Characterisation of the Spindle Assembly Checkpoint in Mammalian Oocytes

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1 Zusammenfassung / Summary

Die Fehlverteilung von Chromosomen, die auf vorzeitiger Trennung von Chromatiden und Non-Disjunction beruhen, erhöht sich dramatisch in Oozyten der Frau mit zunehmendem Alter und eine geringe, wenn auch nicht so auffällige Erhöhung, beobachtet man ebenfalls bei Eizellen einiger Mausstämme. Untersuchungen zur relativen Menge von Boten-RNA (messenger RNA, mRNA) bei diesen Eizellen führte zu der Hypothese, dass das Altern der Eizelle mit einer veränderten Genexpression, z.B. von Zellzyklus-regulierenden Kinasen, Motorproteinen und Kontrollpunkt-Komponenten, assoziiert ist. Desweiteren wurde vorgeschlagen, dass ein Verlust der Kohesinproteine, welche die Homologe zusammenhalten, als auch der Phosphoproteine von den Chromosomen zusätzlich dazu beiträgt, dass ältere Eizellen während der langen Verweildauer in der Meiose anfällig für Chromosomenfehlverteilung werden. Treten Störungen in der Mitochondrienfunktion und in dem Zusammenhalt von homologen Chromosomen auf, ist es für eine Zelle äußerst wichtig, sich mit Hilfe von Schützmechanismen gegen Chromosomenfehlverteilung zu schützen, weil besonders in gealterten Eizellen die Anhäufung von mehr als einer Störung letztendlich das Risiko für Chromosomenfehlverteilung in der Oogenese bestimmt. Das Erfassen und Korrigieren von Fehlern bei der Anheftung von Spindelfasern oder Mikrotubuli an die Chromosomen wird in der Mitose von Mitgliedern des Spindel-Kontrollpunktes (spindle assembly checkpoint, SAC) und Mitgliedern des „chromosomal passenger complex“ (CPC) ausgeführt, um die ordnungsgemäße Anordnung der Chromosomen in der Metaphaseplatte zu gewährleisten und damit den korrekten Chromosomensatz bei der Segregation zu erhalten (Vogt et al., 2008).

Die Aurora Kinase B (AURKB) ist Teil des CPC und es gibt nur wenig Information darüber, was die funktionelle Bedeutung von AURKB und dessen Substrate, wie z.B. das Motorprotein MCAK (Mitotic Centromere Associated Kinesin), welches in der Lage ist, Mikrotubuli zu depolymerisieren, in der Meiose betrifft. Mad2 (Mitotic arrest deficient 2) ist eine hoch konservierte Komponente des SAC, welche zuerst in der Hefe entdeckt wurde. Als Teil des sogenannten „mitotic checkpoint complex“ (MCC) inhibiert es den Anaphase-fördernden Komplex (anaphase promoting complex, APC), wodurch der Eintritt in die Anaphase gehemmt wird. Dies geschieht dann, wenn der Kinetochor-Proteinkomplex des Zentromers nicht mit Mikrotubuli gesättigt ist bzw. keine ausreichenden Zugkräfte am Kinetochor vorhanden sind, nachdem es zur Anbindung von Mikrotubuli und dem Einordnen der Chromosomen zur Metaphase gekommen ist. Die Transkriptmenge von mindestens drei wichtigen Komponenten des Zellzyklus und der Spindelregulation scheint bei gealterten

Eizellen im Vergleich zu jungen Eizellen verändert zu sein: die Boten-RNA von Mad2 und MCAK sind in geringerer Menge vorhanden, während AURKB in gealterten Eizellen der Maus überexprimiert ist (Steuerwald et al., 2001;Hamatani et al., 2004;Pan et al., 2008).

Um die Bedeutung der veränderten Genexpression und die Funktion dieser drei Genprodukte für die Oogenese zu beurteilen, habe ich in dieser Arbeit *in vitro* reifende Mausoozyten wie folgt analysiert: 1) Bestimmung der Reifungsrate und Verteilung von Chromosomen nach Behandlung mit Inhibitoren und Mikrotubuli-depolymerisierenden Chemikalien als auch nach Knockdown mittels der RNAi-Methode, 2) Lokalisierung von Proteinen und Darstellung der Spindel als auch Chromosomen mit spezifischen Antikörpern für die Immunfluoreszenz, 3) Ermitteln der Zellzykluskinetik mit nicht-invasiver Polarisationsmikroskopie und 4) Bestimmung der chromosomalen Konstitution nach Spreitung und C-Banding.

Der erste Teil meiner Arbeit zeigt, dass die Behandlung von Mausoozyten mit Mikrotubuli-depolymerisierenden Substanzen, wie z.B. Nocodazole, die Polymerisationsdynamik der Mikrotubuli und deren Anbindung an die Kinetochore verändert. Mad2 reichert sich an diesen Kinetochoren an, wodurch der SAC aktiviert wird und ein „Wartesignal“ an die Komponenten des Zellzyklus gesendet wird, was zu einem robusten Arrest in der 1. Meiose führt und den Eintritt in die Anaphase hemmt, wie es auch typischerweise in der Mitose auftritt. Die Mikroinjektion von siRNAs zeigt, dass der meiotische Arrest vom SAC vermittelt wird, weil nur das spezifische Ausschalten von Mad2 in Anwesenheit von Nocodazole zu einer Abschnürung des Polkörpers führt. Solche Eizellen sind nicht mehr vor Fehlverteilung geschützt und sehr anfällig für Aneuploidie, was an dem signifikanten Anstieg in der Anzahl von hypoploiden und hyperploiden gespreiteter Metaphase II Oozyten im Vergleich zur Kontrolle deutlich wird. Dagegen kommt es in Abwesenheit von Nocodazole bei Mad2-depletierten Eizellen zu keinem signifikanten Anstieg der Aneuploidie, auch wenn reduzierte Mengen von Mad2 eine Beschleuigung der ersten meiotischen Teilung verursachen und eine verfrühte Anaphase I einleiten. Gesunde, junge Eizellen der Maus sind daher in der Lage, homologe Chromosomen früh genug in eine bipolare Ausrichtung zu bringen, um eine normale Verteilung der Chromosomen stattfinden zu lassen.

Der zweite Teil meiner Arbeit konzentrierte sich auf die Rolle von AURKB in der Spindelbildung und Chromosomensegregation während der Mausoogenese (Vogt et al., 2009). Die Immunfluoreszenzanalyse dieser Untersuchung zeigt, dass AURKB mit den Chromosomen nach Auflösung der Kernmembrane (germinal vesicle breakdown, GVBD) assoziiert ist, sich ab der Prometaphase I an den Zentromeren anreichert und zur Anaphase I in den zentralen Bereich der Spindel (spindle midzone) gewandert ist. Die Nähe von AURKB zu MCAK am Zentromer, teilweise auch überschneidend, kann sowohl in der 1. Meiose als

auch in der 2. Meiose deutlich nachgewiesen werden. Merotelische Bindungen (ein Zentromer oder die Geschwisterzentromere eines Homologs sind mit beiden Spindelpolen anstatt mit einem alleine verbunden) werden normalerweise von Mitgliedern des CPC durch die Aktivität von Depolymerasen, wie z.B. MCAK, aufgelöst, wobei MCAK wiederum negativ durch die Phosphorylierung von AURKB reguliert ist. Die chemische Inhibition von AURKB mit niedriger Konzentration von ZM447439 bei Wiederaufnahme der Reifung führt zu einem Block in der Zytokinese und verhindert die ordnungsgemäße Verteilung der Chromosomen in der 1. Meiose. Vielmehr scheint es eine Aktivierung bzw. Verlängerung des SAC in Eizellen zu geben, die dem Aurora Kinase Inhibitor ausgesetzt sind, weil Kontrollpunktproteine des SAC, wie z.B. BubR1, an Zentromeren solch arretierter Eizellen angereichert werden. Der SAC scheint durchlässig in solchen Eizellen ohne Polkörper zu sein, wodurch Eizellen entweder zwei Sätze von Metaphase II Chromosomen, nur Bivalente oder Bivalente zusammen mit Metaphase II Chromsomen besitzen. Im Moment ist es nicht eindeutig geklärt, ob die Verzögerung bzw. der meiotische Arrest mit einer fehlenden Phosphorylierung des Kohesinproteins Rec8 an den Zentromeren als auch an den Chromosomenarmen zu tun hat, was notwendig ist, damit die Auflösung der Chromosomenkohäsion stattfinden kann, oder mit der fehlenden Inaktivierung von MCAK durch AURKB Phosphorylierung zusammenhängt. Allerdings scheint die Aktivität von AURKB bei der Modifikation von Histonproteinen an den Zentromeren und den Chromosomenarmen eine Rolle zu spielen, was bei der Kondensation der Chromosomen als auch bei der Organisation und Funktion des Zentromers von Bedeutung sein mag. Eine Reduzierung in der Trimethylierung des Histons H3 an Lysin9, wie es in ZM-behandelten Eizellen beobachtet werden konnte, könnte auf eine fehlende Phosphorylierung des Histons H3 an Serin10 hindeuten wie auch eine spezifische Störung in der reifungsabhängigen Konformationsänderung von H3 Histonen durch AURKB darstellen.

Der letzte Teil meiner Arbeit befasste sich mit der Analyse zur Verteilung und Aktivität von MCAK in der weiblichen Meiose. Vorherige Untersuchungen haben gezeigt, dass diesem Mitglied der Kinesin-13-Familie eine meiosis-spezifische Lokalisation in Spermatozyten zugewiesen werden kann (Parra et al., 2006). Daher konzentrierte sich die anfängliche Analyse mit der örtlichen Bestimmung von MCAK in der Oogenese. Es kann gezeigt werden, dass sich MCAK am Zentromer von der Prometaphase I bis zur Telophase I befindet. Der bipolare Spindelaufbau wird nicht durch den Knockdown von MCAK mittels RNAi verhindert und Chromosomen sind in der Lage, sich am Spindeläquator einzuordnen. Die Fokussierung der Spindelpole ist jedoch beeinträchtigt und der Eintritt in die Anaphase I ist ebenfalls blockiert, welcher jedoch durch einen gleichzeitigen Knockdown von MCAK und

Mad2 überwunden werden kann, was zu einer Auflösung der Chiasma, einer Trennung der Chromosomen und einer Abschnürung des ersten Polkörpers führt. Die Spindeln dieser Metaphase II Oozyten sehen sehr aberrant aus und die Chromosomen liegen verstreut in der Spindel, was diesen Phänotyp im Vergleich zum Knockdown von Mad2 alleine folgenschwerer macht. Während es keinen Anstieg bei der Hyperploidie nach dem Knockdown von MCAK und Mad2 gibt, steigt die Anzahl an hypoploiden Eizellen an, was darauf hindeutet, dass MCAK eine Rolle in der Chromosomentrennung der Oogenese spielt. Diese Annahme wird zusätzlich dadurch unterstützt, dass es bei einer verzögerten Anaphase I zu zurückhängenden Chromosomen (lagging chromosomes) kommt, wenn AURKB mit dem ZM-Inhibitor zur späten Prometaphase I gehemmt wird, was den Beobachtungen in der Mitose entspricht, wo eine Deregulation der MCAK und AURKB Aktivität ebenfalls zu zurückhängenden Chromosomen in der Anaphase führte. Daher scheint MCAK ein Bestandteil der Signalkaskade zu sein, um den Kontrollpunkt nach Einordnung aller Chromosomen aufzulösen und den Eintritt in die Anaphase I der Oogenese freizugeben.

Schlussfolgernd unterstützt meine Arbeit die Annahme, dass Veränderungen in der relativen Menge von Mad2, MCAK und/oder AURKB, wie es typisch für gealterte Eizellen ist, dazu beitragen, dass es zu einem Verlust der Zellzykluskontrolle (SAC) als auch zu Störungen bei der Chromosomentrennung kommt, wodurch gealterte Eizellen anfällig für Aneuploidie werden. Dies kann zu Trisomien, wie z.B. Down Syndrom, nach der Befruchtung führen und zu Störungen bei der Einpflanzung wie auch zu spontanen Aborten beitragen.

Errors in chromosome segregation involving precocious separation of sister chromatids and non-disjunction become dramatically increased with advanced maternal age in oocytes of humans and a small although by far not so prominent increase in aneuploidy is also observed in oocytes of some mouse strains. Several reports analysing relative mRNA abundance suggest that oocyte ageing is associated with altered gene expression, e.g. of cell cycle regulating kinases, motor proteins, and checkpoint components. Furthermore, it has been proposed that transient loss of cohesin proteins and phosphoproteins from chromosomes during a prolonged meiotic arrest contributes additionally to susceptibility to meiotic errors. In presence of disturbed function of mitochondria and feasible physical connections between the homologous chromosomes, it is of utmost importance for a cell to express safeguard and feedback mechanisms to protect from chromosomal errors, and the accumulation of more than one deficiency in aged oocytes (more than one "hit") may ultimately determine risks for chromosome non-disjunction at oogenesis. The detection and correction of microtubule attachment errors by members of the spindle assembly checkpoint (SAC) and chromosomal passenger complex (CPC) are essential safeguards to properly align chromosomes at the metaphase plate for maintenance of correct cell ploidy in mitosis (Vogt et al., 2008).

Aurora kinase B (AURKB) is one member of the CPC and there is only limited information available on the functional significance of AURKB and its substrates like the microtubule depolymerase MCAK (Mitotic Centromere Associated Kinesin), in meiosis. Mad2 (Mitotic arrest deficient 2) is a highly conserved key component of the SAC initially detected in yeast that is contained in the MCC (Mitotic checkpoint complex) and as such inhibits the anaphase promoting complex (APC/C) thus halting anaphase onset in response to unsaturated binding of microtubules to kinetochores and/or missing tension by pulling forces on kinetochores from microtubule attachment at metaphase. Concentration of mRNA of at least three important components of cell cycle and spindle regulation appear altered in aged compared to young oocytes: Mad2 and MCAK appear less abundant while AURKB mRNA was reported to be overexpressed in aged mouse oocytes (Steuerwald et al., 2001;Hamatani et al., 2004;Pan et al., 2008).

In order to assess the relevance of altered expression and functionality of these gene products in oogenesis, I analysed in this thesis *in vitro* maturing mouse oocytes for 1) maturation and chromosome segregation following treatment with inhibitors and microtubule depolymerising chemicals, as well as knockdown by RNAi methodology 2) protein localisation and staining of spindle as well as chromosomes with specific antibodies for immunofluorescence, 3) cell cycle kinetics with non-invasive polarisation microscopy and 4) chromosomal constitution after spreading and C-banding.

The first part of my study shows that alterations in microtubule polymerisation dynamics induced by exposing mouse oocytes to microtubule-depolymerising drugs such as nocodazole, which activate the SAC, bring about a robust meiosis I arrest. Mad2 accumulates at kinetochores that lack proper attachment suggesting that a "wait-anaphase" signal is induced, as is characteristically also found in mitosis. The arrest is SAC-mediated because oocytes depleted of Mad2 by microinjection of specific siRNAs but not unspecific control siRNAs undergo polar body extrusion in the presence of microtubule depolymerising chemicals, e.g. nocodazole. These oocytes are no longer protected from chromosome non-disjunction and are highly prone to aneuploidy, since hypo- and hyperploidy increase significantly in Mad2-depleted metaphase II oocytes exposed to nocodazole compared to controls. In contrast, there is no significant increase in aneuploidy in Mad2-depleted metaphase II oocytes in absence of nocodazole despite an acceleration of meiosis I and precocious anaphase I progression. Thus, early orientation and bipolar attachment of homologues chromosomes to both spindle poles may permit normal chromosome distribution at a precocious anaphase I in healthy young mouse oocytes.

The second part of my study focused on the role of AURKB in spindle formation and chromosome segregation during mouse oogenesis (Vogt et al., 2009). The immunofluorescent analysis performed in this study revealed that AURKB associates with chromosomes after germinal vesicle breakdown, is enriched at centromeres from prometaphase I, and transits to the spindle midzone at anaphase I. AURKB is found in overlapping parts or close proximity with MCAK at centromeres at meiosis I and meiosis II. Merotelic attachments (attachment of one centromere or the sister centromeres in one homologue to both instead of one spindle pole) are usually corrected by members of the CPC involving activity of depolymerases, like MCAK, in mitotic cells, which itself is negatively regulated by AURKB phosphorylation. Chemical inhibition of AURKB by low concentrations of ZM447439 with the resumption of maturation causes a block in cytokinesis and congression failure of chromosomes in meiosis I, but does not prevent recruitment of MCAK to centromere domains of chromosomes in arrested mouse oocytes. Rather, there appears to be an activation/prolongation of the SAC in Aurora kinase inhibitor-exposed oocytes because checkpoint proteins like BubR1 are present on kinetochores. The SAC appears leaky in those oocytes without a polar body, which contain either two sets of metaphase II chromosomes, exclusively bivalents or bivalents plus metaphase II chromosomes. Currently, it is unclear whether the delay or meiotic arrest relates to failure in Rec8 cohesin phosphorylation at centromeres and chromosome arms, required for loss of chromosome cohesion at anaphase I, or to failure in MCAK inactivation by AURKB phosphorylation. However, the activity of AURKB appears to be required for protein

modifications of histones at the arms and centromeres of meiotic chromosomes that may have critical effects on chromosome condensation and centromere organisation and function. The reduction in histone H3 lysine 9 trimethylation that is observed in ZM-exposed oocytes could reflect failed phosphorylation of H3 serine 10 phosphorylation and disturbances in maturation-dependent conformational alterations of H3 histones by AURKB at centromeric heterochromatin.

The last part of my study focused on the analysis of the distribution and activity of MCAK in female meiosis. Previous studies had revealed that this member of the kinesin-13 family attains a meiosis-specific localisation in spermatocytes (Parra et al., 2006). Therefore, the study was initially aimed on analysis of the distribution of MCAK in oogenesis. It could be shown that MCAK localises at centromere domains from prometaphase I until telophase I, placing it in the vicinity of AURKB. In addition, RNAi knockdown of MCAK does not prevent bipolar spindle assembly and eventual alignment of chromosomes at the spindle equator but affects focusing of polar spindle parts and blocks anaphase I progression. Consistent with a role of MCAK in satisfying the SAC at meiosis I, the block in meiosis I by MCAK specific RNAi is overcome by simultaneous knockdown of MCAK and Mad2 causing chiasma resolution, chromosome separation and first polar body formation. However, metaphase II spindles are highly aberrant and chromosomes scattered in MCAK/Mad2 depleted oocytes. The phenotype is more severe under these conditions compared to Mad2 depletion alone. While there is no increase in hyperploidy by the double knockdown, hypoploidy is increased, implying that MCAK has a role in faithful chromosome segregation at oogenesis. In support that deregulation of MCAK activity leads to disturbances in chromosome segregation, inhibition of AURKB by low concentrations of ZM447439 inhibitor at late prometaphase I delayed anaphase I progression in presence of lagging chromosomes, which is similar to studies in mitosis. Thus, MCAK is an integral part of the signaling cascade to release checkpoint arrest after chromosome congression and anaphase I progression in oogenesis.

In conclusion, my study supports the notion that alterations in relative abundance of Mad2, MCAK and/or AURKB, as appears characteristic for aged oocytes, may contribute to loss of cell cycle control (SAC) and disturbances in chromosome separation predisposing aged oocytes to aneuploidy that may lead to trisomies, like Down syndrome, after fertilization and contribute to implantation failure and spontaneous abortion.

Parts of this study are published in

Vogt, E., Kipp, A., and Eichenlaub-Ritter, U. (2009) Inactivation of Aurora kinase B affects epigenetic state of centromeric heterochromatin and chiasma resolution in mammalian oocytes. *Reprod Biomed Online* 19:352-368.

Vogt, E., Kirsch-Volders, M., Parry, C., and Eichenlaub-Ritter, U. (2008) Spindle formation, chromosome segregation and the spindle checkpoint in mammalian oocytes and susceptibility to meiotic errors. *Mutat Res* 651:14-29.

Eichenlaub-Ritter, U., Winterscheidt, U., Vogt, E., Shen, Y., Tinneberg, H.R., and Sorensen, R. (2007) 2-Methoxyestradiol induces spindle aberrations, chromosome congression failure, and nondisjunction in mouse oocytes. *Biol Reprod* 76(5):784-93.

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2 INTRODUCTION

2.1 Oogenesis

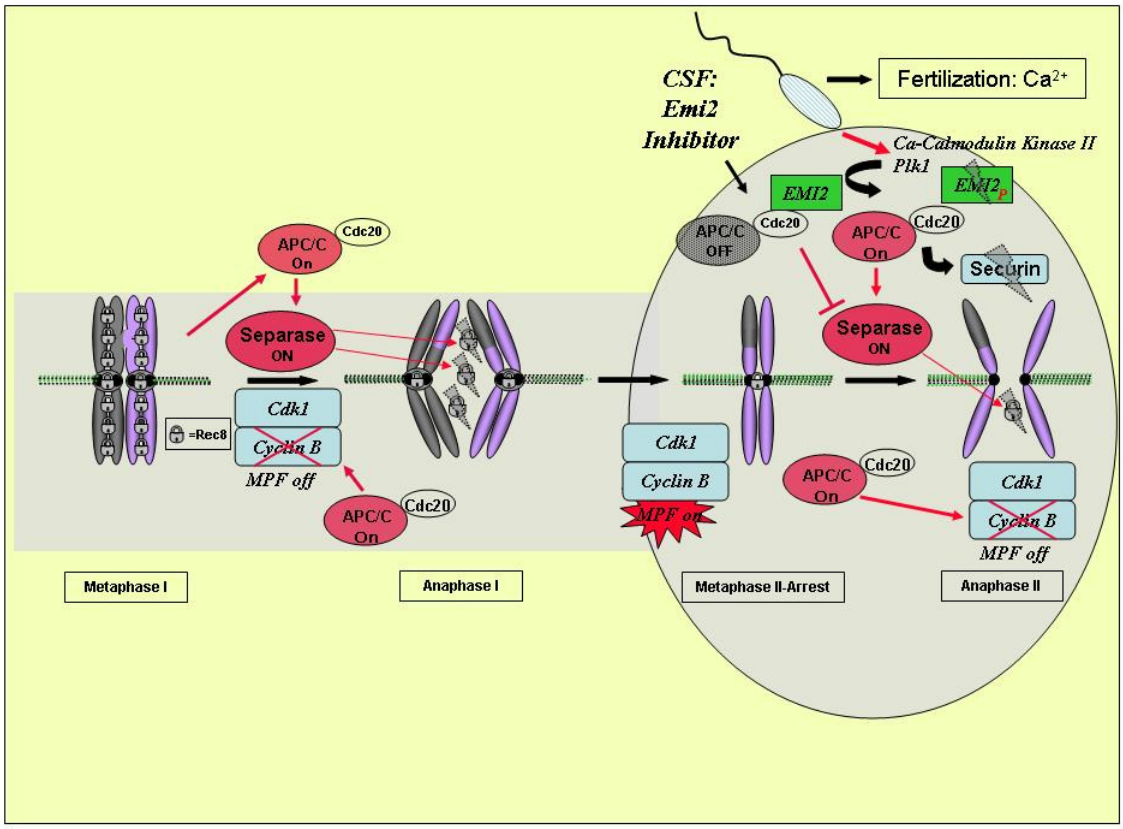
Every egg that a human female will ever produce is already present at birth. Although ovulated at the rate of one or perhaps a few per month for the 35-40 years between puberty and menopause, these eggs will not fully complete their meiotic divisions unless fertilized. Egg formation, also called oogenesis, comprises two cell divisions during meiosis. The reduction of chromosome number in meiosis is a central event in germ cell formation and evolution of most eukaryotes, including humans. Genomic stability and maintenance of a diploid set of chromosomes, one from the father and one from the mother in the zygote, relies on the formation of gametes, which are haploid, so that fertilization produces a diploid zygote. The meiotic cell cycle consists of S-phase and a long meiotic prophase during which pairing and recombination takes place, followed by two consecutive divisions (M-Phases) in the absence of DNA replication (S-Phase), termed meiosis I and meiosis II.

While meiotic progression from S-phase through prophase and both divisions is continuous in spermatogenesis, meiosis of mammalian oocytes is discontinuous with two constitutive phases of arrest. Meiosis begins in the embryonic ovary prior to birth when S-phase, leptotene, zygotene and pachytene commence. Oocytes become then meiotically arrested in diplotene/dictyate stage and remain meiotically-blocked until shortly before ovulation. Before they can resume meiotic maturation primordial follicles have to be recruited and oocytes have to increase significantly in size and volume during an extensive growth phase of folliculogenesis. Once a fully grown oocyte in a dominant follicle has attained meiotic competence it becomes transcriptionally inactive and is inhibited from spontaneous maturation by Maturation Promoting Factor (MPF; Cdk1/cyclin B). MPF activity drives eggs into and through meiosis (reviewed by Masui, 2001) and its oscillating activity with entry and exit from meiosis I and II was shown to be regulated, on the one hand, by cyclin B synthesis, the regulatory subunit of MPF, and, furthermore, by differential phosphorylation of the catalytic subunit of MPF, Cdk1 (Cdc2), as well as by cyclin B degradation in mammalian oocytes (Ledan et al., 2001; Herbert et al., 2003). The initial increase in MPF activity is sufficient to drive chromosome condensation, microtubule polymerisation and germinal vesicle envelope breakdown (GVBD) in prophase I oocytes of the mouse (Brunet and Maro, 2005). The relative level of MPF activity is transiently regulated by activity of APC/C^{Cdh1}-mediated partial degradation of cyclin B (Reis et al., 2007). Finally, at transition to metaphase

I and peak activity of MPF, the loss in MPF activity is associated with cyclin B degradation, which leads to meiosis I exit and cytokinesis with formation of the first polar body. Due to the activity of Emi1, cyclin B is newly synthesized during late meiosis I and meiosis II (Marangos et al., 2007) so that oocytes immediately progress to meiosis II, where they become arrested at metaphase II with aligned chromosomes due to the activity of the cytostatic factor (CSF). The latter comprises activities related to a c-mos kinase phosphorylation cascade including MAPKK, MAPK, p90rsk, and importantly activity of Emi2, an APC/C^{Cdc20} inhibitor (Verlhac et al., 1996; Kalab et al., 1996). Emi2 becomes itself target of APC/C after differential phosphorylation by Polo-like kinase 1 (Plk1) and calcium calmodulin 2 kinase, the latter being activated by a rise in calcium at fertilization (Madgwick et al., 2006; Tang et al., 2008). So, oocytes arrest at meiosis II until fertilization is inducing progression to anaphase II via this cytoplasmic Ca^{2+} signal (Runft et al., 2002) and completion of meiosis is initiated by separation of the sister chromatids, cytokinesis and formation of a second polar body.

As stated above, the metaphase II arrest characteristic for vertebrate oocytes is maintained by the activity of the cytostatic factor (CSF; Masui and Markert, 1971; reviewed by Madgwick and Jones, 2007; Perry and Verlhac, 2008). The identity of the CSF in mouse oocytes has never been fully resolved. An egg-specific protein Emi2 ("early mitotic inhibitor 2") has been identified as a likely candidate to mediate CSF arrest at metaphase II as well as the release from metaphase II upon fertilization separating sister chromatids (also called dyads) at anaphase II (Figure 2.1). Thus, Emi2 degradation is Ca^{2+}-dependent and Emi2 likely functions to both establish and maintain CSF arrest (Madgwick et al., 2006; Tang et al., 2008).

Figure 2.1 In presence of CSF, the mammalian oocyte arrests at metaphase II in spite of aligned chromosomes until fertilization triggers progression into anaphase II for completion of meiosis. CSF component Emi2 inhibitor renders APC/C inactive during metaphase II. The surge of Ca^{2+} induced by fertilization by sperm activates Ca^{2+}-dependent calmodulin kinase II for phosphorylation of Emi2 that is further phosphorylated by Plk1 for subsequent destruction by the proteasome. The degradation of Emi2 liberates and activates APC/C. Cyclin B and securin become degraded and separase liberated to cleave Rec8 at centromeres of sister chromatids for chromosome separation at anaphase II. Image modified from Vogt et al. (2008)



2.2 Aneuploidy in mammalian oocytes

A hallmark of mammalian development is the age-related decrease in fertility, which is largely attributed to depletion of the follicle pool with advancing maternal age in the human and some other mammals with females producing eggs of reduced developmental competence. An increase in aneuploidy is likely the major underlying factor responsible for subfertility with advancing age in human females (Eichenlaub-Ritter, 1998; Hassold and Hunt, 2001; Pellestor et al., 2005). Since aneuploid preimplantation embryos, particularly such carrying trisomies, are not much selected against during preimplantation development, chromosomal imbalance and gene dosage effects appear responsible for implantation failures, congenital abnormalities, abortions, still births and trisomic conceptions, most of which are associated with developmental disabilities, mental retardation, predisposition to diseases like cancer and reduced life expectancy (Patterson and Costa, 2005; Pont et al., 2006). Whereas the incidence of aneuploidy in eggs from women in their 20's is ~2%, it increases dramatically to 35% around 40 years of age and is estimated to be as high as 60% in oocytes of women approaching 50 years of age (Hassold and Jacobs, 1984; Hunt and Hassold, 2002). Studies in trisomic conceptions imply that most errors in chromosome segregation occur at

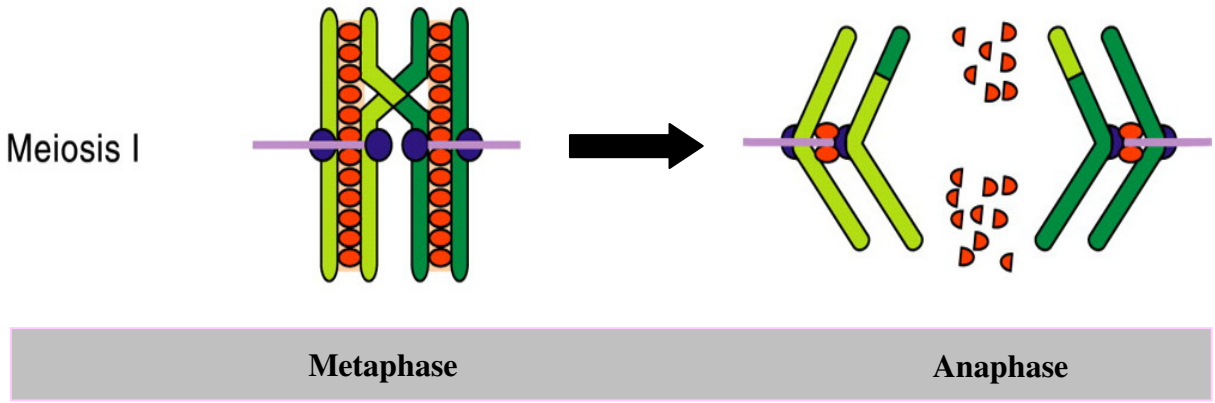
first meiosis of mammalian oogenesis and involve two mechanisms, non-disjunction and precocious chromatid segregation (predivision) prior to anaphase I that can cause first and second meiotic errors (Pellestor et al., 2002; Lamb et al., 2005). Non-disjunction results in the formation of daughter cells with either less or more than the normal haploid number of chromosomes after completion of second meiosis. Humans, particularly the oocytes of women, appear to have the highest frequency of meiotic non-disjunction among mammals. It is estimated that on average (not considering maternal age) aneuploidy may occur in 18-19% of human oocytes, yet only in 4% of human sperm (Martin et al., 1991). By contrast, male and female germ cells of the mouse may only have 1% or less aneuploidy, and there is a small increase in some strains of female mice approaching the end of their reproductive span (Eichenlaub-Ritter, 2005; Adler et al., 2007).

