA for 96 h total time. 48 h after RNAi treatment cells were co-transfected with plasmids encoding H2b-DsRed and RNAi-resistant ZW10-pEGFP-C1. 12h after plasmid transfection, cells were treated for 24 h with aphidicolin and imaged 8 h after aphidicolin release.

Live-cell imaging

Cells were imaged using 20X NA0.75 objective as described in (Meraldi and Sorger, 2005). For following chromosome dynamics objective equipped with an objective heater was used. For high-resolution chromosome tracking, images of mitotic cells were recorded with 100X NA1.4 objective at 45 s intervals. For each time point, a stack of 6 images with 1μm steps in the *z*-direction was obtained. Chromosome tracking was performed manually (Adobe Photoshop). Quantification of directionality of chromosome movement was done as follows: when the trace of chromosome movements from one timeframe to the next through 4 consecutive time frames obtained every 45 s (a total of 3 min corresponding to one data point in Fig.4c) comprised almost a straight line pointing towards the future midzone, the movement was considered 'directed' (Fig.4a). 'No directed movement' was scored otherwise (Fig.4b).

Antibodies

Cells were fixed, permeabilized and blocked as described (Meraldi et al., 2004). Antibodies used were as follows: affinity-purified goat anti-Bub1 (a gift of S. Taylor, University of Manchester, Manchester, UK), anti-Aurora B (AIM-1, BD Transduction Labs), rabbit anti-CENP-E (Meraldi et al., 2004), human anti-CREST (Meraldi et al., 2004), mouse anti-p150Glued (BD Transduction Labs), mouse anti-p50 (BD Transduction Labs), sheep anti-MCAK (a gift of L. Wordeman, University of Washington School of Medicine, Seattle, WA), mouse anti-EB1 (BD Transduction Labs), rabbit anti-CLIP-170 (Santa-Cruz), mouse anti-□-tubulin monoclonal antibodies (Sigma clone Tub2.1). Rabbit antibodies (Covance) were made against His_6 HZW10 (645–779 aa), expressed in *E. coli* and purified on Nickel Sepharose Beads (Qiagen).

Immunofluorescence microscopy

To distinguish between early prometaphase and metaphase cells, cells were co-stained with cyclin A antibody (Sigma). Distributions of attachment defects were analyzed using MatLab. For analysis of MAPs and motors that delocalize from kinetochores following attachment: CENP-E, MCAK, p50, p150Glued and CLIP-170 (Dujardin et al., 1998; Hoffman et al., 2001; King et al., 2000; Moore and Wordeman, 2004), cells were treated with nocodazole for 1 h prior to fixation. EB1 recruitment to kinetochores was tested without nocodazole treatment, since it becomes kinetochore bound after attachment (Tirnauer et al., 2002).Cross-adsorbed secondary antibodies were used (Molecular probes). Images were acquired as described (Martinez-Exposito et al., 1999). For quantification of kinetochore signals, the percentage of protein depletion at kinetochores was quantified with ImageJ software (NIH) using the following formula

$$\%depletion = \frac{\sum_{1\to n} \frac{s_{RNAi}(n) - b(n)}{r_{RNAi}(n) - b(n)}}{\sum_{1\to n} \frac{s_{ctrl}(n) - b(n)}{r_{ctrl}(n) - b(n)}} \times 100$$

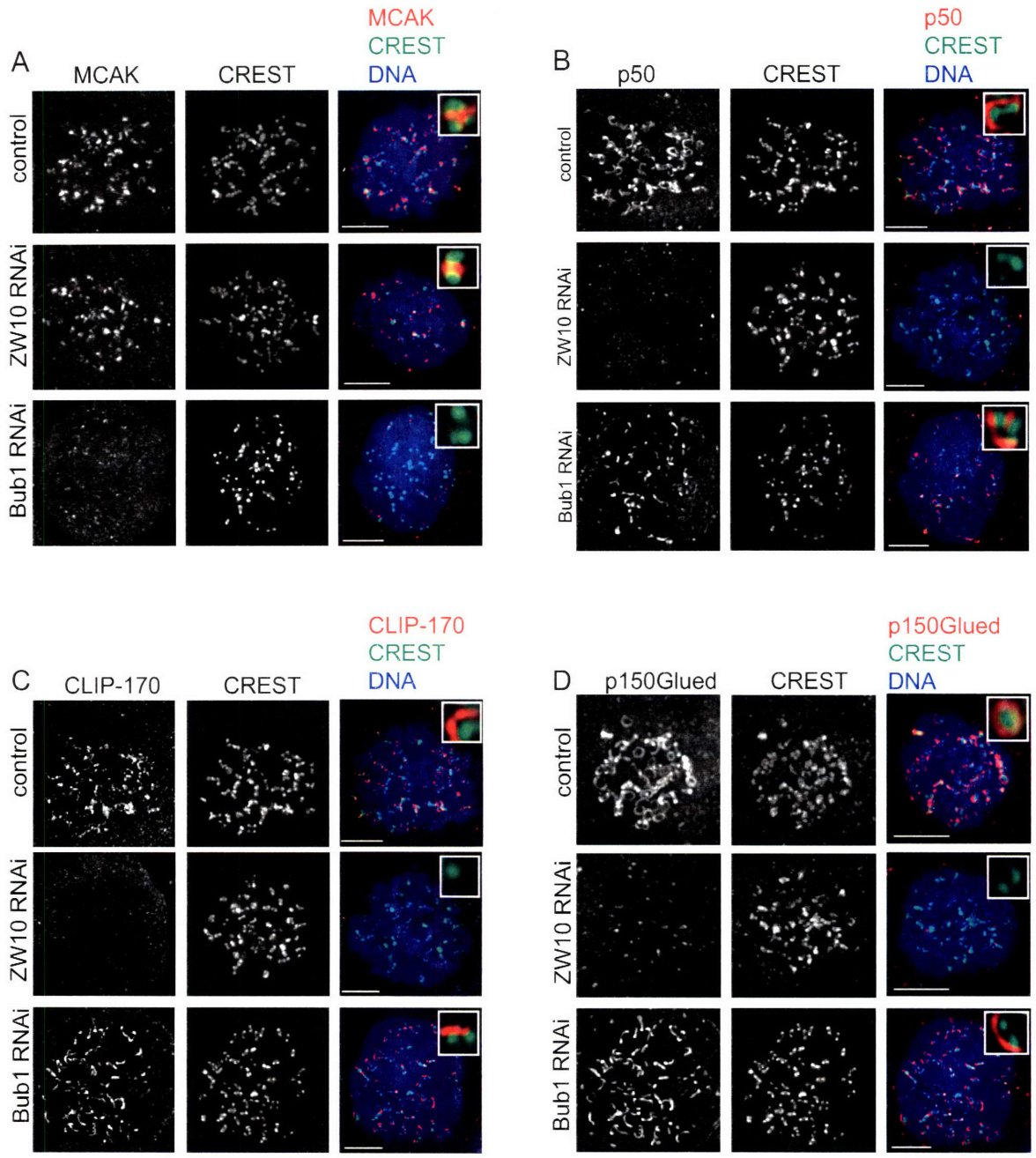
with s (signal), b (background) and r (reference signal). For each measurement, levels in at least 10 cells (50 kinetochores), were determined.

Immunoblotting

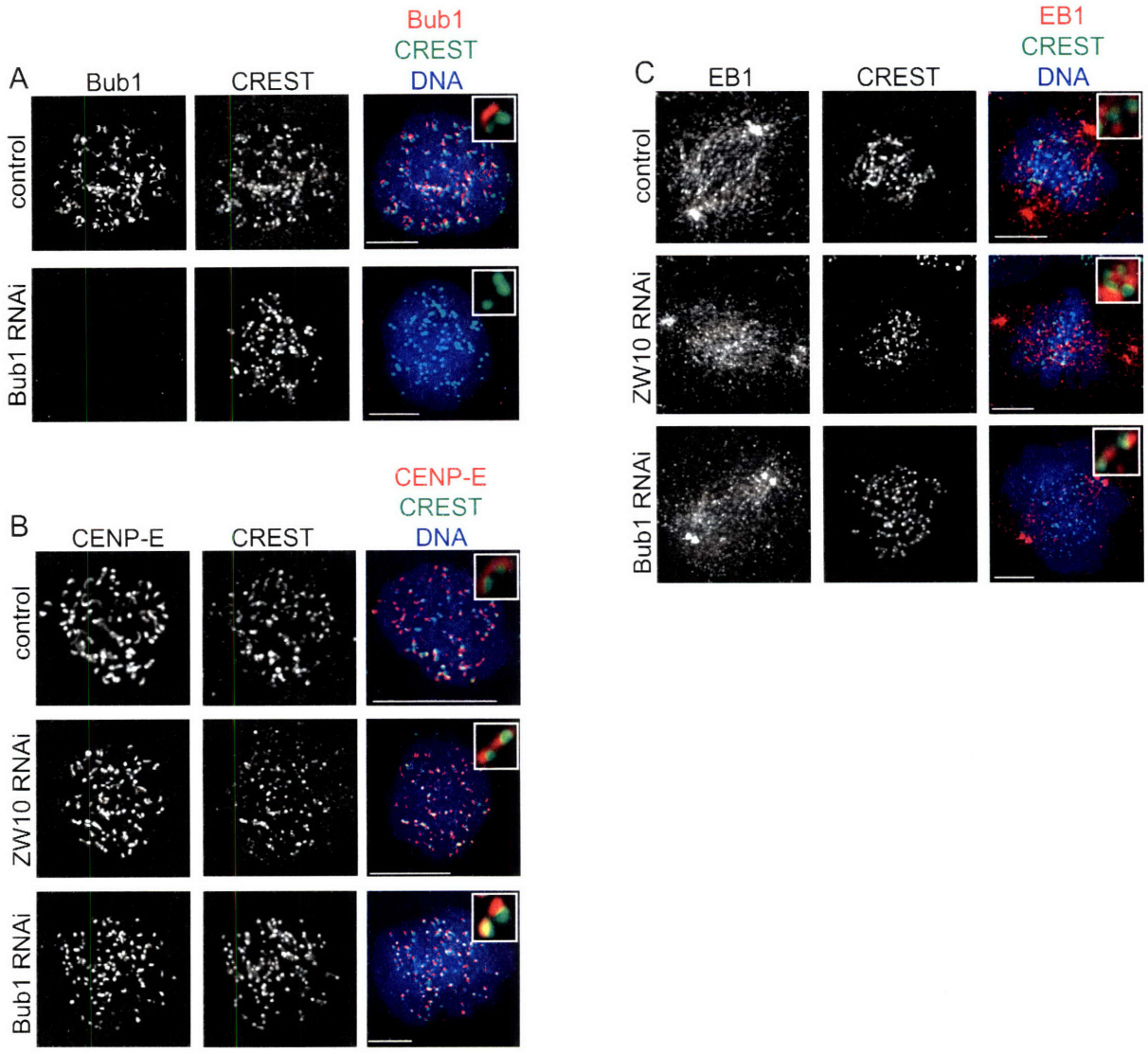
Whole cell extracts were prepared by cell lysis in SDS sample buffer with 15% mercaptoethanol, resolved by SDS-PAGE, transferred to PVDF membranes by semi-dry blotting (Immobilon). Membranes were incubated in blocking buffer (3% low-fat dried milk, PBS, 0.1% Tween-20) and probed with rabbit anti-ZW10 (0.5μg/ml) or mouse anti-β-tubulin (1:1000 dilution; Sigma clone Tub2.1) in blocking buffer. Anti-mouse and anti-sheep HRP-conjugated secondary antibodies (Amersham Pharmacia) were applied in blocking buffer and blots were developed by enhanced chemiluminescence (Supersignal West Femto Maximum kit; Pierce).

3.6 Acknowledgements

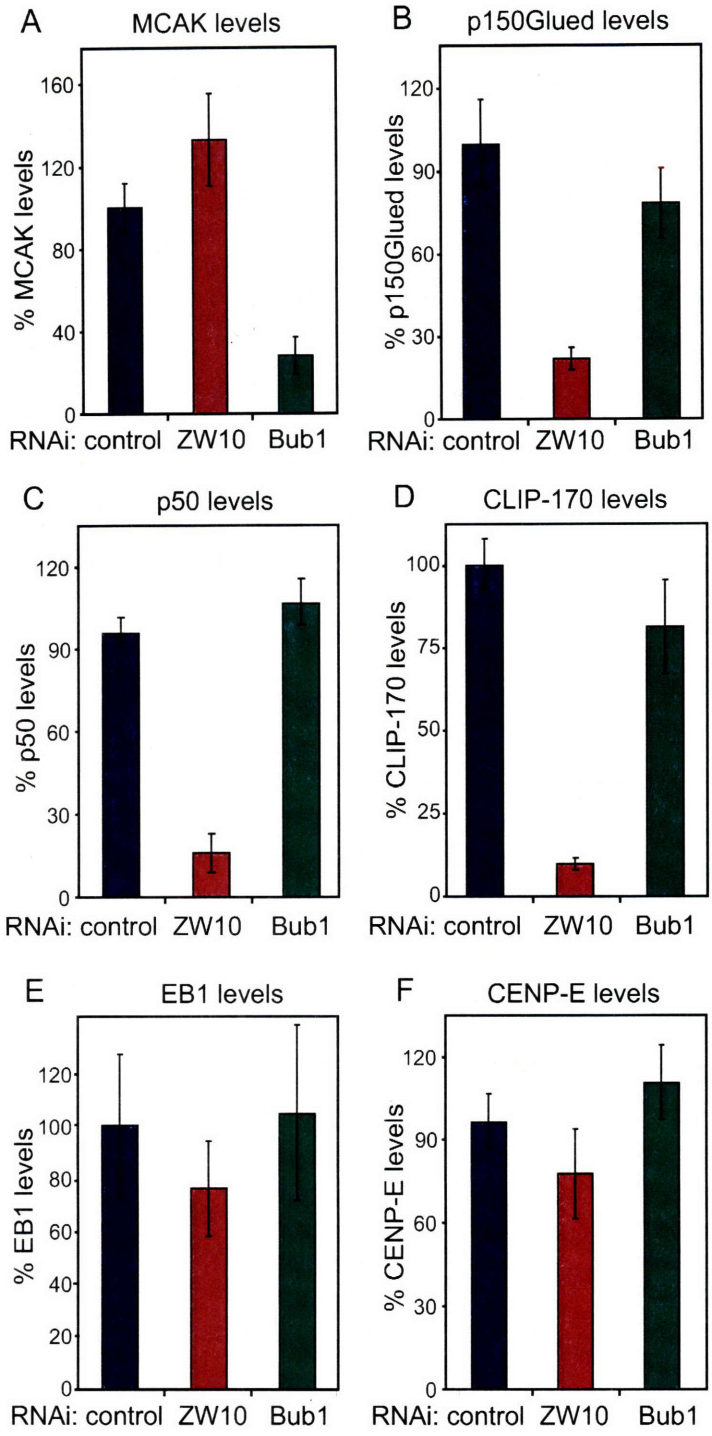
I thank Viji Draviam for inspiration and helpful discussions. I thank B.Williams for ZW10 cDNA, L. Wordeman for anti-MCAK antibody, S.Taylor for anti-Bub1 antibody. I thank Rob Hagan for Mad2-GFP construct. I thank my advisor Peter Sorger and Viji Draviam for critical reading of this chapter. I thank Sorger lab members for reagents and helpful discussions.



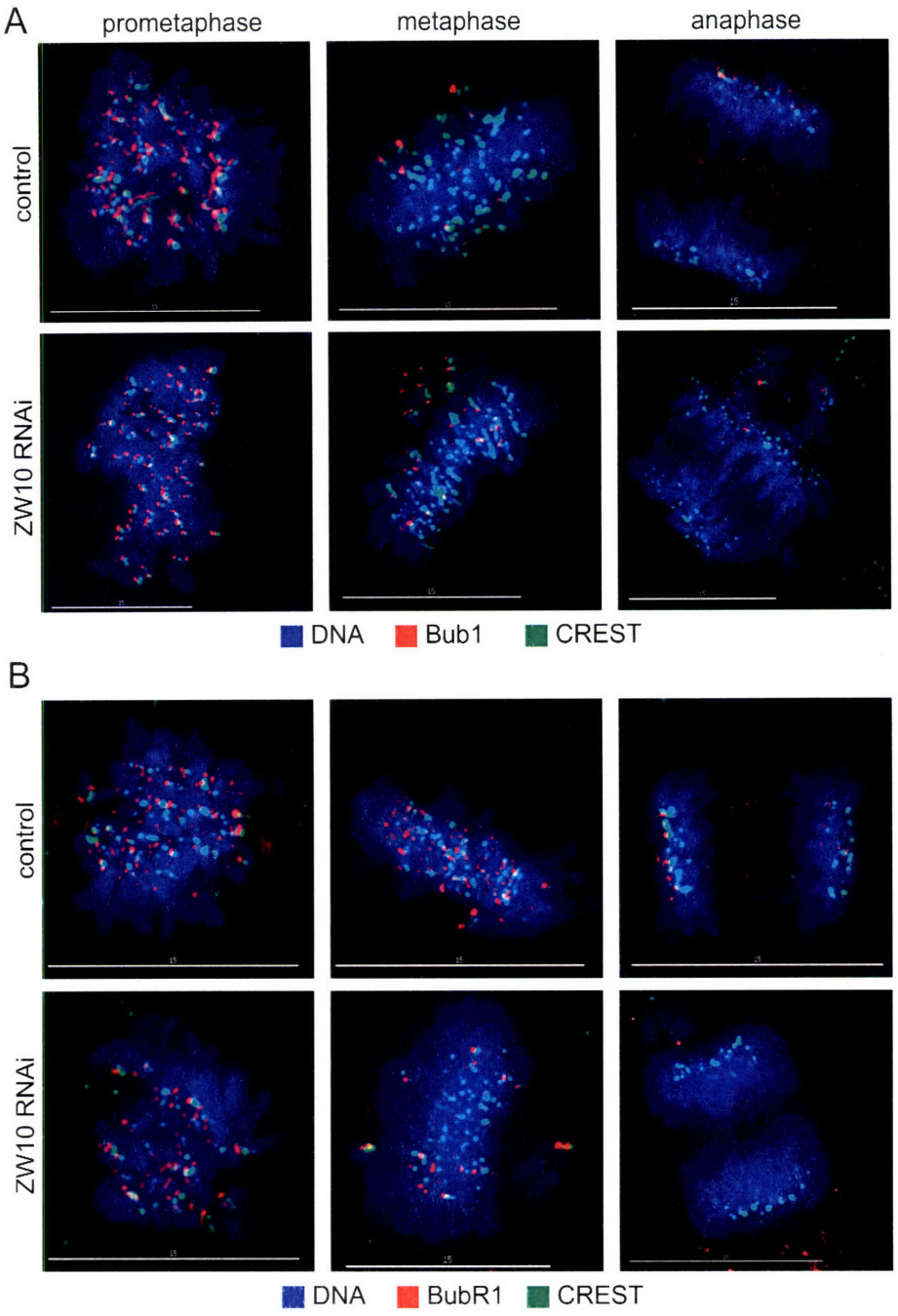
Supplementary Figure 3.1. ZW10 is required for kinetochore localization of p50, p150Glued and CLIP-170, while Bub1 is required for kinetochore localization of MCAK. (A-D) Immunofluorescence images of HeLa cells following transfection with siRNA, as indicated, and co- stained with CREST antisera for kinetochores (green), DAPI for DNA (blue) and one of the following antisera (red): MCAK (A), p50 (B), CLIP-170 (C), p150glued (D). Scale bar: 5 μm. Insets are 3D-rendered and 8X-magnified.



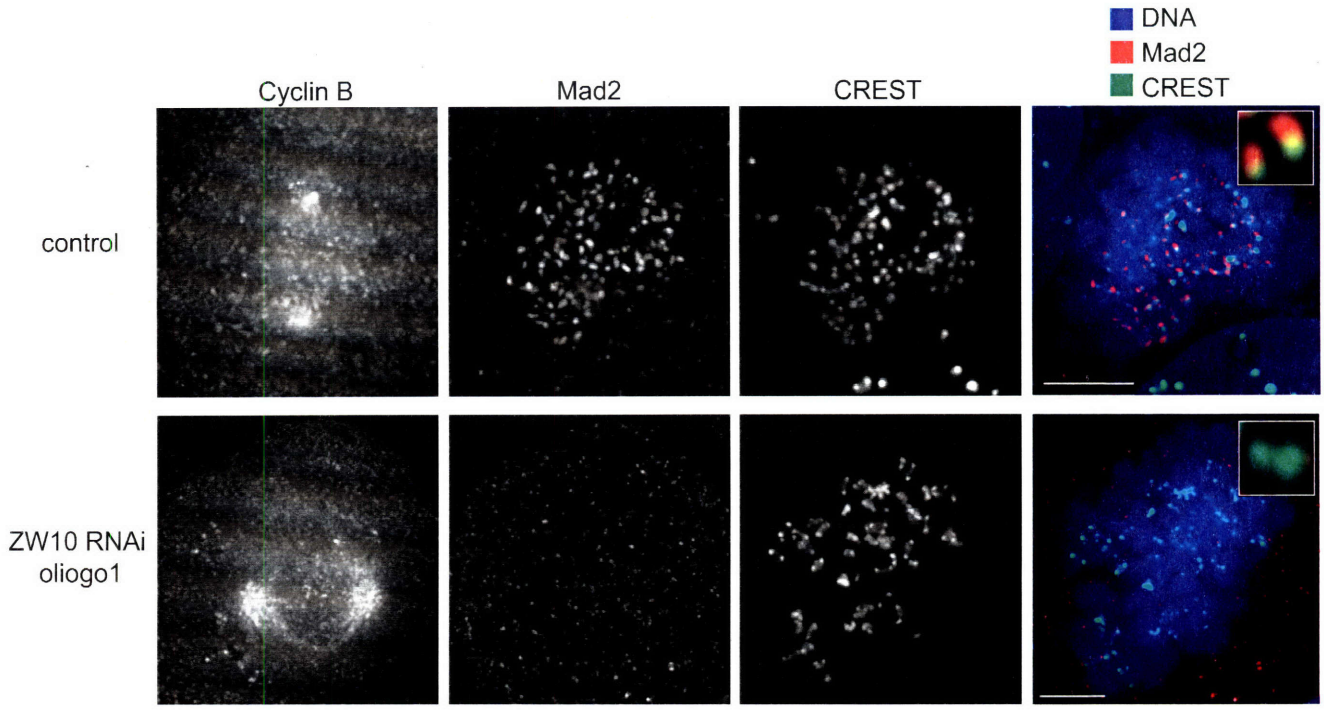
Supplementary Figure 3.2 Kinetochore localization of CENP-E and EB1 does not depend on ZW10 or Bub1. Immunofluorescence images of HeLa cells following transfection with siRNA, as indicated, and co-stained with CREST antisera for kinetochores (green), DAPI for DNA (blue) and one of the following antisera (red): Bub1 (A), CENP-E (B), EB1 (C) Scale bar: 5 μm. Insets are 3D-rendered and 8X-magnified.



Supplementary Figure 3.3. Quantification of immunofluorescence intensities of kinetochochore localized MAPs. (A-F) Plots indicate fluorescence intensity of MCAK **(A)**, p150glued **(B)**, p50 **(C)**, CLIP-170 **(E)**, EB1 **(D)**, CENP-E **(F)** on individual kinetochores in cells transfected with control, ZW10 or Bub1 siRNA, as indicated. Cells were treated with nocodazole for 2 hrs prior to fixation. Error bars show SD from 50 kinetochores in 5 cells. MCAK, p150glued, p50, CLIP-170, EB1 and CENP-E intensity levels were determined from deconvolved 3D reconstructions relative to a CREST reference and corrected for background noise (See Materials and methods for details).



Supplementary figure 3.4. Kinetochore localization of Bub1 and BubR1 do not depend on ZW10. Immunofluorescence images of HeLa cells following transfection with siRNA, as indicated, and co- stained with CREST antisera for kinetochores (green), DAPI for DNA (blue) and one of the following antisera (red): Bub1 (A) and BubR1 (B). Scale bar: 5 μm.



Supplementary Figure 3.5. Kinetochore localization of Mad2 is abrogated in cells depleted of ZW10 using RNAi oligo 1. Immunofluorescence images of HeLa cells following transfection with siRNA, as indicated, and co- stained with CREST antisera for kinetochores (green), DAPI for DNA (blue) and Mad2 (red). Scale bar: 5 μm. Insets are 3D-rendered and 8X-magnified.

3.7 References

Akhmanova, A., and C.C. Hoogenraad. 2005. Microtubule plus-end-tracking proteins: mechanisms and functions. *Curr Opin Cell Biol.* 17:47-54.

Akhmanova, A., A.L. Mausset-Bonnefont, W. van Cappellen, N. Keijzer, C.C. Hoogenraad, T. Stepanova, K. Drabek, J. van der Wees, M. Mommaas, J. Onderwater, H. van der Meulen, M.E. Tanenbaum, R.H. Medema, J. Hoogerbrugge, J. Vreeburg, E.J. Uringa, J.A. Grootegoed, F. Grosveld, and N. Galjart. 2005. The microtubule plus-end-tracking protein CLIP-170 associates with the spermatid manchette and is essential for spermatogenesis. *Genes Dev.* 19:2501-15.

Basto, R., R. Gomes, and R.E. Karess. 2000. Rough deal and Zw10 are required for the metaphase checkpoint in Drosophila. *Nat Cell Biol.* 2:939-43.

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

Canman, J.C., N. Sharma, A. Straight, K.B. Shannon, G. Fang, and E.D. Salmon. 2002. Anaphase onset does not require the microtubule-dependent depletion of kinetochore and centromere-binding proteins. *J Cell Sci.* 115:3787-95.

Chan, G.K., S.A. Jablonski, D.A. Starr, M.L. Goldberg, and T.J. Yen. 2000. Human Zw10 and ROD are mitotic checkpoint proteins that bind to kinetochores. *Nat Cell Biol.* 2:944-7.

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

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

DeLuca, J.G., B.J. Howell, J.C. Canman, J.M. Hickey, G. Fang, and E.D. Salmon. 2003. Nuf2 and Hec1 are required for retention of the checkpoint proteins Mad1 and Mad2 to kinetochores. *Curr Biol.* 13:2103-9.

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

Dujardin, D.L., and R.B. Vallee. 2002. Dynein at the cortex. *Curr Opin Cell Biol.* 14:44-9.

Echeverri, C.J., B.M. Paschal, K.T. Vaughan, and R.B. Vallee. 1996. Molecular characterization of the 50-kD subunit of dynactin reveals function for the complex in chromosome alignment and spindle organization during mitosis. *J Cell Biol.* 132:617-33.

Elbashir, S.M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature.* 411:494-8.

Gorbsky, G.J. 2004. Mitosis: MCAK under the aura of Aurora B. *Curr Biol.* 14:R346-8.

He, X., T.E. Patterson, and S. Sazer. 1997. The Schizosaccharomyces pombe spindle checkpoint protein mad2p blocks anaphase and genetically interacts with the anaphase-promoting complex. *Proc Natl Acad Sci U S A.* 94:7965-70.

Hoffman, D.B., C.G. Pearson, T.J. Yen, B.J. Howell, and E.D. Salmon. 2001. Microtubule-dependent changes in assembly of microtubule motor proteins and mitotic spindle checkpoint proteins at PtK1 kinetochores. *Mol Biol Cell.* 12:1995-2009.

Homer, H.A., A. McDougall, M. Levasseur, K. Yallop, A.P. Murdoch, and M. Herbert. 2005. Mad2 prevents aneuploidy and premature proteolysis of cyclin B and securin during meiosis I in mouse oocytes. *Genes Dev.* 19:202-7.

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

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

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

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

Karess, R. 2005. Rod-Zw10-Zwilch: a key player in the spindle checkpoint. *Trends Cell Biol.* 15:386-92.

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

King, J.M., T.S. Hays, and R.B. Nicklas. 2000. Dynein is a transient kinetochore component whose binding is regulated by microtubule attachment, not tension. *J Cell Biol.* 151:739-48.

Kiseleva, E., S. Rutherford, L.M. Cotter, T.D. Allen, and M.W. Goldberg. 2001. Steps of nuclear pore complex disassembly and reassembly during mitosis in early Drosophila embryos. *J Cell Sci.* 114:3607-18.

Kops, G.J., Y. Kim, B.A. Weaver, Y. Mao, I. McLeod, J.R. Yates, 3rd, M. Tagaya, and D.W. Cleveland. 2005. ZW10 links mitotic checkpoint signaling to the structural kinetochore. *J Cell Biol.* 169:49-60.

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

Lansbergen, G., and A. Akhmanova. 2006. Microtubule plus end: a hub of cellular activities. *Traffic.* 7:499-507.

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

Lin, Y.T., Y. Chen, G. Wu, and W.H. Lee. 2006. Hec1 sequentially recruits Zwint-1 and ZW10 to kinetochores for faithful chromosome segregation and spindle checkpoint control. *Oncogene.* 25:6901-14.

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

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

Martin-Lluesma, S., V.M. Stucke, and E.A. Nigg. 2002. Role of Hec1 in spindle checkpoint signaling and kinetochore recruitment of Mad1/Mad2. *Science.* 297:2267-70.

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

Meraldi, P., V.M. Draviam, and P.K. Sorger. 2004. Timing and checkpoints in the regulation of mitotic progression. *Dev Cell.* 7:45-60.

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

Moore, A., and L. Wordeman. 2004. The mechanism, function and regulation of depolymerizing kinesins during mitosis. *Trends Cell Biol.* 14:537-46.

Murata-Hori, M., and Y.L. Wang. 2002. The kinase activity of aurora B is required for kinetochore-microtubule interactions during mitosis. *Curr Biol.* 12:894-9.

Pierre, P., R. Pepperkok, and T.E. Kreis. 1994. Molecular characterization of two functional domains of CLIP-170 in vivo. *J Cell Sci.* 107 (Pt 7):1909-20.

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

Rieder, C.L. 2005. Kinetochore fiber formation in animal somatic cells: dueling mechanisms come to a draw. *Chromosoma.* 114:310-8.

Rieder, C.L., and S.P. Alexander. 1990. Kinetochores are transported poleward along a single astral microtubule during chromosome attachment to the spindle in newt lung cells. *J Cell Biol.* 110:81-95.

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

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

Savoian, M.S., M.L. Goldberg, and C.L. Rieder. 2000. The rate of poleward chromosome motion is attenuated in Drosophila zw10 and rod mutants. *Nat Cell Biol.* 2:948-52.

Scaerou, F., I. Aguilera, R. Saunders, N. Kane, L. Blottiere, and R. Karess. 1999. The rough deal protein is a new kinetochore component required for accurate chromosome segregation in Drosophila. *J Cell Sci.* 112 (Pt 21):3757-68.

Scaerou, F., D.A. Starr, F. Piano, O. Papoulas, R.E. Karess, and M.L. Goldberg. 2001. The ZW10 and Rough Deal checkpoint proteins function together in a large, evolutionarily conserved complex targeted to the kinetochore. *J Cell Sci.* 114:3103-14.

Shannon, K.B., J.C. Canman, and E.D. Salmon. 2002. Mad2 and BubR1 function in a single checkpoint pathway that responds to a loss of tension. *Mol Biol Cell.* 13:3706-19.