Besides causing developmental disturbances, non-disjunction may be causal to cancer, e.g. aneuploidies are characteristic of many cancer types (Rajagopalan and Lengauer, 2004; Weaver et al., 2007). Since parental chromosomes become physically attached to each other only when they have undergone recombination and possess one or several chiasmata at first meiosis, chromosomes which failed to recombine (univalents) have a high risk for random segregation and errors in chromosome segregation at meiosis I. In fact, molecular studies identified correlations of human aneuploidy with factors such as reduced or altered patterns of meiotic recombination (reviewed by Ferguson et al., 1996; Hassold et al., 2000). Thus, the events of pairing and recombination, which are initiated at early prophase I in the foetal ovary, bear on the risks to undergo non-disjunction at a much later time, in the adult or aged female. Certain chromosomal recombination patterns appear particularly susceptible to non-disjunction in an aged oocyte (first “hit”) (Lamb et al., 1996). Furthermore, proteins involved in the recombination process (Hodges et al., 2001) and chromosome cohesion (Angell, 1991; Hodges et al., 2005) may become lost from chromosomes during the long meiotic arrest or after meiotic resumption thus contributing to predisposition to meiotic error (second “hit”). Finally, spindle abnormalities and faulty chromosome congression on the metaphase plate appear associated with advanced maternal age (Battaglia et al., 1996; Volarcik et al., 1998). In mitotically dividing cells such congression failures and spindle aberrations are sensed by checkpoint mechanisms when they result in incomplete chromosome attachment or loss of tension by spindle fibres. Upon mitotic arrest, such cells are then protected from missegregation. However, there is tentative evidence that aged oocytes may possess only permissive checkpoints, which would pose them at a high risk for errors in chromosome segregation (possible third “hit”) that likely contribute to the observed increased incidence of human aneuploidy (Pacchierotti et al., 2007; Eichenlaub-Ritter et al., 2007a). Alterations or

disturbances in cell cycle control may thereby pose risks for segregation errors in aged oocytes (Eichenlaub-Ritter and Boll, 1989). It therefore appears that ageing causes intrinsic changes in cellular components of regulatory and structural elements expressed at maturation and chromosome segregation in oocytes, which adversely affect fidelity of chromosome separation.

2.3 Chromosome segregation in meiosis

Timing of chromosome attachment and loss of cohesion is essential to faithful chromosome segregation both at mitosis and meiosis (reviewed by Lee and Orr-Weaver, 2001). During meiosis I, the paired parental homologues in the bivalents are held together by chiasmata, which ensure their alignment on the meiosis I spindle (Figure 2.2). Chiasmata are maintained at the site of meiotic exchange by cohesion between sister chromatid arms (Petronczki et al., 2003). Next, sister kinetochores must attach to microtubules (MTs) from the same spindle pole (monopolar attachment, Figure 2.2). Therefore, the spindle creates tension only when homologues are pulled in opposite directions as regions distal to chiasmata physically link them. Chiasmata are then resolved at anaphase I by the loss of cohesion between the arms of sister chromatids in the homologous chromosomes (Figure 2.2). Only then each homologue can move to opposite poles at anaphase I. Cohesion, however, must be maintained at centromeres between sister chromatids beyond meiosis I to prevent premature chromatid separation (predivision) and ensure proper attachment of the sister chromatids to opposite spindle poles of the meiosis II spindle (Nasmyth, 2001; Ishiguro and Watanabe, 2007). Second meiosis therefore resembles mitosis with respect to behaviour of centromeres but not chromosome arms resulting in the separation of sister chromatids (Figure 2.2).



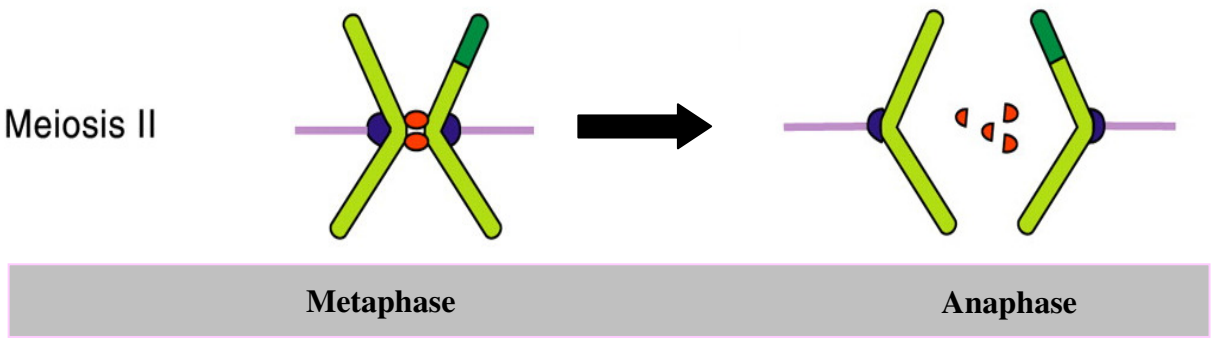


Figure 2.2 Chromosome segregation at meiosis I and II. Homologous chromosomes are held together by chiasmata to ensure alignment at metaphase I. Chiasmata are resolved by loss of cohesion between arms of sister chromatids at anaphase I to allow separation of homologous chromosomes to opposite spindle poles. Cohesion at centromeres is maintained until anaphase II, where sister chromatids segregate. Image modified from Lee and Orr-Weaver (2001).

2.3.1 Chromosome cohesion

The molecular glue between sister chromatids is mediated by the cohesin complex, a protein complex comprised of four core subunits: two subunits belonging to the family of structural maintenance of chromosomes (SMC), Smc1 and Smc3; the kleisin family protein Scc1/Rad21; and an accessory protein Scc3, which has two isoforms in vertebrates, SA1 and SA2 (Michaelis et al., 1997; Sumara et al., 2000). In meiosis, some of the mitotic cohesins are replaced by meiosis-specific cohesins, which appear essential for the sequential loss of cohesion between arms and centromeres of sister chromatids at meiosis I and II, respectively. The mammalian meiosis-specific cohesin subunits are Rec8, which replaces Scc1/Rad21 in some complexes, Stag3, Smc1ß replacing mitotic Smc1α, and SA3 replacing SA1/2 (Watanabe and Nurse, 1999; Prieto et al., 2001; Revenkova et al., 2001; Revenkova et al., 2004). It has been proposed in mitosis that the cohesin complex forms a ring structure around sister chromatids (Nasmyth and Haering, 2005) and becomes associated with chromosomes already prior to S-phase. In mitosis, most cohesin complexes are lost from chromatid arms until prometaphase via the "prophase pathway" (Gimenez-Abian et al., 2004) and relies on phosphorylation of mitotic cohesin proteins SA1 and SA2 by Plk1 and Aurora kinase B (Hauf et al., 2005). At the end of metaphase and at the onset of anaphase, an evolutionary caspase-related protease separase is activated to cleave the remaining Scc1/Rad21 at arms and centromeres in mitosis (Nakajima et al., 2007), which results in the opening of the cohesin

ring and separation of sister chromatids from each other. In contrast, the sister chromatids in meiotic chromosomes remain attached to each other throughout the chromosome length, including arms and centromeres, until anaphase I (Watanabe and Nurse, 1999). At the metaphase I-to-anaphase I transition, separase cleaves the meiotic Rec8 cohesin and releases cohesion between the arms of the sister chromatids and resolution of chiasmata (Figure 4.3; Kudo et al., 2006; Lee et al., 2006). From *C. elegans*, there is some evidence that Rec8 has to be phosphorylated (presumably by the Aurora kinase B ortholog) to be recognised for proteolysis by separase at anaphase transitions (Rogers et al., 2002). Thus, differential phosphorylation and activity of centromere-located phosphatases may regulate differential loss of cohesion at chromosome arms and centromeres in meiosis I.

2.3.2 Regulation of cohesin degradation

To control degradation of cohesin proteins, separase activity is inhibited by complexing with a chaperone securin until the metaphase-to-anaphase transition. Securin, on the other hand, is regulated by the anaphase-promoting complex/cyclosome (APC/C), a large multi-subunit E3 ubiquitin protein-ligase, which transfers ubiquitin to lysine in substrate proteins modifying them in such a way that they are recognised and degraded by the 26S proteasome (Figure 2.3; Peters, 2006). At anaphase onset, securin is marked by the APC/C for degradation releasing active separase and allowing separation of sister chromatids (mitosis; meiosis II) and homologues (meiosis I). Besides securin, the APC/C is also responsible for targeting cyclin B for degradation (Peters, 2006). Small residual levels of arm cohesins maintain arm cohesion up until anaphase onset in unperturbed mitotic cells, but can be induced to dissociate completely in cells which have been delayed sufficiently long by spindle poisons (Gimenez-Abian et al., 2004). The delay or arrest following spindle depolymerisation is characterised by stabilisation of securin and cyclin B due to inhibition of APC/C (Lefebvre et al., 2002; Homer et al., 2005a). The ubiquitin ligase activity of the APC/C is stimulated upon association with two accessory factors: Cdc20 and Cdh1 (Visintin et al., 1997; Peters, 2006). In mitosis, APC/C^{Cdc20} is responsible for securin and cyclin B degradation at the metaphase-anaphase transition (Peters, 2006). Binding of Cdh1 to the APC/C occurs at anaphase onset in mitotically dividing cells and remains bound until G1, where it is involved in the degradation of residual securin and cyclin B as well as Plk1 and Aurora kinase B. In meiosis, high APC/C^{Cdh1} activity is seen early in meiosis I, at prophase I, to maintain GV arrest (Reis et al., 2006), is retained for some time to regulate length of prometaphase I by restricting activity of

MPF through transient cyclin B degradation and finally declines by late prometaphase I (Reis et al., 2007). At that stage, APC/C^{Cdc20} activity begins to rise eventually inducing securin and cyclin B degradation at the metaphase I-anaphase I transition. APC/C^{Cdh1} activity during prometaphase I is important for proper congression of homologous chromosomes, otherwise leading to non-disjunction (Reis et al., 2007).

The protection of centromeric cohesion from the "prophase pathway" during mitotic prometaphase as well as from the separase pathway at the metaphase I-anaphase I transition is accomplished by the centromeric protein shugoshin (Sgo) (Lee et al., 2008). Two shugoshin paralogues have been identified in meiosis, shugoshin 1 and 2 (Sgo1 and 2) (Kitajima et al., 2004). Shugoshin appears necessary for recruiting a phosphatase (PP2A) to the centromeres of sister chromatids, thereby preventing phosphorylation of SA2 in vertebrate mitosis and Rec8 in yeast and vertebrate meiosis (Kitajima et al., 2006; Riedel et al., 2006; Tang et al., 2006b; Lee et al., 2008). Inactivation of PP2A causes premature loss of centromeric cohesion during meiosis I, possibly as a consequence of inappropriate phosphorylation of Rec8, and, in turn, induces missegregation of chromatids at meiosis II in yeast (Kitajima et al., 2006; Riedel et al., 2006), which is similar to the phenotype following depletion of Sgo2 in mouse oocytes (Lee et al., 2008). In accordance, inactivation of PP2A in mouse oocytes, e.g. by exposure to the phosphatase inhibitor ocadaic acid, not only induces precocious loss of contact between sister chromatids but also greatly increases aneuploidy (Mailhes et al., 2003). Bub1, a protein kinase with a conserved function in the spindle assembly checkpoint (see 2.7), is required for the localisation and retention of shugoshin to centromeres in human mitosis as well as in meiosis (Figure 2.3; Tang et al., 2004; Kitajima et al., 2004). Bub1 appears also essential to set up the meiotic pattern of chromosome segregation (Bernard et al., 2001). Therefore, centromeric cohesion in mitosis and meiosis is regulated by essentially the same molecules and mechanisms: the phosphorylation of cohesin and its counteraction by shugoshin-associated PP2A (Lee et al., 2008), while behaviour of sister centromeres to loss of cohesion appears related to there being tension (mitosis) or lack of tension (Meiosis I).

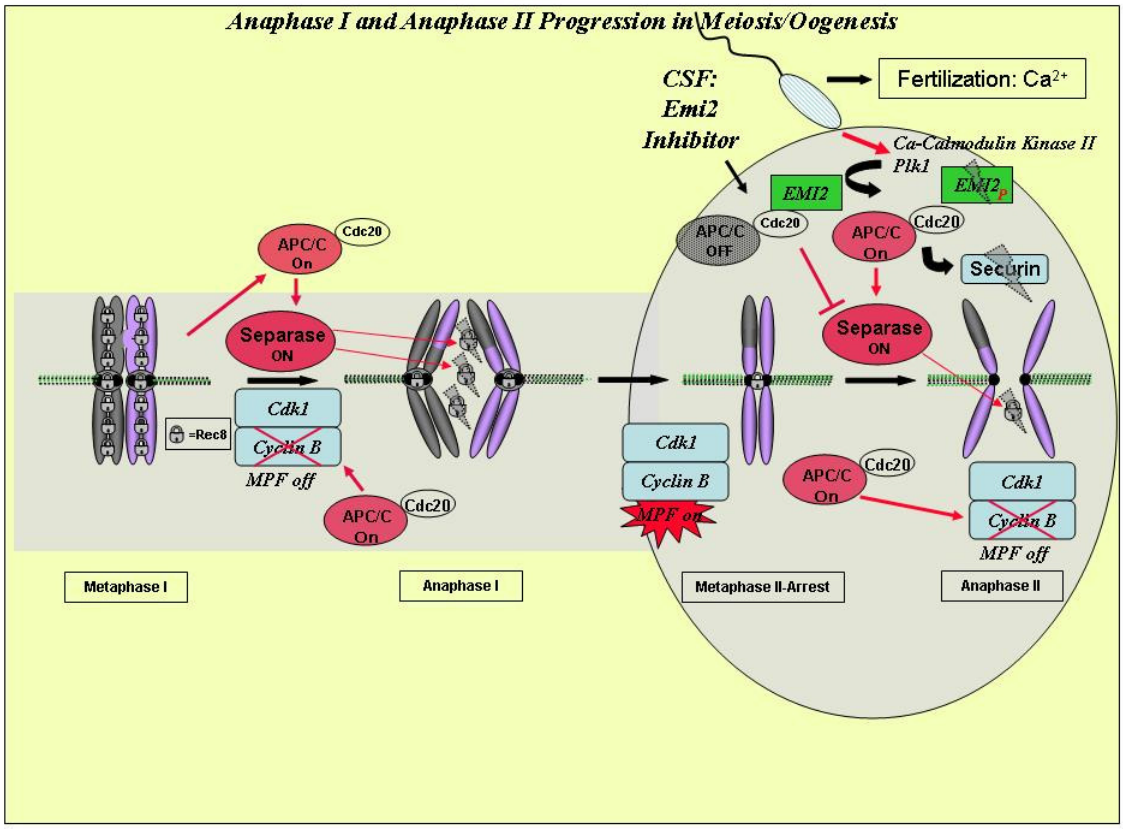


Figure 2.3 During meiosis two consecutive rounds of nuclear division are required to first segregate homologous chromosomes (at anaphase I) followed by the segregation of sister chromatids (at anaphase II) for the formation of a haploid gamete. To pull homologous chromosomes to opposite spindle poles during meiosis I, both sister kinetochores of a homologue must establish attachment to the same pole (monopolar attachment). Sister chromatids of meiotic chromosomes are held together all along the chromosome arms and centromeres by the meiosis-specific cohesin complexes containing Rec8. The kinase Plk1 marks Rec8 at chromatid arms by phosphorylation for degradation by separase. After silencing of the SAC at the metaphase I-to-anaphase I transition, separase cleaves Rec8 to release cohesion between sister chromatid arms while centromeric cohesins are kept unphosphorylated by activity of the phosphatase PP2A. A complex of Shugoshin (Sgo) and PP2A is recruited by Bub1 to centromeres at meiosis I to protect Rec8 from phosphorylation by Plk1. Upon chromosome congression, when chromosomes are under full tension from spindle fibres at metaphase I, cyclin B and securin become degraded after ubiquination by APC/C. Separase cleaves meiotic cohesin Rec8 marked by phosphorylation by Plk1 along chromosome arms. Image adopted from Vogt et al. (2008).

2.4 Spindle assembly in meiosis

During mitosis, centrosomes are the main sites of microtubule polymerisation and thus spindle assembly. They translocate to opposite sides of the nucleus, where they nucleate MTs, which are captured and stabilised by the kinetochore of the sister centromeres of chromosomes after nuclear envelope breakdown, in this way facilitating rapid organisation of

a bipolar spindle. While spindle formation in spermatogenesis involves the activity of centriole-containing microtubule organizing centres (MTOCs) that reduplicate during S-phase like in mitosis, and thus facilitate formation of a bipolar spindle with fusiform, astral poles, oocytes lack centrioles. Instead in the mammalian oocyte, multiple MTOCs with pericentriolar material are present and a bipolar spindle is formed in the ooplasm by the self-organizing capacity of MTs, motor proteins and microtubule-associated factors (Schuh and Ellenberg, 2007). MTOCs are recruited in the vicinity of chromosomes at the onset of meiosis I, just after germinal vesicle breakdown (GVBD), where randomly growing MTs are stabilised and progressively organised into a bipolar spindle (Albertini, 1992; Brunet et al., 1998). Unlike in frog oocytes, the first meiosis of mammalian oocytes does not require a Ran-GTP gradient from chromosomes for bipolar spindle formation (Dumont et al., 2007) although TPX-2 (Targeting Protein for the *Xenopus* kinesin xklp2; Wittmann et al., 1998), a Ran target, is necessary (Brunet et al., 2008). The molecular processes required for acentriolar spindle formation in female meiosis is driven by members of the chromosomal passenger complex (CPC, see 2.6.1) by stabilising the equatorial region of the metaphase I spindle (Colombie et al., 2008)

2.5 Kinetochore – the interface between spindle microtubules and chromosomes

A major site for chromosome motility is the kinetochore, a multi-protein complex at the centromeres of chromosomes that becomes occupied by the dynamic plus ends of emanating MTs and translates the interaction into force necessary to position chromosomes in mitosis and meiosis (for review see Rieder and Salmon, 1998; Maney et al., 2000; Maiato et al., 2004a). Under the electron microscope, the vertebrate kinetochore that forms at centromeres during prophase appears as a trilaminar structure (Figure 2.4). It is situated on opposite sides of the centromeric heterochromatin at the centromeres of each sister chromatid. The latter are glued to each other by inner centromere proteins (INCENPs) and cohesins. The typical trilaminar structure is marked by an electron-dense-layer-appearing inner plate, followed by a light intermediate and a dense outer layer with a fibrous-appearing corona (Brinkley and Stubblefield, 1966). The fibrous corona and the outer plate contain the majority of microtubule-interacting proteins, e.g. motor proteins and checkpoint proteins (Cooke et al., 1997; Jablonski et al., 1998), which monitor the status of microtubule attachment. Conserved

centromere proteins (CENPs) are located in the inner core chromatin, or the inner, middle or outer layer of the kinetochore at M-phase of mitosis as well as meiosis.

Once the chromosomes establish connections to MTs, they oscillate back and forth in mitosis, creating and breaking microtubule attachments until they are correctly bi-oriented (Skibbens et al., 1993; Rieder and Salmon, 1994). Paired sister chromatids must capture MTs emanating from their respective poles and maintain bi-orientation. This process is error prone and misconnections are made. Merotelic attachments arise when the kinetochore of one sister chromatid is attached to both poles, whereas syntelic attachments occur when the kinetochores of both sister chromatids are connected to one pole (Figure 2.4). In contrast to mitosis, sister kinetochores of one homologue need to be oriented to one pole during the first meiotic division (monopolar attachment) to ensure a reductional division. Whereas merotelic attachments have been well characterised in mitosis (Salmon et al., 2005; Cimini, 2008), there is no information and data available on the frequency of merotelic attachments in meiosis. Nevertheless, these erroneous attachments can be detrimental to the cell because they can cause aneuploidy (Cimini et al., 2001). To protect the cell from errors of chromosome segregation the kinetochore is the site of a surveillance system, the spindle assembly checkpoint (SAC), which monitors these erroneous attachments and protects the cell from aneuploidy (see 2.7).

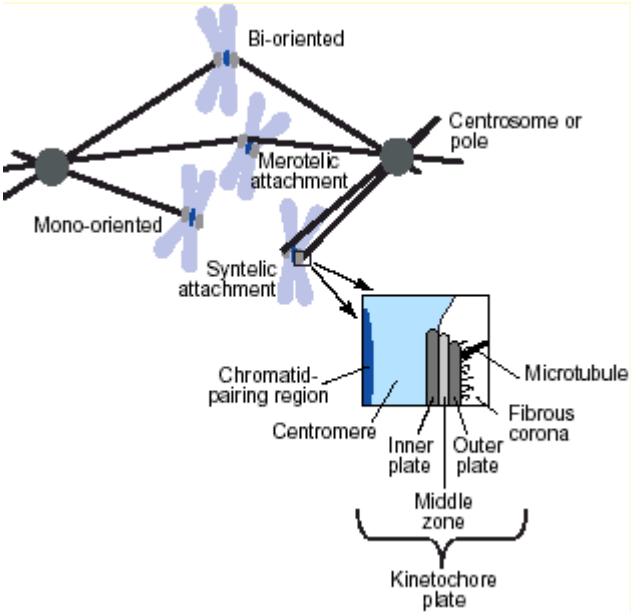


Figure 2.4 The kinetochore, the centromere and microtubule attachment. Erroneous attachments arise prior to the alignment at the metaphase plate. The attachments include merotelic (one sister chromatid attached to both poles) and syntelic (both sister chromatids attached to one pole) attachments. Image adopted from Moore and Wordeman (2004).

2.5.1 Functional role of kinetochore fibers is to power chromosome movement

Kinetochores both capture and stabilise MTs for the formation of kinetochore fibers (or K-fibers), which are typically comprised of 20-30 plus-end MTs (Maiato et al., 2004b). After kinetochores have become competent for anchoring K-fibers and have connected both kinetochores of one chromosome with their minus end to opposite spindle poles in mitosis, they are capable of chromosome bi-orientation during metaphase and chromosome segregation during anaphase of mitosis and meiosis (Maiato et al., 2004b). Despite their attachment to kinetochores, plus-end MTs remain dynamic, either continuously adding tubulin subunits to their plus ends or disassembling tubulin subunits during poleward chromosome movement (Gorbsky et al., 1987; Mitchison and Salmon, 1992). This lead to the "Pac-man"-model which posits that chromosomes move poleward along stationary MTs as kinetochores follow the depolymerising plus-ends of their attached MTs (Inoue and Salmon, 1995). These processes are mediated by motor proteins on the kinetochore and involve either polymerisation/depolymerisation prior to anaphase, or a continuous depolymerisation at both kinetochores for poleward chromosome movement during anaphase. In mouse oocytes, the kinetochores of bivalent chromosomes are not competent for making stable connections with MTs, and hence K-fiber formation, during most of the first meiotic M-phase (Brunet et al., 1999).

2.5.2 Functional roles of poleward microtubule flux

Poleward microtubule flux has been proposed to be a force-generating mechanism to power poleward chromosome movement, especially during anaphase (Mitchison, 1989). This has come from observations that spindle MTs continuously translocate poleward and disassemble their minus ends at spindle poles as well. In turn, inhibition of microtubule flux decreased chromosome velocity (Ganem et al., 2005).

Even though poleward microtubule flux and the role of K-fibers may be viewed as redundant mechanism during the process of chromosome segregation, the inhibition of poleward microtubule flux has revealed additional functional roles next to chromosome movement. For example, microtubule flux has been proposed to direct chromosome alignment to the equator of the spindle (Kapoor and Compton, 2002). Microtubule flux has also been implicated in regulating spindle length by continuously removing tubulin subunits from MT minus ends to counter tubulin subunit addition at plus end kinetochores (Rogers et al., 2005). A more recent

proposal is that flux plays a role in correcting errors in chromosome attachment to spindle MTs, since somatic cells exhibit a significant increase in both the frequency and the number of lagging chromatids during anaphase following the progression through mitosis in the absence of flux (Ganem et al., 2005). These lagging chromatids are most likely caused by failure to correct merotelic chromosome attachment prior to anaphase onset (Cimini et al., 2001). Finally, poleward microtubule flux appears to regulate kinetochore activity during mitosis (Maddox et al., 2003). Generation of tension at centromeres requires that kinetochore-bound MT plus-ends are maintained in a polymerisation state to prevent their detachment.

2.6 Motor proteins involved in chromosome segregation

The accuracy of chromosome segregation relies on the function of motor proteins, which are present on chromosome arms, the kinetochore, and the spindle poles (Table 2.1). The presence of motors at these different subcellular regions indicates that they not only function in a conventional way, i.e. generating force involved in chromosome movement during mitosis and meiosis, but are also involved in chromosome alignment, spindle assembly and checkpoint activity (Brunet and Vernos, 2001). Microtubule-based motors form two families of ATP-dependent force-generating enzymes, the kinesins and dyneins. The common feature of kinesins is a conserved catalytic core, the motor domain, which contains both the microtubule- and ATP binding sites (for review see Vale and Fletterick, 1997). Kinesin-related motors are categorised by the location of the motor domain within their amino acid sequence, which is predictive of the motor's directionality. Motors that have an N-terminal domain (Kin-N) move predominately towards the MT plus end, whereas those with a C-terminal motor domain (Kin-C) generally move towards the minus end. Motors belonging to the Kin-I subfamily, which contain internally located motor domains, do not exhibit gliding motility; instead, they appear to be specialised in destabilising MT ends (Desai et al., 1999). Although dyneins are structurally unrelated to kinesins (Samso et al., 1998), they use ATP hydrolysis to power gliding motility along MTs. However, dyneins move only in a minus-end-directed manner and differ from kinesins in their mode of microtubule translocation (Wang et al., 1995). The organisation of spindle MTs has implications for how motors position chromosomes: plus-end-directed motors could drive chromosomes towards the metaphase plate while minus-end-directed motors could drive them toward the spindle poles.

At least three conserved motors are known to localise to kinetochores: dynein and two kinesin-like proteins, CENP-E and MCAK (Maney et al., 2000). Whereas dynein, a minus-

end-directed gliding motor, powers chromosome movement to spindle poles directly (Sharp et al., 2000; Savoian et al., 2000), CENP-E, a plus-end-directed gliding motor, is essential for chromosome alignment (Wood et al., 1997; Schaar et al., 1997). MCAK (Mitotic Centromere Associated Kinesin), on the other hand, is an ATP-dependent microtubule-depolymerising motor which promotes disassembly of K-fibers for chromosome segregation at anaphase (Maney et al., 1998; Hunter et al., 2003). MCAK targets MT ends by a "diffusion and capture" mechanism, whereby it associates weakly with the MT, diffuses and then binds to its end at the depolymerisation process (Helenius et al., 2006). The advantage of this strategy as opposed to a directed motility mechanism is that it allows very rapid binding to the MT end for inducing microtubule depolymerisation at prometaphase of mitosis and meiosis as well as during anaphase. Rapid binding to the ends may also be important for destabilising improper kinetochore-microtubule attachments to prevent segregation defects at prometaphase and anaphase, thus avoiding aneuploidy (Kline-Smith et al., 2004).

The depolymerisation of MT minus ends at spindle poles is performed by the kinesin Kif2a, a member of the kinesin-13 family of microtubule-depolymerising proteins (Rogers et al., 2004). Knockdown of Kif2a in human somatic cells results in monopolar spindles suggesting that poleward MT flux is due to Kif2a at spindle poles (Ganem and Compton, 2004). There also appears to be functional relationship between Kif2a and the earlier mentioned kinesin-related protein, MCAK, in human somatic cells to assure proper bipolar spindle assembly. Their activities are spatially restricted and appropriately balanced in spindles with MCAK regulating MT plus ends at kinetochores and Kif2a regulating MT minus ends at spindle poles.

It is also clear that chromosome positioning is mediated by polar ejection forces generated directly on chromosome arms (Rieder and Salmon, 1994). The presence of polar ejection forces lead to the identification of several kinesin-like proteins (KLPs) that are associated with chromosome arms and considered to play an important role in chromosome alignment on the metaphase plate (Vernos et al., 1995; Antonio et al., 2000; Funabiki and Murray, 2000; Bringmann et al., 2004). These include Kif4 and Kif22, the respective homologues of the *Xenopus* kinesin-like proteins Xklp1 and Xkid.

Table 2.1 Sites and mechanism of action of motor proteins

Position	Motor family	Mechanism of action
Kinetochore	Dynein	Kinetochore transport
Kinetochore	CENP-E	Kinetochore transport/MT plus-end anchor
Kinetochore	MCAK/XKCM1	Microtubule disassembly
Spindle pole	Kif2a	Microtubule disassembly
Chromosome	Kif4/Xklp1	Chromosome positioning
Chromosome	Kif22/Xkid	Chromosome positioning

2.6.1 The regulation of MCAK by Aurora kinase B

MCAK belongs to the subfamily of depolymerising Kin-I kinesins where the motor domain is located in the middle of the peptide sequence rather than at one end, as it is in other kinesins. The motor domain is flanked by the neck at the N-terminal end and the tail at the C-terminus (Figure 2.5). The centromere-binding domain is located in the N-terminal neck region and centromere-targeting does not depend on the motor domain (Maney et al., 1998). It is a two-headed molecule that does not associate with any extrinsic factors.

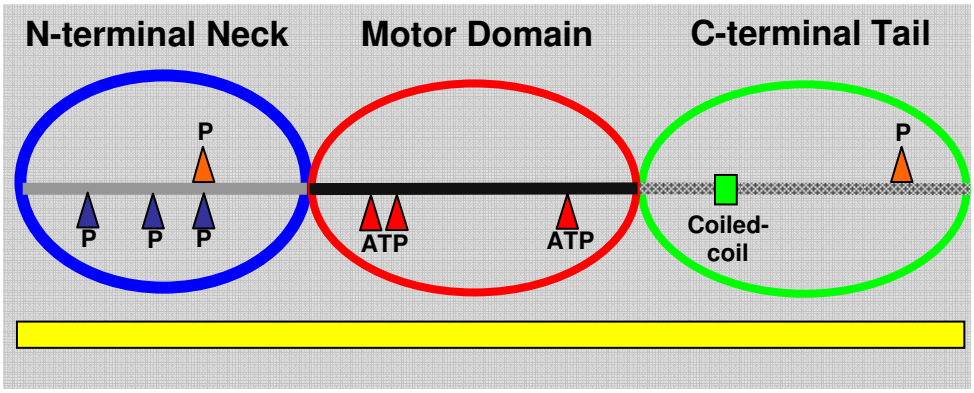


Figure 2.5 Schematic drawing of a MCAK molecule (yellow) defining the N-terminal neck domain containing one Aurora kinase A (orange) and several Aurora kinase B

phosphorylation sites (blue; also see Figure 2.6), the central ATP-hydrolysing motor domain (red) and the C-terminal tail inclusive of the predicted coiled-coil (green) and a second Aurora kinase A phosphorylation site (orange).