Sharp, D.J., G.C. Rogers, and J.M. Scholey. 2000. Cytoplasmic dynein is required for poleward chromosome movement during mitosis in Drosophila embryos. *Nat Cell Biol.* 2:922-30.

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

Starr, D.A., B.C. Williams, Z. Li, B. Etemad-Moghadam, R.K. Dawe, and M.L. Goldberg. 1997. Conservation of the centromere/kinetochore protein ZW10. *J Cell Biol.* 138:1289-301.

Tai, C.Y., D.L. Dujardin, N.E. Faulkner, and R.B. Vallee. 2002. Role of dynein, dynactin, and CLIP-170 interactions in LIS1 kinetochore function. *J Cell Biol.* 156:959-68.

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

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

Walczak, C.E. 2003. The Kin I kinesins are microtubule end-stimulated ATPases. *Mol Cell.* 11:286-8.

Wang, H., X. Hu, X. Ding, Z. Dou, Z. Yang, A.W. Shaw, M. Teng, D.W. Cleveland, M.L. Goldberg, L. Niu, and X. Yao. 2004. Human Zwint-1 specifies localization of Zeste White 10 to kinetochores and is essential for mitotic checkpoint signaling. *J Biol Chem.* 279:54590-8.

Williams, B.C., Z. Li, S. Liu, E.V. Williams, G. Leung, T.J. Yen, and M.L. Goldberg. 2003. Zwilch, a new component of the ZW10/ROD complex required for kinetochore functions. *Mol Biol Cell.* 14:1379-91.

Xiang, X. 2006. A +TIP for a smooth trip. *J Cell Biol.* 172:651-4.

Yang, Z., U.S. Tulu, P. Wadsworth, and C.L. Rieder. 2007. Kinetochore dynein is required for chromosome motion and congression independent of the spindle checkpoint. *Curr Biol.* 17:973-80.

Chapter 4

Additional considerations regarding ZW10 localization and function.

Note:

All experiments described in this chapter are the author's own work.

Outline

4.1 Abstract

Understanding the function of kinetochore components is crucial to our understanding of the highly regulated process of kinetochore-MT attachment and spindle checkpoint signaling. Here I attempt to dissect a molecular structure of ZW10, a member of the RZZ checkpoint complex discovered in metazoans. I perform a mutational analysis of ZW10 and identify regions of the protein required for its kinetochore localization, viability and nuclear export. I also report a novel interaction of ZW10 with BubR1, a key component of the spindle checkpoint. With the role of ZW10 in the checkpoint signaling remaining controversial in regards to Mad2, association of ZW10 and BubR1 might have implications for checkpoint function of ZW10.

4.2 Introduction

The structure and function of metazoan kinetochores is more complex than the budding yeast ones where main kinetochore components, like Mads and Bubs proteins, have been originally discovered (Hoyt et al., 1991; Li and Murray, 1991). Multiple reasons account for increased kinetochore complexity in higher eukaryotes. While budding yeast kinetochores attach to only one microtubule (MT), metazoan kinetochores bind 20-40 MTs (Brinkley and Cartwright, 1971). *S.cerevisiae* pursue a closed mitosis when the nucleus stays intact during chromosome division, while in higher eukaryotes mitosis is open and the nuclear envelope breaks down before assembly of the mitotic spindle thus making kinetochore-MT attachment more difficult. Mechanics of the attachment process is also different: assembly and attachment of budding yeast kinetochores starts right after S phase, in contrast, metazoan kinetochores bind MTs only

after nuclear breakdown thus leaving a short period of time, duration of mitosis, for kinetochores to achieve a bipolar attachment (Brinkley and Cartwright, 1971; Winey et al., 1995). It is not surprising that additional kinetochore components that do not have clear yeast orthologs are still being discovered in higher eukaryotes. Currently yeast kinetochore is thought to consist of about 60 proteins while mammalian one already has ~100 components (Cheeseman et al., 2004; De Wulf et al., 2003; Liu et al., 2006). Additional components have to be integrated into a mechano-signaling network that ensures proper chromosome segregation in mitosis, basic structure of which we know from yeast.

Kinetochore proteins ZW10, Rod and Zwilch were discovered in metazoans and are essential for proper function of the spindle assembly checkpoint (Scaerou et al., 1999; Williams et al., 1992; Williams et al., 2003). These proteins have no clear yeast orthologues. ZW10 is an 85kDa protein that localizes to kinetochores in prometaphase and is redistributed to the spindle after establishment of bipolar attachment (Williams et al., 1992). Rod has a molecular mass of 240kDa and exhibits the same localization pattern as ZW10 (Scaerou et al., 1999). Depletion of ZW10 or Rod causes chromosome missegregation with unaligned chromosomes in metaphase and lagging chromatids in anaphase ((Scaerou et al., 1999; Williams et al., 1992); chapter 3 of this thesis). Since both proteins colocalize in mitosis, their behavior was examined in reciprocal *Drosophila* mutants. Rod function was assessed in *zw10* mutant cells, while ZW10 function was investigated in *rod* mutants (Scaerou et al., 2001; Williams and Goldberg, 1994). Two proteins were shown to require each other for kinetochore localization and co-immunoprecipitate from both fly and human cell extracts (Scaerou et al., 2001). Gel

exclusion chromatography demonstrated that Rod and ZW10 are members of 800kDa protein complex. A third member of the complex Zwilch, molecular weight 75kDa, was identified by immunoaffinity chromatography with anti-ZW10 antibodies (Williams et al., 2003). *zwilch* mutations produce the same mitotic phenotype as *zw10* and *rod* mutants and all three proteins depend on each other for kinetochore recruitment. Affinity tag chromatography on ZW10 in human cells identified ZW10, Rod and Zwilch to be in a stable complex and no other stable components have been found (Kops et al., 2005; Williams et al., 2003).

ROD, ZW10 and Zwilch therefore appear to form a core complex, called RZZ, that performs essential functions at the kinetochore but the biochemical nature of this complex remains unclear. None of the proteins have identifiable protein domains that may suggest a hint to their function. The combined mass of ROD, Zw10 and Zwilch (240, 85 and 75 kDa respectively) is half of the RZZ mass that is ~800kDa (Williams et al., 2003). This suggests that either the complex is a stable dimer or that there are two copies of each protein in the complex. There is an urgent need to understand a biochemical composition of RZZ since mutations in ZW10, ROD and Zwilch have been found in human colorectal cancers (Wang et al., 2004b), implying that RZZ dysfunction might be a cause of chromosomal instability and cancer.

While some studies suggest that RZZ complex plays a structural role at kinetochore by providing a platform for kinetochore localization of Mad1/Mad2 and dynein/dynactin (Buffin et al., 2005; Kops et al., 2005), others point to Mad2 independent checkpoint signaling role for RZZ (Lin et al., 2006). Despite recruitment interdependency, all efforts to demonstrate interaction of ZW10 with Mad2 by co-immunoprecipitation analysis,

immunoaffinity chromatography and gel filtration remained unsuccessful (Buffin et al., 2005; Kops et al., 2005; Williams et al., 2003). This suggests that checkpoint function of ZW10 might be fulfilled through interactions with other key checkpoint components, for example BubR1.

BubR1 is a mitotic kinase that localizes to unattached kinetochores in prometaphase and whose levels at kinetochores are only moderately reduced in metaphase after completion of kinetochore-MT attachment (Hoffman et al., 2001). BubR1 is a member of a mitotic checkpoint complex (MCC), although it is capable of inhibiting APC/C activity on its own (Sudakin et al., 2001; Tang et al., 2001). Both Mad2 and BubR1 bind Cdc20 directly but have distinct binding sites; therefore it is thought that Mad2 and BubR1 have synergistic effects on APC/C inhibition (Fang, 2002). BubR1 interacts with CENP-E, a kinesin-like MT plus-end-directed motor important for chromosome alignment (Mao et al., 2003; Yao et al., 2000). BubR1 localizes to the outer kinetochore where it is postulated to act as a mechanosensor that monitors activity of CENP-E (Chan et al., 1999). *In vitro* kinase assays have demonstrated that kinase activity of BubR1 is stimulated by interaction with CENP-E (Mao et al., 2003).

Here I perform a structure-function analysis of ZW10. I use live-cell imaging and immunofluorescence analysis to address localization and function of a full length wild type ZW10, a panel of ZW10 deletion mutants and point mutants of ZW10 found in colorectal cancers. I find a region of ZW10 required for its kinetochore localization in mitosis and cytoplasmic localization in interphase. Analysis of ROD and Mad2 localization in cells overexpressing ZW10 deletion mutants suggested that the region of ZW10 required for its kinetochore localization is probably also necessary for assembly of

RZZ complex. I also uncover a novel interaction of ZW10 with BubR1 that might potentially explain the checkpoint function of ZW10.

4.3 Results

4.3.1 Dynamic localization of ZW10 in mitosis.

Dynamic behavior of RZZ complex has been examined by fixed cell analysis in *Drosophila* spermatocytes and neurocytes and in HeLa cells (Scaerou et al., 1999; Williams et al., 1992). Using live cell imaging localization of Rod-GFP has been assessed in *Drosophila* cells (Basto et al., 2004; Buffin et al., 2005). Mitotic behavior of other RZZ members in mammalian systems, including humans, is poorly understood. To better visualize localization of ZW10-GFP, I used a high-resolution deconvolution microscopy to capture 3D image stacks of Histone2B-RFP expressing HeLa cells (n=10) coexpressing ZW10-GFP every 60 sec for a period of 20-30 min (Figure 4.1). ZW10-GFP exhibited cytoplasmic localization during interphase. After nuclear breakdown (NBD), ZW10-GFP moved to the vicinity of chromosomes and bright GFP signal was visible at kinetochores followed by redistribution of ZW10-GFP to the spindle and spindle poles in prometaphase. A weak kinetochore staining was still observed at kinetochores in metaphase while GFP signal was prominent at spindle poles. After anaphase onset, ZW10-GFP could not be detected at kinetochores anymore but GFP signal was still visible at spindle poles in the early anaphase and disappeared as anaphase progressed (Figure 4.1). Immunofluorescence analysis described previously revealed that ZW10 remains associated with kinetochores till the end of anaphase and then can be seen in the midzone during telophase (Scaerou et al., 2001). We did not observe any specific

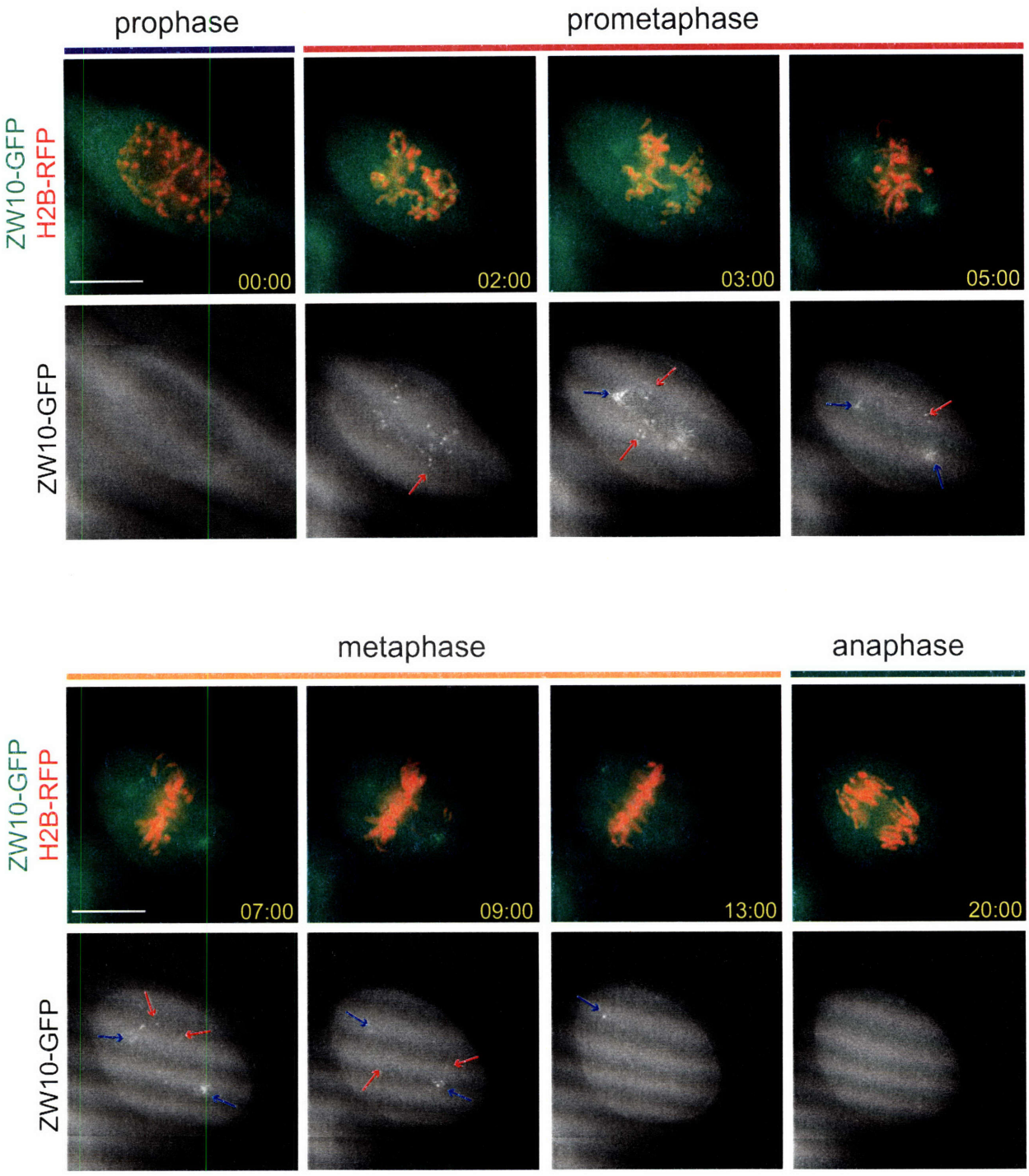


Figure 4.1. Analysis of ZW10-GFP localization in HeLa cells.
Time stills from live-cell movies of ZW10-GFP in HeLa cells expression H2B-RFP. Overlay of GFP anf RFP signals is shown in the upper row. GFP signal alone is shown in the bottom row. NBD is set as t=0. Red and blue arrows mark kinetochores and spindle poles, respectively. Bar: 10 μm.

localization of ZW10-GFP in late anaphase or telophase, ZW10-GFP signal was uniformly distributed throughout the cell (Figure 4.1). In summary, the live-cell analysis of ZW10-GFP localization in human cells revealed that ZW10-GFP associates with kinetochores in prometaphase, delocalizes to the spindle and spindle poles in metaphase and early anaphase, and becomes cytoplasmic as anaphase progresses.