The activity of MCAK is regulated by several factors, for instance, by ICIS (an inner centromere protein) that stimulates MCAK activity (Ohi et al., 2003). Furthermore, centromeric MCAK activity is influenced by Aurora kinase B-directed phosphorylation that appears involved in sequential phosphorylation of MCAK causing changes in localisation, conformation and activity of MCAK in cultured cells and *Xenopus* egg extract (Ohi et al., 2004; Lan et al., 2004; Andrews et al., 2004; Zhang et al., 2007b). The phosphorylation sites that have been identified are highly conserved between human, mouse and *Xenopus* MCAK (Figure 2.6) and some of these sites are on the conserved neck domain. In addition, another phosphoregulatory networks exits on MCAK (Figure 2.5), in which Aurora kinase A controls MCAK targeting to spindle poles and proper spindle pole focusing, as shown in centrosome-depleted and chromatin-free cytoplasmic extracts of *Xenopus* eggs (Zhang et al., 2008). Aurora kinase B (AURKB) inhibits MCAK's depolymerising activity at appropriate stages of mitosis e.g. when bi-orientation of chromosomes is to be completed (Ohi et al., 2004; Lan et al., 2004; Andrews et al., 2004; Zhang et al., 2007b). AURKB is a component of the chromosomal passenger complex (CPC) consisting of a number of proteins like INCENP, survivin, and Dasra/borealin (reviewed by Ruchaud et al., 2007). The CPC exhibits a characteristic cell cycle-dependent redistribution from chromosomes to the interpolar spindle. The CPC thus associates with the centromeres of chromosomes at prometaphase to metaphase of mitosis and translocates to the spindle midzone at anaphase, telophase and cytokinesis (Ruchaud et al., 2007). AURKB is a critical component of the error correction machinery at kinetochores that monitors defective attachments (Tanaka et al., 2002; Pinsky et al., 2006; Cimini et al., 2006). AURKB colocalises with MCAK in chromosomes that are unattached or mono-oriented during prometaphase in mammalian cells (Andrews et al., 2004). In *Xenopus* egg extracts, colocalisation is seen at mitotic centromeres and spindle midzones (Lan et al., 2004). Throughout mitosis, the extent of colocalisation seems to decrease as result of microtubule attachment and tension on the chromosomes. This suggests that phosphorylation has a role in regulating MCAK activity during the capture and bi-orientation of kinetochores in mitosis. AURKB appears to become spatially separated from MCAK when chromosomes are fully saturated with MTs and tension is generated on centromeres in mitosis (Andrews et al., 2004). AURKB occupies a more central, inner centromere domain in prometaphase of mitosis while MCAK attached to the centromere is pulled away towards the polar region.

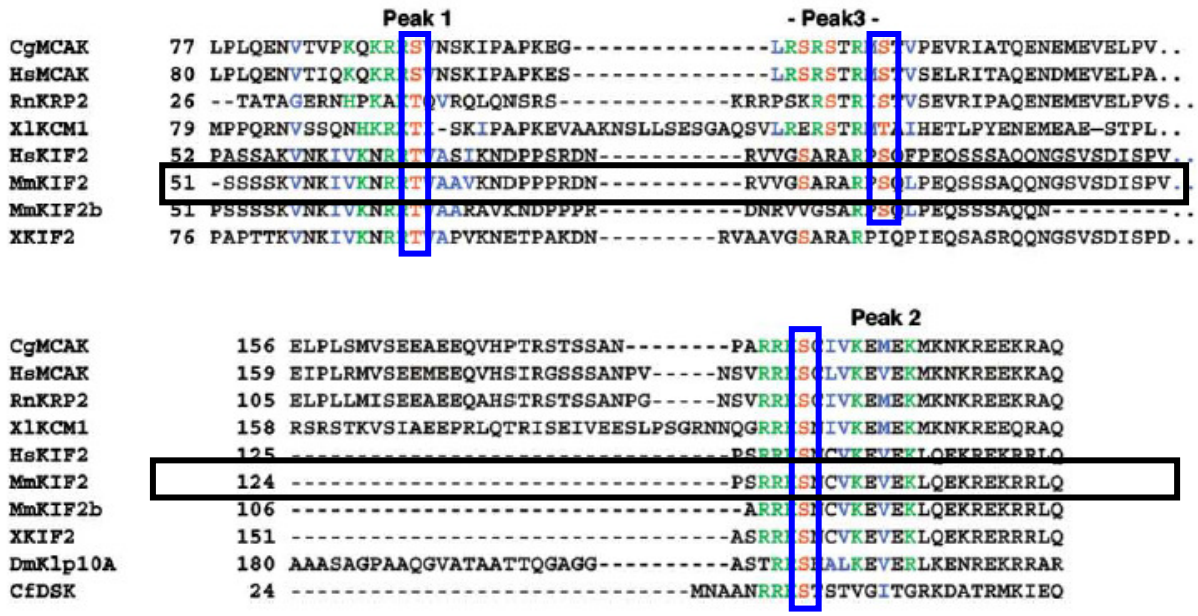


Figure 2.6 Alignment of conserved Aurora kinase B phosphorylation sites (blue) in Kin-I kinesins of different species. Mouse MCAK is shown in black box. Image modified from Andrews et al. (2004).

Faulty microtubule-kinetochore attachments would thus localise MCAK away from AURKB at inner centromeres, and MCAK's depolymerising activity is possibly activated to destabilise these faulty attachments (Knowlton et al., 2006), presumably after dephosphorylation of inhibitory sites by protein phosphatase 1 (PP1) (Murnion et al., 2001; Trinkle-Mulcahy et al., 2003). Once bipolarity is achieved MCAK's affinity for kinetochores is probably important for generating tension across centromeres (Kline-Smith et al., 2004; Andrews et al., 2004). Disruption of AURKB function decreases MCAK phosphorylation and also leads to a loss of centromeric MCAK in mitotic divisions (Andrews et al., 2004) suggesting complex mechanisms regulating acquisition, retention and activity of MCAK.

2.7 The spindle assembly checkpoint (SAC) protects from aneuploidy

In eukaryotes, the spindle assembly checkpoint (SAC) is a surveillance mechanism that ensures the fidelity of chromosome segregation in M-phase (reviewed by Musacchio and Salmon, 2007; Vogt et al., 2008). The SAC prevents chromosome missegregation and aneuploidy by halting cells at the transition from metaphase to anaphase and prolonging prometaphase until all chromosomes have become bi-oriented between separated spindle

poles at the metaphase plate. The molecular identity of key checkpoint components was already determined over a decade ago by screening mutations in budding yeast, which affect checkpoint signaling. Such screens identified *MAD* ("mitotic arrest deficient") (Hoyt et al., 1991) and *BUB* ("budding uninhibited by benzimidazole") (Li and Murray, 1991) mutations in mitotic checkpoint genes. The ubiquitous core checkpoint proteins of the SAC in mitosis and meiosis are Mad1, Mad2, BubR1 (Mad3 in yeast), Bub1, Bub3, and Mps1. Several additional checkpoint components were found to be well conserved from yeast to humans to plants, including other newly identified checkpoint components (for a detailed listing see Table 2.2), which appear essential in establishing tension, sensing disturbances or passing signals to halt the cell cycle such as kinases, motor proteins (Yao et al., 2000; Abrieu et al., 2001; Mao et al., 2003; Karess, 2005), and components of the chromosomal passenger complex (Vagnarelli and Earnshaw, 2004; Pinsky et al., 2006). Complete loss of checkpoint through inactivation in *D. melanogaster*, Mad2 in *C. elegans* and Mad2 or Bub3 in mice leads to early embryonic lethality due to chromosome missegregation events and associated apoptosis (Kitagawa and Rose, 1999; Basu et al., 1999; Dobles et al., 2000; Kalitsis et al., 2000). Furthermore, depletion of Mad2 increased the incidence of premature centromere separation in human somatic cells (Michel et al., 2004). Thus, it is believed that the checkpoint proteins in higher eukaryotes are required in every cell cycle to prevent missegregation of chromosomes.

2.7.1 The SAC signaling pathway

Converging genetic, cell biological and biochemical studies have begun to shed light on how the SAC components work on the molecular level. When the components of the checkpoint are all present on the kinetochore, unattached chromosomes or such not properly saturated by microtubule attachment for bipolarity and thus lacking tension create a "wait-anaphase" signal in mitosis as well as in meiosis (Figure 2.7; reviewed by Musacchio and Salmon, 2007; Vogt et al., 2008). SAC proteins are thus targeted to unattached or improperly attached kinetochores, where they remain, interact and are modified to create an inhibitory signal that is diffusible into the entire cell (for a detailed listing of SAC components as well interactions and functions see Table 2.2). Interference with microtubule dynamics, e.g. by addition of spindle poisons, activate the SAC (Rieder and Maiato, 2004). It is now clear that one consequence of SAC activation is the inhibition of the APC/C by complexing with its activator Cdc20 to prevent securin and cyclin B degradation (see 2.3.1-2). The nature of the diffusible inhibitory "wait-anaphase" signal has not been clearly established. However, the

ultimate mediators of the SAC are the checkpoint proteins Mad2 (Fang et al., 1998; Wassmann and Benezra, 1998) and BubR1 (Chan et al., 1999; Li et al., 1999), most potently as part of a direct inhibitory complex, the Mitotic Checkpoint Complex (MCC). The MCC consists of the checkpoint proteins BubR1, Bub3, Mad2 and Cdc20 (Sudakin et al., 2001). Unattached kinetochores recruit checkpoint kinases, such as Bub1, BubR1, and Mps1 that phosphorylate the APC/C for stable interaction with the MCC (Sudakin et al., 2001; Herzog et al., 2009). The turnover of MCC components is rapid (Howell et al., 2000; Howell et al., 2004; Shah et al., 2004) supporting the model whereby unattached kinetochores facilitate the formation and release of the APC/C inhibitory complex into the cytosol. On the other hand, there are also kinetochore-independent mechanisms for MCC formation (Fraschini et al., 2001; Poddar et al., 2005). *In vitro* and *in vivo* studies suggest that kinetochore-bound Mad2 undergoes a conformational change when binding to Cdc20 (reviewed by Yu, 2006). Structural studies have shown that recombinant Mad2 can adopt an open form, O-Mad2 (also known as N1), and a closed form, C-Mad2 (also known as N2) (Luo et al., 2004; DeAntoni et al., 2005). In solution, free Mad2 adopts an open conformation (O-Mad2), but on binding to Cdc20 Mad2 is modified into a stable, more potent closed conformation (C-Mad2) (DeAntoni et al., 2005). The release of C-Mad2 and its blocking of APC/C activity constitute a possibly important part of the SAC prior to metaphase to regulate cell cycle progression. At late prometaphase and metaphase of mitosis and meiosis the SAC can become prolonged or reactivated in response to aneugenic exposures and disturbances in spindle formation and chromosome attachment. The kinetochore is believed to act as a catalytic site for the production of the “wait-anaphase” signal. In fact, it was shown that all of the vertebrate Mad and Bub checkpoint proteins localise to unattached kinetochores (Chen et al., 1996; Chan et al., 1999; Nicklas et al., 2001), consistent with the proposed role of kinetochores in generating the inhibitory checkpoint signal.

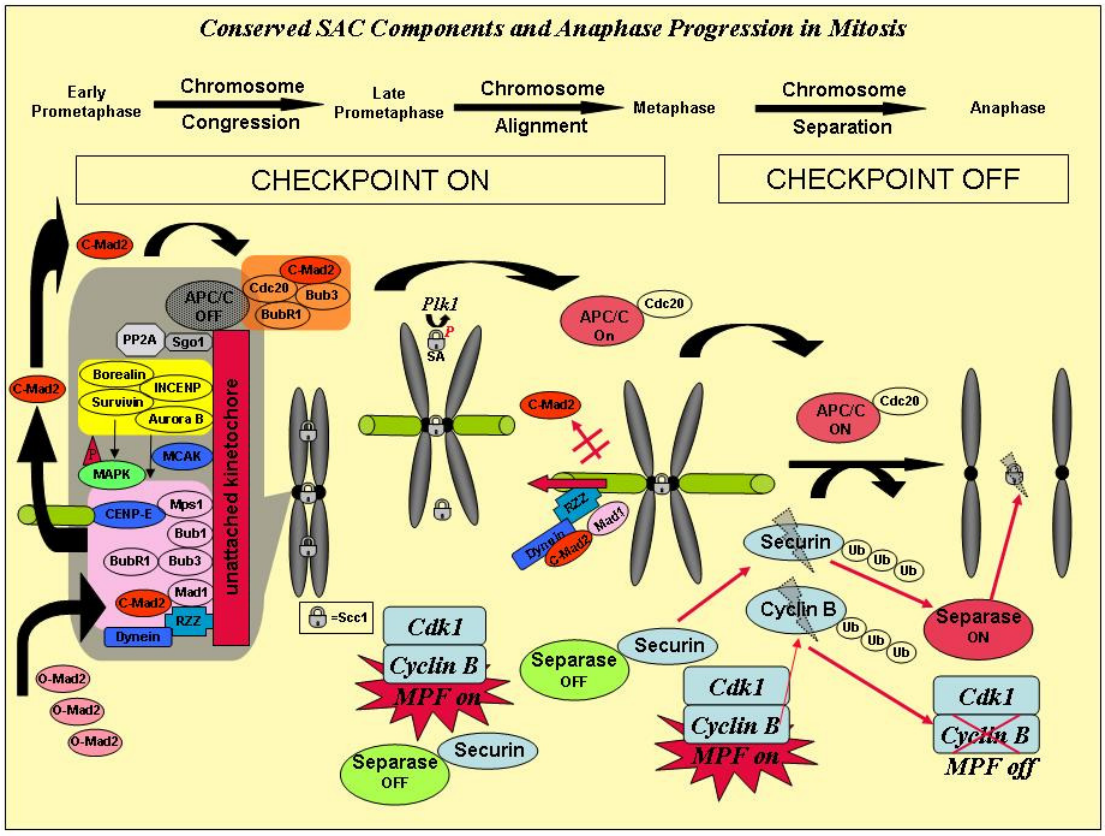


Figure 2.7 Conserved signaling cascade of the spindle assembly checkpoint (SAC) for normal chromosome segregation during mitosis and meiosis. In mitosis, the checkpoint is turned on at early prometaphase when chromosomes have not made bipolar attachment with spindle microtubules for alignment at the spindle equator. Core checkpoint proteins (Mad1, Mad2, BubR1, Bub1, Bub3 and Mps1; in light purple box), motor proteins (CENP-E, MCAK, Dynein, blue), kinases (MAPK, green; Aurora B, yellow) and components of the chromosomal passenger complex (INCENP, Borealin and Survivin, in yellow box) are recruited to unattached kinetochores. Open-Mad2 (O-Mad2, light purple) binds to the kinetochore, where it changes its conformation to Closed-Mad2 (C-Mad2, red) to be released into the cytosol for the formation of active MCC (C-Mad2, BubR1, Bub3 and Cdc20, in orange box). MCC interacts with Cdc20 and the APC to render it inactive. In mitosis cohesion between centromeres of sister chromatids (padlock) containing mitotic cohesin proteins like Scc1 is maintained since separase is inactive when associated with its inhibitory factor securin. Only at centromere arms cohesion may be lost independent of activity of APC/C and separase by phosphorylation of mitotic cohesin proteins like SA1/2 by Plk1 kinase. Securin is under the control of APC/C-Cdc20 and will not be degraded when APC/C is inactive by binding to the MCC. When all chromosomes have made proper microtubule attachment and have aligned at the spindle equator at metaphase APC/C-Cdc20 inhibition is released by silencing of the SAC. Under these conditions, the checkpoint proteins are transported away from the kinetochore towards the spindle pole by the RZZ/dynein complex. When the checkpoint is silenced securin can be ubiquitinated by APC/C and degraded, and separase is released and activated. Active separase can now cleave mitotic cohesins at centromeres and those remaining at chromosome arms to cause chromosome separation while APC/C induced degradation of cyclin B causes inactivation of MPF. This results in chromosome separation at anaphase and progression from M-phase to interphase. Image adopted from Vogt et al. (2008).

In addition, Cdc20 is also enriched at kinetochores, turning over rapidly along with other checkpoint proteins (Fang et al., 1998). The exact role of the kinetochore in contributing to checkpoint signaling and in the formation of distinct checkpoint protein complexes is still not fully resolved. However, the formation a functional centromere requires the participation of AURKB-mediated phosphorylation of centromere proteins and histone H3 (Wang et al., 2006a; Slattery et al., 2008; Swain et al., 2008). This suggests that multiple epigenetic modifications and changes in the constitution of pericentromeric heterochromatin could possibly affect centromere activity, recruitment of checkpoint proteins to kinetochores and chromosome segregation.

Table 2.2 Proteins involved in the signaling cascade of the spindle assembly checkpoint (SAC) during mitosis.

Core SAC proteins	**Interactions and functions**
Mad1*	Binds to Mad2 for kinetochore recruitment.
Mad2*	Binds to Mad1 for kinetochore recruitment. After a conformational change to C-Mad2, it interacts with Cdc20, BubR1 and Bub3 to form the MCC which inhibits the APC/C.
Mad3 (BubR1*)	Binds to Bub3. Interacts with C-Mad2 and Cdc20 to form the MCC for APC/C inhibition.
Bub1*	Protein kinase that binds to Bub3. Required for recruiting other SAC proteins to the kinetochore. Component of cytostatic factor in *Xenopus* but probably not in mammalian oocytes. Required for SAC in meiosis I and meiosis II.
Bub3*	Binds to Bub1 and BubR1 for kinetochore localisation. Interacts with C-Mad2 and Cdc20 to form the MCC for APC/C inhibition.
Motor proteins	
CENP-E*	Captures and stabilises microtubules at the kinetochore for association of checkpoint proteins. Necessary for chromosome alignment.
Dynein*	Transports C-Mad2 away from kinetochore to deactivate checkpoint. Also required for chromosome movement.
RZZ complex	Complex of Rod, ZW10 and Zwilch to recruit dynein and Mad1/Mad2 to kinetochore.
MCAK*	Depolymerises microtubules in response to a lack of tension. Also required for poleward chromosome movement.
Protein kinases	
Aurora B*/Aurora C*	Chromosomal passenger proteins that bind survivin, INCENP and borealin. Recruit Mps1, BubR1, CENP-E, Bub3, Mad1/Mad2 to kinetochores. Respond to a lack of tension phosphorylating MCAK for depolymerase activity.
Mps1	Required for CENP-E interaction with kinetochore.
MAP kinase*	Phosphorylates Mps1 for CENP-E to associate with

	kinetochore.
Targets and Components Regulated by SAC	
APC/C*	E3 ubiquitin ligase that targets mitotic and meiotic cyclins and regulatory proteins like securin for destruction by the proteasome.
Cdc20*	Binds to APC/C; interaction with the MCC inhibits APC/C activity.
Securin*	Binds and inhibits separase.
Separase*	Protease that cleaves the cohesin subunit Scc1 (Rec8 in meiosis) for chromatid separation.
Cohesins (Scc1/Rec8*)	Cohesions of mitosis/meiosis, respectively that are proteolytically cleaved by separase after release from SAC. Rec8 in concert with shugoshin required for monopolar attachment of kinetochores of sister chromatids to only one spindle pole.
Shugoshin (Sgo1/Sgo2) *	Centromeric protein protecting centromeric cohesin in concert with PP2A phosphatase from Plk-dependent phosphorylation and thereby inhibiting dissociation of cohesin from centromeres in APC/C-independent fashion at prophase of mitosis and at meiosis I. Promoting bipolar attachment of sister chromatids at meiosis I.
Polo-like kinase I (Plk1) *	Kinase phosphorylating proteins of the mitotic cohesin complex for separase-independent dissociation of cohesins from chromatid arms at metaphase of mitosis. Phosphorylates meiotic cohesin proteins targeting them for proteolytic cleavage by separase along arms of sister chromatids for chiasma resolution at anaphase I, after release from SAC. Together with CamKII involved in release from CSF arrest at fertilization by triggering proteolysis of Emi2 inhibitor of APC/C.
Emi1/2*	Component of CSF that inhibits APC/C activation. Target of calcium calmodulin-dependent kinase II (CamKII) and of Plk1 that target Emi2 for degradation after calcium release by fertilization leading to activation of APC/C and anaphase II progression in meiosis II of oogenesis.
Other Functional Components of Centromeres	
Centromere Proteins (CENPs)	Heterogeneous group of proteins required for structural and functional integrity of centromeres and kinetochore function.
Chromosomal Passenger Protein Complex (CPC)	Aurora B, inner centromere protein (INCENP), survivin and Dasra (borealin) localised to centromeres at metaphase and to interpolar microtubules at anaphase; may be required to recruit shugoshin to centromeres.

*****Also reported for mammalian meiosis. For references, see text (adopted from Vogt et al., 2008).

2.7.2 The SAC in oocytes

Many previous studies indicate that SAC protein members are present and functionally expressed in the mammalian oocyte (Kallio et al., 2000; Wassmann et al., 2003; Brunet et al., 2003; Homer et al., 2005a). However, there are subtle differences in the requirement for checkpoint components between female and male germ cells. For instance, male meiosis does not appear to require activity of Mad2 and Bub3, while BubR1 is essential (Jeganathan and van Deursen, 2006). Recently, Aurora C kinase (AURKC) was identified in mouse male meiosis (Tang et al., 2006a), where it may contribute together with AURKB to the regulation of spindle dynamics and checkpoint control (Wang et al., 2006b). Since AURKC was not found in mitotic spermatocytes, it may therefore possess a meiosis-specific function. Furthermore, studies in mice showed that a meiotic arrest can be induced by exposure of oocytes to chemicals interfering with microtubule dynamics at meiosis I and II suggesting that the SAC exists in mammalian oocytes (e.g. (Eichenlaub-Ritter and Boll, 1989; Wassmann et al., 2003; Eichenlaub-Ritter et al., 2007b), and reacts to loss of microtubule attachment (e.g. in presence of microtubule-depolymerising chemicals). Mad2 and Bub1 are retained at the kinetochores of meiosis I or II in arrested mouse oocytes upon exposure to nocodazole, a microtubule-depolymerising drug (Wassmann et al., 2003; Brunet et al., 2003). Therefore, oocytes appear to mount a robust SAC. However, the SAC may be weaker in old oocytes coming from observations that old mouse oocytes enter meiosis I earlier than young oocytes (Eichenlaub-Ritter and Boll, 1989) and that the expression of SAC components is altered in old human oocytes (Steuerwald et al., 2001).

On the other hand, studies from mouse models implicate that SAC signaling in the mammalian oocyte may not respond to cues in the same stringent manner as it does in mitosis, i.e. attachment status of kinetochores and tension across sister kinetochores. XO mice possess a univalent X chromosome, which lacks a pairing partner during recombination (non-exchange homologue). XO oocytes fail to arrest in meiosis I, but undergo anaphase I progression and polar body extrusion (LeMaire-Adkins et al., 1997). Similarly, *SYCP3*$^{-/-}$ oocytes, which possess achiasmate chromosomes due to reduced crossover events in response to a compromised synaptonemal complex, also do not arrest in meiosis I in presence of univalents (Kouznetsova et al., 2007). In contrast, *MLH1*$^{-/-}$ oocytes with reduced recombination due to lack of the DNA mismatch repair gene arrest at the end of meiosis I in presence of misaligned chromosomes (Woods et al., 1999).

Unlike mitosis, tension on chromosomes at meiosis I is not directly generated at the sister kinetochores of homologues but rather by the physical connection between homologues

mediated by chiasmata (Petronczki et al., 2003). Sister kinetochores of each homologue face the same spindle pole during meiosis I (monopolar attachment) and act as one functional unit. When the homologues are thus bipolarly attached, tension is generated and causes a visible stretching of the chromosome arms up to the most proximal chiasma on a bivalent. The arms adjacent to this site are usually much more condensed and contracted, irrespective of absence or presence of another chiasma. The physical connection by the first chiasma is thus thought to create tension, which can be sensed by kinetochores and signalled to the SAC. In summary, the data indicate that the SAC is present and functional in mammalian oocytes, but the mechanism of SAC signaling may be unique and different from mitosis and male meiosis.

3 AIM OF THE WORK

Early studies on *in vitro* maturation and aneuploidy in oocytes of such strains of mice that exhibit moderate age-related increases in oocyte aneuploidy have shown that there is a link between loss of cell cycle control and maternal age (Eichenlaub-Ritter and Boll, 1989). In particular, the SAC may be weakened in aged mammalian oocytes due to reduced expression of checkpoint components. Steuerwald et al. (2001) were the first to show that transcript message of *Mad2* appears lower in metaphase II-arrested human oocytes from aged women. In accordance, studies in aged oocytes of the mouse are consistent that checkpoint mRNAs are also less abundant (Hamatani et al., 2004; Pan et al., 2008).

At the beginning of this study, the SAC component Mad2 was not well characterised during mouse oogenesis. One report existed on the localisation of Mad2 in mouse oocytes (Kallio et al., 2000). However, the localisation of Mad2 was only demonstrated for metaphase II and not investigated during meiosis I. For a more comprehensive functional analysis of Mad2 in mouse oocytes, a GFP-Mad2 construct was prepared during my diploma thesis. I demonstrated that GFP-Mad2 localised to the meiotic spindle and that overexpression of Mad2 leads to a cell cycle arrest at meiosis I (unpublished results), which was shortly thereafter confirmed by another lab (Wassmann et al., 2003). Therefore, one aim of this study was to continue the functional analysis of Mad2 in mouse oogenesis, with a particular focus on the consequences of altered Mad2 expression on SAC strength and meiotic progression, which could be a contributing factor to the increased incidence of aneuploidy, causative to disorders such as Down Syndrome and associated with increasing maternal age (Shonn et al., 2000).

To ensure correct cell ploidy both in mitosis and meiosis, correction of attachment errors is as essential as the detection of such errors by the SAC. This is carried out by the chromosomal passenger complex (CPC). AURKB is one member of the CPC and there is only limited information available on the functional significance of AURKB and its substrates, like the depolymerase MCAK, in mouse oogenesis. Early studies of MCAK in human somatic cells and *Xenopus* demonstrated that this depolymerase is important in regulating key events during progression through mitosis (Moore and Wordeman, 2004). In particular, MCAK is required for anaphase chromosome segregation (Maney et al., 1998) and is also involved together with AURKB in the correction of microtubule misattachments to prevent aneuploidy (Kline-Smith et al., 2004; Lan et al., 2004). Since MCAK's depolymerising activity is regulated by AURKB phosphorylation at appropriate stages of mitosis and kinase activity, in turn, appears to be implicated in SAC function (Murata-Hori and Wang, 2002; Kallio et al., 2002; Pinsky et

al., 2006), AURKB may act in concert with MCAK upstream of the SAC in response to lack of bi-orientation and tension. To what extent the various functions of AURKB and MCAK described in human somatic cells and *Xenopus* are conserved in mouse oocytes was a further aim of this study, especially because AURKB and MCAK expression is also altered in aged oocytes (Hamatani et al., 2004; Pan et al., 2008).

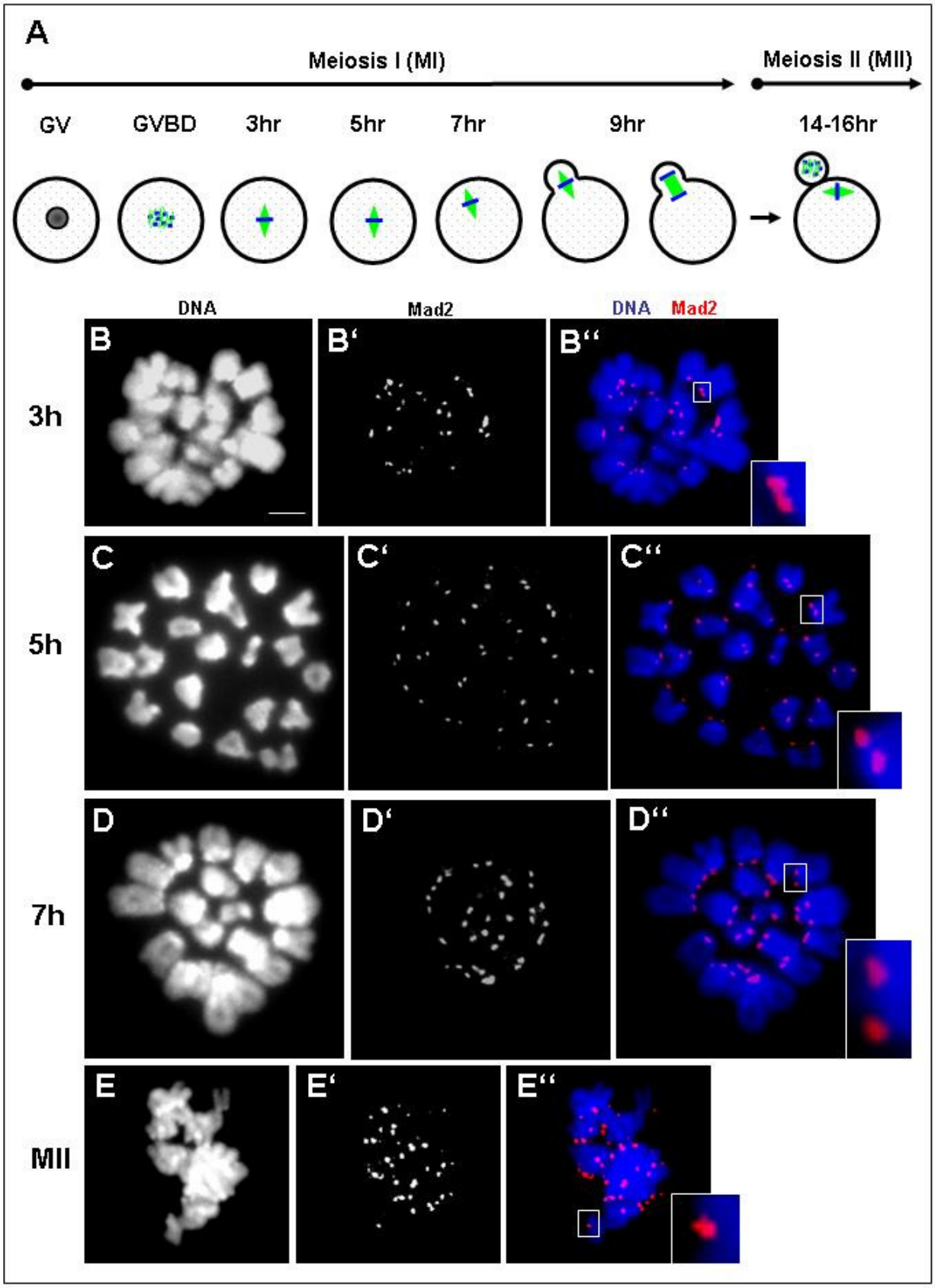
4 RESULTS

4.1 Distribution of Mad2 in mouse oocytes

4.1.1 Mad2 localises to kinetochores during meiosis I and II

Since the subcellular distribution of SAC proteins is tightly coupled to their biochemical and morphological functions, the distribution of Mad2 was determined in maturing mouse oocytes using a specific antibody. The oocyte spends several hours of meiotic M-phase in prometaphase I depending on the mouse strain (Brunet and Maro, 2005). Figure 4.1A illustrates the time scale for meiosis I, as representative of the MF1 mouse strain used in this study, where prometaphase I can last 3-4 hours after germinal vesicle breakdown (GVBD). To determine the localisation domain of Mad2 during meiosis I, oocytes spreads were prepared at 3, 5 and 7 hours after the resumption of maturation. Gentle spreading to dispose kinetochores to antibody showed that Mad2 is present at kinetochores from early prometaphase I (Figure 4.1B-C'', insets) to late prometaphase I / early metaphase I (Figure 4.1D-D'', inset). At metaphase II, Mad2 is also present at kinetochores (Figure 4.1E-E''). These results suggest that Mad2 becomes recruited to unattached kinetochores early in meiosis I and remains there until early metaphase I. Furthermore, Mad2 is also associated with kinetochores during the CSF-mediated metaphase II arrest.