4.3.2 Localization and function of ZW10 deletion and point mutants.

The absence of obvious yeast homologues and the lack of recognizable protein domains left the structure of RZZ complex poorly understood, despite the continuous efforts to investigate its function that have been conducted for more than a quarter of a century (Karess, 2005). To map structural domains of ZW10, I have generated a panel of deletion mutants of ZW10 and analyzed their localization and function using high resolution live cell imaging. Fragments of ZW10 of various lengths were fused to GFP and their localization was assessed in Hela cells overexpressing Histone2B fused to RFP (H2b-RFP) to visualize DNA (For the full list of fragments analyzed see Figure 4.2A). Imaging was performed in the presence of nocadazole to enhance kinetochore localization of ZW10. I found that 80 N-terminal amino acids of ZW10 (1-80aa) are dispensable for its kinetochore localization (Figure 4.2B), in contradiction with previously published results (Wang et al., 2004a). Interestingly, ZW10 Δ120 fragment (121-779aa) localized to kinetochores weakly when expressed in HeLa H2B-RFP cells, but its kinetochore recruitment was much more prominent in HeLa H2B-RFP cells depleted of endogenous ZW10 by RNAi treatment (Figure 4.2C). I conclude that the

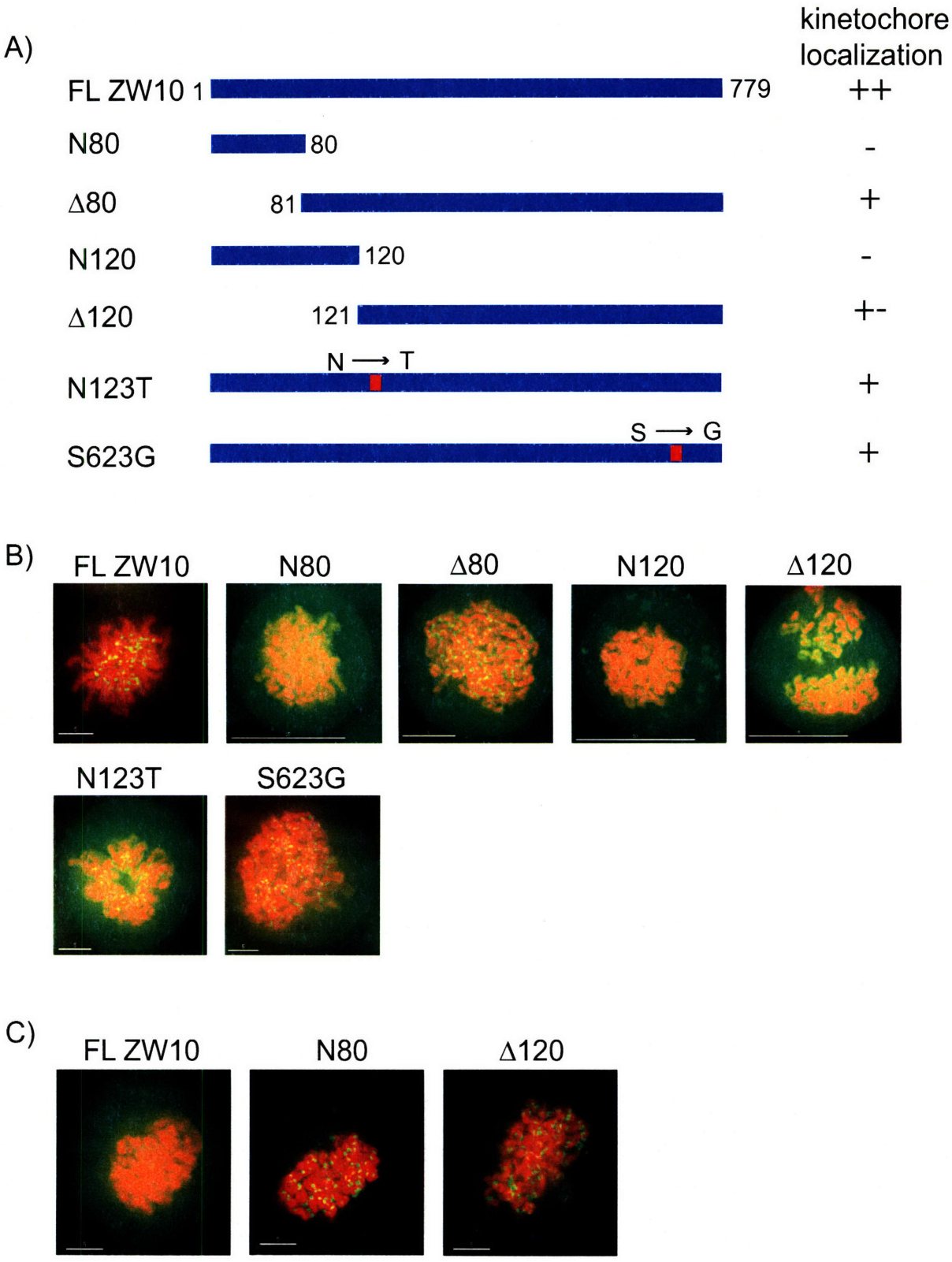


Figure 4.2. Effect of ZW10 mutants expression on kinetochore localization.
A) Schematic representation of ZW10 constructs. Amino acid numbers are indicated at fragment extremities. (++) indicates a bright kinetochore signal; (+) indicates a moderate kinetochore signal; (+-) indicates a weak kinetochore signal; (-) indicates no kinetochore signal.
B) and C) Live-cell images of HeLa cells expressing H2B-RFP (shown in red) and one of the ZW10 mutants fused to GFP (shown in green) as indicated. C) HeLa cells were treated with RNAi agains ZW10 for 96h before imaging.

region required for kinetochore localization of ZW10 resides in the C-terminal part of the protein.

Two point mutations in ZW10 were identified in colorectal cancers (Wang et al., 2004b). While checkpoint proteins have been implicated in tumorigenesis, mutations in the proteins per se were rarely observed in tumors (Draviam et al., 2004). I have generated GFP fusions of these ZW10 point mutants using site-specific mutagenesis (Wang et al., 2004b). ZW10 (N123T) and ZW10 (S623G) localized to kinetochores in HeLa H2B-RFP cells as observed by high resolution imaging (Figure 4.2B). To investigate the effect of point mutations of ZW10 on chromosome segregation and checkpoint function, ZW10 (N123T) and ZW10 (S623G) were expressed in HeLa H2B-GFP cells and imaged for the period of 6-8 hours in the presence or absence of nocodazole. Cells arrested in mitosis when treated with nocodazole indicating an intact checkpoint and chromosome segregation was not perturbed in the absence of nocodazole cells (data not shown). Thus point mutations N123T and S623G do not perturb localization and function of ZW10. At the moment, it is unclear how these mutations could have contributed to tumorigenesis.

In attempt to find a dominant/negative mutant of ZW10 I assessed checkpoint integrity and fidelity of chromosome segregation in HeLa cells expressing 80 N-terminal aa of ZW10 (1-80aa) or ZW10 Δ80 (81-779aa) fragment (Figure 4.3 A,B). HeLa cells (n~100) expressing H2B-RFP and Full-length ZW10 (FL) or one of the deletion mutants were imaged every 3min for 8hrs. Chromosomes that did not congressed to the metaphase plate prior to anaphase, or that remained away from the metaphase plate at the time of anaphase onset, were scored as unaligned; those that remained stranded at the

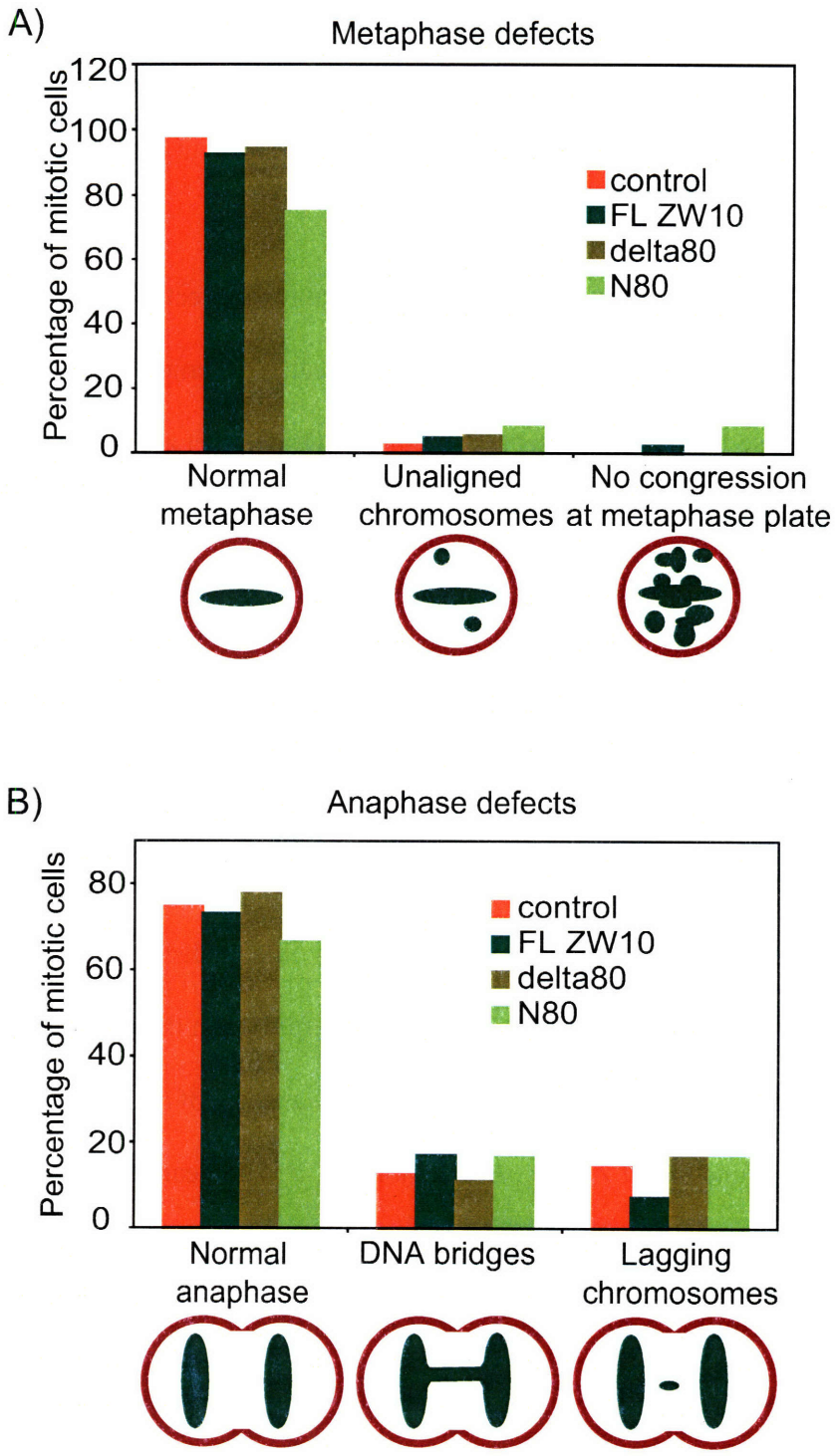


Figure 4.3. Analysis of mitotic outcome in HeLa cells expressing ZW10 mutants.
A) Metaphase defects in control or cells expressing FL ZW10 or mutants of ZW10. Misalignment was visually scored as no chromosome away from the metaphase plate (normal metaphase), 1–2 (unaligned chromosomes) or >2 (no congression). Schematic depicts defects scored.
B) Anaphase defects in control or cells expressing FL ZW10 or mutants of ZW10. Segregation errors were visually scored as chromosomes separate in two equal masses (normal anaphase), DNA bridges or laggin chromsomes. Schematic depicts defects scored.

spindle equator after anaphase A (chromatid disjunction) were scored as lagging. Expression of various mutants of ZW10 as well as FLZW10 did not significantly change the amount of congression errors in metaphase or segregation errors in anaphase compared to control (Figure 4.3 A,B). Cells expressing deletion fragments of ZW10 as well as FL ZW10 arrested in mitosis in the presence of nocodazole indicating a functional checkpoint (data not shown). Thus none of the mutants analyzed behaves as a dominant/negative since their overexpression does not interfere with chromosome congression or checkpoint function, phenotypes associated with the depletion of ZW10.

4.3.3 Search for kinetochore localization domain of ZW10.

N-terminal 80 aa fragment of ZW10 has been proposed to comprise its kinetochore localization domain (Wang et al., 2004a), however in my analysis it was not recruited to kinetochores (Figure 4.2B). I attempted to narrow down a region of ZW10 required for its kinetochore localization, therefore additional deletion mutants of ZW10 fused to GFP were generated and their kinetochore recruitment was assessed in H2B-RFP expressing HeLa cells treated with nocadazole (Figure 4.4). 421-779aa fragment of ZW10 was the smallest region found in this analysis to localize to kinetochores (Figure 4.4). Expression of smaller fragments of ZW10 was toxic to cells causing massive cell death. This result is in agreement with Ala scan and transposon-based insertion mutagenesis analysis of ZW10 reported by the Chan lab (Famulski J. and Chan G., Abstract book, 46th ASCB meeting, San Diego, CA, December 9-12, 2006). I conclude that kinetochore localization domain of ZW10 resides in the C-terminal half of the protein, but not in the N-terminal part as shown before (Wang et al., 2004a).

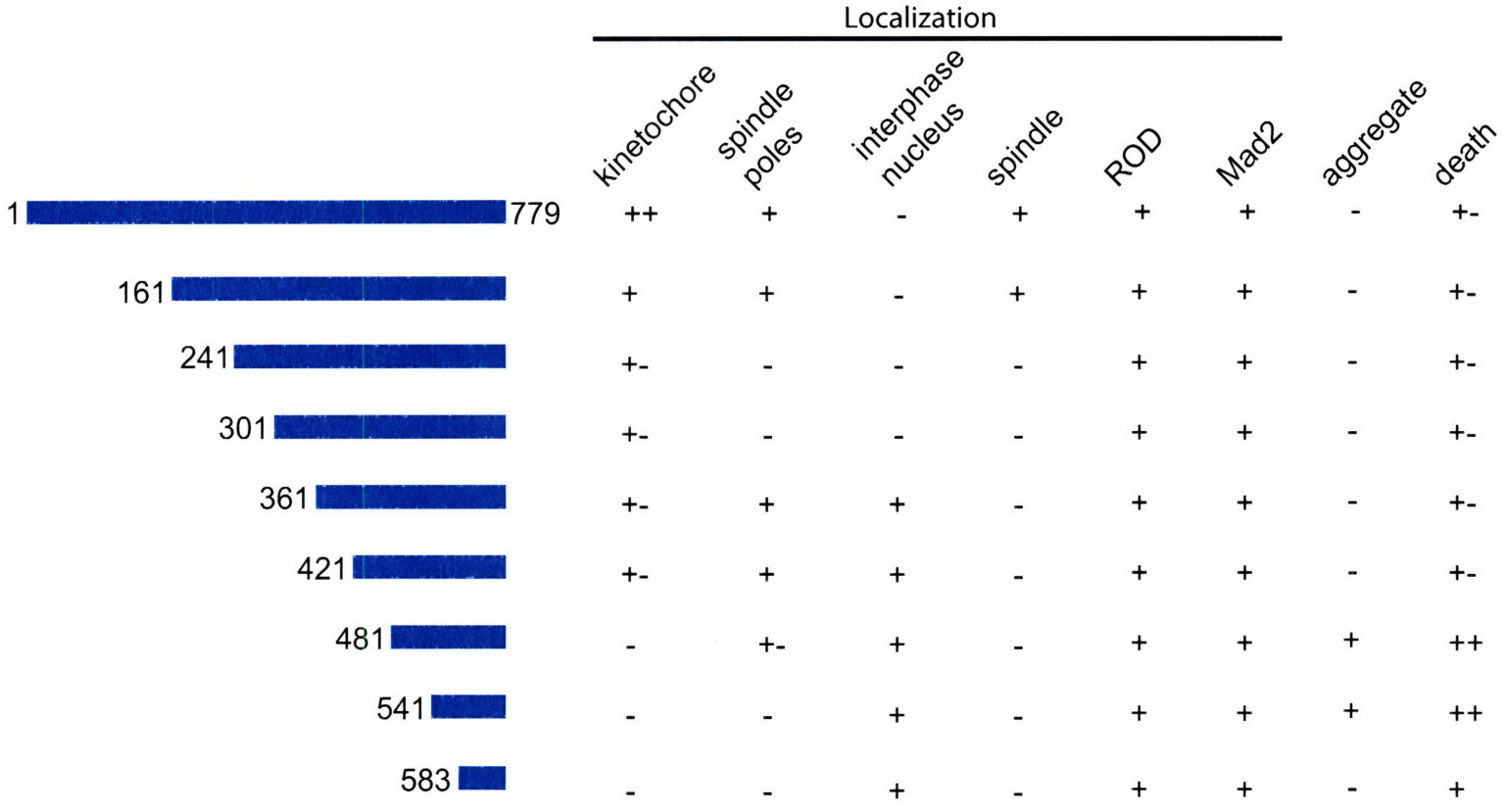


Figure 4.4 Schematic representation of deletion mutants of ZW10 and analysis of their function. Amino acid numbers are indicated at fragment extremities. (++) indicates a bright signal or a strong effect; (+) indicates a moderate signal or effect; (+-) indicates a weak signal or effect; (-) indicates no signal or effect.

Deletion mutants of ZW10 were also tested for nuclear localization in interphase. 361-779 aa fragment of ZW10 and all smaller fragments were present in the nucleus during interphase while the full length protein is sequestered from the nucleus. It remained unclear whether nuclear localization in interphase interferes with the role of ZW10 in maintaining integrity of Golgi, endosomes and lysosomes as well as in trafficking between endoplasmic reticulum and Golgi (Hirose et al., 2004; Varma et al., 2006). I also tested whether expression of ZW10 C-terminal regions of various lengths perturbs kinetochore localization of ROD and Mad2. None of the deletion mutants analyzed displaced ROD or Mad2 from kinetochores independent of their own kinetochore localization (Figure 4.4). I conclude that the fragment of ZW10 required for its binding to kinetochores is also necessary for assembly of RZZ complex and Mad2 recruitment.

A 583-779 aa region of ZW10 has been suggested in the literature to act as a dominant/negative (Varma et al., 2006). Cells expressing this fragment exhibited low mitotic index in the presence of nocodazole indicating a defective checkpoint (Varma et al., 2006). I tested kinetochore localization of the 583-779 aa fragment of ZW10 fused to GFP using high-resolution imaging. Kinetochore GFP signal was absent in cells expressing 583-779 aa region of ZW10 (Figure 4.4). I also tested whether expression of 583-779 aa region of ZW10 affected checkpoint function and fidelity of chromosome segregation. HeLa cells (n~100) expressing H2B-RFP and ZW10(583-779aa)-GFP were imaged every 3min for 8hrs in the presence or absence of nocodazole (data not shown). Nocodazole treatment caused a prolonged mitotic arrest indicating that the checkpoint was intact. Chromosome segregation was not perturbed in untreated cells. Thus

expression of 583-779 aa fragment of ZW10 does not have a dominant/negative effect in agreement with results reported by Chan G.K. (personal communication).

4.3.4 ZW10 interacts with BubR1 and CENP-E.

Although ZW10 has been proposed to play a role in Mad2 recruitment to unattached kinetochores thus fulfilling its checkpoint function (Buffin et al., 2005; Kops et al., 2005), all efforts aimed to demonstrate an interaction between these two proteins remained unsuccessful (Buffin et al., 2005; Kops et al., 2005). I sought to test whether ZW10 interacts with other checkpoint proteins that potentially may explain the role of ZW10 in checkpoint signaling. HeLa cell lysates were immunoprecipitated with antibodies against BubR1, CENP-E, Dynein Intermediate Chain (DIC) and Cdc20 and analyzed by Western blotting with an anti-ZW10 antibody (Figure 4.5A). I found that ZW10 associated not only with DIC, as has been reported previously (Starr et al., 1998), surprisingly, ZW10 also associated with BubR1 and CENP-E but not with Cdc20 (Figure 4.5A). To confirm these results, I performed a reciprocal experiment. HeLa cell lysates were immunoprecipitated with an anti-ZW10 antibody and analysed by Western blotting with antibodies against CENP-E, DIC and Cdc20 (Figure 4.5B). I observed association of ZW10 with CENP-E and DIC but not with Cdc20. Interaction of ZW10 and BubR1 was not confirmed in the reciprocal experiment probably because of the nature of anti-BubR1 antibody. Nevertheless, I conclude that ZW10 interacts with both BubR1 and CENP-E.

To determine whether association of ZW10 with BubR1 and CENP-E depends on the previously reported interaction between BubR1 and CENP-E (Chan et al., 1999), I tested association of these proteins in HeLa cells treated with siRNA against BubR1 or

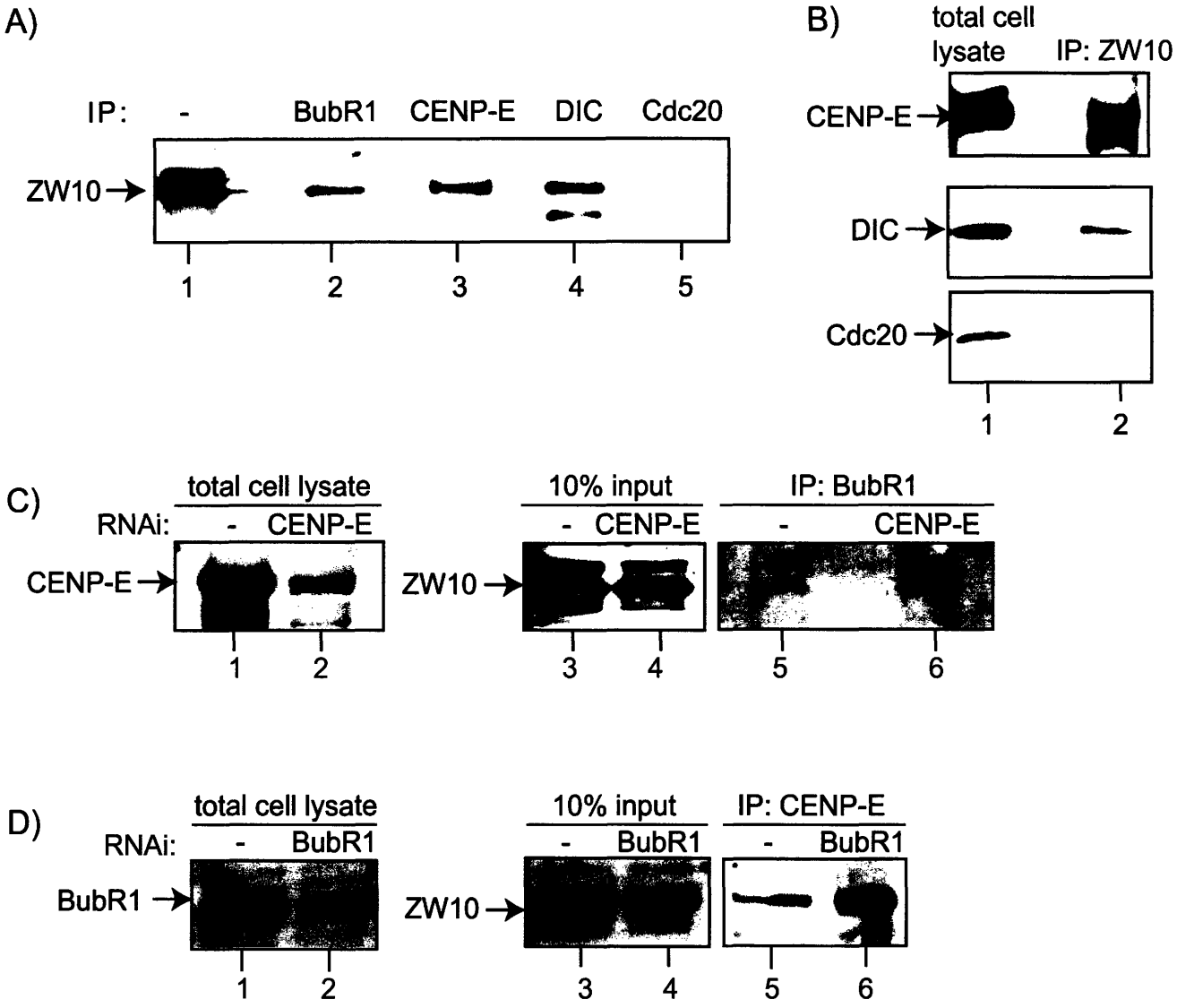


Figure 4.5. ZW10 associates with BubR1 and CENP-E.
A) Lysates of HeLa cells were immunoprecipitated with anti-BubR1 (lane 2), anti-CENP-E (lane 3), anti-DIC (lane 4) or anti-Cdc20 (lane 5) antibodies, and the immunoprecipitates were analyzed by Western blotting with an anti-ZW10 antibody. Lane 1 - total cell lysate. **B)** Lysates of HeLa cells were immunoprecipitated with anti-ZW10 antibody, and the immunoprecipitates were analyzed by Western blotting with anti-CENP-E, anti-DIC and anti-Cdc20 antibodies, as indicated. Lane 1- total cell lysate; lane 2- immunoprecipitates. **C)** Control and RNAi-treated total cell lysates were probed with anti-CENP-E (lanes 1,2) and anti- ZW10 (lanes 3,4) antibodies. Lysates of control cells and cells depleted of CENP-E by RNAi treatment (lanes 5,6) were immunoprecipitated with anti-BubR1 antibody and the immunoprecipitates were analyzed by Western blotting with anti-ZW10 antibody. **D)** Control and RNAi-treated total cell lysates were probed with anti-BubR1 (lanes 1,2) and anti- ZW10 (lanes 3,4) antibodies. Lysates of control cells and cells depleted of BubR1 by RNAi treatment (lanes 5,6) were immunoprecipitated with anti-BubR1 antibody and the immunoprecipitates were analyzed by Western blotting with anti-ZW10 antibody.

CENP-E (Figure 4.5 C,D). When cell lysates depleted of CENP-E by RNAi treatment were immunoprecipitated with anti-BubR1 antibodies and analyzed by Western blotting with antibody against ZW10, I still observed BubR1 association with ZW10 (Figure 4.5C). Using the same methodology I observed association of ZW10 and CENP-E in cells depleted of BubR1 by RNAi treatment (Figure 4.5D). Thus ZW10 interacts with BubR1 and CENP-E independently of each other.

Interaction between ZW10, BubR1 and CENP-E and the fact that all three proteins are recruited to kinetochores in mitosis (Hoffman et al., 2001) suggested that they might require each other for kinetochore localization. Immunofluorescence analysis in HeLa cells depleted of BubR1 by RNAi treatment with antibodies against ZW10 and CENP-E revealed the presence of both proteins at kinetochores (Figure 4.6A). Moreover, depletion of ZW10 by RNAi treatment did not perturb kinetochore localization of BubR1 or CENP-E (Figure 4.6B). I conclude that ZW10, BubR1 and CENP-E are recruited to kinetochores independently of each other.

4.3.5 ZW10 and BubR1 associate in a complex.

To determine the extent to which ZW10, BubR1 and CENP-E are associated with each other and with other proteins, I performed a sucrose-gradient density ultracentrifugation of HeLa cell lysates. Fractions were analyzed by a quantitative Western blotting with antibodies against BubR1, CENP-E and ZW10 (Figure 4.7A). Molecular weight standards were separated on an identical gradient that was ran in parallel. BubR1, CENP-E and ZW10 sedimented as separate peaks around 5,7 and 10 $X10^{-13}$s respectively. Since size exclusion chromatography was not performed, I could

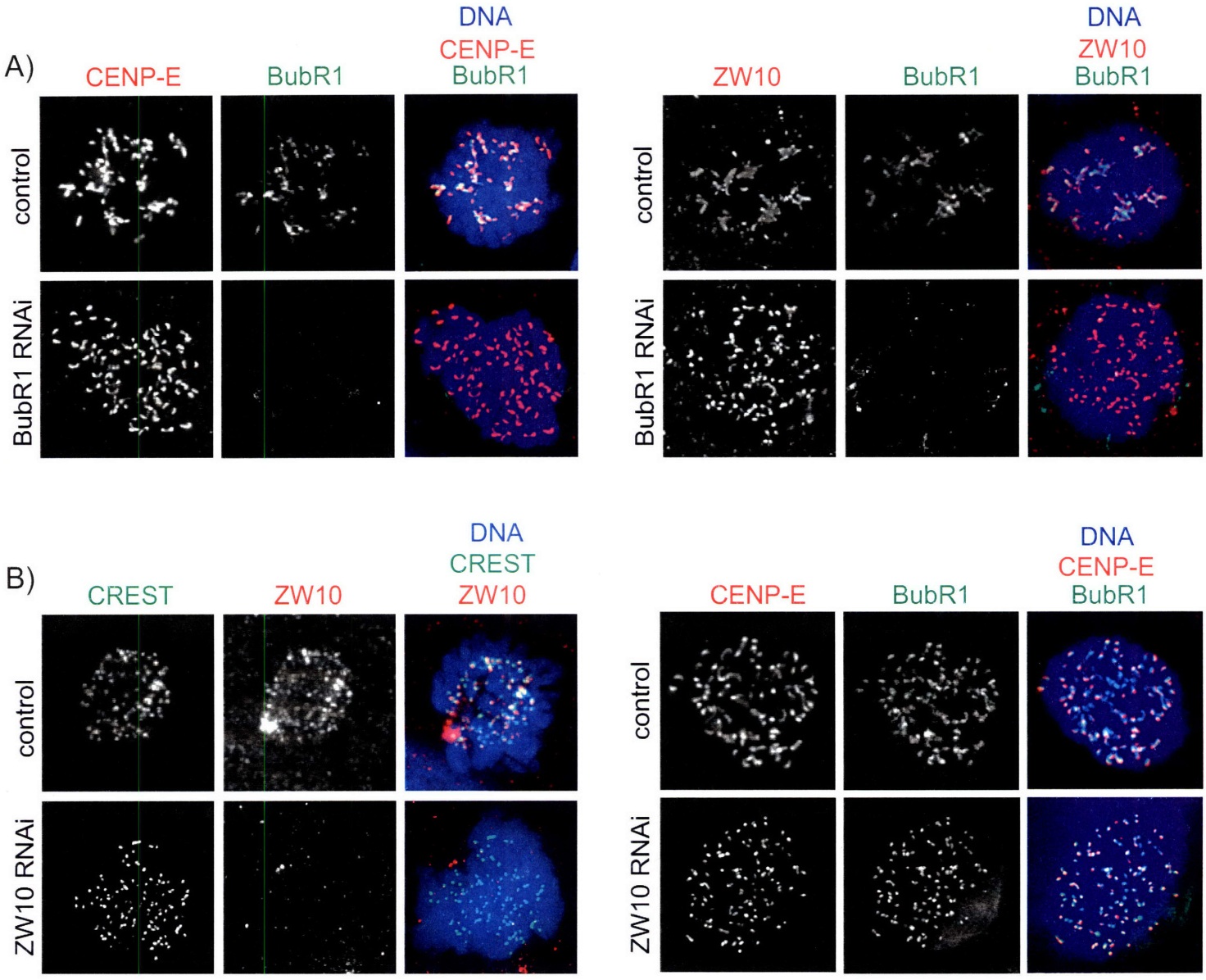


Figure 4.6. ZW10, BubR1 and CENP-E localize to kinetochores independently of each other. Images of HeLa cells treated with BubR1 siRNA (A) and ZW10 siRNA (B) and stained with anti-CENP-E, anti-BubR1, anti-ZW10 antibodies or CREST sera, as indicated. DAPI stain was used to visualize DNA.

not determine the native molecular weight of observed protein complexes. But because Svedberg coefficients corresponding to BubR1, CENP-E and ZW10 peaks were quite low, we assumed that BubR1, CENP-E and ZW10 sedimented as monomers in fractions 4-13 (Figure 7a). Residual amounts of BubR1, CENP-E and ZW10 were observed in fractions 15-20 corresponding to Svedberg coefficients of 18-27X10^{-13}s, indicating a presence of a high molecular weight complex that might contain all three proteins (Figure 4.7A).