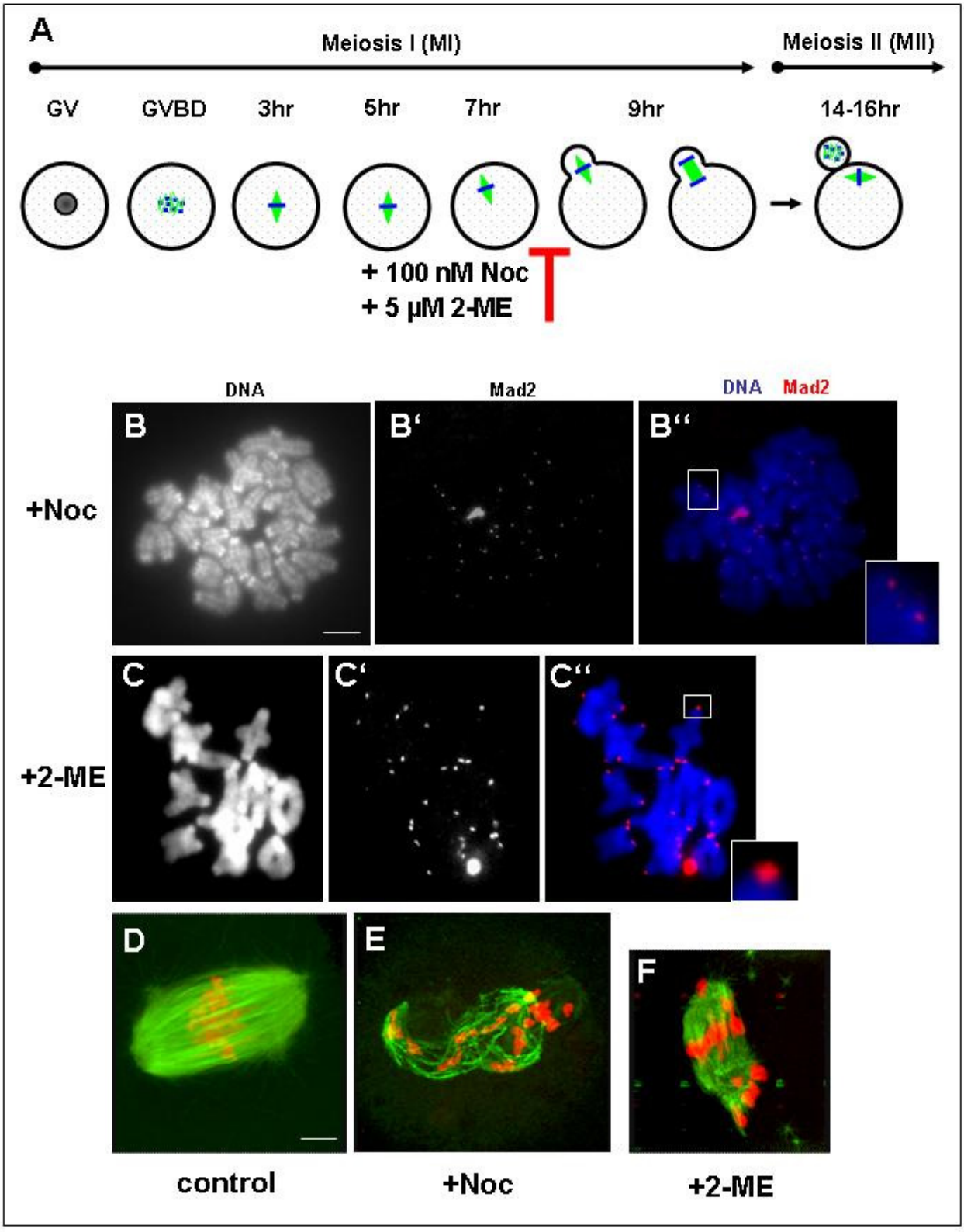
Figure 4.1 Distribution of Mad2 in maturing oocytes. Oocytes undergo GVDB within the first 2 hours after the resumption of maturation and initiate polar body extrusion at 9 hours, arresting at metaphase II in meiosis II (**A**). Image modified from Wassmann et al. (2003). Mad2 is localised at kinetochores during prometaphase I as shown at 3 hours (**B-B',** red spot in **B''**, **inset**) and 5 hours after the resumption of maturation (**C-C'**, red spot in **C''**, **inset**). Mad2 is also enriched at kinetochores at metaphase I (**D-D'**, red spot in **D''**, **inset**) and metaphase II (**E-E'**, red spot in **E''**, **inset**).
Bar in B: 10 µm.


A
Meiosis I (MI)
Meiosis II (MII)
GV
GVBD
3hr
5hr
7hr
9hr
14-16hr
DNA
Mad2
DNA Mad2
3h
5h
7h
MII
B
B'
B''
C
C'
C''
D
D'
D''
E
E'
E''

4.1.2 Mad2 localises to kinetochores following disturbances in spindle formation

In mitotic cells, the localisation of Mad2 to kinetochores depends on microtubule attachment (Waters et al., 1998). Therefore, Mad2 is lost from kinetochores of Ptk1 cells as they accumulate kinetochores and Mad2 rebinds previously attached kinetochores after MTs are depolymerised with nocodazole (Chen et al., 1996; Waters et al., 1998). To assess the distribution of Mad2 in mouse oocytes following disturbances in spindle formation, microtubule depolymerisation was induced with 100 nM nocodazole. Most oocytes treated with 100 nM nocodazole for 14-16 hours did not extrude a polar body and arrested in meiosis I (92.4%; $p<0.001$; Table 4.1). Furthermore, oocytes, which matured in presence of 100 nM nocodazole, contained aberrant spindles and unaligned chromosomes (Figure 4.2E) in contrast to untreated oocytes with a bipolar spindle and aligned chromosomes (Figure 4.2D). Hence, Mad2 localised to kinetochores of unaligned bivalents when oocyte spreads were prepared (Figure 4.2B-B'', inset) indicating that the SAC was induced in response to not properly attached kinetochores and that the SAC can be sustained for prolonged periods consistent with previous reports (Soewarto et al., 1995; Shen et al., 2005; Homer et al., 2005a). In addition to antimitotic chemicals, such as nocodazole, naturally occurring metabolites and components of the follicular fluid, such as 2-Methoxyestradiol (2-ME) may be altered in their concentrations in response to environmental changes leading to alterations in the cytoskeleton which causes cell cycle delay and M-phase arrest (Figure 4.2A; Eichenlaub-Ritter et al., 2007b). 5 µM 2-ME induced spindle aberrations and chromosome congression failure (Figure 4.2F). Hence, Mad2 was also present at kinetochores of these meiosis I-blocked oocytes (Figure 4.2C-C'', inset). The results demonstrate that Mad2 is retained at improperly attached kinetochores of meiosis-I arrested oocytes and the SAC reacts to disturbances in spindle dynamics as well as loss of microtubule attachment.

Figure 4.2 Distribution of Mad2 in maturing oocytes following disturbances in spindle formation and arrest at meiosis I (**A**). Mad2 is localised at kinetochores of meiosis I-arrested oocytes treated with either 100 nM nocodazole (Noc) (**B-B',** red spot in **B''**, **inset**) or 5 µM 2-ME for 16 hours (**C-C'**, red spot in **C''**, **inset**). Barrel-shaped meiosis I spindle with aligned chromosomes in control (**D**). Oocytes with aberrant spindles and scattered chromosomes after maturation in the presence of 100 nM Noc (**E**) or 5 µM 2-ME (**F**). Bar in B+D: 10 µm.


A
Meiosis I (MI)
Meiosis II (MII)
GV
GVBD
3hr
5hr
7hr
9hr
14-16hr
+ 100 nM Noc
+ 5 µM 2-ME
DNA
Mad2
DNA Mad2
B
B'
B''
+Noc
C
C'
C''
+2-ME
D
E
F
control
+Noc
+2-ME

4.2 Mad2 is an essential component of the SAC

4.2.1 Knockdown of Mad2 by siRNA does not interfere with meiotic progression to metaphase II, but with spindle formation and chromosome congression

To analyse the function of the SAC protein Mad2 in spindle formation, chromosome congression and cell cycle regulation, I knocked down Mad2 by microinjecting specific small interfering RNAs (siRNAs) into GV-staged mouse oocytes. Real-time RT-PCR revealed that *Mad2* transcript levels were significantly reduced by 87.3% ($p<0.001$; Figure 4.3B) after a 6-hour block in phosphodiesterase 3 (PDE3)-specific inhibitor cilostamid following specific RNAi. Immunostaining confirmed that Mad2 protein was depleted from kinetochores of metaphase II chromosomes, unlike in control oocytes injected with unspecific RNAi, while CREST signal was normal (Figure 4.3C,C', insets). Quantitative analysis showed that Mad2 staining intensity was significantly reduced by 83.0% relative to CREST following specific RNAi ($p<0.001$), whereas Mad2 and CREST levels were nearly equal following control RNAi (Figure 4.3D). After release from the meiotic inhibitor the majority (77.4%, n=318) of Mad2-depleted oocytes progressed to anaphase I and subsequently to metaphase II (Figure 4.3A) suggesting that Mad2 is not required for resumption of maturation and subsequent cell cycle progression to metaphase II, as control-injected oocytes matured at a similar rate to metaphase II (74.9%, n=221). However, the frequency of metaphase II oocytes showing aberrant spindles and chromosome congression failure increased significantly from 0% and 6.7% in control-injected oocytes (n=15), respectively, to 34.4% and 56.2% in Mad2-depleted oocytes (n=32, $p<0.01$, Figure 4.3F+G). The observed defect in spindle morphology and chromosome congression indicates that knockdown of Mad2 perturbs spindle formation and function at meiosis II.

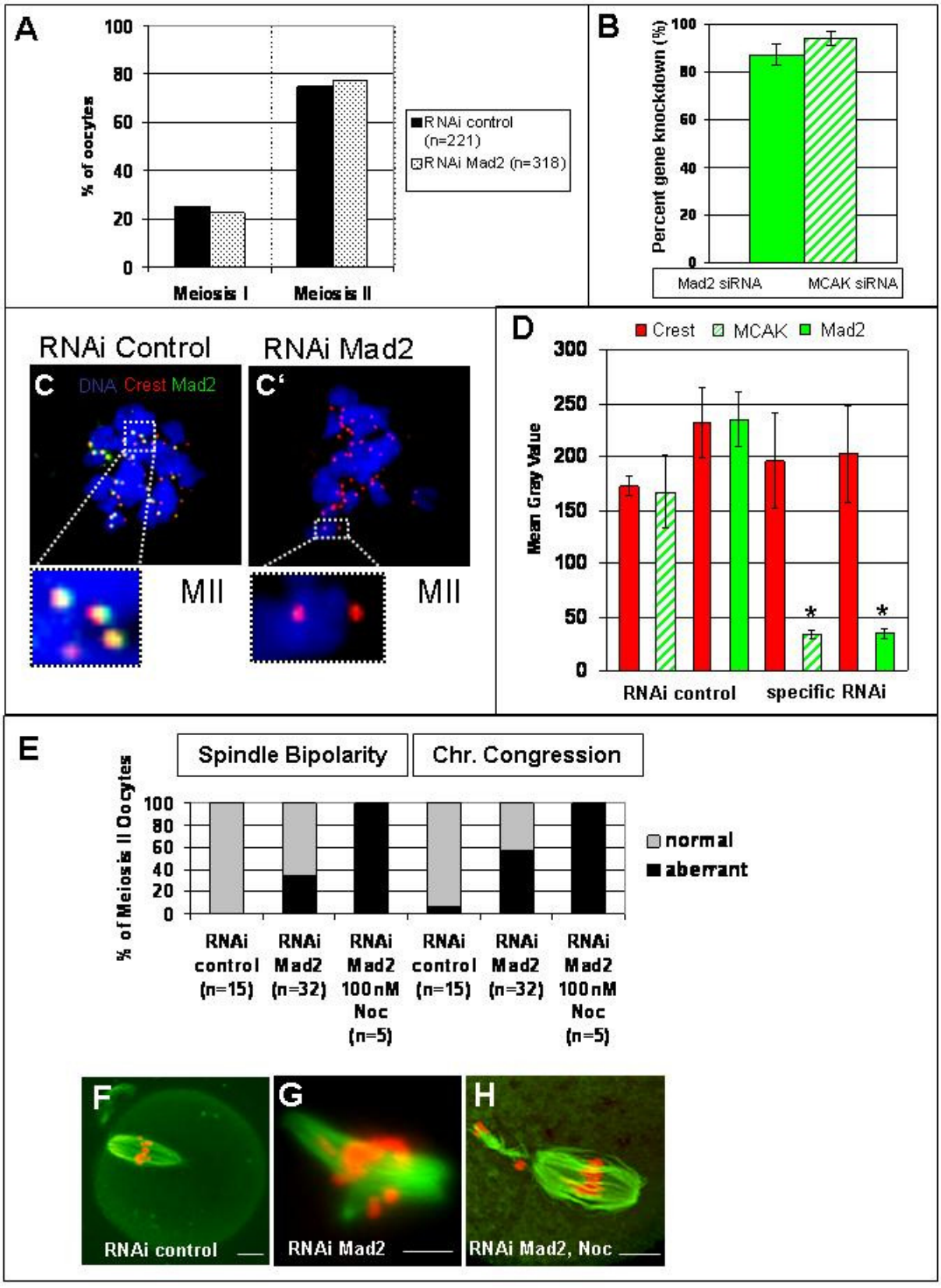


Figure 4.3 Maturation of oocytes following the knockdown of Mad2. (**A**) Depletion of Mad2 by specific siRNA (RNAi Mad2) does not interfere with first polar body formation and progression to meiosis II. (**B**) Oocytes were analysed by real-time RT-PCR. Expression ratio of *Mad2* mRNAs in oocytes injected with specific siRNAs relative to negative control siRNAs was calculated and normalised to *ß-actin* with REST software and converted to

percentage of gene knockdown. Mad2 expression was significantly reduced following specific RNAi ($p<0.001$). (**C**) Mad2 is present at kinetochores in meiosis II oocytes (RNAi Control, green spots in **C, inset**) but absent in meiosis II, Mad2-depleted oocytes (RNAi Mad2, **C', inset**) cultured for 16 hours following microinjection. CREST staining is present in both control and Mad2-depleted oocytes (red spots in **C**,**C'**, **insets**). Meiosis II oocytes with aberrant spindles and unaligned chromosomes (**D**) after specific RNAi (**F**) and exposure to 100 nM nocodazole (**G**) in contrast to controls with normal, bipolar spindles and aligned chromosomes (**E**). Bar in C,E,F: 10 µm.

4.2.2 Mad2 is required for inhibition of anaphase I progression following spindle depolymerisation

I have shown that disturbances in spindle formation by exposing oocytes to 100 nM nocodazole produces a robust meiosis I arrest and that Mad2 is localised to these unattached or misattached kinetochores (see 4.1.2) The results suggested that the meiosis I arrest following spindle depolymerisation was mediated by a SAC-dependent response. If the SAC prevents segregation of homologues in meiosis I in response to chromosome congression failure, I reasoned that homologues should not disjoin after prolonged nocodazole treatment. Therefore, air-dried chromosome spreads of oocytes were prepared that had been arrested in meiosis I for ~12 hours post GVBD. C-banding revealed that only bivalents were present in arrested oocytes, i.e. homologous pairs did not disjoin, indicating that arm cohesion was maintained (Figure 4.5B). To confirm that the SAC was induced upon nocodazole treatment, I depleted the SAC protein Mad2 in mouse oocytes utilising specific siRNAs and continuously exposed oocytes to 100 nM nocodazole with the resumption of maturation. After 16 hours of culture, 91.8% of control-injected oocytes ($n=110$, $p<0.001$, Table 4.1) did not extrude a polar body in presence of 100 nM nocodazole and arrested at the GVBD stage with uncongressed chromosomes, as viewed by *in vivo* DNA staining (Figure 4.4A). However, following the depletion of Mad2, 17.8% of oocytes ($n=326$, $p<0.001$, Table 4.1) progressed to meiosis II and emitted a polar body in presence of nocodazole. These oocytes contained aberrant spindles and uncongressed chromosomes (Figure 4.3G and 4.4B). To increase the maturation rate of oocytes to meiosis II, nocodazole exposure was reduced to 40 nM. Indeed, more oocytes emitted a polar body in comparison to oocytes exposed to 100 nM nocodazole, both in control-injected (51.8%, $n=108$) and Mad2-depleted oocytes (55.7%, $n=140$), even though a significant number of oocytes remained arrested at GVBD in both groups, 38.0% and 37.9% ($p<0.001$), respectively (Table 4.1). The number of Mad2-depleted PB oocytes was 3-fold

higher in 40 nM nocodazole in comparison to 100 nM nocodazole indicating that the SAC-induced meiosis I arrest responds to spindle poisons in a dose-dependent manner.

Table 4.1 Meiotic resumption and nuclear maturation of mouse oocytes injected with specific or unspecific Mad2 siRNAs cultured in M2 medium with either 40 nM or 100 nM Nocodazole.

	Meiotic progression				Nuclear Maturation		
	n*	GV (%)	GVBD (%)	PB (%)	n#	Biv. (%)	MII (%)
Solvent control	126		19 (15.1)	107 (84.9)	23	0	23 (100)
100 nM Noc	133		123[a] (92.4)	4[a] (3.0)	n/d	n/d	n/d
RNAi control (40 nM Noc)	108	11 (10.2)	41 (38.0)	56 (51.8)	92	36 (39.1)	56 (60.9)
RNAi Mad2 (40 nM Noc)	140	9 (6.4)	53[b] (37.9)	78[b] (55.7)	130	50 (38.5)	80 (61.5)
RNAi control (100 nM Noc)	110	8 (7.3)	101 (91.8)	1 (1.0)	20	17 (85.0)	3 (15.0)
RNAi Mad2 (100 nM Noc)	326	18 (5.5)	250[c] (76.6)	58[c] (17.8)	153	122 (76.3)	31 (19.4)

GV=germinal vesicle; GVBD=germinal vesicle breakdown; PB=polar body; Noc=nocodazole; n/d=not determined

*all isolated oocytes; #all maturing oocytes

χ^2-test: Significantly different from solvent control, a+b: $p<0.001$; RNAi control, c: $p<0.001$

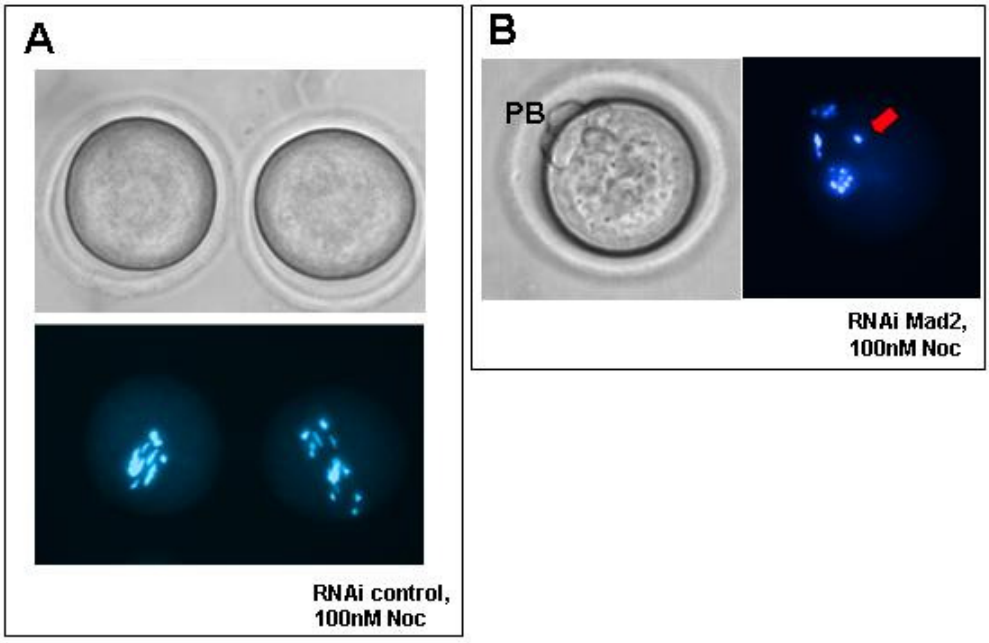


Figure 4.4 Spindle depolymerisation inhibits polar body extrusion, which is overcome upon Mad2 depletion. (**A**) Mouse oocytes arrest at meiosis I following microinjection of unspecific siRNAs and prolonged exposure to 100 nM nocodazole. Chromosomes are not aligned. (**B**) Mouse oocytes extrude a polar body and progress to meiosis II in presence of 100 nM nocodazole following microinjection of specific siRNAs against Mad2. Chromosomes fail to congress to the metaphase II plate (red arrow). DNA stained *in vivo* by Hoechst 33342; Upper panel (**A**) and left panel (**B**), phase contrast image.

4.2.3 Mad2 prevents aneuploidy following spindle depolymerisation

A complete absence of Mad2 during meiosis I in yeast (Shonn et al., 2000) and reduced levels of Mad2 during somatic mitosis (Michel et al., 2004) result in chromosome missegregation. If Mad2 was important during meiosis I of mammalian oogenesis, I reasoned that depletion of Mad2 would induce aneuploidy in mouse oocytes.

To examine the impact of Mad2 depletion on the chromosomal constitution, air-dried chromosome spreads of metaphase II oocytes were prepared (Table 4.2). Control oocytes of the solvent group had a normal chromosome compliment of 20 monovalents (Figure 4.5A). Most oocytes (86.0%, n=86) injected with control siRNAs and cultured in nocodazole-free medium also had a normal chromosomal constitution (Figure 4.5C), with only three oocytes (3.5%) being hyperploid through gain of chromosomes. Following depletion of Mad2, 88.4% (n=129) of oocytes contained 20 monovalents (Figure 4.5D), whereas only two oocytes (1.6%) were hyperploid. The number of hypoploid cells was similar in both groups, 10.5%

and 10.1%, respectively. The elevated incidences of hyper- and hypoploidy in treated oocytes, irrespective of the injected type of siRNA, suggest that handling of the oocyte during and past the microinjection procedure may affect oocyte quality susceptible to chromosomal malsegregation. Thus, in contrast to yeast meiosis and mouse somatic cells (Shonn et al., 2000; Bernard et al., 2001; Michel et al., 2004), Mad2 depletion did not lead to a significant increase of aneuploidy in undisturbed, young, healthy mouse oocytes. Altering microtubule dynamics by exposing oocytes to 40 nM nocodazole also did not affect chromosomal constitution with no hyperploid oocytes and only two of 50 (4.0%, n=50) being hypoploid through loss of chromosomes (Figure 4.5F). Due to the vast majority of oocytes arresting at meiosis I in presence of 100 nM nocodazole, no spreads were performed on oocytes injected with control siRNAs. In contrast, following Mad2 depletion, hyperploidy rose significantly in 35.0% of oocytes (n=20, $p<0.05$), when exposed to 100 nM nocodazole (Figure 4.5E), yet only to 3.2% (n=62) in presence of 40 nM nocodazole. Assuming an almost equal incidence of hypoploidy, six hypoploid oocytes (30.0%) were indeed observed in the 100 nM nocodazole group. In contrast, the frequency of hypoploidy in the 40 nM nocodazole group was nearly 5 times higher in comparison to hyperploidy (16.1% vs. 3.2%). The data indicate that Mad2 prevents aneuploidy following severe spindle depolymerisation in mouse oocytes, whereas aneuploidy through loss rather than gain of chromosomes appears to occur preferentially when low concentrations of nocodazole are affecting spindle integrity. So far, it is not possible to determine whether the rise in hypoploidy might relate to spreading artefact or a disturbance in chromosome segregation associated with preferential migration of chromosomes to the first polar body due to chromosome lagging. However, the significant increase in the proportion of hypoploid oocytes following Mad2 depletion in comparison to controls suggests the latter to be likely.

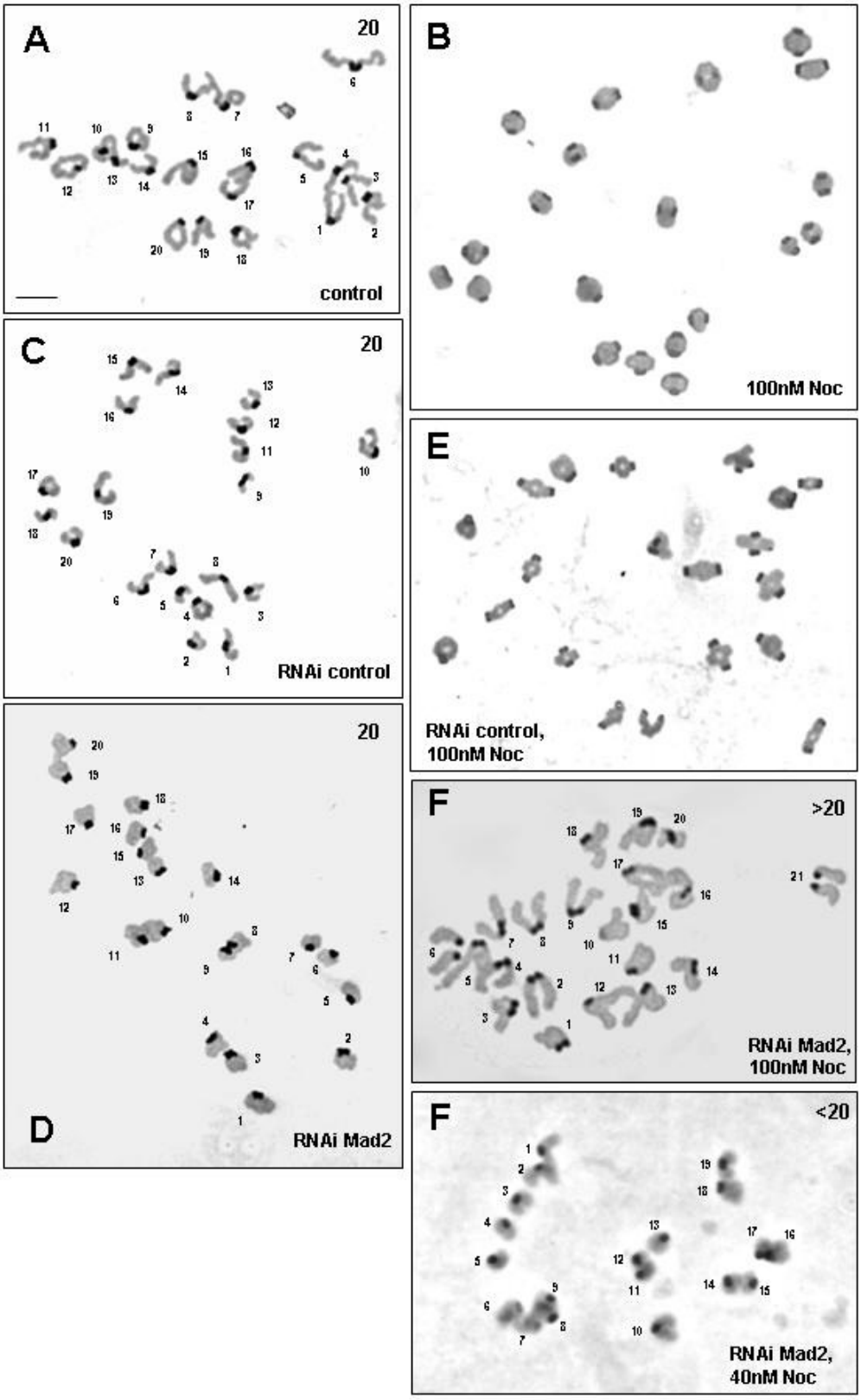
Table 4.2 Chromosomal constitution of spread and C-banded controls and oocytes injected with specific or unspecific Mad2 siRNAs cultured in M2 medium with either 40 nM or 100 nM nocodazole.

	Aneuploidy			
	n*	20 (%)	<20 (%)	>20 (%)
Solvent control	20	20 (100)	0	0[b]
RNAi control (-Noc)	86	74 (860)	9 (10.5)	3 (3.5)
RNAi Mad2 (-Noc)	129	114 (88.4)	13 (10.1)	2 (1.6)
RNAi control (40 nM Noc)	50	46 (92.0)	2 (4.0)	0
RNAi Mad2 (40 nM Noc)	62	49 (79.0)	10 (16.1)	2 (3.2)
RNAi control (100 nM Noc)	-	-	-	-
RNAi Mad2 (100 nM Noc)	20	7 (35.0)	6 (30.0)	7[b] (35.0)

*all PB oocytes with countable metaphase II chromosomes
χ^2-test: Significantly different from solvent control, b: $p<0.05$

Figure 4.5 Depletion of Mad2 results in aneuploidy following spindle depolymerisation. Chromosomal constitution of spread, C-banded mouse oocytes exposed to nocodazole with or without knockdown of Mad2. **(A+C)** Control and RNAi control metaphase II oocyte usually possess 20 chromosomes (dyads). **(B)** Oocyte arrested at meiosis I following exposure to 100nM nocodazole containing 20 homologous chromosomes (bivalents). Many RNAi Mad2 oocytes are euploid containing 20 dyads **(D)**, whereas following the exposure to 100nM

nocodazole oocytes are also hyperploid (>20 dyads) **(E)**. Hypoploid oocyte (<20) after exposure to 40nM nocodazole **(F)**. Chromosome numbering relates to sequential order and not the karyotypic nomenclature. Bar: 10µm.



4.3 Distribution of Aurora kinase B in mouse oocytes

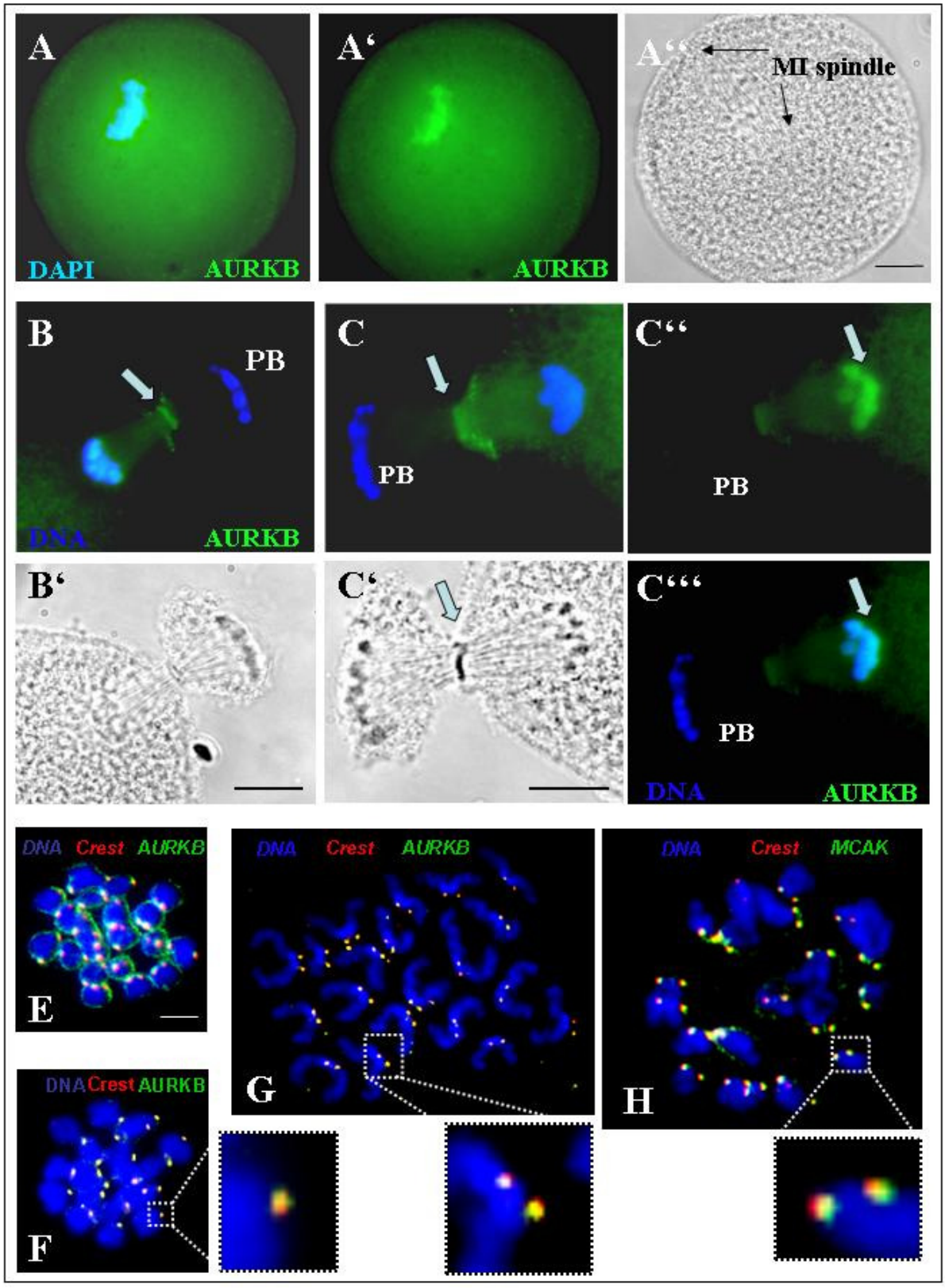
4.3.1 Aurora kinase B localises to chromosomes, centromeres and the mid-spindle

Aurora kinase B (AURKB) is a critical component of the error correction machinery at kinetochores that monitors defective attachments (Tanaka et al., 2002; Pinsky et al., 2006; Cimini et al., 2006). Since subcellular distribution of Aurora kinases may be tightly coupled to their biochemical and morphological functions, e.g. by targeting proteins for phosphorylation and activation/deactivation, I initially determined the subcellular distribution of AURKB in maturing mouse oocytes using specific antibodies (Vogt et al., 2009). Conventional immunofluorescence on cells fixed by ice-cold methanol after extraction in microtubule-stabilising solution revealed that AURKB initially becomes associated with bivalent chromosomes after GVBD (Figure 4.6A-A"). Gentle spreading to dispose centromeres to antibody showed that AURKB is present on the chromosomes (Figure 4.6E) and preferentially colocalises to the centromere domain during metaphase I of meiosis with CREST antibody (Figure 4.11C-C’’’, 4.6E and F, insets) similar to MCAK, which is regulated by AURKB phosphorylation (Ohi et al., 2004; Lan et al., 2004; Andrews et al., 2004; Zhang et al., 2007b). Likewise, in MG132-arrested metaphase I oocytes AURKB colocalises with CREST-reactive proteins (Figure 4.11D-D’’’, inset) similar to MCAK, consistent with a colocalisation of MCAK and AURKB (Figure 4.11E-E’’’, inset).