To test whether BubR1, CENP-E and ZW10 interact as a part of a high molecular weight complex, we performed an immunoprecipitation analysis of fractions 5-12 and 15-18 with anti-BubR1 antibody followed by Western blotting with antibodies against BubR1, CENP-E and ZW10 (Figure 4.7B). Association of BubR1 and CENP-E was observed in fractions 5-12, while fractions 15-18 did not reveal this interaction (Figure 4.7B). Interestingly, a weak association of BubR1 with ZW10 was observed in fractions 15-18 (Figure 4.7B). I conclude that BubR1 but not CENP-E might be a part of RZZ complex or some other high molecular weight complex containing ZW10.

4.4 Discussion

Here I combine molecular biological analysis with live cell imaging to dissect functional domains of ZW10. I identify regions of ZW10 required for its localization to kinetochores, for nuclear export in interphase and for cell viability. I find that expression of none of ZW10 deletion mutants perturbs kinetochore localization of Mad2 and ROD. Biochemical approaches allowed me to identify a novel interaction between ZW10 and

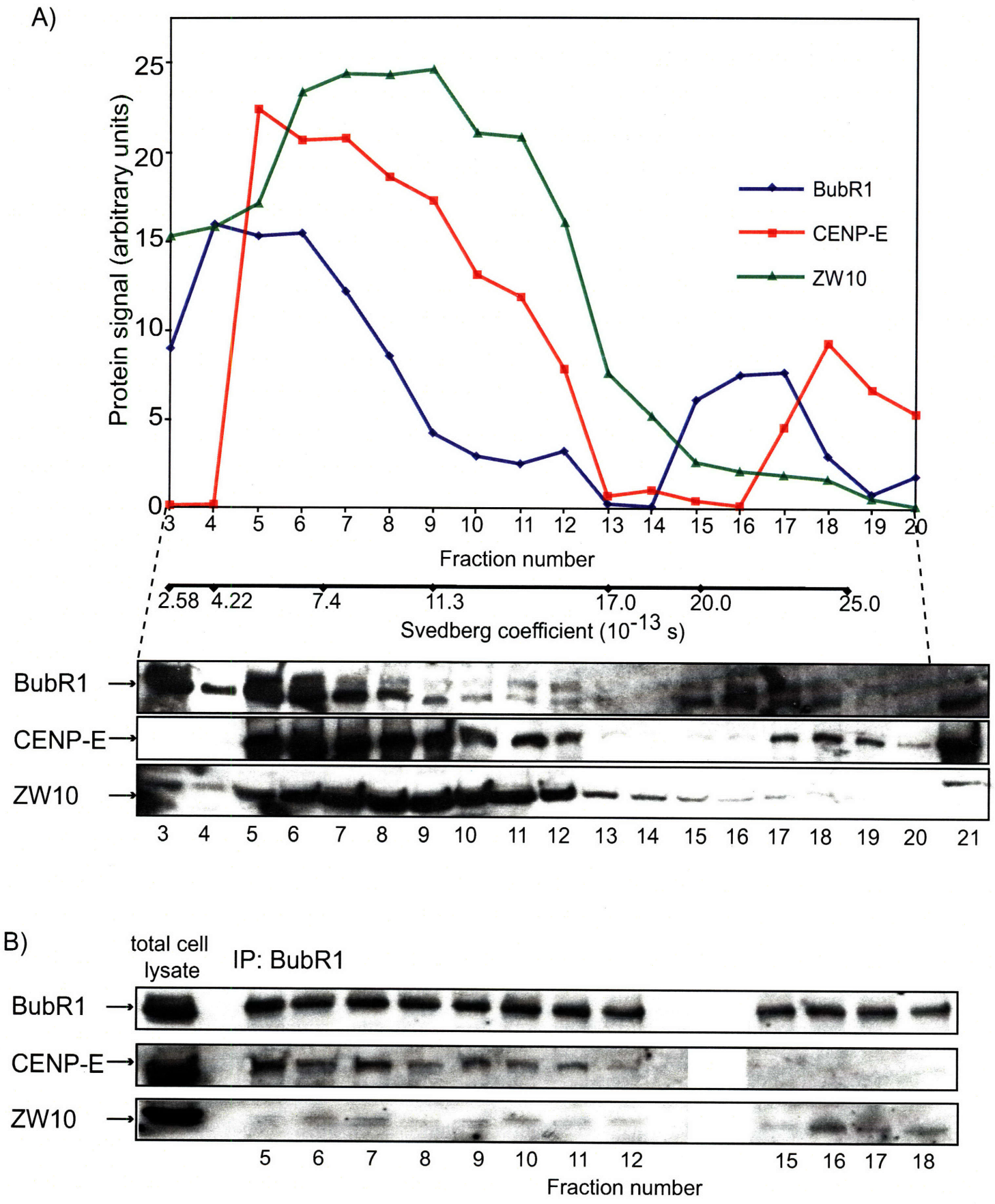


Figure 4.7. ZW10 and BubR1 interact as a part of high molecular weight complex.
A) Velocity sedimentation analysis of HeLa cell lysate in 5-25% sucrose gradient. Fractions were immunoblotted with anti-ZW10, anti-CENP-E and anti-BubR1 antibodies, as indicated. Graph depicts protein signal intensity quantified using Image Quant. Numbers on horizontal line indicate Svedberg coefficient values. B) Co-immunoprecipitation analysis of selected fractions that were immunoprecipitated with anti-BubR1 antibody and probed with anti-BubR1, anti-ZW10 or anti –CENP-E antibodies, as indicated.

BubR1. Further investigation is required to support these data; however, if proved to be true, this interaction may have implications for ZW10 checkpoint function.

4.4.1 Mutational analysis of ZW10

Kinetochore localization domain of ZW10 has been previously mapped to the N-terminal 80 amino acids of ZW10 and the same region has been suggested to bind to Zwint-1 that correlated well with the observation that Zwint-1 is required for ZW10 recruitment to kinetochores (Wang et al., 2004a). However, my analysis does not support this conclusion. I identified a 421-779aa region in the C-terminus of ZW10 to be required for its kinetochore localization. This data is in agreement with observations by Chan lab, which narrowed kinetochore localization domain even more proposing amino acids 536-586 to perform the same function (Famulski, J. and Chan, G., Abstract book, 46th ASCB meeting, San Diego, CA, December 9-12, 2006). 421-779aa region of ZW10 that I identified includes 536-686aa fragment reported by Chan lab (Famulski, J. and Chan, G., Abstract book, 46th ASCB meeting). Thus kinetochore localization domain of ZW10 is located in the C-terminal part of the protein. The difference between my and Chan labs maps of the region required for kinetochore recruitment of ZW10 probably originated from the methodology employed. While I generated a panel of deletion mutants that were 60aa apart resulting in a robust mapping of kinetochore-binding region of ZW10 to a C-terminal part of the protein, Chan lab performed a very careful analysis that included Ala scan of the whole ZW10 sequence (Famulski, J. and Chan, G., Abstract book, 46th ASCB meeting). It is likely that I could have achieved the same result by generating additional

deletion mutants of ZW10 of intermediate lengths and analyzing their localization in cells depleted of endogenous ZW10 by RNAi treatment.

Point mutations have been identified in ROD, ZW10 and Zwilch in a panel of colorectal cancers (Wang et al., 2004b). Interestingly, expression of point mutants of ZW10 in HeLa cells did not affect localization of ZW10, checkpoint integrity or fidelity of chromosome segregation. It is even more surprising, since one of the mutations, N123T, resulted in the formation of a potential phosphorylation site on ZW10, while the other one, S623G, resulted in its disappearance. It is possible that these mutations are recessive and their effect can be observed only in cells depleted of endogenous ZW10 by RNAi treatment. A more favorable explanation is that these mutations result in subtle defects in chromosome segregation that are not detected by the spindle checkpoint or that they impair a checkpoint function of ZW10 but do not generate a massive chromosome missegregation. Reasoning behind this hypothesis is that gross disruption of checkpoint proteins function results in mitotic catastrophe and cell death (Draviam et al., 2004). That is why only few human tumors have been found to carry mutations in Mad and Bub checkpoint genes (Cahill et al., 1998; Yamaguchi et al., 1999). Therefore ZW10 mutations found in colorectal tumors likely cause subtle defects that probably can not be analyzed in a highly aneuploid HeLa cell line and should be tested in primary cells.

4.4.2 On a search for a dominant/negative.

A C-terminal 583-779aa fragment of ZW10 has been proposed to act a dominant/negative (Varma et al., 2006). In a fixed cell analysis, Varma D *et al.* has observed a low mitotic mitotic index in HeLa cells expressing 583-779aa of ZW10 and

treated with nocodazole (Varma et al., 2006). My results contradict this data. I observed that expression of 583-779aa region of ZW10 caused massive cell death and did not perturb checkpoint function or chromosome segregation in live-cell analysis. Therefore, the discrepancy may be explained by the fact that dead cells appear rounded up to a naïve eye and might be easily confused with mitotic cells in a fixed cell assay. Moreover, 583-779aa fragment of ZW10 did not localize to kinetochores in my study that would be expected from a dominant/negative mutant, and only partially overlaps with 536-686aa kinetochore localization domain proposed by Chan lab (Famulski, J. and Chan, G., Abstract book, 46th ASCB meeting).

Could a dominant/negative mutant of ZW10 be ever isolated? It seems that this question can not be answered until the structure of ZW10 and other RZZ components are better understood. Since RZZ complex composition has not been studied apart from identification of its core subunits, it is unclear how many copies of each protein are in a complex or whether the complex is a dimer itself. The molecular interphase among ZW10, ROD and Zwilch has not been mapped yet. Therefore, in a mutational analysis of ZW10 it is unclear which interactions are being disrupted. Moreover, it is still not understood whether RZZ is assembled in the cytoplasm or whether it requires a kinetochore platform for assembly. Since a random mutagenesis analysis did not identify a dominant/negative mutant of ZW10, a substantial amount of structural data on RZZ would help to determine whether such a mutant exists.

4.4.3 ZW10-BubR1 interaction: implications for a checkpoint control.

My preliminary analysis revealed that ZW10 associates with BubR1 in co-immunoprecipitation experiments and possibly in sucrose-gradient density ultracentrifugation. Additional experiments are required to prove existence of such interaction. GST-pull downs of BubR1 and ZW10 could be used to assess the possibility of this interaction in vitro. Since total HeLa cell lysates were utilized for this analysis it would be necessary to determine whether ZW10 associates with BubR1 in mitotic cells or whether this association is not cell cycle specific.

In case ZW10-BubR1 interaction is mitosis-specific, it would be interesting to determine whether these proteins associate in the cytoplasm or at the kinetochore. Independence of kinetochore localization of ZW10 and BubR1 favors cytoplasmic interaction hypothesis. On the other hand, kinetochore may serve as a platform for their association. Since kinetochore is a densely populated protein structure, many indirect interactions can be uncovered using mild co-immunoprecipitation conditions. However, interaction of BubR1 and Mad2 have not been demonstrated by inmmunoprecipitation analysis, although Mad2 depends on BubR1 for its kinetochore localization (Fang, 2002; Meraldi et al., 2004) and both of them are part of the mitotic checkpoint complex (MCC) (Fang, 2002; Sudakin et al., 2001). BubR1 and ZW10 interaction at kinetochores may be transient, since in metaphase ZW10 delocalizes to the spindle while BubR1 levels slightly decrease at kinetochores (Fang, 2002; Hoffman et al., 2001). In case ZW10 and BubR1 interact in the cytoplasm, it is unclear why BubR1 plays a role in anaphase timing while ZW10 does not (Figure 3.1; (Meraldi et al., 2004)).

I have proposed in the previous chapter that checkpoint function of ZW10 is independent of Mad2 (see Chapter 3 of this thesis). It is possible that ZW10-BubR1 interaction plays a role in checkpoint signaling thus fulfilling checkpoint function of ZW10. For example, ZW10 might alter BubR1 kinase activity as it has been reported for CENP-E (Mao et al., 2003). Alternatively, ZW10 may change BubR1 binding properties as a component of MCC thus preventing or altering MCC assembly. Future biochemical analysis will clarify the role ZW10 association with BubR1 plays in checkpoint signaling.

4.5 Materials and methods

Cell culture, cDNA

HeLa cells were grown as described (Meraldi et al., 2004). H2B-GFP has been described previously (Meraldi et al., 2004). For transient expression of full length HZW10 and ZW10 mutants, ZW10 cDNA was PCR amplified from ZW10-S11/pBS-KS (a gift of B.Williams, Cornell University, Ithaca, NY) and subcloned into pEGFP-C1.

Live-cell imaging

Cells were imaged using 20X NA0.75 objective as described in (Meraldi and Sorger, 2005). For following ZW10-GFP dynamics and imaging of ZW10 mutants localization a 100X NA1.4 objective equipped with an objective heater was used.

Antibodies and Immunofluorescence

Cells were fixed, permeabilized and blocked as described (Meraldi et al., 2004). Antibodies used were as follows: affinity-purified goat anti-BubR1 (BD Biosciences), rabbit anti-CENP-E (Meraldi et al., 2004), human anti-CREST (Meraldi et al., 2004).

Rabbit antibodies (Covance) were made against His_6 HZW10 (645–779 aa), expressed in *E. coli* and purified on Nickel Sepharose Beads (Qiagen).

Immunoblotting

Whole cell extracts were prepared by cell lysis in SDS sample buffer with 15% mercaptoethanol, resolved by SDS-PAGE, transferred to PVDF membranes (Immobilon) by semi-dry blotting (Hoefer). Membranes were incubated in blocking buffer (3% low-fat dried milk, PBS, 0.1% Tween-20) and probed with rabbit anti-ZW10 (0.5μg/ml), mouse anti-BubR1 (0.5μg/ml) or rabbit anti-CENP-E (0.5μg/ml) in blocking buffer. Anti-mouse and anti-sheep HRP-conjugated secondary antibodies (Amersham Pharmacia) were applied in blocking buffer and blots were developed by enhanced chemiluminescence (Supersignal West Femto Maximum kit; Pierce).

Sucrose Gradient Sedimentation

Gradients were prepared in 4-ml volumes by layering 2 successive 2-ml aliquots of column buffer containing 5% or 25% sucrose prepared in 100mM NaCl and incubating the gradient at 4°C for 2min on a gradient-maker (Biocomp Instruments) to equilibrate. HeLa cell lysates were sedimented in 5-25% gradients, the standards were run separately. Gradients were centrifuged at 50,000 rpm for 4hrs at 4°C in a TL-S55 swinging bucket rotor (Beckman Instruments) and fractionated using an automated fractionator (Biocomp Instruments). Fractions were then analyzed by TCA precipitation followed by immunoblotting. Protein standards (Boehringer Mannheim) chymotrypsinogen A (molecular mass = 25 kD, s = 2.58), bovine serum albumin (molecular mass = 68 kD, s = 4.22), aldolase (molecular mass = 158 kD, s = 7.4), catalase (molecular mass = 240 kD, s = 11.3), and ferritin (molecular mass = 440 kD, s = 17) were separated on SDS-

polyacrylamide gels, stained with Coomassie blue and quantified using Image Quant software. All samples were analyzed at least two times, and sedimentation coefficients varied by no more than 15% between experiments.

4.6 Acknowledgements

I would like to thank Viji Draviam for inspiration, helpful discussions and critical reading of this chapter. I thank Peter De Wulf for reagents and assistance with velocity sedimentation experiments. I thank Byron Williams for HZW10 cDNA. I thank Sorger lab members for reagents and helpful discussions.

4.7 References

Basto, R., F. Scaerou, S. Mische, E. Wojcik, C. Lefebvre, R. Gomes, T. Hays, and R. Karess. 2004. In vivo dynamics of the rough deal checkpoint protein during Drosophila mitosis. *Curr Biol.* 14:56-61.

Brinkley, B.R., and J. Cartwright, Jr. 1971. Ultrastructural analysis of mitotic spindle elongation in mammalian cells in vitro. Direct microtubule counts. *J Cell Biol.* 50:416-31.