On transition to anaphase I and during telophase I and cytokinesis, AURKB was associated with the mid-spindle (Figure 4.6B, B', C-C"), consistent with its localisation in mitotic cells as part of the CPC (Ruchaud et al., 2007). Analysis of spread oocytes revealed that AURKB remains colocalised with CREST signals at early anaphase I (Figure 4.10I, I’, inset) similar to MCAK, but disappeared from centromeres at telophase I (Figure 4.10K, inset). The spatial separation of AURKB from MCAK residing at centromeres at anaphases might contribute to support microtubule depolymerisation during chromosome segregation at anaphase I/telophase I. Unlike mitotic cells progressing to interphase, in which AURKB is degraded, staining was again found on chromosomes at telophase I, once homologues had separated to opposite spindle poles (Figure 4.6C"). At this stage AURKB was preferentially observed at chromosomes retained in the oocyte with no (Figure 4.6B, C-C'") or only faint staining by antibody of chromosomes in the first polar body. In metaphase II-arrested mouse oocytes AURKB occupied a centromere domain overlapping with CREST-positive foci (Figure 4.6G and inset). MCAK was closely associated with the centromere and also occupied sites

recognized by CREST antibody (Figure 4.6H and inset), consistent with some overlap in localisation of AURKB and MCAK. This might regulate phosphorylation and inactivation of MCAK, which in its active form can contribute to rapid microtubule turnover as is characteristic for spindles in metaphase II-arrested oocytes (Gorbsky et al., 1990; Schuh and Ellenberg, 2007).

Figure 4.6 Distribution of AURKB in maturing oocytes (n=91) oocytes fixed for whole mount immunofluorescence in prometaphase I, metaphase I and ana- to telophase I). AURKB (**A'**, green) is enriched on chromosomes at prometaphase I and metaphase I (**A**, blue) that are assembled at the spindle equator (**A''**, arrows depict spindle poles) in meiosis I. At telophase I AURKB is present in the midzone of the spindle (arrow in **B,B',C,C'**) and also associated with chromosomes of the oocyte (arrows in **C''**, **C'''**) with little or no label of chromosomes in the first polar body (**C''**). At first metaphase anti-AURKB antibody reacts with the surface of bivalent chromosomes (**E**). Labelling CREST-reactive sites in red (red, in **E-H**) and AURKB-reactive sites in green (green spots in **E,F** and **G** and insets) shows that the label overlaps to produce yellow spots showing that AURKB is enriched and stably associated with the same centromere domains of sister chromatids at metaphase I and metaphase II occupied by CENP-A/C proteins recognized by CREST autoimmune antibody. This places AURKB into the vicinity to MCAK (**H**, green signal), which appears also to occupy a position at a CREST reactive site recognising centromere proteins CENPA/C. Bar in **A**'' for **A-A''** and in **B'** and **C'** for **B-B'** and **C-C'''**, respectively: 10 µm. Bar in **E for E-H**: 10 µm. Image adopted from Vogt et al. (2009).


A
DAPI
AURKB
A‘
AURKB
A‘‘
MII spindle
B
PB
DNA
AURKB
C
PB
C‘‘
PB
B‘
C‘
C‘‘‘
PB
DNA
AURKB
DNA Crest AURKB
E
DNA Crest AURKB
G
DNA Crest MCAK
H
DNA Crest AURKB
F

4.4 Aurora kinase B in regulation of maturation, spindle formation, chromatin constitution and chiasma resolution in oocytes

4.4.1 Chemical Inhibition of Aurora kinase by ZM447439 causes a block in cytokinesis and prolonged spindle assembly checkpoint (SAC)

Alexandra Kipp showed in her diploma thesis that, consistent with a mild or no effect of low concentrations of the Aurora kinase inhibitor ZM447439 on AURKA activity, resumption of meiosis was not affected by 1 µM ZM inhibitor (Vogt et al. 2009). However, most inhibitor-treated oocytes (50.2% of all oocytes resuming maturation) arrested after GVBD and only few emitted a polar body (49.8% in treated versus 82.9% in control group, n=269 and 249, respectively; $p<0.001$; Figure 4.7A). Maturation rate dropped further with increased ZM concentration (1.5 µM) to 33.2% oocytes with first polar body in treated versus 88.3% in controls (n = 271 and 309, respectively; $p<0.001$). There was only a minor effect on meiotic progression, however, when oocytes matured for 7h to prometaphase I without inhibitor (ZM t=7), followed by exposure to 1.5 µM ZM until 16 h (91.4% PB formation versus 80% in control and ZM group, respectively; Table 4.6).

The inhibition of AURKB not only blocked cytokinesis but additionally appeared to prolong the spindle assembly checkpoint (SAC), and those oocytes progressing to anaphase I and cytokinesis tended to emit the first polar body with a delay (Figure 4.7D'), as I determined by time-lapse polarisation microscopy. 50% of the ZM group initiated polar body extrusion with a delay of approximately 16 min in comparison to the control group as derived from the logarithmic function of polar body formation kinetics (Figure 4.7D'). Additionally, Alexandra Kipp could show on spread oocytes that nuclear maturation and/or chiasma resolution was arrested in a large number of oocytes as suggested by increased numbers of oocytes with bivalents in GVBD oocytes (Table 4.3). Low concentrations of ZM did not interfere with expression of some MCAK at centromeres of sister chromatids in meiosis I mouse oocytes when I stained spread oocytes with specific antibodies (Figure 4.7B'). Characteristically, fully congressed chromosomes had MCAK localised overlapping or close to centromeric CREST positive sites (yellow stained area in lower inset, Figure 4.7B'), while those chromosomes oriented with their axis perpendicular to the presumptive division axis displayed some MCAK not totally overlapping with the CREST-stained part of the centromere domain (non-overlapping green and red signals in upper inset of Figure 4.7B'). The delay or meiotic arrest might relate to failure in Rec8 cohesin phosphorylation at centromeres and chromosomes or to

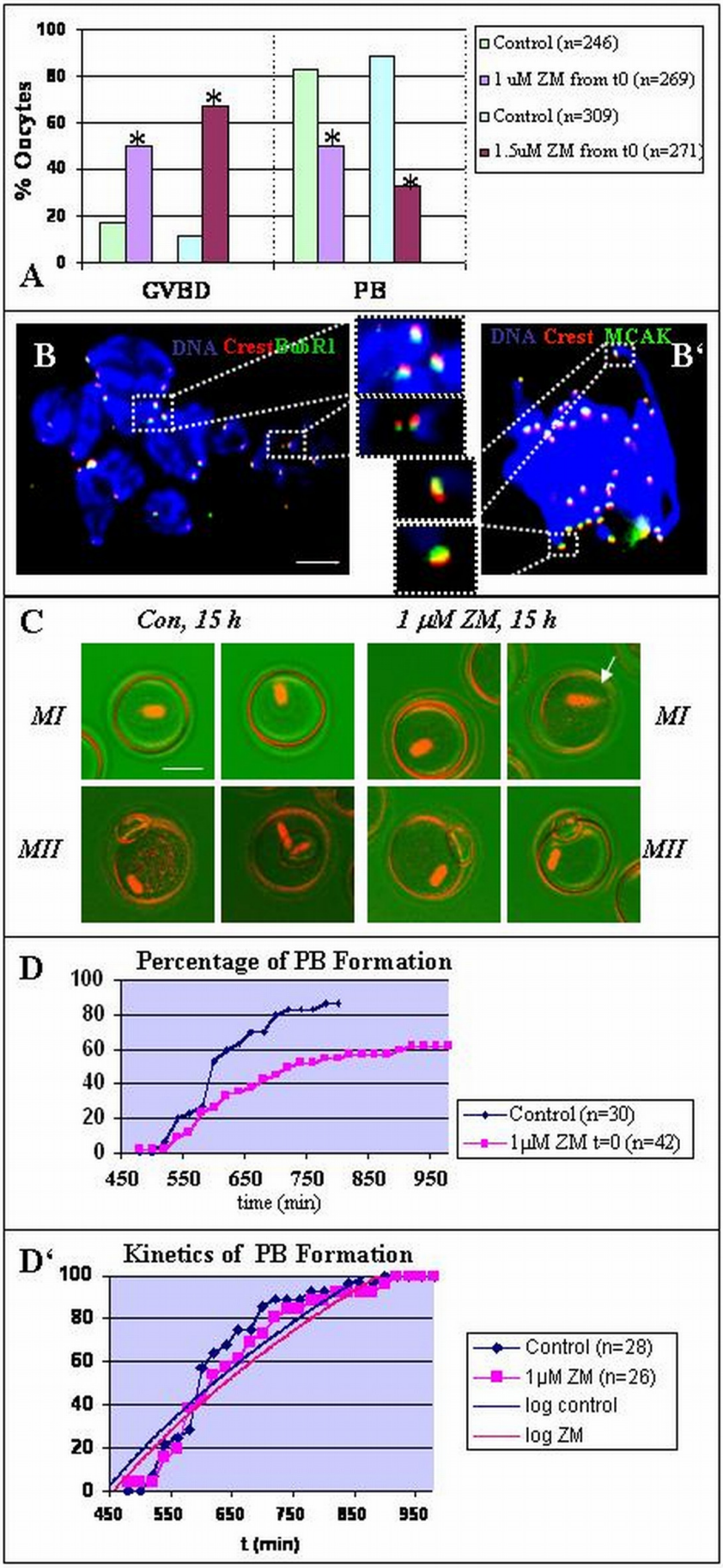
failure in MCAK inactivation by AURKB phosphorylation, which, in turn, might be responsible for unstable spindle attachments, chromosome congression failure and prolongation of the SAC in the inhibitor-exposed oocytes. In support of this, I detected the BubR1 checkpoint protein at centromeres of bivalent chromosomes in ZM-exposed meiotically blocked oocytes (Figure 4.7B and upper two insets).

Table 4.3 Chromosomal constitution of spread and C-banded controls and oocytes exposed to 1µM ZM throughout maturation (16h).

	Meiotic Maturation			Ploidy		Aneuploidy			Predivision	
	n*	Bivalents (%)	Dyads (%)	n**	Polyploids (%)	n***	Euploids (%)	Hyperploids (%)	n**	MII with Chromatids. (%)
Control	195	24[a] (12,3)	171[a] (87,7)	104	1[a] (1)	85	83 (97,6)	2 (2,4)	104	4 (3,8)
1µMZM	182	67[a] (36,8)	115[a] (63,2)	86	17[a] (19,8)	52	51 (98,1)	1 (1,9)	86	8 (9,3)

n*: All GVBD and PB oocytes with recognizable chromosomes; n**: All GVBD and PB oocytes containing dyads; N***: PB oocytes with countable dyads.
Significantly different from control, a: $p<0.001$. Table adopted from Vogt et al. (2009).

Figure 4.7 Maturation of oocytes in presence of ZM inhibitor. Rate of GVBD and PB in oocytes exposed to 1 or 1.5 µM ZM (**A**); Stars: Significantly different from control. BubR1 checkpoint protein (green in **B** and insets) and MCAK microtubule depolymerase (green in **B'** and insets) at centromeres of chromosomes (DAPI, blue) of meiosis I blocked oocytes exposed to 1 µM ZM for 16h. Images by polarising microscopy (OCTAX EyeWear) of spindles of in vitro maturing control (Con) and ZM-exposed oocytes at meiosis I (MI) and meiosis II (MII) (**C**); arrowhead depicts aberrantly shaped spindle in ZM-exposed MI-blocked oocyte. Percentage of oocytes undergoing cytokinesis and formation a first polar body (**D**) and kinetics of formation of the first polar body (PB) (**D'**) in control oocytes (blue dots and lines) and oocytes exposed to 1 µM ZM (pink lines). Bar in **B** and **B'**: 10 µm. Bar in **C** for all images in **C**: 50 µm. Image adopted from Vogt et al. (2009).


A
% Oocytes
100
80
60
40
20
0
GVBD
PB
Control (n=246)
1 uM ZM from t0 (n=269)
Control (n=309)
1.5uM ZM from t0 (n=271)
B
DNA Crest Bub1R1
DNA Crest MCAK
B'
C
Con, 15 h
1 µM ZM, 15 h
MI
MII
D
Percentage of PB Formation
time (min)
Control (n=30)
1µM ZM t=0 (n=42)
D'
Kinetics of PB Formation
t (min)
Control (n=28)
1µM ZM (n=26)
log control
log ZM

4.4.2 Chemical Inhibition of Aurora kinase leads to spindle aberrations and chromosome congression failure

Alexandra Kipp's immunofluorescent analysis revealed that the majority of meiosis I-arrested oocytes treated with 1.5 μM ZM for 16 h had aberrant spindles (Vogt et al. 2009). Whereas only 15.4% (n=13; Table 4.4) in the few partially immature, meiosis I-arrested oocytes showed aberrations, 63.5% in the ZM group contained aberrant spindles (n=96; $p<0.005$; Table 4.4). Furthermore, more than two thirds of this group (71.6%) failed to align chromosomes at the spindle equator (13.3% in controls; $p<0.001$; Table 4.4). The number of meiosis II oocytes with failure in chromosome congression was twice as high as in the controls (15.4% versus 7.3%, n=165 and 52, respectively; Table 4.5) and the percentage of aberrant spindles was increased by over fourfold but the differences did not reach statistical significance and were not nearly as dramatic as in the meiotically-arrested GVBD oocytes (Table 4.4, 4.5).

To visualise spindles three-dimensionally I prepared whole oocytes for confocal microscopy, which confirmed the findings obtained with conventional fluorescence microscopy (Figure 4.8) and furthermore revealed that some meiosis I spindles contained many unaligned and more than 20 bivalent chromosomes (Figure 4.8B) as might be expected when all cytokinesis arrested oocytes had also a block in nuclear maturation. In contrast, the analysis suggested that transient separation of homologues took place in spite of the cytokinesis arrest and some chromosomes appeared in the process of separation (arrowhead in Figure 4.8B). Chromosomes seemed to be delayed or arrested in migration to spindle poles and/or congression at the spindle equator. In addition, some chromosomes might possibly only attach to only one spindle pole and were located at the spindle periphery close to one pole (arrow in Figure 4.8B). Meiosis II spindles had mostly normal morphology, occasionally loosely arranged chromosomes in the equatorial plane but rarely totally unaligned chromosomes (Figure 4.8D, Table 4.5)

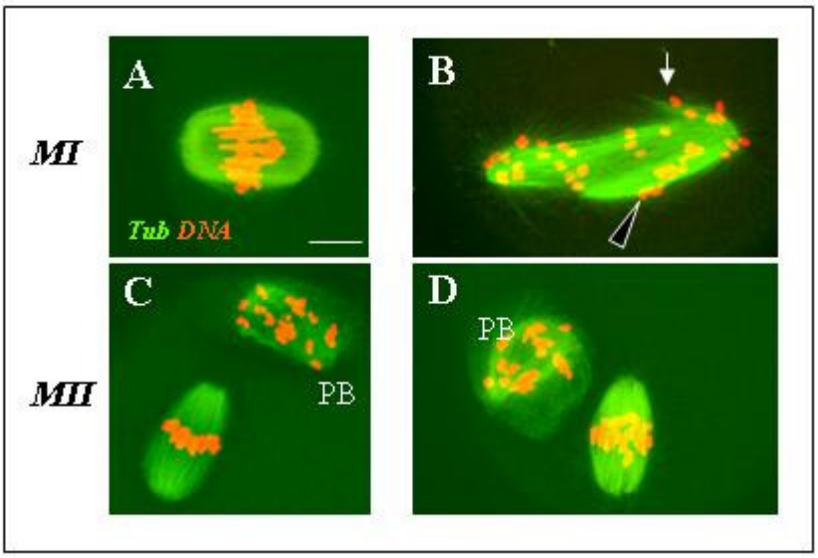


Figure 4.8 Spindles and chromosomes in controls and ZM-exposed oocytes as viewed by confocal microscopy. Chromosomes (propidium iodide, red/yellow) align on meiosis I and II spindle (green) in controls (**A,C**) while more than 20 bivalent chromosomes are frequently present and scattered on the spindle of arrested ZM-exposed oocytes (**B**). Some precociously divided chromosomes appear monopolarly attached (arrow) while other bivalent-like chromosomes are still present (arrowhead, **B**). Bar: 10 µm. Image modified from Vogt et al. (2009).

Table 4.4 Spindle aberrations and failure in chromosome congression in control and ZM-exposed GVBD oocytes.

GVBD Oocytes	Spindles			Chromosomes		
	n	normal (%)	aberrant (%)	n	aligned (%)	unaligned (%)
Control	13	11[a] (84,6)	2[a] (15,4)	15	13[b] (86,7)	2[b] (13,3)
1,5µMZM	96	35[a] (36,4)	61[a] (63,5)	95	27[b] (28,4)	68[b] (71,6)

Significantly different from control, a: $p<0.005$; b: $p<0.001$.
Table adopted from Vogt et al. (2009).

Table 4.5 Spindle aberrations and failure in chromosome congression in control and ZM-exposed PB oocytes.

PB Oocytes	Spindles			Chromosomes		
	n	normal (%)	aberrant (%)	n	aligned (%)	unaligned (%)
Control	169	166 (98,2)	3 (1,8)	165	153 (92,7)	12 (7,3)
1,5µMZM	52	47 (90,4)	5 (9,6)	52	44 (84,6)	8 (15,4)

Table adopted from Vogt et al. (2009).

4.4.3 Epigenetic modifications of histones following Aurora kinase inhibition

Centromeric heterochromatin is usually tightly condensed at M-phase, and there is evidence from artificial chromosomes that epigenetic alterations affecting recruitment of centromeric proteins, and chromosome condensation state are essential for functionality of centromeres of eukaryotic chromosomes (reviewed by Ruchaud et al., 2007). Inhibition of Aurora kinases by high concentrations of ZM block has been shown to reduce histone H3 serine 10 and serine 28 phosphorylation and interferes with condensation of chromatin (Swain et al., 2008). To assess further disturbances in heterochromatin I examined distribution of H3 lysine 9 trimethylation in controls and oocytes exposed to low concentrations of ZM AURKB inhibitor (Vogt et al., 2009). Antibody reacted with chromosomes in control metaphase I and anaphase I oocytes, showing particular strong staining of centromeric heterochromatin (arrows in Figure 4.9A, B). Distinct staining of centromeres of sister chromatids was also observed in spread, meiosis II arrested control oocytes (arrows in Figure 4.9C, C'). Importantly, ZM caused alterations in epigenetic constitution of heterochromatin since centromeric heterochromatin in oocytes exposed to 1.5 µM ZM lacked trimethylated histone H3 lysine 9 (Figure 4.9D, D') or there was only faint staining of centromeres in the meiosis II oocytes (Figure 4.9E, E'). Furthermore, chromosomes appeared less condensed and had a fluffy appearance (Figure 4.9D, E). Frequently telomeres or chromatid arms appeared to cluster and stick to each other (arrows in Figure 4.9D, E). In contrast, GVBD in absence of inhibitor with subsequent exposure to ZM (from 2 h of maturation) did not cause this severe interference with modification of H3 at centromeric heterochromatin (Figure 4.9F, F').

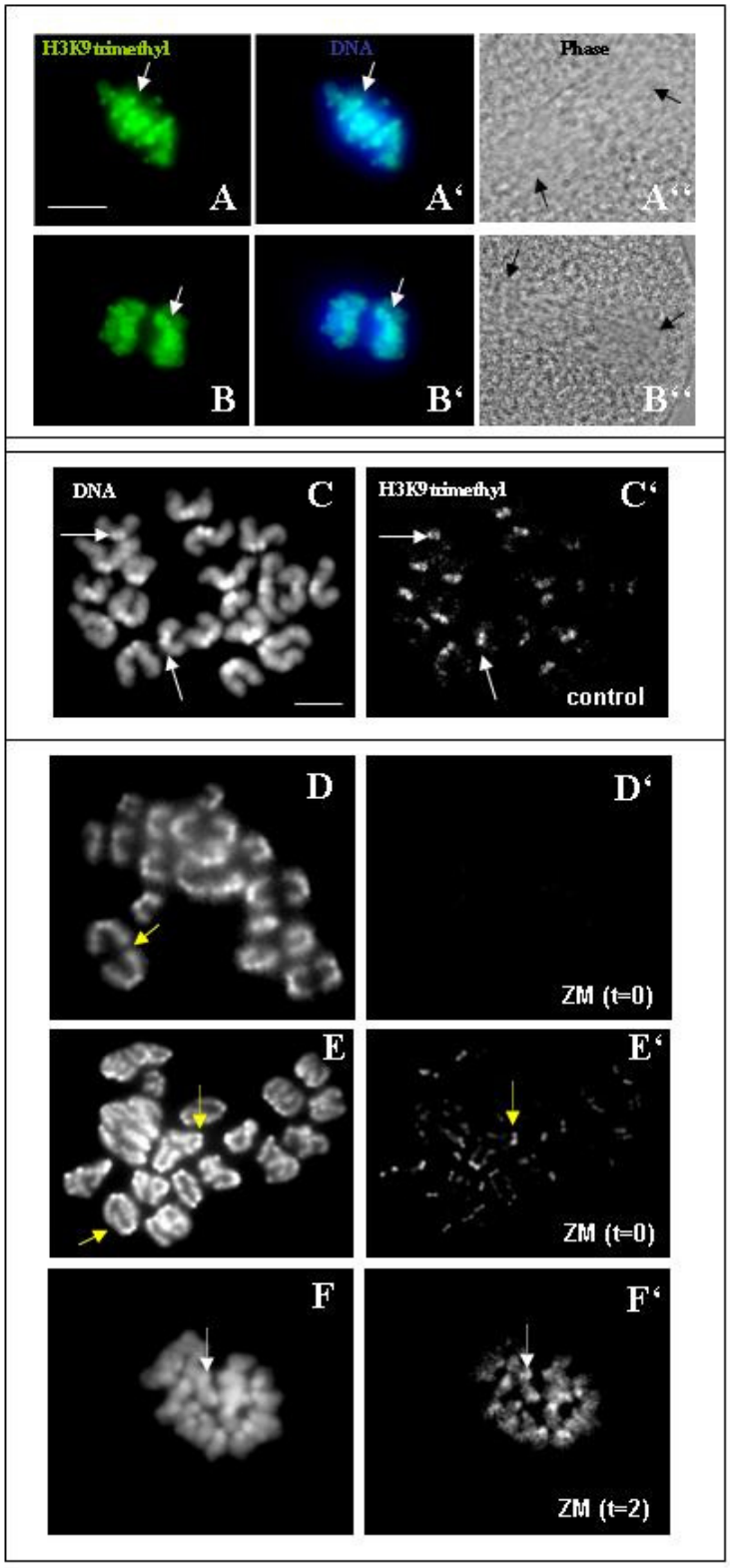


Figure 4.9 Distribution and presence of trimethylated histone H3 lysine 9 (H3K9trimethyl) (**A,B,C',D',E',F'**) on chromosomes of control (**A-C**) and ZM-exposed oocytes (**D,E,F**). Arrows in **A,B,A',B'** depict centromeric heterochromatin on metaphase I (**A-A''**) and anaphase I (**B-B''**) chromosomes and centromeres of sister chromatids at meiosis II of control (**C,C'**) and ZM-exposed oocytes (**E,E',F,F'**). Yellow arrows in **D** depicts telomeric or arm attachment between chromatids. Bar in **A''** and **B''** for **A-B''**: 10 µm; in **D** for **C-F'**: 10 µm. Image adopted from Vogt et al. (2009)

4.5 Distribution of MCAK in mouse oocytes

4.5.1 MCAK is recruited to chromosome arms after GVBD and localises to centromere domains from prometaphase I to metaphase II

The kinesin-13 MCAK was originally described as a motor protein, which localises to centromeres in mitotic cells (Wordeman and Mitchison, 1995) and is recruited to centromeres in prophase I spermatocytes, where it remains until anaphase I (Parra et al., 2006). The present study shows that MCAK is initially recruited to chromosome arms after GVBD in *in vitro* maturing mouse oocytes (Figure 4.10A). Later at prometaphase I stage MCAK is no longer found at chromosome arms but rather at centromeric sites from prometaphase I to mid metaphase I as well as at metaphase II stage (Figure 4.10B and C). Double immunolabelling of MCAK and human CREST serum that recognizes centromere proteins (CENP-A and CENP-C) reveals that MCAK occupies foci overlapping with CREST staining at metaphase II, (Figure 4.10D-D’’’, inset) similar to the distribution of AURKB (Figure 4.10E-E’’’, inset). While MCAK appears as a pericentromeric ring below kinetochores at metaphase II of male meiosis in the mouse (Parra et al., 2006), it is either concentrated at distinct foci at centromere domains in metaphase II-arrested mouse oocytes (Figure 4.10D’’) or in a cone-like structure, presumably beneath the kinetochore proper (Figure 4.10C and C’). Since chromosomes usually become deposited with their long axis and chromosome arms on the surface of the slide during the spreading procedure, it is not possible to unambiguously determine whether the cone of MCAK staining actually depicts a ring-like structure beneath the kinetochore as is recognized in side view of squashed spermatocytes. However, despite possible subtle differences in distribution of MCAK at centromeres in oocytes compared to male meiosis, the observations suggest that MCAK becomes recruited to a position within the centromere domain at metaphase II of oogenesis similar to its localisation in male meiosis.

In control oocytes that spontaneously resume maturation MCAK occupies a focal centromere domain at late metaphase I of oogenesis, which is recognised by CREST antibody (Figure 4.11A-A’’’, inset). Furthermore, MCAK remains colocalised with CREST stained centromeres when oocytes are blocked from progression into anaphase I by exposure to the proteasome inhibitor MG132 from late prometaphase I stage (7h) for 4 hours, consistent with an extended metaphase I arrest, (Figure 4.11B-B’’’, inset). MCAK persisted at centromeres at early anaphase I (Figure 4.10H, H’, inset) and telophase I (Figure 4.10J, inset). Therefore, no clear shift away from CREST-positive sites is observed in the majority of chromosome spreads of mouse oocytes irrespective of meiotic stage.

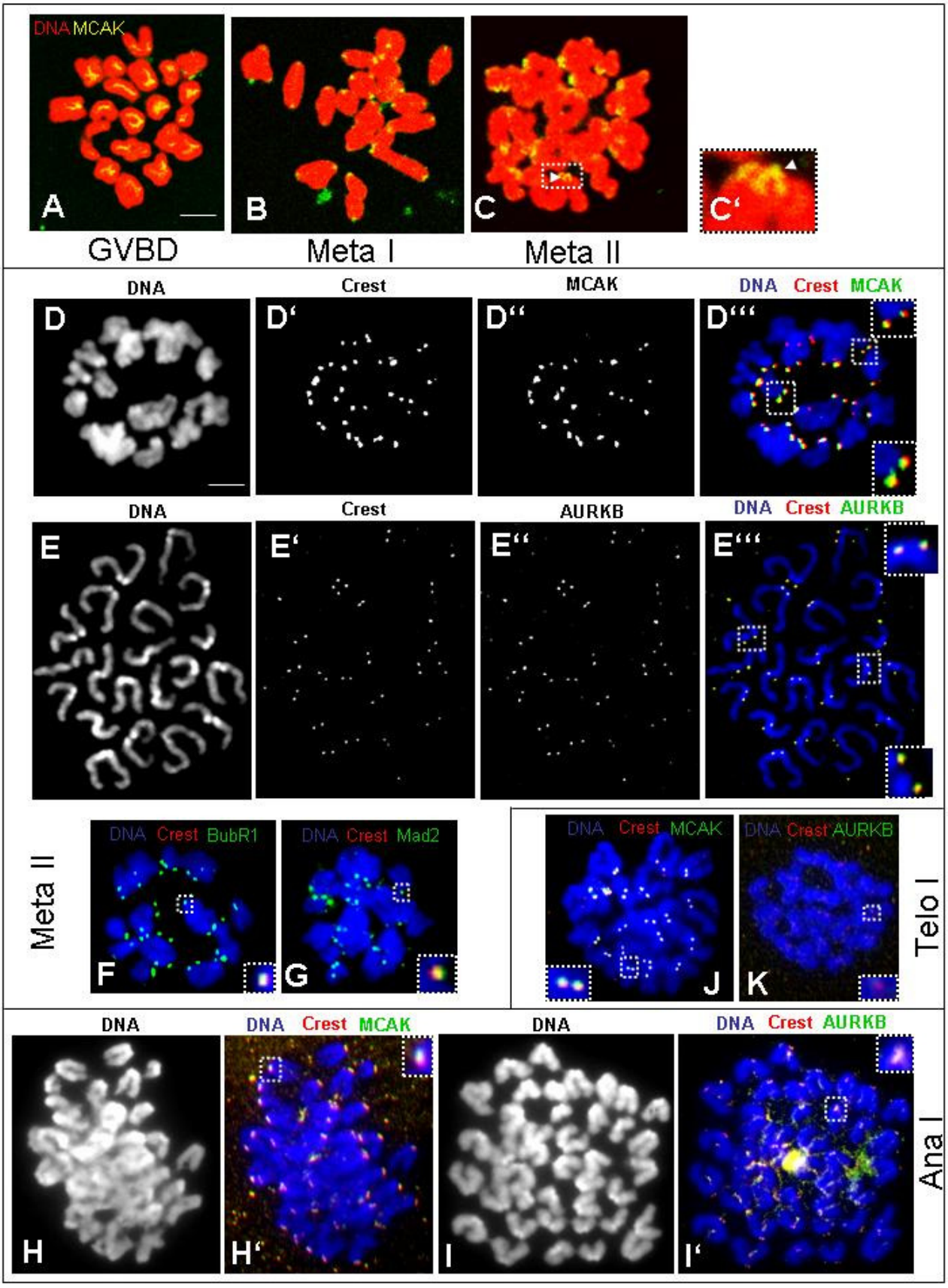
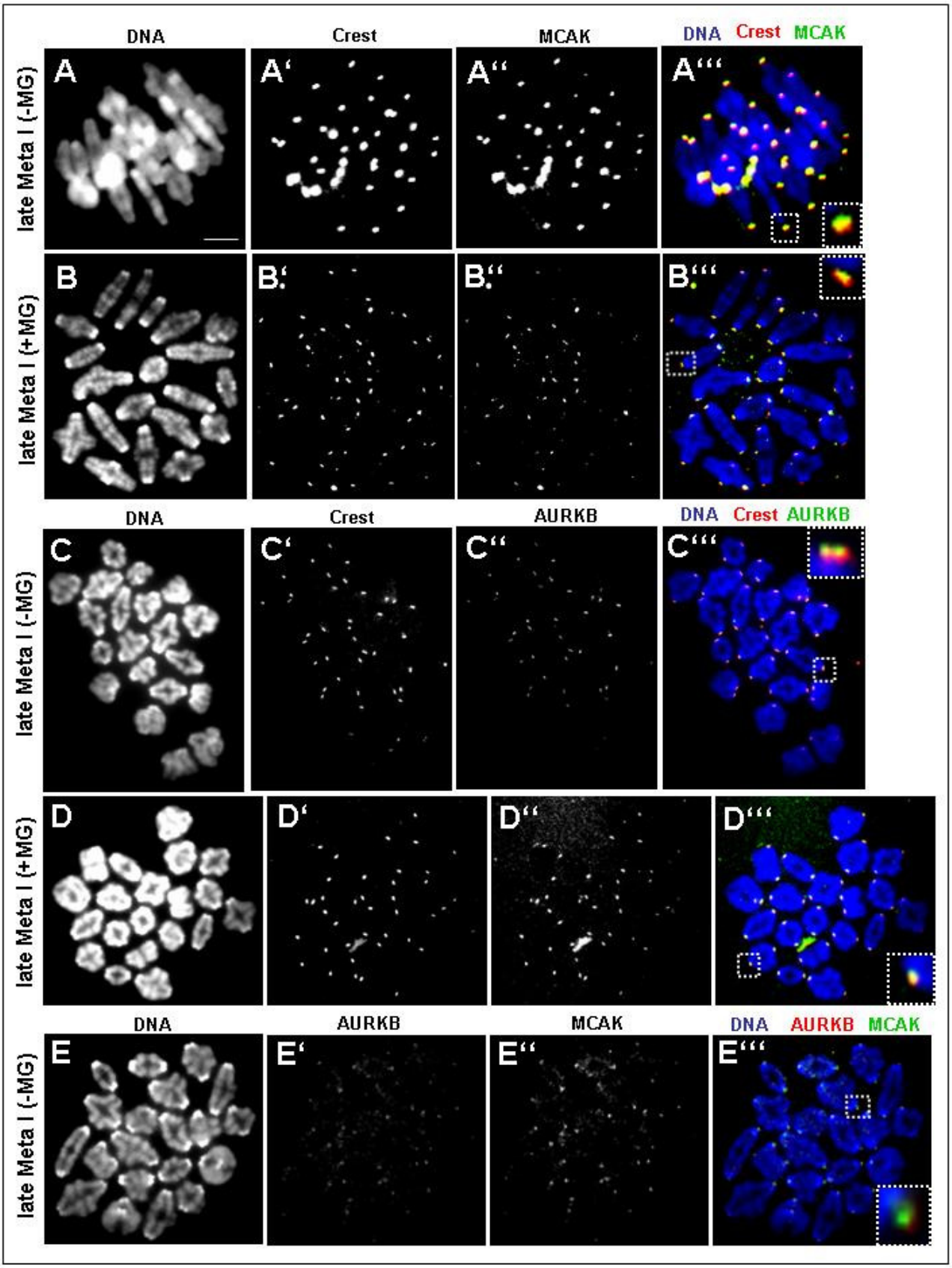


Figure 4.10 Distribution of MCAK in maturing oocytes. MCAK (**A-C'**, yellow) is localised on arms of sister chromatids after germinal vesicle breakdown (**A,** 3 hours after the resumption of maturation), and at centromeres at metaphase I (**B**) and metaphase II (**C-C'**;

arrows depict cone-like distribution of MCAK beneath kinetochores of sister chromatids in dyads at meiosis II). MCAK is enriched at centromere domains of sister chromatids at metaphase II (green spots in **D''', insets**) overlapping with centromere proteins as recognised by CREST antibody (red spots in **D''', insets**), placing it into the vicinity to AURKB (green spots in **E''', insets**), which occupies a position at CREST-reactive centromere proteins (red spots in **E''', insets**). A metaphase II checkpoint proteins BubR1 (**F**, green) and Mad2 (**G**, green) are present at kinetochores of sister chromatids. At early anaphase I and telophase I MCAK (green spot in **H'+J**, **insets**) is localised at centromere domains of sister chromatids colocalising with CREST-reactive sites (red spot in **H'+J**, **insets**). AURKB staining (green spot in **I'**, **inset**) overlaps with CREST (red spot in **I'+K**, **insets**) at early anaphase I, but disappears from centromeres at telophase I (**K, inset**). In merged image overlapping regions appear in yellow (**D'''-J, insets**).
Bar in **A-K**: 10µm.

Figure 4.11 Distribution of MCAK and AURKB in maturing oocytes at late metaphase I. MCAK (**A'',** green spots in **A''', inset**) and AURKB (**C'',** green spots in **C''', inset**) are enriched at centromere domains of sister chromatids at metaphase I overlapping with centromere proteins as recognised by CREST antibody (red spots in **A'''+C''', inset**). Inhibition of anaphase I progression with the proteasome inhibitor MG132 places MCAK (**B'',** green spot in **B''', inset**) and AURKB (**D''**, green spots in **D''', inset**) to the centromere domain as recognised by CREST antibody (red spots in **B'''+D''', insets**). This places MCAK (**E'',** green spots in **E''', inset**) and AURKB (**E',** red spots in **E''', inset**) to the same centromere domain at late metaphase I. In merged images overlapping regions appear in yellow (**A'''-E''', insets**). Bar in **A-E'''**: 10µm.


DNA
Crest
MCAK
DNA Crest MCAK
late Meta I (-MG)
A
A'
A''
A'''
late Meta I (+MG)
B
B'
B''
B'''
DNA
Crest
AURKB
DNA Crest AURKB
late Meta I (-MG)
C
C'
C''
C'''
late Meta I (+MG)
D
D'
D''
D'''
DNA
AURKB
MCAK
DNA AURKB MCAK
late Meta I (-MG)
E
E'
E''
E'''

4.6 MCAK is involved in the metaphase I-anaphase I transition

4.6.1 Knockdown of MCAK by siRNA induces a meiotic arrest

To analyse the function of MCAK in spindle formation, chromosome congression and cell cycle regulation, MCAK was initially knocked down by Mourad Sanhaji during his diploma thesis microinjecting siRNAs specific to the Kin-I kinesin. Real time RT-PCR revealed that *MCAK* mRNA was significantly reduced (94.1%, $p<0.001$) relative to *beta-actin* mRNA after microinjection of specific message into GV-staged oocytes (Figure 4.12B), followed by a 6 hour meiotic arrest of oocytes at the GV stage by the specific phosphodiesterase 3 (PDE3)-inhibitor cilostamid. Arrest was overcome by washing and transfer to cilostamid-free medium (Figure 4.12A). After release from the meiotic arrest the majority of the denuded GV-staged oocytes of the controls injected with unspecific RNAi underwent GVBD and emitted a first polar body and matured to metaphase II within 16h of culture (Figure 4.12A; Table 4.6). Immunostaining of siRNA-treated spread mouse oocytes verified that MCAK protein was depleted from centromeres while CREST signal was normal (Figure 4.12C,C', insets). Quantitative analysis revealed that depletion of MCAK resulted in a significant reduction in MCAK staining intensity by 82.7% relative to CREST following specific RNAi ($p<0.001$), compared to 3.0% following control RNAi (Figure 4.12D). Knockdown of MCAK inhibited meiotic progression and 77.8% of oocytes (Figure 4.12A, $n=194$, $p<0.001$) arrested at meiosis I, after GVBD. Still the oocytes possessed aligned bivalent chromosomes, as confirmed by air-dried chromosome spreads and Giemsa staining (Figure 4.12E), after extended meiosis I arrest at 16h past resumption of maturation and possessed a fairly normal appearing bipolar spindle (Figure 4.13A+B) indicating that MCAK may be dispensable for eventual chromosome assembly at the spindle equator. Spindle poles were frequently less focused than in control meiosis I oocytes. Thus, MCAK was not required to achieve spindle bi-polarity and chromosome congression in oocytes possessing bivalent chromosomes but MCAK was obviously indispensable for tight focusing of spindle poles and for the metaphase I-to-anaphase I transition.

4.6.2 Double knockdown of MCAK and Mad2 overcomes the meiotic arrest leading to spindle and chromosome congression defects at metaphase II

To further assess whether the meiotic arrest was mediated by prolongation of the SAC, the checkpoint pathway regulating the metaphase I-anaphase I transition (Wassmann et al., 2003; Homer et al., 2005a), chromosome spreads of arrested oocytes were immunostained for the SAC proteins Mad2 and BubR1. Mad2 and BubR1 both localised at centromere domains in MCAK-depleted oocytes (Figure 4.13D, D', insets). Thus, alterations in microtubule dynamics of spindle MTs causing an absence of full tension on kinetochores might be at the basis of the meiotic arrest and suggests a role of MCAK upstream of satisfying the SAC at metaphase I of mammalian oogenesis.

RNAi knockdown of Mad2 in mouse oocytes has been previously performed (see 4.2). Mad2-depleted oocytes progressed to meiosis II in the presence of the microtubule-depolymerising drug nocodazole. Therefore, MCAK and Mad2 were double-depleted in oocytes by microinjection of specific siRNAs to test whether the MCAK-induced meiotic arrest is caused by prolongation of the SAC and thus would be expected to be released by Mad2 depletion. Following the successful knockdown of MCAK and Mad2, as confirmed by real-time RT-PCR and immunostaining (Figure 4.12B, C', C''', insets) the majority (82.9%, n=217) of double-depleted oocytes, unlike in the MCAK-depleted oocytes, progressed to anaphase I and subsequently to metaphase II (Figure 4.12A) supporting the notion that MCAK depleted meiotic arrest is dependent on the SAC and that inactivation of the SAC by RNAi reverses the arrest. Therefore, it appears that MCAK is involved in the regulation of microtubule dynamics at the metaphase I-anaphase I transition and indirectly or directly in the satisfying of the SAC.

A series of mouse genetic studies during the past several years has demonstrated that deficiencies of major components of the SAC predispose cells to spindle abnormalities and segregation defects (Babu et al., 2003; Baker et al., 2004; Baker et al., 2006). When Mad2 was ablated in mouse oocytes, they progressed to meiosis II in spite of severe spindle aberrations induced by an exposure to nocodazole (see 4.2.2). Therefore, spindle and chromosome morphology of those oocytes, which progressed to metaphase II after double knockdown of MCAK and Mad2 or single knockdown of MCAK or Mad2, were analysed. The frequency of oocytes showing aberrant spindles and chromosome congression failure at metaphase II increased from 5.0% and 9.0% in control-injected oocytes (n=58), respectively, to 53.8% and 41.0% in double-depleted oocytes (n=39, $p<0.005$), respectively (Figure 4.13E+I). Analysis of the few MCAK-depleted oocytes, which escaped the meiotic block and

progressed to metaphase II (22.2%, n=194; Figure 10.3A) revealed the same defects in 43% of the oocytes (n=7; Figure 4.13H).

Taken together, the observed spindle morphology and chromosome congression defects in both single or double depletion experiments indicate that knockdown of MCAK perturbs spindle formation and function at meiosis II when the SAC is overcome in meiosis I. Aneuploidy was increased in somatic cells of homozygous *Mad2* knockout mice or haplo-insufficient *Mad2* animals (Dobles et al., 2000; Michel et al., 2001). Metaphase II oocytes of heterozygous *Mad2*$^{+/-}$ mice have increased levels of aneuploidy (Niault et al., 2007), and mouse oocytes, in which Mad2 was knocked down by specific RNAi, progressed to meiosis II in spite of severe nocodazole- induced spindle aberrations (see 4.2.3). There was no increase in hyperploidy in young mouse oocytes progressing to meiosis II after Mad2 depletion by RNAi (see 4.2.3), possibly due to the existence of multiple feedback controls in healthy, young and largely unstressed oocytes. Therefore, I analysed chromosomal constitution of metaphase II mouse oocytes, which progressed to meiosis II after double depletion of MCAK and Mad2. Unexpectedly, only one of the 58 double-depleted metaphase II oocytes (corresponding to 1.7%) was hyperploid. None of the 59 control-injected oocytes possessed more than 20 metaphase II oocytes. Thus, there was no significant increase in the hyperploidy rate at metaphase II following the knockdown of MCAK and Mad2 in mouse oocytes (Table 4.6, Figure 4.12E). However, the hypoploidy rate (< 20 dyads) was increased from 6.8% in controls to 13.8% in RNAi-exposed oocytes (Table 4.6). So far, it is not possible to determine whether the rise in hypoploidy, also observed in Mad2-depleted, nocodazole-treated oocytes (see 4.2.3), might relate to spreading artefact or a disturbance in chromosome segregation associated with preferential migration of chromosomes to the first polar body due to chromosome lagging.

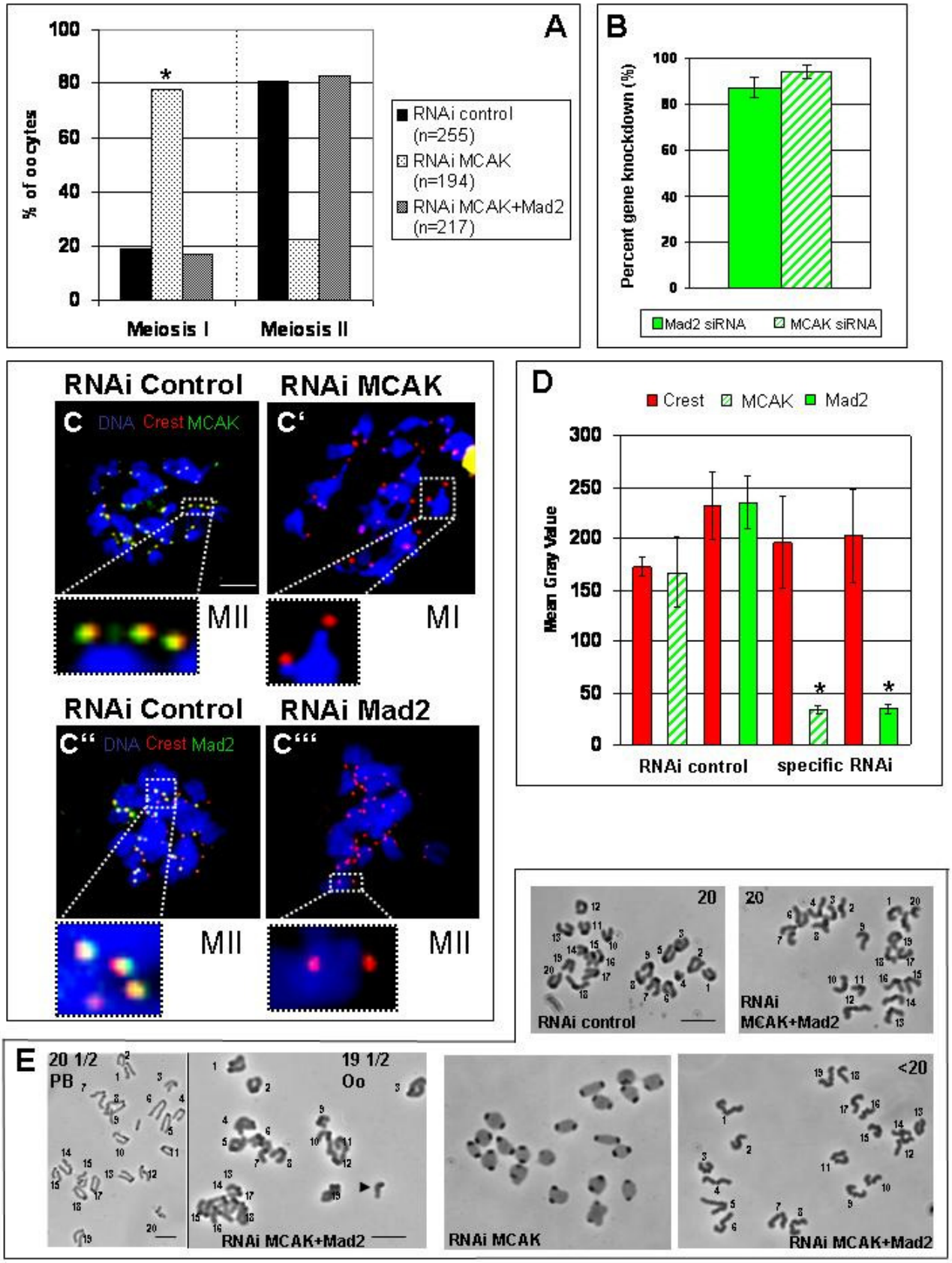
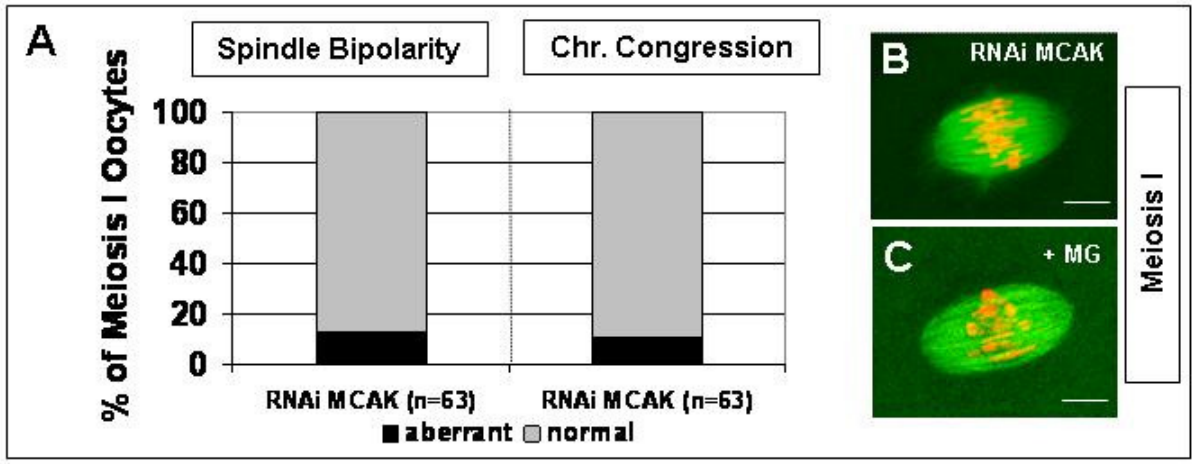


Figure 4.12 Maturation of oocytes following the knockdown of MCAK or double-knockdown of MCAK and Mad2. (**A**) Depletion of MCAK by specific RNAi (RNAi MCAK) leads to a meiosis I arrest, which is released after double-depleting MCAK and Mad2, first

polar body formation and progression to meiosis I; star: significantly different from control ($p<0.001$) (**B)** Oocytes were analysed by real-time RT-PCR for *MCAK* and *Mad2*. Expression ratios of *MCAK* and *Mad2* mRNAs in oocytes injected with specific siRNAs relative to negative control siRNAs were calculated and normalised to *ß-actin* with REST software and converted to percentage of gene knockdown. Mad2 and MCAK expression was significantly reduced following specific RNAi ($p<0.001$). MCAK is present in meiosis II oocytes (RNAi Control, green spots in **C, inset**) but absent in meiosis I arrested, MCAK-depleted oocytes (RNAi MCAK, **C', inset**) cultured for 16 hours following microinjection. Similarly, Mad2 (RNAi Control, green spots in **C'', inset**) is expressed at centromeres of untreated metaphase II oocytes of the control but not at the centromeres of meiosis II oocyte depleted of Mad2 (RNAi Mad2, **C''', inset**). **(D)** Protein expression following RNAi was examined by analysing mean gray values of CREST, MCAK, and Mad2 foci. MCAK and Mad2 were significantly reduced following specific RNAi in contrast to CREST. *: significantly different from control ($p<0.001$). (**E**) Chromosomal constitution of spread, C-banded metaphase II oocytes with our without knockdown of MCAK, or double knockdown of MCAK and Mad2. RNAi control meiosis II oocytes usually possess 20 metaphase chromosomes, but few are also hypoloid (<20 dyads). Oocyte arrested at meiosis I following RNAi MCAK containing 20 homologous chromosomes (bivalents). Many RNAi MCAK+Mad2 oocytes are euploid containing 20 dyads, but few are also hypoploid. One oocyte (Oo) possesses 19 dyads and one chromatid, while the polar body (PB) contains the other chromatid. Chromosome numbering relates to sequential order and not to the karyotypic nomenclature.
Bar in **C-E**:10µm.

Figure 4.13 Spindles and chromosomes following the knockdown of MCAK and Mad2 meiosis I and II. The majority of spindles are bipolar and chromosomes align at the spindle pole in meiosis I-arrested oocytes after knockdown of MCAK (**A,B**) although poles of MCAK-depleted meiosis I oocytes are unfocussed (**B**) in comparison to MG-arrested metaphase I control oocytes (**C**). Metaphase-I arrested oocytes depleted of MCAK (RNAi MCAK) express Mad2 (green spots in **D, inset**) and BubR1 (green spots in **D', inset**) at centromeres. Numbers of meiosis II oocytes with bipolar spindle and aligned chromosomes are decreased in oocytes escaping a meiotic block and progressing to metaphase II after knockdown of Mad2 (**E**, **G**, RNAi Mad2), of MCAK (**E,H**, RNAi MCAK) or of double knockdown of MCAK and Mad2 (**E, I**, RNAi MCAK+Mad2, *: $p<0.005$) in comparison to oocytes injected with unspecific RNAi (**E**, **F**, RNAi control). **B-C and F-I**: Tubulin-immunofluorecent images of spindle, green; propidium iodide stained chromosomes, red. Bar: 10µm.


A
Spindle Bipolarity
Chr. Congression
% of Meiosis I Oocytes
100
80
60
40
20
0
RNAi MCAK (n=63)
RNAi MCAK (n=63)
aberrant
normal
B
RNAi MCAK
C
+ MG
Meiosis I

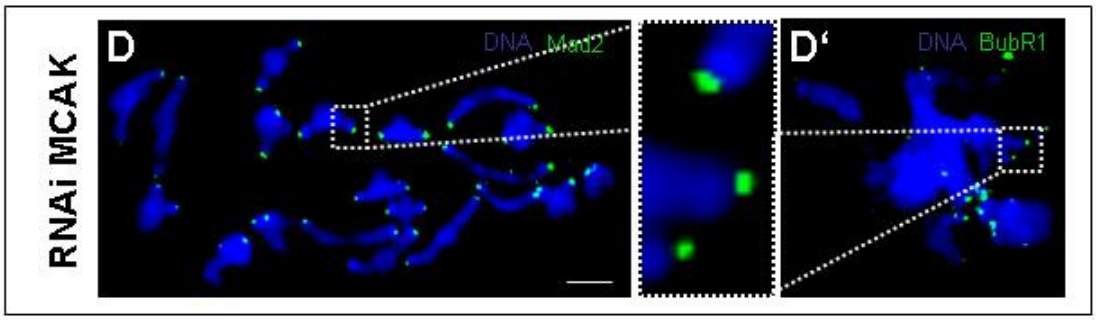

RNAi MCAK
D
DNA Mad2
D'
DNA BubR1

Fig. 4

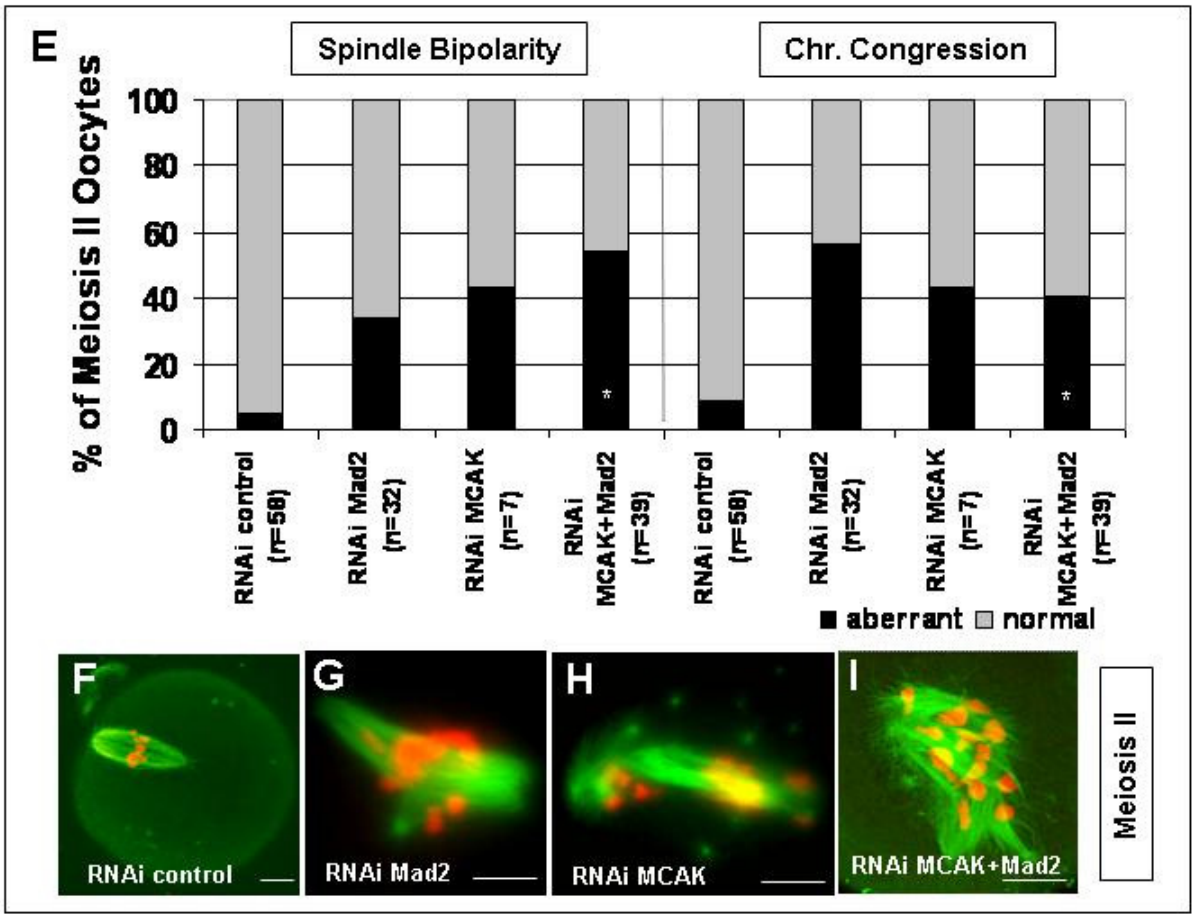

E
Spindle Bipolarity
Chr. Congression
% of Meiosis II Oocytes
100
80
60
40
20
0
RNAi control (n=58)
RNAi Mad2 (n=32)
RNAi MCAK (n=7)
RNAi MCAK+Mad2 (n=39)
RNAi control (n=58)
RNAi Mad2 (n=32)
RNAi MCAK (n=7)
RNAi MCAK+Mad2 (n=39)
aberrant
normal
F
RNAi control
G
RNAi Mad2
H
RNAi MCAK
I
RNAi MCAK+Mad2
Meiosis II

Table 4.6 Chromosomal constitution of spread and C-banded controls and oocytes following microinjection of unspecific and specific siRNAs (Mad2 and MCAK+Mad2) and exposed to 1.5µM ZM after 7 hours of resumption of maturation.

	Nuclear Maturation				Ploidy		Aneuploidy			
	n	GV (%)	GVBD (%)	PB (%)	n*	~40	n**	20 (%)	<20 (%)	>20 (%)
Control (no RNAi)	152	0 (0)	13 (8.6)	139 (91,4)	128	2 (1.6)	126	119 (94.4)	7[b] (5.6)	0 (0)
1.5µM ZM t=7 (no RNAi)	180	2 (1.1)	34 (18.9)	144 (80.0)	125	19[a] (15.2)	104	88 (84.6)	16[b] (15.4)	0 (0)
RNAi control (MCAK+Mad2)	265	10 (3.8)	49 (18.5)	206[a] (77.7)			59	55 (93.2)	4 (6.8)	0 (0)
RNAi Mad2	334	16 (4.8)	72 (21.6)	246 (73.3)			129	114 (88.4)	13 (10.1)	2 (1.6)
RNAi MCAK	212	18 (8.5)	151 (71.2)	43[a] (20.3)			n/d	n/d	n/d	n/d
RNAi MCAK+Mad2	223	6 (2.7)	37 (16.6)	180 (80.7)			58	49 (84.5)	8 (13.8)	1 (1.7)

n*: All GVBD and PB oocytes containing dyads; n**: All PB oocytes with countable dyads; n/d=not determined
Significantly different from control, a: $p<0.001$; b: $p<0.05$

4.6.3 Delay in anaphase I progression and anaphase lagging after chemical inhibition of Aurora kinase by ZM447439 from prometaphase I

We have previously cultured oocytes in presence of the Aurora kinase (AURK) inhibitor ZM447439 (ZM; see 4.4). Treatment with low ZM concentrations (1.5 µM) should preferentially inhibit Aurora kinase B (and possibly also Aurora kinase C) but have little effect on Aurora kinase A activity. Culture of oocytes from the resumption of maturation blocked cytokinesis and caused arrest after GVBD (Vogt et al., 2009). The inhibition of AURKB not only blocked cytokinesis, but additionally appeared to prolong the SAC and those oocytes progressing to anaphase I and cytokinesis tended to emit the first polar body

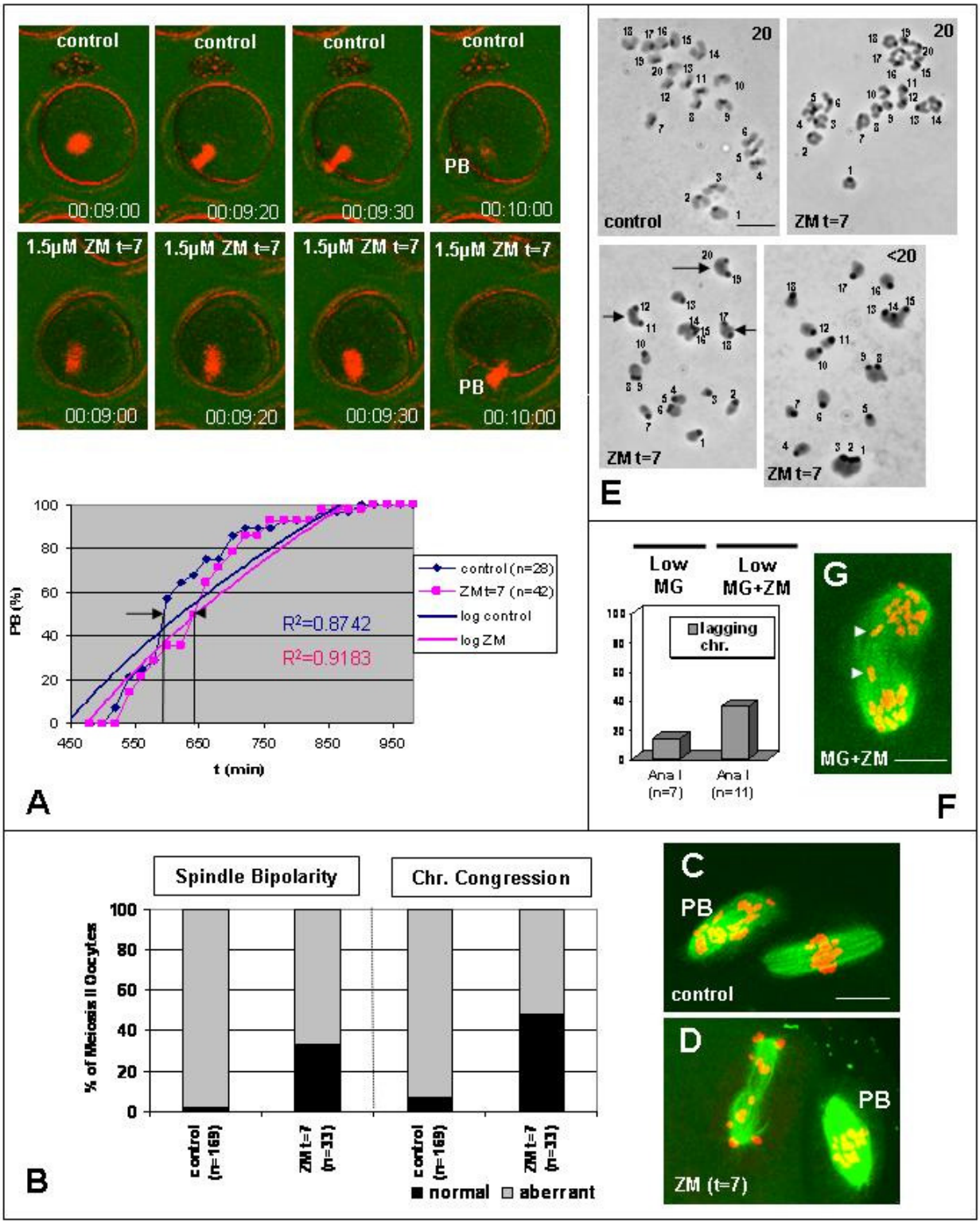
with a delay. Exposure of oocytes did not prevent recruitment of MCAK to centromere domains in meiosis I-arrested mouse oocytes. Since AURKB has so many targets, it is currently unclear whether the delay or meiotic arrest in presence of inhibitor relates to failure in Rec8 cohesin phosphorylation at centromeres and chromosome arms or to failure in MCAK inactivation by AURKB phosphorylation. Therefore, I reasoned that exposure of oocytes to ZM inhibitor at prometaphase I might alter MCAK activity more specifically.