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

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

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

Cheeseman, I.M., S. Niessen, S. Anderson, F. Hyndman, J.R. Yates, 3rd, K. Oegema, and A. Desai. 2004. A conserved protein network controls assembly of the outer kinetochore and its ability to sustain tension. *Genes Dev.* 18:2255-68.

De Wulf, P., A.D. McAinsh, and P.K. Sorger. 2003. Hierarchical assembly of the budding yeast kinetochore from multiple subcomplexes. *Genes Dev.* 17:2902-21.

Draviam, V.M., S. Xie, and P.K. Sorger. 2004. Chromosome segregation and genomic stability. *Curr Opin Genet Dev.* 14:120-5.

Fang, G. 2002. Checkpoint protein BubR1 acts synergistically with Mad2 to inhibit anaphase-promoting complex. *Mol Biol Cell.* 13:755-66.

Hirose, H., K. Arasaki, N. Dohmae, K. Takio, K. Hatsuzawa, M. Nagahama, K. Tani, A. Yamamoto, M. Tohyama, and M. Tagaya. 2004. Implication of ZW10 in membrane trafficking between the endoplasmic reticulum and Golgi. *Embo J.* 23:1267-78.

Hoffman, D.B., C.G. Pearson, T.J. Yen, B.J. Howell, and E.D. Salmon. 2001. Microtubule-dependent changes in assembly of microtubule motor proteins and mitotic spindle checkpoint proteins at PtK1 kinetochores. *Mol Biol Cell.* 12:1995-2009.

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

Karess, R. 2005. Rod-Zw10-Zwilch: a key player in the spindle checkpoint. *Trends Cell Biol.* 15:386-92.

Kops, G.J., Y. Kim, B.A. Weaver, Y. Mao, I. McLeod, J.R. Yates, 3rd, M. Tagaya, and D.W. Cleveland. 2005. ZW10 links mitotic checkpoint signaling to the structural kinetochore. *J Cell Biol.* 169:49-60.

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

Lin, Y.T., Y. Chen, G. Wu, and W.H. Lee. 2006. Hec1 sequentially recruits Zwint-1 and ZW10 to kinetochores for faithful chromosome segregation and spindle checkpoint control. *Oncogene.* 25:6901-14.

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

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

Meraldi, P., V.M. Draviam, and P.K. Sorger. 2004. Timing and checkpoints in the regulation of mitotic progression. *Dev Cell.* 7:45-60.

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

Scaerou, F., I. Aguilera, R. Saunders, N. Kane, L. Blottiere, and R. Karess. 1999. The rough deal protein is a new kinetochore component required for accurate chromosome segregation in Drosophila. *J Cell Sci.* 112 (Pt 21):3757-68.

Scaerou, F., D.A. Starr, F. Piano, O. Papoulas, R.E. Karess, and M.L. Goldberg. 2001. The ZW10 and Rough Deal checkpoint proteins function together in a large, evolutionarily conserved complex targeted to the kinetochore. *J Cell Sci.* 114:3103-14.

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

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

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

Varma, D., D.L. Dujardin, S.A. Stehman, and R.B. Vallee. 2006. Role of the kinetochore/cell cycle checkpoint protein ZW10 in interphase cytoplasmic dynein function. *J Cell Biol.* 172:655-62.

Wang, H., X. Hu, X. Ding, Z. Dou, Z. Yang, A.W. Shaw, M. Teng, D.W. Cleveland, M.L. Goldberg, L. Niu, and X. Yao. 2004a. Human Zwint-1 specifies localization of Zeste White 10 to kinetochores and is essential for mitotic checkpoint signaling. *J Biol Chem.* 279:54590-8.

Wang, Z., J.M. Cummins, D. Shen, D.P. Cahill, P.V. Jallepalli, T.L. Wang, D.W. Parsons, G. Traverso, M. Awad, N. Silliman, J. Ptak, S. Szabo, J.K. Willson, S.D. Markowitz, M.L. Goldberg, R. Karess, K.W. Kinzler, B. Vogelstein, V.E. Velculescu, and C. Lengauer. 2004b. Three classes of genes mutated in colorectal cancers with chromosomal instability. *Cancer Res.* 64:2998-3001.

Williams, B.C., and M.L. Goldberg. 1994. Determinants of Drosophila zw10 protein localization and function. *J Cell Sci.* 107 (Pt 4):785-98.

Williams, B.C., T.L. Karr, J.M. Montgomery, and M.L. Goldberg. 1992. The Drosophila l(1)zw10 gene product, required for accurate mitotic chromosome segregation, is redistributed at anaphase onset. *J Cell Biol.* 118:759-73.

Williams, B.C., Z. Li, S. Liu, E.V. Williams, G. Leung, T.J. Yen, and M.L. Goldberg. 2003. Zwilch, a new component of the ZW10/ROD complex required for kinetochore functions. *Mol Biol Cell.* 14:1379-91.

Winey, M., C.L. Mamay, E.T. O'Toole, D.N. Mastronarde, T.H. Giddings, Jr., K.L. McDonald, and J.R. McIntosh. 1995. Three-dimensional ultrastructural analysis of the Saccharomyces cerevisiae mitotic spindle. *J Cell Biol.* 129:1601-15.

Yamaguchi, K., K. Okami, K. Hibi, S.L. Wehage, J. Jen, and D. Sidransky. 1999. Mutation analysis of hBUB1 in aneuploid HNSCC and lung cancer cell lines. *Cancer Lett.* 139:183-7.

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

Chapter 5

Conclusions and Future Directions

Outline

Kinetochores play a key role in ensuring a faithful segregation of chromosomes during cell division. They perform at least two crucial functions: establishment of kinetochore-microtubule (MT) attachment and monitoring the state of attachment to rapidly inhibit progression through anaphase, in case errors are present. The process of kinetochore-MT attachment is complex, involving the action of multiple MT-binding proteins and is tightly regulated. Kinetochore structure and function have been originally studied in *S.cerevisiae* where classical kinetochore and checkpoint proteins have been identified through genetic screens (Hoyt et al., 1991; Li and Murray, 1991). Implementation of RNAi technology coupled to live cell imaging allowed to expand investigation of kinetochore structure and function to mammalian cells. However, before I began this work, establishment of kinetochore-MT attachment in mammals was poorly understood and the function of kinetochore components, found only in metazoans, in attachment and checkpoint signaling, was unclear. In the previous chapters, I answer basic questions regarding the role of MAPs and motors in chromosome segregation and analyze biochemical and cell biological function of checkpoint complex RZZ. As will be described below, my findings also reveal a close link between establishment and monitoring of attachment at the kinetochore, in contrary to classical thinking, and suggest future avenues of research.

5.1 Analysis of MAPs and motors.

MAPs and motors work together at the kinetochore to regulate MT dynamics in a spatially controlled fashion and to generate forces necessary for directed movement (Howard and Hyman, 2003). The establishment of bipolar attachment is thought to

depend on the capture of MTs by kinetochores and the subsequent regulation of MT +end polymer dynamics by kinetochore-bound MAPs and motors. When I began this work, kinetochore localization and functions in regulation of MT dynamics were known for some MAPs and motors, but their role in chromosome segregation remained poorly understood. Mitotic functions of LIS1 and CLIP-170 were unclear, although they had been demonstrated to associate with kinetochore indirectly through dynein/dynactin motor complex (Coquelle et al., 2002; Tai et al., 2002). Reports addressing the role of dynein/dynactin in mitosis and MT binding have been controversial (Echeverri et al., 1996; Howell et al., 2001). Overexpression of dynamitin(p50) subunit of dynactin caused defects in chromosome congression (Echeverri et al., 1996), while injection of the purified p50 protein or anti-dynein antibody 70.1 did not interfere with chromosome alignment at the metaphase plate (Howell et al., 2001). My results emphasize the importance of dynein/dynactin, LIS1 and CLIP-170 for chromosome congression and kinetochore-MT attachment. Since functional analysis of these MAPs is obscured by their localization to multiple subcellular locations (Vallee and Tsai, 2006), I selectively depleted them from kinetochores by interfering with the function of ZW10. I resolved the controversy associated with dynein function in congression and demonstrated that dynein/dynactin and CLIP-170 are necessary for initial kinetochore-MT attachment. My data supports earlier observations that indicated the role of dynein in poleward chromosome movement and hypothesized its possible role in chromosome congression and attachment (Rieder and Alexander, 1990). It is also in agreement with the recent report suggesting the role for CLIP-170 in formation of kinetochore-MTs (Tanenbaum et al., 2006).

Investigation of the role of MAPs and motors in chromosome segregation poses several important questions: when a number of MAPs and motors localize to kinetochores, to establish a bipolar attachment, how are their functions integrated together to achieve simultaneous binding of 20-40 MTs to each kinetochore in a timely manner? It will be necessary to dissect functions of all MAPs and motors at the kinetochore by combining biochemical and cell biological methods, including point mutations resulting in the loss of protein function and high resolution light microscopy methods. Another fundamental question that has remained is how MT binding sites are organized so that MTs exhibit dynamic instability without 'falling off' the kinetochore? Recent studies of Dam1 complex in yeast suggest topological coupling of the MT +end to the kinetochore (Miranda et al., 2005; Westermann et al., 2006). Dam1 assembles in rings around + ends of MTs and can remain on the end of shrinking MTs by 'gliding' along its surface (Wang et al., 2007; Westermann et al., 2006). Dam1 bound to MTs is connected to kinetochore by Ndc80 complex, which, in turn has been demonstrated to be directly involved in attachment (Cheeseman et al., 2006; DeLuca et al., 2006; Shang et al., 2003). Homologues of Dam1 components still have to be identified in higher eukaryotes. And even then, a ring complex formation around MT (+)end suggests repetitive structure of the MT binding site at metazoan kinetochores. However, recent EM analysis of the kinetochore outer plate suggests that kinetochore corona is comprised of fibrous network with extending rods of Hec1/Ndc80 that attach to MTs (Dong et al., 2007). Moreover, functions of MAPs and motors that also connect kinetochores and MTs have to be integrated with the function of Dam1 in maintaining dynamic interaction with MTs.

5.2 The role of RZZ complex in early attachment.

Recently additional role for some checkpoint proteins started to emerge in regulation of kinetochore-MT attachment. Aurora B functions in the correction of syntellic malorientations by destabilizing incorrect configurations and activating the checkpoint (Pinsky et al., 2006). Depletion of Bub1 leads to formation of side-on kinetochore-MT binding; therefore Bub1 has been suggested to play role in formation of end-on attachments (Meraldi and Sorger, 2005). Others and I have shown that Aurora B and Bub1 are required for kinetochore localization of MCAK (Andrews et al., 2004; Liu et al., 2006). Abrogation of BubR1 function causes congression failure (Lampson and Kapoor, 2005). BubR1 is proposed to monitor the function of CENP-E at kinetochores, which, in turn, is involved in congression of monooriented chromosomes to metaphase plate (Chan et al., 1999; Kapoor et al., 2006). Thus, before I began this work, some checkpoint proteins have been implicated in chromosome alignment and possibly in regulation of attachment but later in mitosis. How initial capture of MTs by kinetochores is established and regulated remained an open question in the field.

Using RNAi-mediated depletion of ZW10 and live-cell imaging I discovered a novel role for RZZ complex in initial attachment at the kinetochore. My results indicate that the attachment function of RZZ is mediated by at least two MT-binding proteins, CLIP-170 and dynein/dynactin. This data is in agreement with the recent report by Yang Z. *et al.* that demonstrated abrogation of dynein function by antibody injection or siRNA treatment of ZW10 inhibits poleward chromosome movement after initial capture (Yang et al., 2007). Astral MTs were observed to contact kinetochores in cells injected with anti-dynein antibody, but chromosomal movement towards the pole was blocked.

Therefore, dynein motor at the kinetochore has been proposed to empower a poleward motion but not to participate in the initial capture (Yang et al., 2007). Congression defects that arose from depletion of ZW10 have been explained by lack of poleward chromosome movement associated with dynein dysfunction (Yang et al., 2007). However, my data indicates that RZZ complex plays a key role in initial capture at kinetochores, since depletion of ZW10 displaces not only dynein/dynactin, but also CLIP-170 from kinetochores. CLIP-170 has been implicated in the initial kinetochore-MT encounter because its dysfunction completely abrogates kinetochore-MT attachment (Tanenbaum et al., 2006). Moreover, we can not exclude a possible role for LIS1 in initial attachment, since LIS1 interacts with the motor domain of dynein and might regulate dynein activity (Tai et al., 2002).

My results indicate that the whole module of MT-binding proteins recruited to kinetochore by RZZ, including dynein/dynactin, CLIP-170, LIS1 and possibly others, participates in the initial kinetochore-MT binding. One future approach that may contribute to our understanding of the mechanics of initial attachment is to dissect individual functions of these proteins. Since RNAi depletion of dynein, CLIP-170 and LIS1 abrogates their function in multiple subcellular locations making it difficult to carefully address the role of these proteins at kinetochores, mutational analysis could be employed. MT capture and poleward chromosomes movement can be studied in cells expressing non-interacting mutants of dynein, CLIP-170 and LIS1. For example, a C-terminal fragment of LIS1 containing WD repeats 5, 6 and 7 can be used, since it interacts with the distal zinc finger domain of CLIP-170 and its overexpression has been shown to displace CLIP-170 from kinetochores (Coquelle et al., 2002; Tai et al., 2002).

EM analysis will be helpful to assess the state of kinetochore- MT attachment in these cells. An important advancement in our understanding of a sensoring function of the spindle checkpoint would be to determine whether RZZ complex monitors the function of dynein/CLIP-170/LIS1 at kinetochores. For this purpose a ZW10 mutant that is checkpoint proficient but defective in binding dynein may be employed. In case separation of function mutants can not be designed for ZW10 they might probably be generated for other RZZ members, ROD or Zwilch. If RZZ senses functionality of MAPs at the binding site and reports to the checkpoint, expression of this mutant will trigger a mitotic arrest.

5.3 RZZ complex and checkpoint signaling.

RZZ complex has been proposed to play a structural role at kinetochores by recruiting dynein/ dynactin and Mad2 to unattached kinetochores in prometaphase (Buffin et al., 2005; Kops et al., 2005; Starr et al., 1998). The absence of Mad2 at kinetochores in cells depleted of RZZ has been suggested to explain a checkpoint deficient phenotype associated with the depletion of RZZ (Buffin et al., 2005; Kops et al., 2005). However, several lines of evidence indicate that RZZ complex may be involved in the checkpoint signaling in parallel or downstream of Mad2 thus playing an important role in the control of metaphase to anaphase transition. First, my studies revealed that a partial depletion of ZW10 does not displace Mad2 from kinetochores; moreover, Mad2 accumulates at unattached kinetochores to a control prometaphase levels that diminish but do not disappear completely in anaphase. It is currently unclear why Mad2 accumulation at anaphase kinetochores in ZW10 depleted cells is not sufficient to

activate the checkpoint. As has been demonstrated previously, Mad2 overexpression causes a prolonged mitotic arrest that has been explained by sequestration of Cdc20 by excessive amounts of Mad2 (He et al., 1997; Homer et al., 2005). I observed that depletion of ZW10 by RNAi treatment rescues an arrest imposed by disproportional amounts of Mad2 and cells proceed to anaphase. I propose that ZW10 role in the checkpoint signaling is independent of Mad2 function, although structurally, as a component of the kinetochore corona, ZW10 might increase stability of Mad2 at kinetochores in agreement with previous studies (Buffin et al., 2005; Kops et al., 2005). To collect additional evidence supporting this finding, it will be interesting to test Mad2 localization in cells expressing a non-Zwint-1 interacting mutant of ZW10, that has been shown to localize to kinetochores but abrogate the checkpoint (Famulski J. and Chan, G, Abstract book, 46th ASCB meeting, San Diego, December 9-12, 2006).