In contrast to oocytes exposed to ZM with the resumption of maturation, addition of the inhibitor at late prometaphase I stage (from 7h of maturation; ZM t=7, Table 4.6) did not prevent first polar body formation, and the majority of oocytes (80%, n=180) matured to metaphase II. About one-third of these failed to align chromosomes at the spindle equator and spindles were aberrant (Figure 4.14B+D). Polarisation microscopy revealed that ZM-exposed oocytes emitted the first polar body with a delay in comparison to controls (Figure 4.14A). 50% of oocytes in the control initiated polar body extrusion by 590min of culture (Figure 4.14A, arrow), whereas only less than 40% of the ZM group underwent cytokinesis by that time. By 640min 50% of the ZM group initiated polar body extrusion (Figure 4.14A, arrowhead) indicating that the timing of anaphase I progression was altered by approximately 30min in response to AURKB inhibition at late stages of meiosis I. Furthermore, when logarithmic plots and functions were generated for each experimental condition, maturation with and without AURK inhibition, the maturation kinetics were well fit by a logarithmic curve (Fig 4.14A). The time when 50% of oocytes progressed to anaphase I and extruded their first polar body differed by approximately 22 min between the control and ZM group. This is consistent with a transient delay of <20 min following the depletion of MCAK in HeLa cells due to defective kinetochore attachments resulting in anaphase lagging (Huang et al., 2007).

To assess the consequences of transient inactivation of AURKB on the chromosomal constitution metaphase II oocytes were analysed for numerical aberrations. From all the oocytes containing metaphase II chromosomes, a significantly higher number in the ZM-group compared to controls contained twice the number of metaphase II and were polyploid (Table 4.6). Oocytes with 40 metaphase II chromosomes did not possess a polar body implying that segregation of homologous chromosome was disturbed by 1.5 µM ZM, and anaphase I progression was uncoupled from cytokinesis. Accordingly, polyploidy was significantly increased from 1.6% in the control to 15.2% in the ZM group, respectively ($p<0.001$; Table 4.6). Analysed PB oocytes exposed to ZM inhibitor from 7 h of maturation had “sticky” chromosomes with arms of chromatids attached to each other (Figure 4.14E). However, most possessed normal chromosome numbers (84.6%, n=104) and there was no increase in hyperploids (> 20 dyads). However, similar to the MCAK and Mad2 knockdown

situation, hypoploidy rate (< 20 dyads) was increased from 5.6% in controls to 15.4% in ZM-exposed oocytes (Table 4.6, Figure 4.14E) suggesting that de-regulation of MCAK activity, either by depletion or loss of down regulation by AURKB, may disturb chromosome segregation at anaphase I and preferential inclusion of chromosomes in the first polar body. Apart from the increase in hypoploidy, ZM inhibitor also interfered with normal loss of chromosome cohesion as there were oocytes with less than 20 dyads possessing bivalents (Figure 4.14E). In order to analyse chromosome behaviour at anaphase I when AURKB was inhibited I matured oocytes for 7 h before continuing culture in the presence of 1.5 µM ZM and 1 mM MG132 or 1 mM MG132 alone and fixed oocytes 2 hours later for tubulin-immunofluoresecence. In order to induce a leaky meiotic arrest, the concentration of MG132 was kept purposefully low and thus allowing some oocytes to progress to anaphase I. While most MG control oocytes showed no signs of lagging chromosomes at anaphase I, the group exposed to both ZM and MG132 inhibitor contained single chromosomes, which were lagging behind (14% versus 36% in the MG control and ZM/MG group, respectively; Figure 4.14F+G).

Figure 4.14 Maturation of oocytes exposed to AURK inhibitor ZM prior to metaphase I. **(A)** Images by polarisation microscopy (OCTAX EyeWear) of spindles in *in vitro* maturing control and ZM-exposed oocytes (**1.5 µM ZM t=7**). Dynamics of anaphase I progression and first polar body formation in 50% of control (arrow, blue dots) and ZM-exposed oocytes (arrow head, pink dots). The line and function was generated by logarithmic curve fitting with Microsoft Excel software. R^2 values for each fit are reported at the right side of the graph. Anaphase I progression is delayed by approximately 22 min following ZM treatment, as represented by 50% of oocytes. (**B-D**) Most control oocytes contain metaphase II spindles that are bipolar with aligned chromosomes (**B,C**), whereas ZM-exposed oocytes show spindle aberrations and unaligned chromosomes (**B,D**). (**E**) Control meiosis II oocytes usually possess 20 metaphase chromosomes, whereas a significant number of ZM-exposed oocytes are hypoploid (<20) and some contain condensed bivalents (arrowheads), bivalents or dyads attached to each other as well as dyads and are therefore aneuploid**.** Chromosome numbering relates to sequential order and not to the karyotypic nomenclature. (**F,G**) ZM-exposed oocytes contained more single chromosomes, which were lagging behind at anaphase I in comparison to controls**.** Tubulin-immunofluorecent images of spindle, green; propidium iodide stained chromosomes, red. Bar in **A:** 50 µm. Bar in **C,D,E,G**: 10 µm.


control
00:09:00
00:09:20
00:09:30
PB
00:10:00
1.5µM ZM t=7
A
PB (%)
t (min)
control (n=28)
ZM t=7 (n=42)
log control
log ZM
R²=0.8742
R²=0.9183
B
Spindle Bipolarity
Chr. Congression
% of Meiosis II Oocytes
control (n=169)
ZM t=7 (n=33)
normal
aberrant
C
control
D
ZM (t=7)
E
20
<20
F
Low MG
Low MG+ZM
lagging chr.
Anal (n=7)
Anal (n=11)
G
MG+ZM

5 Discussion

5.1 Mad2 is localised at kinetochores for proper SAC function during meiosis I

Chromosomes utilise a bipolar spindle for the distribution of the genome crosslinking their kinetochore structures to the emanating plus ends of microtubule arrays in mitosis and meiosis (Rieder and Salmon, 1994; Brunet et al., 1999). The interaction between the kinetochore and spindle MTs is central to the equatorial alignment and the subsequent segregation of chromosomes on the spindle. Following breakdown of the nuclear envelope in mitosis, kinetochores start to interact both laterally and in an end-on fashion with spindle MTs during prometaphase until stable attachments are made between the kinetochore and MTs (Maiato et al., 2004b). By metaphase, all chromosomes become bi-oriented with sister kinetochores exclusively connected to MTs from opposite spindle poles. However, during the progression from prometaphase to metaphase, some chromosomes may be delayed in connecting stably to spindle MTs, while others may be inappropriately attached (merotelic or syntelic) lacking the bipolar tension by pulling forces from MTs of opposite spindle poles (Salmon et al., 2005). To prevent errors in chromosome segregation the metaphase-anaphase transition is guarded by the spindle assembly checkpoint (SAC) to protect cells from precocious progression into anaphase without saturated kinetochore-microtubule attachment in mitosis and meiosis (reviewed by Musacchio and Salmon, 2007; Vogt et al., 2008), which upon anaphase progression may otherwise generate an aneuploid chromosome number in the progeny of somatic or germ cells, potentially causing predisposition to tumour progression and in induction of cancer (Kops et al., 2005; Weaver and Cleveland, 2006; Sotillo et al., 2007; Cimini, 2008), or an abnormal chromosome number in the embryo derived by aneuploid sperm and oocytes leading to spontaneous abortions, pregnancy loss or birth defects in humans (Bond and Chandley, 1983; Pont et al., 2006; Eichenlaub-Ritter et al., 2007a). The SAC halts cell cycle progression in response to attachment and tension defects (Pinsky and Biggins, 2005) and provides in this way time for correcting these defects prior to anaphase progression. Checkpoint proteins accumulate at unattached kinetochores in prometaphase, but become translocated from kinetochores late in mitosis or meiosis (Chen et al., 1996; Chan et al., 1999; Chan et al., 2000; Nicklas et al., 2001). The kinetochores therefore act as the catalytic sites for generating the inhibitory checkpoint signal (Maney et al., 2000). Consistent with the expression of SAC proteins in undisturbed mitotic cells (Chen et al., 1996; Chan et al., 1999; Chan et al., 2000), I demonstrated that Mad2 is localised at

kinetochores of mouse oocytes from early prometaphase I until early metaphase I. In nocodazole-treated mitotic cells with depolymerised MTs, checkpoint components accumulate at each of the unattached kinetochore due to the activation of the SAC (Waters et al., 1998; Chan et al., 1999; Chan et al., 2000). The addition of spindle poisons inhibits the initial steps leading to proteolysis of cylin B and securin blocking in this way the onset of anaphase (Rieder and Maiato, 2004). I also exposed oocytes to nocodazole to examine the sensitivity of the SAC to disrupted kinetochore attachments and found Mad2 at kinetochores of meiosis I-arrested oocytes demonstrating that the kinetochores were indeed not properly attached at that stage. Furthermore, inhibition of anaphase I onset was indicated by the presence of only bivalent chromosomes in arrested oocytes demonstrating that, unlike in mitosis, arm cohesion was maintained during the meiosis I arrest. Thus, not only degradation of securin was inhibited but apparently also the loss of cohesion along chromosome arms and phosphorylation-controlled proteolytic cleavage of meiotic cohesin, like Rec8, remained arrested. In mitosis, resolution of arm cohesion is governed by activity of kinases (Polo-like kinase 1 and Aurora kinase B) and does not require activation of APC/C and protein proteolysis, which is entirely needed for loss of cohesion at centromeres at transition to anaphase (Gimenez-Abian et al., 2004). On a molecular level, the arrest following spindle depolymerisation is characterised by stabilisation of securin and cyclin B due to inhibition of APC/C^{Cdc20} in mitosis and meiosis (Lefebvre et al., 2002; Homer et al., 2005a). The nocodazole-induced meiosis I arrest in mammalian oocytes is reversible because the release of oocytes into nocodazole-free medium leads to anaphase I onset in presence of a bipolar spindle resulting in the correct segregation of homologous chromosomes (Brunet et al., 1999; Wassmann et al., 2003). Thus, the addition of drugs altering spindle dynamics activates/prolongs the SAC in response to unaligned or unattached chromosomes leading to the accumulation of Mad2 at improperly attached kinetochores and arrest at meiosis I (Wassmann et al., 2003; Shen et al., 2005; Homer et al., 2005a; Eichenlaub-Ritter et al., 2007b).

At odds with my findings, which suggest a rather rigid checkpoint control in healthy, young oocytes, prolonged exposure of mouse oocytes to nanomolar concentrations of nocodazole (400 nM) leads to 40-60% polar body extrusion after a transient delay, which was interpreted to that a leaky checkpoint operates in mammalian oocytes (Wassmann et al., 2003). Whereas micromolar concentrations of nocodazole depolymerise the spindle completely and the oocyte responds with a robust SAC-mediated meiosis I arrest lasting over 18 hours (Homer et al., 2005a), I believe that nanomolar concentrations of the microtubule-depolymerising drug nocodazole (100 nM) are sufficient to mount a robust SAC response in meiosis I when

exposed chronically throughout the resumption of maturation, as shown previously (Shen et al., 2005). This may be different when exposure occurs only hours after GVBD when a bipolar spindle has already assembled (Wassmann et al., 2003) and when some critical stages sensitive to checkpoint delay/arrest have already been passed. Exposing GV-staged oocytes to concentrations of nocodazole as low as 30-40 nM induces a transient meiotic arrest after the resumption of maturation (Shen et al., 2005), reminiscent of the arrest described by Wassmann et al. (2003). Brunet et al. (1999) demonstrated that incubations in nocodazole for 2 or 3 hours do not induce a delay in polar body extrusion, whereas longer incubations of 4 to 6 hours result in significant delays. Furthermore, spindle destruction before 5 hours after GVBD does not delay polar body extrusion suggesting the following: 1) a transient arrest prior to polar body extrusion depends on the time of nocodazole exposure; 2) spindle assembly does not determine the duration of meiosis I since the minimum time needed for the formation of a functional spindle and polar body extrusion after spindle disruption by nocodazole is 2.5 hours; 3) the delays after nocodazole are likely under the control of stable kinetochore-microtubule attachments, which are set up late in meiosis I possibly influencing the sensitivity and functionality of checkpoint control. While the transient arrest in low doses of nocodazole does not protect such oocytes from chromosome missegregation (Shen et al., 2005) or rectifies congression defects at metaphase II (Wassmann et al., 2003), it appears that the SAC is susceptible to mitotic slippage (Elhajouji et al., 1998; Rieder and Maiato, 2004) when faced with certain types of defects. It is known that high concentrations of nocodazole not only depolymerise spindle MTs, but also affect the structure of the kinetochore producing defects in kinetochore-microtubule attachment/tension. Therefore, the complete absence of attachment and tension resulting from high doses of nocodazole generates a cumulative stronger SAC response and a more robust meiosis I arrest than low doses mainly causing alterations in polymerisation dynamics (Homer et al., 2005a). In presence of low doses of nocodazole, which preserve the spindle sufficiently well, MTs may still be capable of anchoring at kinetochores, but in such a fashion that one single kinetochore of a sister chromatid is attached to MTs extending from both poles (merotelic). Since merotelically attached chromosomes may align on the spindle (even when not assembling at the equatorial plate), tension can be generated on such chromosomal configurations, from which it was suggested that they may escape checkpoint control under these conditions (Cimini et al., 2001). Activity of proteins like Aurora kinase B and MCAK can possibly rescue cells with merotelic attachments even in anaphase of mitosis (Cimini, 2007) and appears essential for monotelic attachment of homologues in meiosis (Hauf et al.,2007). When examined in detail different checkpoint proteins are recruited successively in meiosis as has been demonstrated

in spermatocytes (Parra et al., 2009). Furthermore, certain SAC proteins are removed after microtubule attachment and formation of structurally normal kinetochore MTs (Chen et al., 1996; Waters et al., 1998). For example in mitosis, the amount of Mad2 becomes highly reduced at metaphase kintochores and checkpoint inactivation compared with unattached prometaphase kinetochores. Because Mad2 accumulates at unattached kinetochores and is removed on attachment, their kinetochore localisation is interpreted to signify a lack of attachment in mitosis (Waters et al., 1998; Skoufias et al., 2001). In contrast to mitotic cells, where loss of Mad2 staining is correlated with initial microtubule attachment, loss of Mad2 staining in plant meiosis appears to be tension-dependent (Yu et al., 1999). In mouse oocytes, the Mad2-derived immunofluorescent signal begins to progressively disappear from chromosomes some hours before anaphase I, with no Mad2 detectable around polar body extrusion (Wassmann et al., 2003). I did not analyse the level of Mad2 quantitatively during the course of meiosis I, but observed a substantial staining for Mad2 at kinetochores until late prometaphase I/early metaphase I. Since stable microtubule-kinetochore attachments are only formed late in meiosis I in mouse oocytes to allow the final alignment of chromosomes on the metaphase plate (Brunet et al., 1999), Mad2 signals at kinetochores would not be expected to decline significantly hours before anaphase I. Consistent with the notion of stable microtubule-kinetochore attachment in the generation of pulling tension forces and SAC inactivation, I found that Mad2 appears no longer present at kinetochores of mouse oocytes, which have entered anaphase I and are about to extrude a polar body (data not shown). This is consistent with a model whereby Mad2 is efficiently transported away from the centromeres towards the centrosomes/spindle poles via dynein/dynactin/dynein light chain once firm attachment and saturation of centromeres with MTs has occurred (Sivaram et al., 2009).

5.2 Loss of Mad2 function does not predispose mammalian oocytes to aneuploidy

Depletion of Mad2 during meiosis I in yeast results in chromosome missegregation (Shonn et al., 2000; Bernard et al., 2001) indicating that meiosis I possesses a checkpoint. Knockdown of Mad2 in mouse oocytes using morpholino oliogonucleotides produces a significant increase in the rate of chromosome non-disjunction (~32%), which has been taken as an indicator that Mad2 is required for normal segregation of homologous chromosome pairs (Shonn et al., 2000; Homer et al., 2005b). In that study the authors display the total incidence of aneuploidy, i.e. both hyper- and hypoploidy are used in the calculation. In fact, the observed percentage of hyperploidy was merely 16.3% (Homer et al., 2005b). Unlike these

reports I did not detect a significant increase in the number of aneuploid/hyperploid oocytes following the knockdown of Mad2 using siRNAs, although the numbers of hypoploids were increased in comparison to solvent control (but not unspecific RNAi). Rather, my data indicate that knockdown of Mad2 interferes with spindle formation and chromosome congression at meiosis II, similar to a previous RNAi study in mouse oocytes (Wang et al., 2007). Likewise, a dominant negative hMad2 has no effect on the metaphase I-anaphase I transition in mouse oocytes in the absence of nocodazole (Wassmann et al., 2003). Even though my findings and the findings by others (Wassmann et al., 2003; Wang et al., 2007) appear at odds with the study by Homer et al. (2005b), there is evidence that hyperploidy is influenced by PMS (pregnant mare serum) hormone priming (Geert Michel, unpublished results), which is routinely applied to increase the yield of large oocytes resuming maturation by many scientists in reproductive biology. Whereas Homer et al. (2005b) hormonally stimulated mice with PMS, I obtained oocytes from young, healthy mice at the diestrous stage corresponding to unstimulated, spontaneous cycles to affect oocyte quality as little as possible prior to resumption of maturation. However, oocytes from stimulated cycles compared to spontaneous cycles have been shown to contain more hyperploids suggesting a link between hormonal homeostasis prior to resumption of maturation and fidelity of chromosome segregation in oocytes (Geert Michel, unpublished results). Assuming that handling of the oocyte may affect oocyte quality in addition to the hormonal stimulus, thus making the oocyte per se susceptible to aneuploidy, would provide one explanation why Homer et al. (2005b) observed the rise in non-disjunction following Mad2 knockdown by morpholino-oligonucleotides. One would expect that a disturbance in the absence of functional SAC has immediate effects while a mere disruption of the SAC may not be initially critical. Even studies showing that chromosome segregation returns to normal by restoring the SAC by re-expression of Mad2 in morpholino-oligonucleotide injected oocytes would be thus not very convincing since the restitution of controls by the SAC may overcome slight disturbances by handling/hormonal stimulation or the morpholino-oligonucleotides per se. It cannot be excluded that increased aneuploidy in Mad2 heterozygous knockout mice (Niault et al., 2007) might also be related to errors in chromosome segregation and aneuploidy/apoptosis of the rapidly dividing granulosa cells during folliculogenesis, which in turn indirectly affects oocyte quality and susceptibility to meiotic errors.

Previous studies in mouse oocytes to analyse the function of Mad2 were conducted in the absence of MT inhibitors (Wassmann et al., 2003; Homer et al., 2005b; Wang et al., 2007). Overexpression of hMad2 produces a meiosis I arrest in the absence of nocodazole (Wassmann et al., 2003; Homer et al., 2005b), whereas depletion of Mad2 or a dominant-

negative hMad2 advances the meiosis I-to-meiosis II progression without affecting meiotic progression to metaphase II (Wassmann et al., 2003; Homer et al., 2005b; Wang et al., 2007), which is consistent with my findings. However, meiotic progression is accelerated by ~2 hours in mouse oocytes following depletion of Mad2 with morpholinos (Homer et al., 2005b), which is similar to mitosis (Meraldi et al., 2004) reflecting overlapping functions for Mad2 in timing of anaphase progression in female meiosis I and mitosis. Although I did not analyse cell cycle kinetics in Mad2-depleted oocytes during meiosis I quantitatively, it appeared that oocytes emitted a polar body precociously when injected with specific siRNA (data not shown). Whether the loss of timing function is independent of Mad2's classic role in the SAC or the consequence of a comprised SAC is still unclear. However, studies from mouse models indicate that meiotic progression is likely accelerated in the face of a compromised SAC. Oocytes from *MAD2*$^{+/-}$ mice (Niault et al., 2007), which are viable in contrast to its *MAD2*$^{-/-}$ counterparts (Dobles et al., 2000), extrude their polar body on average 33 minutes earlier than wild type oocytes. Importantly, *MAD2*$^{+/-}$ oocytes are aneuploid at elevated rates (22.5%) due to chromosome missegration in meiosis I. The large discrepancy in the time of meiotic acceleration (2 hours vs. 33 minutes) may reflect the degree of efficiency of the knockdown, depending on the reverse-genetic approach, and therefore also the susceptibility of the oocyte to aneuploidy. Utilising morpholino oliogonucleotides, the level of Mad2 protein depletion was at least by 85% as determined by Western blotting (Homer et al., 2005b) compared to at least 83% in this study using siRNAs as determined by immunofluorescence, whereas no information is given on the protein level in *MAD2*$^{+/-}$ mice (Niault et al., 2007). Whereas morpholinos sterically block translation initiation by complementary binding in the region of the AUG translation start site, the likelihood of an efficient knockdown is high due to the restricted region of mRNA and the high affinity of morpholinos for mRNA. In contrast, the efficiency of the most potent siRNA duplex has to be determined in a pre-screen due to the importance of the target site (Amanai et al., 2006). Alternatively, multiple siRNAs targeting the same mRNA could be introduced into the oocyte to enhance the efficiency of RNAi. Long double stranded RNAs (dsRNA), following cleavage by the Dicer endoribonuclease, generate a variety of siRNAs with a greater chance of producing an efficient knockdown than a single siRNA duplex. Mouse oocytes have been shown to possess the machinery for RNAi-mediated gene suppression using long dsRNAs (Svoboda et al., 2000; Wianny and Zernicka-Goetz, 2000). A very sophisticated approach was taken by Baker et al. (2006) employing transgenic mice to investigate the role of the checkpoint component BubR1. They generated mice that reduced the expression of BubR1 in a graded fashion from normal levels to zero demonstrating a graded response to differing degrees of loss of BubR1 function. It may be

that the same holds true for Mad2 with evidence coming from mitosis. Following 30% reduction of Mad2 levels in HeLa cells, spindle dynamics and cell cycle progression is unperturbed (Michel et al., 2001). Following 90% Mad2 depletion, however, spindle assembly and chromosome condensation are grossly perturbed and mitosis is accelerated by 40% (Michel et al., 2004). In fact, like in mitosis, mouse oocytes appear to demonstrate a graded response to threshold amounts of Mad2. Whereas 85% depletion of Mad2 advances the onset of cyclin B and securin destruction (Homer et al., 2005b), no difference in securin levels between control and *MAD2*$^{+/-}$ oocytes is observed (Niault et al., 2007) suggesting that ≥ 85% Mad2 reduction deregulates the cell cycle in prometaphase and metaphase of meiosis I, whereas ≤ 85% leads to a general acceleration. It cannot be excluded that my siRNA duplex produced a less efficient knockdown than the morpholino-based approach despite the significant reductions on the transcript and protein level, as determined by quantitative real-time RT-PCR and immunofluorescence, respectively. Nevertheless, I conclude from my data that young, healthy mouse oocytes are capable of normal chromosome segregation in the absence of an essential SAC component (Mad2) and in the face of an accelerated cell cycle at meiosis I provided that oocytes are obtained from unstimulated mice and handled gently.

Interestingly, a permissive checkpoint has been proposed to operate in mammalian oocytes of advanced maternal age coming from evidence that aged human oocytes contain reduced transcript levels of the checkpoint genes *Mad2* and *Bub1* (Steuerwald et al., 2001) and that oocytes from aged CBA/Ca mice progress faster to anaphase I than oocytes from young mice as well as being significantly higher aneuploid (Eichenlaub-Ritter and Boll, 1989). CBA/Ca females have a small primordial follicle pool that is depleted at the end of the reproductive span, similar to the human, making it a suitable model for studying maternal age-effects (Eichenlaub-Ritter, 1998). It is tempting to speculate that declining oocyte SAC function, which does not delay anaphase I onset effectively and concomitantly accelerates progression through meiosis I, could contribute to the observed rise in aneuploidy as women get older. A recent study tested this hypothesis using a different mouse strain as a model of natural reproductive ageing (Duncan et al., 2009). Aneuploidy levels were higher in old oocytes compared to young oocytes (Duncan et al., 2009), consistent with earlier reports (Zuccotti et al., 1998; Pan et al., 2008). Following individual oocytes, rather than cohorts of oocytes (Eichenlaub-Ritter and Boll, 1989), through meiosis I by time-lapse microscopy revealed that, in contrast to oocytes from 9-10 month old CBA/Ca mice (Eichenlaub-Ritter and Boll, 1989), the duration of meiosis I was similar in young and old oocytes indicating that SAC deterioration does not correlate with premature anaphase I entry and may not be the primary cause of aneuploidy with increased age (Duncan et al., 2009). On the other hand, an increase

in aneuploidy has been discussed as the major factor responsible for the increase in infertility with advancing age in human females (Hassold and Hunt, 2001; Pellestor et al., 2005) suggesting a possible correlation between SAC failure and an accumulation of early ageing-related phenotypes (Baker et al., 2005). The kinds of chromosome segregation defects seen in oocytes of mutant mice expressing low levels of BubR1 imply that to date only the progressive decline of BubR1 activity might play a causal role in age-related female sterility (Baker et al., 2006). The rise of infertile *MAD2*$^{+/-}$ female mice was suggested to be due to chromosome missegregation (Niault et al., 2007). However, in contrast to BubR1-deficient mice, which develop typical age-related phenotypes next to infertility and the accumulation of aneuploid cells (Baker et al., 2004; Baker et al., 2006), no further age-related phenotypes were described in *Mad2*$^{+/-}$ mice. Therefore, it is only speculation that loss of Mad2 function besides BubR1 might be involved in the decline of fertility with age. There is evidence in humans and in the mouse that physiological ageing of the ovary associated with depletion of follicle pool rather than chronological age of the female may relate to aneuploidy in oocytes (Brook et al., 1984; Eichenlaub-Ritter et al., 1988), which would explain the correlation between the decline of the ovarian reserve associated with reduced follicles and the increased risk for trisomy (Freeman et al., 2000). Instead, the decline in fertility may relate to post-meiotic factors, such as a disturbed hormonal feedback or mitochondrial dysfunction (Kevenaar et al., 2006; Trifunovic and Larsson, 2008). Rather, there is possibly a link between acceleration in meiotic progression, reduced expression of SAC components and aneuploidy in oocytes with advanced female age, though not in a linear fashion, i.e. that other factors likely contribute to the induction of aneuploidy such as genetic background and threshold levels of checkpoint proteins. Unlike shown for Mad2 depleted oocytes, heterozygosity for *Bub1*, another gene and gene product involved in cell cycle and chromosome segregation in meiosis, causes increases in aneuploidy involving precocious chromatid separation (Leland et al., 2009), while morpholino-based depletion of Mad2 involves homologue segregation errors (Homer et al., 2005b). Since both chromosome segregation errors and precocious chromatid separation are observed in aged human oocytes, alterations in expression of several rather than a single gene may be responsible for maternal age-related non-disjunction in the human.

5.3 Loss of Mad2 function makes mammalian oocytes highly susceptible to aneuploidy when exposed to spindle poisons

One model that is proposed to explain how age-associated increases in aneuploidy could occur, is the “two-hit” model in which the first hit occurs during fetal development when the

oocytes are entering meiosis and undergoing recombination (Lamb et al., 1996). This first "hit" would generate a bivalent that is at risk for giving rise to aneuploidy, thus being age-independent. The second "hit" would occur in the aged ovary, likely during oocyte development and/or maturation, resulting in an oocyte that improperly processes the susceptible bivalent. This would be age-dependent and could be a consequence of hormonal imbalances, reduced peri-follicular microcirculation, dysfunctional mitochondria, reduced oxygen supply or reduced chromosome cohesion (Eichenlaub-Ritter et al., 2004; Pellestor et al., 2005). Furthermore, the formation of normal spindles derived from old females appears compromised (Battaglia et al., 1996; Volarcik et al., 1998). Such spindles may favour missegregation of chromosomes with susceptible configurations consistent with the observation that chromosomes fail to congress to the MI plate in oocytes from old females (Volarcik et al., 1998). Another view of the concept of a "second hit" is that the quality of the oocyte, including the transcriptome, is compromised in old eggs. In line with this view, transcript levels of *Mad2* and *Bub1* are reduced in metaphase II-arrested human oocytes from aged women (Steuerwald et al., 2001). In accordance, studies in aged oocytes of the mouse are consistent that checkpoint mRNAs are also less abundant (Hamatani et al., 2004; Pan et al., 2008). Therefore, checkpoints may be rather permissive in oogenesis, and, possibly, also early embryogenesis (Harrison et al., 2000). Whether loss of checkpoint control is a general and/or the most important 'hit' in induction of aneuploidy in old oocytes needs to be further explored as recent studies in oocytes of one mouse strain did not find a correlation between timing of anaphase I progression, maternal age and errors in chromosome segregation (Duncan et al., 2009). However, this study observed oocytes from hormonally primed animals which might obliterate the intrinsic differences in cell cycle regulation at maturation *in vivo*.