Second, in a recent study, Zwint1 siRNA treatment that completely displaces ZW10 from kinetochores has been shown to reduce Mad2 kinetochore signal to half of its control levels (Lin et al., 2006). In nocodazole treated cells depleted of Hec1 by siRNA treatment that completely blocks Mad2 recruitment to kinetochores (Meraldi et al., 2004), the checkpoint failure is observed. In contrast, in nocodazole treated cells depleted of Zwint-1 that abolishes ZW10 kinetochore recruitment, while only partially affecting Mad2 localization, mitotic index is higher than in Hec1 depleted cells but not as high as in control indicating a partial loss of checkpoint. This indicates that the spindle checkpoint is only partially abrogated in Zwint-1 depleted cells (Lin et al., 2006). Altogether these data suggests that a functional checkpoint depends on both checkpoint proteins, Mad2 and ZW10.

Current models of checkpoint activation include the activity of mitotic checkpoint complex (MCC) and Mad2 'template model' that proposed an explanation for signal amplification away from kinetochores (De Antoni et al., 2005). My work imposes an important question: what is the function of RZZ in the checkpoint signaling and how can it be incorporated into current models? FRAP analysis of Mad2 dynamics at kinetochores revealed biphasic kinetics of Mad2 localization that indicated a presence of two pools of Mad2, a stably bound pool and a rapidly exchanging pool (Shah et al., 2004). A stable pool has been accounted for a Mad1-Mad2 complex that forms a scaffold for recruitment and 'activation' of rapidly exchanging Mad2 in a form that inhibits Cdc20, as proposed by the 'template' model of checkpoint signaling (De Antoni et al., 2005; Shah et al., 2004). One possibility for RZZ function in the checkpoint is to inhibit formation of 'active' pool of Mad2, which will not affect kinetochore localization of Mad2 but will prevent formation of inhibitory Mad2-Cdc20 complex. FRAP studies of Mad2 dynamics in cells depleted of ZW10 by RNAi treatment could clarify this hypothesis. Alternatively, RZZ might contribute to inhibition of APC/C activity by some other mechanism. It will be useful to address the requirement for ZW10 in APC/C function using *in vitro* reconstituted APC/C-mediated ubiquitination assays of Xenopus egg extracts (Fang et al., 1998) immuno-depleted of ZW10. My data also suggest that ZW10 may interact with BubR1. As I discussed in chapter 4, additional experiments are required to prove this interaction and to determine whether it is mitosis-specific and direct. The study of ZW10-BubR1 interaction may explain a checkpoint role of ZW10, since recent studies in budding yeast demonstrated the key function for BubR1/Mad3 in APC/C inhibition (Burton and Solomon, 2007; King et al., 2007). Destruction boxes found in Mad3 are

critical for MCC formation and Mad3 inhibits APC/C by blocking substrate binding to Cdc20, an activity of Mad3 that also depends on the destruction box (Burton and Solomon, 2007). Therefore, ZW10 may potentially alter BubR1 activity thus contributing to checkpoint signaling.

5.4 Kinetochore- MT attachment and checkpoint signaling at the kinetochore.

Checkpoints have been originally defined as transition points in the cell cycle where the progression to the next phase is inhibited until all upstream events are completed successfully (Weinert and Hartwell, 1988). It has been proposed that a checkpoint has to consist of at least three modules: a system for detecting the failure to complete a particular event (a sensor), a signal that is generated by the incomplete event (a transducer), and a mechanism by which this signal inhibits a biochemical reaction (an effector) (Li and Murray, 1991). In the case of spindle assembly checkpoint, a sensor that monitors completion of kinetochore-MT attachment is still unclear, the nature of the signal generated by unattached kinetochores to inhibit progression through anaphase is also poorly understood, an effector, APC/C, is well established and a mechanism by which APC/C inhibits metaphase to anaphase transition in case errors are present is also well defined (Cohen-Fix et al., 1996). Based on the original Rad9 study of the feedback control in S phase, it was postulated that checkpoints do not participate in particular cell cycle events but only monitor their completion (Weinert and Hartwell, 1988). Consecutive studies that contributed to the discovery of spindle checkpoint proteins and to analysis of their function, considered checkpoint proteins the part of sensoring/signaling module of the spindle checkpoint that do not participate in the actual

kinetochore-MT attachment, chromosome movements and physical separation of sister chromatids associated with cell division.

My data and multiple studies from other groups (Lampson and Kapoor, 2005; Lampson et al., 2004; Meraldi and Sorger, 2005; Pinsky et al., 2006) suggest that the sensoring module of the checkpoint and proteins that make structural links between kinetochores and MTs are intrinsically connected to each other. Proteins that have always been thought of as checkpoint regulators, like BubR1 and Bub1, are required for chromosome congression at the metaphase plate (Lampson and Kapoor, 2005; Meraldi and Sorger, 2005). Aurora B/ Ipl1 kinase, that was originally identified in budding yeast screen for increase-in-ploidy mutants (Chan and Botstein, 1993), destabilizes improper attachments and engages the checkpoint (Pinsky et al., 2006). My findings demonstrate that RZZ complex is required for initial kinetochore-MT binding and checkpoint activity. Altogether this data suggests that there is no clear separation of function at the kinetochore as has been previously thought (Weinert and Hartwell, 1988).

Recently a lot of structural data has been obtained regarding kinetochore assembly and architecture (Cheeseman et al., 2006; Liu et al., 2006), but no function has been assigned yet to many of kinetochore proteins. A limited number of biochemical methods make it difficult to dissect functions of kinetochore proteins with the kinetochore reconstitution *in vitro* being an ultimate goal of kinetochore biochemistry. Understanding of the checkpoint signaling currently includes only functions of classical checkpoint proteins (Yu, 2006), while the role of many additional proteins critical for kinetochore-MT binding and checkpoint integrity has not been addressed.

5.5 Aneuploidy and kinetochore dysfunction.

Observations of Theodor Boveri defined aneuploidy as a common characteristic of solid tumors (Boveri, 1914). Tumor aneuploidy may be caused by genomic instability resulted from chromosome missegregation or it may reflect deregulation of the sell cycle after cellular transformation. Spindle checkpoint dysfunction has been considered for a long time a cause for tumorigenesis but the link between aneuploidy and cancer is still unclear. After initial reports of checkpoint genes being mutated in human cancers (Cahill et al., 1998) further studies have found that only a few tumors harbor mutations in spindle checkpoint genes (Yamaguchi et al., 1999). Targeted deletion of checkpoint genes reported so far causes embryonic lethality, as the massive chromosome loss leads to mitotic catastrophe and a p53-dependent apoptosis (Burds et al., 2005; Dobles et al., 2000; Kalitsis et al., 2000). Heterozygous deletion of Mad2 in mice leads to lung pappilary adenocarcinoma late in life (Michel et al., 2001). Bub3 heterozygous mice exhibit chromosomal instability but do not develop tumors (Kalitsis et al., 2000). A hypomorphic allele of BubR1 in mice causes accelerated aging and infertility but does not lead to tumor formation (Baker et al., 2004). These studies suggest that a complete loss of checkpoint proteins is not permissive for survival. How then defects in spindle checkpoint signaling or kinetochore function may contribute to tumor development?

A sporadic chromosome loss occurring as a result of subtle defects in a kinetochore dysfunction or checkpoint slippage that do not trigger mitotic arrest may cause chromosomal instability (CIN) and promote tumorigenesis. Several studies provide experimental evidence for this hypothesis. Mutations in ROD, ZW10 and Zwilch have been found in a panel of aneuploid colorectal cancers (Wang et al., 2004). My

experimental results indicate that expression of point mutants of ZW10 found in colorectal tumors does not affect checkpoint signaling or fidelity of chromosome segregation despite their localization to kinetochores in HeLa cells. I suggest that these mutations do not grossly disrupt the function of ZW10 but slightly alter it, thus the effect of expression of these mutants can not be observed in aneuploid HeLa cell line with the robust checkpoint. These mutants should be tested in primary cells where subtle lesions in chromosome segregation or checkpoint slippage caused by altered function of ZW10 would be more obvious.

Another example of lesions that escape checkpoint control is caused by inactivation of APC and EB1 (Draviam et al., 2006). In cells depleted of EB1 or APC chromosomes align at metaphase plate but exhibit misorientation of centromeric axes that arises from insufficient pulling forces on sister kinetochores from the mitotic spindle. Occasionally misorientations of centromeric pairs lead to missegregation that escapes the checkpoint control (Draviam et al., 2006). In both cases, subtle lesions that do not cause a massive chromosome loss but just a few missegregated chromosomes do not interfere with cell viability but may cause CIN. Identification of additional proteins whose disruption causes lesions that are invisible to the spindle checkpoint would be useful for understanding how kinetochore dysfunction might contribute to tumorigenesis.

5.6 Conclusions

Overall, my work contributes to the understanding of the kinetochore function by analyzing kinetochore components involved in both kinetochore-MT attachment and checkpoint signaling. I discover a novel role for RZZ complex in initial attachment at

kinetochores and suggest a Mad2 independent checkpoint function for RZZ. My work suggests a close link between establishment of attachment and its monitoring at kinetochores. I also propose several avenues for future research, including investigation of the checkpoint role of RZZ complex that may advance our understanding of spindle checkpoint signaling mechanisms and a careful analysis of initial kinetochore-MT encounter that may define how defect monitoring and signaling are associated at kinetochores.

5.7 References

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

Baker, D.J., K.B. Jeganathan, J.D. Cameron, M. Thompson, S. Juneja, A. Kopecka, R. Kumar, R.B. Jenkins, P.C. de Groen, P. Roche, and J.M. van Deursen. 2004. BubR1 insufficiency causes early onset of aging-associated phenotypes and infertility in mice. *Nat Genet.* 36:744-9.

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

Burds, A.A., A.S. Lutum, and P.K. Sorger. 2005. Generating chromosome instability through the simultaneous deletion of Mad2 and p53. *Proc Natl Acad Sci U S A.* 102:11296-301.

Burton, J.L., and M.J. Solomon. 2007. Mad3p, a pseudosubstrate inhibitor of APCCdc20 in the spindle assembly checkpoint. *Genes Dev.* 21:655-67.

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

Chan, C.S., and D. Botstein. 1993. Isolation and characterization of chromosome-gain and increase-in-ploidy mutants in yeast. *Genetics.* 135:677-91.

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

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

Cohen-Fix, O., J.M. Peters, M.W. Kirschner, and D. Koshland. 1996. Anaphase initiation in Saccharomyces cerevisiae is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. *Genes Dev.* 10:3081-93.

Coquelle, F.M., M. Caspi, F.P. Cordelieres, J.P. Dompierre, D.L. Dujardin, C. Koifman, P. Martin, C.C. Hoogenraad, A. Akhmanova, N. Galjart, J.R. De Mey, and O. Reiner. 2002. LIS1, CLIP-170's key to the dynein/dynactin pathway. *Mol Cell Biol.* 22:3089-102.

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

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

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

Dong, Y., K.J. Vanden Beldt, X. Meng, A. Khodjakov, and B.F. McEwen. 2007. The outer plate in vertebrate kinetochores is a flexible network with multiple microtubule interactions. *Nat Cell Biol.* 9:516-22.

Draviam, V.M., I. Shapiro, B. Aldridge, and P.K. Sorger. 2006. Misorientation and reduced stretching of aligned sister kinetochores promote chromosome missegregation in EB1- or APC-depleted cells. *Embo J.* 25:2814-27.

Echeverri, C.J., B.M. Paschal, K.T. Vaughan, and R.B. Vallee. 1996. Molecular characterization of the 50-kD subunit of dynactin reveals function for the complex in chromosome alignment and spindle organization during mitosis. *J Cell Biol.* 132:617-33.

Fang, G., H. Yu, and M.W. Kirschner. 1998. The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. *Genes Dev.* 12:1871-83.

He, X., T.E. Patterson, and S. Sazer. 1997. The Schizosaccharomyces pombe spindle checkpoint protein mad2p blocks anaphase and genetically interacts with the anaphase-promoting complex. *Proc Natl Acad Sci U S A.* 94:7965-70.

Homer, H.A., A. McDougall, M. Levasseur, K. Yallop, A.P. Murdoch, and M. Herbert. 2005. Mad2 prevents aneuploidy and premature proteolysis of cyclin B and securin during meiosis I in mouse oocytes. *Genes Dev.* 19:202-7.

Howard, J., and A.A. Hyman. 2003. Dynamics and mechanics of the microtubule plus end. *Nature.* 422:753-8.

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

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

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

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

King, E.M., S.J. van der Sar, and K.G. Hardwick. 2007. Mad3 KEN boxes mediate both Cdc20 and Mad3 turnover, and are critical for the spindle checkpoint. *PLoS ONE.* 2:e342.

Kops, G.J., Y. Kim, B.A. Weaver, Y. Mao, I. McLeod, J.R. Yates, 3rd, M. Tagaya, and D.W. Cleveland. 2005. ZW10 links mitotic checkpoint signaling to the structural kinetochore. *J Cell Biol.* 169:49-60.

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

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

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

Lin, Y.T., Y. Chen, G. Wu, and W.H. Lee. 2006. Hec1 sequentially recruits Zwint-1 and ZW10 to kinetochores for faithful chromosome segregation and spindle checkpoint control. *Oncogene.* 25:6901-14.

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

Meraldi, P., V.M. Draviam, and P.K. Sorger. 2004. Timing and checkpoints in the regulation of mitotic progression. *Dev Cell.* 7:45-60.

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

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

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

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

Rieder, C.L., and S.P. Alexander. 1990. Kinetochores are transported poleward along a single astral microtubule during chromosome attachment to the spindle in newt lung cells. *J Cell Biol.* 110:81-95.

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

Shang, C., T.R. Hazbun, I.M. Cheeseman, J. Aranda, S. Fields, D.G. Drubin, and G. Barnes. 2003. Kinetochore protein interactions and their regulation by the Aurora kinase Ipl1p. *Mol Biol Cell.* 14:3342-55.

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

Tai, C.Y., D.L. Dujardin, N.E. Faulkner, and R.B. Vallee. 2002. Role of dynein, dynactin, and CLIP-170 interactions in LIS1 kinetochore function. *J Cell Biol.* 156:959-68.

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

Vallee, R.B., and J.W. Tsai. 2006. The cellular roles of the lissencephaly gene LIS1, and what they tell us about brain development. *Genes Dev.* 20:1384-93.

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

Wang, Z., J.M. Cummins, D. Shen, D.P. Cahill, P.V. Jallepalli, T.L. Wang, D.W. Parsons, G. Traverso, M. Awad, N. Silliman, J. Ptak, S. Szabo, J.K. Willson, S.D. Markowitz, M.L. Goldberg, R. Karess, K.W. Kinzler, B. Vogelstein, V.E.

Velculescu, and C. Lengauer. 2004. Three classes of genes mutated in colorectal cancers with chromosomal instability. *Cancer Res.* 64:2998-3001.

Weinert, T.A., and L.H. Hartwell. 1988. The RAD9 gene controls the cell cycle response to DNA damage in Saccharomyces cerevisiae. *Science.* 241:317-22.

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

Yamaguchi, K., K. Okami, K. Hibi, S.L. Wehage, J. Jen, and D. Sidransky. 1999. Mutation analysis of hBUB1 in aneuploid HNSCC and lung cancer cell lines. *Cancer Lett.* 139:183-7.

Yang, Z., U.S. Tulu, P. Wadsworth, and C.L. Rieder. 2007. Kinetochore dynein is required for chromosome motion and congression independent of the spindle checkpoint. *Curr Biol.* 17:973-80.

Yu, H. 2006. Structural activation of Mad2 in the mitotic spindle checkpoint: the two-state Mad2 model versus the Mad2 template model. *J Cell Biol.* 173:153-7.