Our finding that solely the loss of Mad2 function does not predispose mammalian oocytes to aneuploidy makes the following assumptions: 1) oocytes are obtained from young, healthy mice which have not been stimulated by hormonal priming, 2) oocytes are handled gently during culturing, 3) oocytes are cultured in the absence of MT inhibitors. It appears that simply depleting a checkpoint component like Mad2 does not lead to a significant increase in non-disjunction or, to argue along the line of the "two-hit " model, that the introduction of one artificial "second hit" is not strong enough to deregulate chromosome segregation in young mouse oocytes, as opposed to aged ones. Therefore I tested the hypothesis that loss of Mad2 function and consequently a compromised SAC would make mammalian oocytes more susceptible to non-disjunction when possessing aberrant spindles, a condition reminiscent of aged oocytes. Spindle poisons have been utilised on numerous occasions to delay or arrest meiosis I in mouse oocytes. Both short-term and long-term spindle depolymerisation induced

a meiosis I arrest due to unaligned chromosomes (Soewarto et al., 1995; Brunet et al., 1999; Lefebvre et al., 2002; Homer et al., 2005a) suggesting that the arrest was mediated by the SAC to protect oocytes from chromosome missegregation. I could demonstrate the requirement for Mad2 in maintaining the meiosis I arrest following the depletion of Mad2 using siRNAs in presence of the spindle poison nocodazole, as ~ 18% of oocytes undergo polar body extrusion, which is similar to the data (15%) of a previous study (Homer et al., 2005a). Oocytes which progress to metaphase II under such disturbed circumstances are highly aneuploid, both in hyper- and hypoploidy, reaching 65% of numerical aberrations. With keeping in mind that chromosome identity has not been analysed, even some of the oocytes with 20 dyads may be aneuploid in addition. Treating oocytes from *Mad2*$^{+/-}$ mice with a pulse of nocodazole also leads to a significant rise in aneuploidy (Niault et al., 2007) indicating that SAC control is severely impaired when oocytes lack full Mad2 function in the face of spindle disturbances (possible third "hit"). Mammalian oocytes are then no longer protected from aneuploidy. Aged oocytes are likely confronted with multiple defects, such as susceptible chromosome configurations, aberrant spindles, hormonal imbalances, reduced oxygen supply, reduced chromosome cohesion or reduced expression of key components as consequence of ageing and genetic background (Warren and Gorringe, 2006). My results provide experimental evidence that more than one hit is needed to deregulate chromosome segregation in mammalian oocytes. In particular, a reduction in Mad2 expression together with spindle aberrations puts mammalian oocytes at high risk for aneuploidy.

5.4 Aurora kinase B promotes bipolar attachment

During mitosis, kinetochores encounter MTs by chance and attachment errors are common in mitosis (Maiato et al., 2004a; Salmon et al., 2005). The SAC detects kinetochores on mono-oriented chromosomes that are either unattached or have syntelic attachment (Figure 5.1), delaying activation of the APC/C and therefore the onset of anaphase (reviewed by Musacchio and Salmon, 2007; Vogt et al., 2008). The checkpoint is sensitive to the level of kinetochore occupancy by MTs. There is still debate about the precise regulation of the checkpoint exerted by spindle microtubule tension force on kinetochores (Pinsky and Biggins, 2005; Yang et al., 2009), in part because the tension generated by bi-orientation stabilises microtubule attachment (Nicklas et al., 2001). The activation of the SAC in the presence of syntelic attachments reflects the fact, though, that syntelic attachments are unable to generate tension between sister kinetochores. Other studies suggest that transient creation of

monopolar attachments and generation of “free” kinetochores upon correction of wrong attachments may be necessary to prolong the SAC (Yang et al., 2009). Merotelic attachments arise in mitosis when the kinetochore of one sister chromatid is attached to both poles (Figure 5.1), which puts the bi-oriented chromosome under tension despite incorrectly attached kinetochores (Salmon et al., 2005). Merotelic kinetochores are a major source of aneuploidy in mammalian tissue cells and differ from other attachment errors in that they are not sensed by the SAC (Cimini et al., 2001; Cimini, 2008). As a result, cells with merotelic kinetochores are not delayed in metaphase by the SAC and enter anaphase with timing similar to controls (Cimini et al., 2002). For such cells activity of the depolymerase MCAK and corrections of merotelic attachments on lagging chromosomes may be essential to prevent mitotic errors (Cimini et al., 2002). Promoting the accumulation of merotelic connections by inhibiting aurora kinase B (AURKB) with ZM447439 does not arrest mitotic culture cells (Ditchfield et al., 2003; Cimini et al., 2006). However, it delays anaphase or the mean rate of anaphase chromosome movement (Huang et al., 2007; Wordeman et al., 2007) consistent with our observations in ZM-treated mouse oocytes (Figure 4.14A). Generally, bi-oriented chromosomes differ from syntelic chromosomes in the level of tension, as determined by their interkinetochore distance, which can be shown by utilising markers of the centromere such as the CREST antibody in fixed preparations. Importantly, a study in budding yeast indicated that a circular unreplicated chromosome carrying two single kinetochores widely separated from each other preferentially establishes bipolar attachment stressing the importance of tension sensing to ensure proper alignment (Dewar et al., 2004). Therefore, any physical connection, which supports the development of tension and can be sensed by the kinetochore, would facilitate bi-orientation. Normally, as bi-oriented chromosomes are facing opposite spindle poles at metaphase, the centromere is stretched by microtubule pulling tension forces, resulting in an increase in sister centromere separation and stabilisation of microtubule attachments at the kinetochore. In contrast, erroneous attachments result in a decline of sister centromere separation exhibiting decreased tension. In this way, tension discriminates between bi-oriented and syntelic attachments. Hence, unattached kinetochores have a high concentration of checkpoint proteins, which becomes reduced as these kinetochores become saturated with MTs (Waters et al., 1998; Skoufias et al., 2001). On the other hand, the concentration of Mad2 is reduced to the same low level at merotelic kinetochores in anaphase as in bi-oriented kinetochores on late metaphase aligned chromosomes suggesting that merotelic kinetochores have bound a similar number of MTs with normal tension forces (Cimini et al., 2001) and are therefore not detected by the SAC.

Unlike mitosis, the sister kinetochores of each homologue must orient to the same pole (monopolar orientation) in meiosis I and therefore need to maintain cohesion at sister centromeres (reviewed by Lee and Orr-Weaver, 2001; Ishiguro and Watanabe, 2007). In that conformation, sister kinetochores are not under tension by pulling of sister chromatids to opposite poles and the two closely associated sister kinetochores of each homologue can be visualised as a single unit (Figure 4.11B') such as in *Drosophila* and mouse (Goldstein, 1981; Parra et al., 2004). Electron microscopy in mouse spermatocytes revealed that both sister kinetochores indeed attach to MTs excluding the possibility that inactivation of one sister kinetochore results in monopolar attachment (Parra et al., 2004). Monopolar attachment in fission yeast depends on both the meiosis-specific cohesin Rec8 and the meiosis-specific protein Moa1 (Watanabe and Nurse, 1999; Yokobayashi and Watanabe, 2005), whereas in budding yeast a different set of proteins, called monopolins, is required (Toth et al., 2000). Lack of Rec8 leads to bipolar attachments of sister kinetochores and equational, rather than reductional division at meiosis I in yeast (Yokobayashi et al., 2003). Mutations in Rec8 homologues in maize and *Arabidopsis* cause similar equational division at meiosis I suggesting that the mechanism is conserved in plants (Yu and Dawe, 2000; Chelysheva et al., 2005). Importantly, Rec8 is a highly conserved meiotic cohesin protein, which contributes to centromeric cohesion throughout meiosis I until metaphase II (Revenkova and Jessberger, 2005). Proteolysis at sister chromatid arms at anaphase I appears to be an essential step in chiasma resolution and homologue separation (Kudo et al., 2006; Lee et al., 2006). Centromeric Rec8 is protected by shugoshin from cleavage by separase (Watanabe and Kitajima, 2005), to ensure reductional division of homologous chromosomes during meiosis I as demonstrated in yeast and mouse (Watanabe and Nurse, 1999; Lee et al., 2006). There are two shugoshin-like proteins, Sgo1 and Sgo2 (Kitajima et al., 2004). Depletion of shugoshins in mouse oocytes did not affect alignment of bivalents at metaphase I indicating that shugoshins are largely dispensable for monopolar attachment of homologues in mammalian oocytes (Lee et al., 2008). However, misalignment of chromosomes increased at metaphase II presumably due to the failure of correcting defective attachments in meiosis I. Whereas the misalignment was modest in Sgo1-depleted oocytes (~40%), Sgo-2 depleted oocytes showed a high frequency in disordered chromosome misalignment (100%) in addition to separated single chromatids demonstrating that Sgo2 alone plays a predominant role in protecting centromeric cohesion in meiosis I in oocytes (Lee et al., 2008). Sgo1, on the other hand, is mostly dispensable for this function and might be involved in promoting bipolar attachment coming from evidence that budding yeast Sgo1 is required for sensing loss of tension in mitosis (Indjeian et al., 2005) and *Xenopus* Sgo1 was identified as a factor to bind MTs (Salic

et al., 2004). Accordingly, one model proposes that shugoshin might bind at the connection between MT and kinetochore, and thereby functions as a mechanical sensor of tension to centromeres (Indjeian et al., 2005). Surprisingly, fission yeast Sgo2 is also required for promoting tension-generating bipolar attachment of kinetochores in mitosis and meiosis by localising the Aurora kinase complex to centromeres (Kawashima et al., 2007). Aurora- and Sgo2-deficient yeast cells show similar chromosome segregation defects at anaphase I, like lagging chromosomes and non-disjunction of homologues. Accordingly, centromeric localisation of Aurora kinase at meiosis I was reduced in Sgo2-depleted cells demonstrating the specific interaction of Sgo2 and Aurora kinase, which is required for bipolar attachment in yeast meiosis (Kawashima et al., 2007). AURKB plays an essential role at centromeres in destabilising erroneous attachments, where it is enriched at merotelic kinetochores (Knowlton et al., 2006), thereby promoting bipolar connections (Tanaka et al., 2002; Pinsky et al., 2006; Cimini et al., 2006). The destabilisation of attachment by AURKB would create unattached kinetochores, which simultaneously activates the SAC (Pinsky et al., 2006). Hence, in Aurora-deficient yeast cells, erroneous merotelic attachments are prematurely stabilised with the checkpoint being silenced, resulting in missegregation of chromosomes (Hauf et al., 2007; Kawashima et al., 2007). My results support the observations in yeast, and as most previously shown also in mouse oocytes by another study (Shuda et al., 2009). Inhibiting AURKB with low concentrations of ZM447439 at late prometaphase I did not affect progression to meiosis II. However, there was an increase of misaligned chromosomes and aberrant spindles at metaphase II, likely due to failure of destabilising erroneous attachments in meiosis I. The presence of lagging chromosomes at anaphase I and the rise in hypoploidy at metaphase II following AURKB inhibition further imply that proper establishment of bipolarity was likely disturbed by merotelic attachments in meiosis I. These are not sensed by the SAC (Cimini et al., 2001; Cimini, 2008) eventually releasing cells from checkpoint control. The requirement of AURKB for regulating bipolar chromosome alignment during meiosis I in mouse oocytes was elegantly demonstrated recently (Shuda et al., 2009). Overexpression of AURKB, but not of the other two aurora kinases, rescued the chromosome alignment defect in ZM-treated oocytes. The authors also confirmed our assumption that low concentrations of the inhibitor specifically affect Aurora kinas B activity (Shuda et al., 2009).

Even though the monopolar orientation is not disrupted in fission yeast depleted of Aurora kinase due to still functional Rec8 and Moa1, sister kinetochores on one homologue become attached merotelically, which gives rise to non-disjunction of homologues in the absence of error correction (Hauf et al., 2007). Accordingly, it is proposed that Aurora kinase controls sister kinetochore mono-orientation by preventing merotelic attachments, which in turn

ensures the bi-orientation of homologous chromosomes in meiosis I. As attachment of both sister kineotochores to MTs from one pole at meiosis I is also observed in the mouse (Parra et al., 2004), the mechanism proposed in yeast may also be functional in mammals. Even though AURKB inhibition does not prevent the kinase from localising to centromeres in the present study (data not shown), the lack of kinase activity may affect the spatio-temporal shifts in localisation and/or activites of its substrates. The relocation of shugoshins, which are also *in vitro* substrates of AURKB (Pouwels et al., 2007), could be affected in response to loss of AURKB-dependent phosphorylation. The misalignment phenotypes at metaphase II in Sgo1- and Sgo2-depleted oocytes (Lee et al., 2008) resemble those of AURKB-inhibited oocytes (Figure 4.14D). Whereas separated single chromatids were prevalent in Sgo2-depleted oocytes, Sgo1-depleted oocytes exhibited largely intact cohesion of sister chromatids similar to our findings in ZM-treated oocytes, which possess dyads but not chromatids (monads) consistent with retained cohesion at sister chromatids' centromeres. It appears, however, that AURKB inhibition might cause a loosening of chromatin, e.g. by preventing condensin phosphorylation (data not shown). AURKB-mediated phosphorylation may be important for the timely relocation of shugoshins in response to tension, but not prior to metaphase II. Otherwise, precocious relocation of shugoshins would no longer protect Rec8 from phosphorylation, which is essential for centromeric cohesion and monopolar attachment of bivalent chromosomes in meiosis I. Given that lagging chromosomes originate from merotelic attachment and AURKB is involved in the correction and control of monopolar orientation, AURKB-deficient cells are unable to correct merotely, which in consequence alters the monopolar attachment of sister kinetochores to promote bi-orientation of bivalent chromosome in meiosis I.

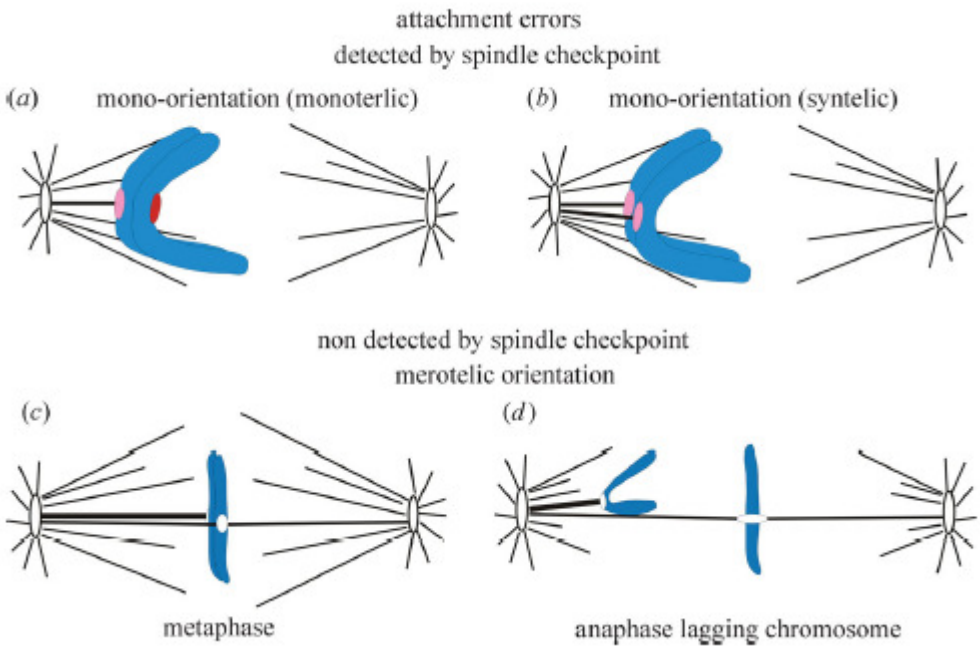


Figure 5.1 Attachment errors of kinetochores to spindle microtubules during mitosis. See text for details. Image adopted from Salmon et al. (2005).

5.5 Role of Aurora kinase B in chiasma resolution and depolymerisation of microtubules in the midbody

The majority of oocytes failed to progress to metaphase II and did not extrude a polar body when exposed to the Aurora kinase inhibitor ZM447439 (ZM) from the resumption of maturation (Vogt et al., 2009), confirming three other studies in mouse oocytes which also used the inhibitor (Wang et al., 2006a; Swain et al., 2008; Shuda et al., 2009). Oocytes arrested at meiosis I showing aberrant spindles, unaligned bivalent chromosomes, and the checkpoint protein BubR1 expressed at kinetochores, indicating a prolonged SAC response (Vogt et al., 2009). Alexandra Kipp already observed in her diploma thesis that some of the ZM-exposed GVBD oocytes contained bivalents as well as metaphase II chromosomes, some of which were polyploid, suggesting a leaky meiotic arrest. Furthermore, it suggests that AURKB activity appears required for loss of cohesion between sister chromatids and in this way contributes/regulates chiasma resolution. Currently, we hypothesise that AURKB directly or indirectly affects Rec8 cohesin phosphorylation, which targets the cohesin protein along chromosome arms for proteolysis by separase, similar to what has been proposed for *C. elegans* meiosis (Kaitna et al., 2002; Rogers et al., 2009). However, we cannot exclude that vice versa AURKB inhibition leads to loss of cohesion between sister chromatid arms in all or some bivalent chromosomes such that polyploid oocytes arise by total loss of chromatid

cohesion rather than a silenced SAC, meiotic congression and activation of APC/C and seaparase activity. Further experiments, e.g. in oocytes expressing GFP-tagged cyclin B might be helpful to distinguish between these two alternatives. Since some oocytes that become exposed to ZM at late prometaphase I progress to meiosis II and undergo cytokinesis also contain bivalent-like chromosomes next to dyads, it is more likely that AURKB activity promotes chiasma resolution as progression to meiosis II is essentially accompanied by activity of APC/C and separase.

Furthermore, this study can show for the first time that preferential inhibition of AURKB by low dose of ZM causes specific changes in heterochromatin constitution, which are not only related to posttranslational phosphorylation events of histone H3 serine residues (Vogt et al., 2009). The alterations in H3K9 trimethylation might influence centromere function and recruitment of M-phase heterochromatin proteins (Fischle et al., 2005; Terada, 2006) as well as deposition of essential passenger and centromere regulatory proteins (Hauf et al., 2007) after nuclear envelope breakdown and such required for correcting erroneous attachments. In fact, it appears that oocytes acquiring H3K9 trimethylation during the initial first hours of maturation post GVBD are competent to progress to meiosis II when exposure to ZM occurs only from late metaphase I stage (Vogt et al., 2009). Since the majority of oocytes will go into anaphase I when ZM inhibitor is given after GVBD, which does not much interfere with H3K9 trimethylation, this study suggests a link between histone modification, chromatin condensation and timed recruitment of proteins to the centromeres and to the central spindle that facilitate chromosome congression, bi-orientation and separation in oocytes.

5.6 Tension facilitates bi-orientation to silence the SAC

Despite the monopolar orientation of sister kinetochores in meiosis I, tension is generated in bivalents by physical connections of homologues through chiasmata (Petronczki et al., 2003), which can be sensed by the sister kinetochores and facilitate alignment of bivalents at metaphase I. Because chiasmata provide the physical linkage between homologues needed for their proper alignment, chromosomes that undergo reciprocal crossovers normally disjoin properly (reviewed by Lee and Orr-Weaver, 2001). Thus, in recombination-deficient mutants, non-exchange chromosomes frequently do not disjoin, whereas those that still undergo exchange segregate properly. In accordance, oocytes from *MLH1* null females never have a normal metaphase configuration, a disturbance which is detected by the SAC leading to an arrest at meiosis I (Woods et al., 1999). Even after an extensive period of time, the orientation

of the majority of univalent chromosomes on the meiosis I spindle is random suggesting that most chromosomes were unable to form stable bipolar attachments necessary for congression to the spindle equator. Furthermore, *MLH1*$^{-/-}$ oocytes exhibited extremely long spindles and precocious spindle pole formation. Hence, it was suggested that bipolar attachment of most bivalents occurs concurrently with spindle pole formation in female meiosis (Woods et al., 1999). Otherwise, a stable metaphase spindle with proper tension forces on aligned chromosomes cannot be assembled and anaphase onset is prevented due to an active SAC. These observations are consistent with a number of previous studies demonstrating that exposure of MI oocytes to spindle-disrupting drugs prevents or significantly delays anaphase onset (Eichenlaub-Ritter and Boll, 1989; Wassmann et al., 2003; Shen et al., 2005; Homer et al., 2005a; Shen et al., 2008). Other mouse models for univalent meiotic segregation of non-exchange chromosomes have shown that oocytes are not arrested at meiosis I and capable of segregation (LeMaire-Adkins et al., 1997; Kouznetsova et al., 2007). Therefore, it has been proposed that mammalian oocytes lack a SAC and univalents bypass checkpoint control owing to the large volume of the cell (LeMaire-Adkins et al., 1997). A more detailed analysis of univalents in *SYCP3*$^{-/-}$ oocytes has revealed that univalents do form bipolar attachments (Kouznetsova et al., 2007). In this way, tension could develop across the sister kinetochore satisfying the requirements of the SAC. Accordingly, Mad2 signals are lost from kinetochores when both bivalents and univalents align at the metaphase plate (Kouznetsova et al., 2007), consistent with our analysis of Mad2 distribution. In molecular terms, the tension on bi-oriented univalents in *SYCP3*$^{-/-}$ oocytes induces a relocation of Sgo2-PP2A away from centromeric cohesin ensuing separase-induced cleavage of Rec8 (Lee et al., 2008). Whereas tension does not develop across sister kinetochores of bivalents in meiosis, because they are not attached to opposite spindle poles, Rec8 is protected by Sgo2 and PP2A preventing its phosphorylation from degradation in such a way maintaining centromeric cohesion (Lee et al., 2008). The bipolar orientation of univalents neatly explains how tension satisfies the SAC in meiosis I. Rather than indicating a lack of SAC activity in mammalian oocytes, the studies on univalents or non-exchange chromosomes demonstrate therefore the susceptibility of mammalian oocytes to chromosome missegregation when facing such chromosomal configurations. Whereas the SAC responds robustly to the lack of bi-orientation of the numerous unaligned univalents in the *MLH1*$^{-/-}$ oocytes (Woods et al., 1999), the SAC fails to detect the presence of a single or few univalents in the *SYCP3*$^{-/-}$ or XO mice, respectively, due to their tension-generating attachments predisposing oocytes to aneuploidy. Hence, the absence of *Sycp3* increases the number of oocytes with an abnormal karyotype at both meiosis I and II (Kouznetsova et al., 2007), and the absence of a pairing partner in the XO mice

frequently leads to misaligned chromosomes as well as to precocious chromatid segregation at meiosis I (LeMaire-Adkins et al., 1997). Instead of the observed bipolar attachments in *SYCP3*$^{-/-}$ oocytes (Kouznetsova et al., 2007), it could also be conceivable that some univalents exhibit merotelic attachments since lagging chromosomes, which are frequently produced by merotelic kinetochores in mitosis (Salmon et al., 2005), were observed at anaphase I in more than half of analysed *SYCP3*$^{-/-}$ oocytes (Kouznetsova et al., 2007). Notably, merotelic kinetochores are not detected by the SAC and contribute to aneuploidy in mammalian tissue cells (Cimini et al., 2001; Salmon et al., 2005; Cimini, 2008). Irrespective of whether kinetochores of univalents are predominantly attached in a bipolar or merotelic manner, the fact that achiasmate chromosomes are capable of attaining an orientation which produces the tension force necessary to satisfy the SAC has important implications for human aneuploidy. The "two-hit" model (see 5.3), to which much attention has been drawn to explain the age-associated increases in human aneuploidy makes one important prediction in that recombination should be similarly altered in non-disjunctional meiosis from young and older women (Hassold and Hunt, 2001). However, there is no evidence to support this prediction. On the contrary, trisomy 21 cases in older women account for a lower proportion of susceptible exchange patterns than in cases from younger women (Lamb et al., 2005). In fact, several classes of non-disjoined chromosomes contribute to aneuploidy/trisomy in that non-exchange chromosomes and those with a telomeric exchange are always at risk for meiotic errors, whereas those with one single pericentromeric exchange have increased risk for random segregation at meiosis II with advancing age (Allen et al., 2009; Oliver et al., 2009). From this it can be inferred that in aged oocytes the chromosome segregation process is deregulated on a global scale, which impairs the ability to accurately disjoin bivalents with even stable chiasmata. In particular, an acceleration in meiotic progression would leave insufficient time for the establishment of stable kinetochore-MT attachments, which occurs late in meiosis I (Brunet et al., 1999), and could possibly interfere with resolution of a very proximal chiasma. Reduced levels of Mad2 (Homer et al., 2005a; Niault et al., 2007) and reduced APC/C^{Cdh1} activity (Reis et al., 2007) cause the oocyte to extrude the first polar body much earlier than controls as result of premature APC/C^{Cdc20} activation (Homer et al., 2005b) leading to missegregation of homologues. Reduced transcript levels of SAC components have also been reported in human oocytes from older women (Steuerwald et al., 2001) supporting the notion that an altered cell cycle among other changes like transient loss of chromatid cohesion could account for the second "hit" by which certain chromosomes become susceptible to aneuploidy with advancing female age (Lamb et al., 1996).

5.7 MCAK is not primarily involved in the correction of kinetochore-microtubule attachment errors during meiosis I

Attachments between MTs and kinetochores are error prone (Cimini et al., 2003). The detection and correction of microtubule attachment errors is an essential process to ensure maintenance of correct cell ploidy and chromosome stability thus also protecting from cancer (Cimini et al., 2003; Salmon et al., 2005; Ganem et al., 2009; Silkworth et al., 2009). What is clear, however, is that merotelic attachments of bi-oriented chromosomes are presumably not detected by the SAC progressing beyond anaphase in mitosis (Figure 5.1; Cimini et al., 2001; Cimini et al., 2002). One well-established mitotic error produced by merotelic kinetochores is that of lagging chromosomes near the spindle equator at anaphase due to almost equal forces exerted by MTs from opposite spindle poles on one kinetochore (Figure 5.1; Salmon et al., 2005). Nevertheless, most merotelic attachments are corrected (Cimini et al., 2003). There appear to be two major correction mechanisms: one that functions before anaphase to reduce the number of merotelic kinetochores and one that functions after anaphase onset to prevent most merotelic kinetochores from producing lagging chromosomes (Salmon et al., 2005). Correction before anaphase requires destabilisation of kinetochore-microtubule attachments to the wrong pole and involves the chromosomal passenger complex (CPC), which consists of the kinase Aurora B (AURKB), its targeting and activation subunit INCENP, and two other subunits, survivin and Dasra/borealin (reviewed by Ruchaud et al., 2007). Correction during anaphase does not occur by detachment, but by differences in the polymerisation dynamics of MTs to the correct versus incorrect pole (Cimini et al., 2004).

Many studies have shown that AURKB is required to destabilise improper kinetochore-microtubule attachments. Initially, studies in budding yeast indicated that correction of misattachments depends on both the activity of AURKB (Ipl1 in yeast) and kinetochore tension (Biggins et al., 1999; Tanaka et al., 2002). Normally, stable kinetochore-microtubule attachment is achieved when chromosomes bi-orient generating tension across kinetochores, which are pulled towards opposite spindle poles and restrained by the centromeric cohesion holding sisters together in mitosis. This stretches the centromere, inactivates AURKB/Ipl1 activity, and stabilises microtubule attachment. When syntelic attachments are made in mitosis, these kinetochores are not under high tension, AURKB/Ipl1 is active and attachments are unstable. Hence, Ipl1 yeast mutants have been shown to maintain stable syntelic attachments at lower tension (Biggins et al., 1999; Tanaka et al., 2002). Inhibiting AURKB in mammalian tissue cells, *Drososphila* and *C. elegans* by dominant-negative mutants, RNAi, antibody microinjection, or selective drug targeting promotes stability of microtubule

attachments and errors in segregation, like anaphase lagging, accumulate (Adams et al., 2001; Murata-Hori and Wang, 2002; Kallio et al., 2002; Kaitna et al., 2002; Cimini et al., 2003; Ditchfield et al., 2003; Hauf et al., 2003). It has been proposed that AURKB is normally located at the inner centromere beneath the kinetochore in mitosis so that it can no longer phosphorylate key targets at the kinetochore when sister kinetochores are stretched apart by tension (Andrews et al., 2004). Indeed, AURKB associates with the centromere at prometaphase to metaphase of mitosis (Ruchaud et al., 2007). Similarly, AURKB occupies a site on the centromere domain at metaphase I in spermatocytes and oocytes (Parra et al., 2006; Vogt et al., 2009). In the absence of tension, AURKB phosphorylates several kinetochores proteins. Two important kinetochore-localised microtubule-capture factors, the Hec1/Ndc80 and Dam1 complex, were shown to be AURKB/Ipl1 substrates (Cheeseman et al., 2001; Cheeseman et al., 2002; DeLuca et al., 2006). Phosphorylation of Hec1/Ndc80 reduces its affinity for MTs *in vitro* and mutation of the putative AURKB phosphorylation sites stabilises kinetochore-microtubule interaction *in vivo*. Members of the Ndc80 complex are conserved from fungi to humans (Kline-Smith et al., 2005), whereas no homologues of the Dam1-complex have been found in organisms outside budding and fission yeast. Phosporylation has been shown to destabilise kinetochore-microtubule attachments, whereas dephosporylation produces stabilisation.

Besides the Hec1/Ndc80 and Dam1 complexes, AURKB also influences kinetochore-microtubule attachment by a different mechanism. The Kin-I kinesin MCAK (Wordeman and Mitchison, 1995) appears to be involved in the correction of attachment errors during chromosome alignment in mitosis (Kline-Smith et al., 2004; Knowlton et al., 2006). Perturbations of MCAK function lead to increases in the frequency of anaphase lagging chromosomes (Maney et al., 1998; Kline-Smith et al., 2004; Ganem et al., 2005; Huang et al., 2007) suggesting that MCAK utilises MT depolymerase activity to destabilise inappropriate MT attachments at kinetochores. Phosphorylation of MCAK by AURKB inhibits its ability to promote MT disassembly (Ohi et al., 2004; Lan et al., 2004; Andrews et al., 2004; Zhang et al., 2007b)., while ICIS, a protein that stimulates MCAK activity is centrally located on the centromeres and thus would be able to stimulate depolymerisation of merotelically attached MTs. With respect to polar attachments, AURKB and MCAK largely colocalise at centromeres that are not under tension, but MCAK moves away from AURKB and becomes more closely associated with kinetochores as chromosomes achieve bi-orientation and their centromeres are stretched due to increased tension in mitosis (Andrews et al., 2004). The phosphatase PP1 resides within the outer kinetochore domain (Murnion et al., 2001; Trinkle-Mulcahy et al., 2003), where it counterbalances AURKB activity in the activation of MCAK.

My study demonstrates that AURKB and MCAK occupy the same site on the centromere domain at metaphase I in oocytes, similar to its position in spermatogenesis (Parra et al., 2006). While it is impossible to precisely map AURKB or MCAK distribution in terms of kinetochore geometry on spread chromosomes in the present study, as is recognised in side view of squashed spermatocytes depicting a ring-like structure beneath the kinetochore (Parra et al., 2006), my findings place the kinase into the vicinity of MCAK at metaphase I. This may seem counter-intuitive since colocalisation of AURKB and MCAK would result in inactivation of MCAK by phosphorylation. One explanation could be that the principal function of MCAK is to correct merotelic attachments, which persist beyond anaphase onset after tension has been established in mitosis (Cimini et al., 2003). Prolonging metaphase by an additional two hours in mammalian tissue culture cell, to allow more time for error correction, does not reduce the number of merotelic kinetochores before anaphase significantly, although it does produce a four-fold decrease in anaphase lagging chromosomes (Cimini et al., 2003) suggesting that error correction is a time-dependent process and is not only going on before but also during anaphase (Cimini et al., 2004). When I delayed metaphase I with the proteasome inhibitor MG132, distribution of MCAK to the centromere domain was retained, similar to a control metaphase I, indicating that MCAK does not translocate away from the centromere and AURKB at that meiotic stage. The spatial association of MCAK and AURKB at metaphase I centromeres in presence or absence of the proteasome inhibitor MG132 suggests that active MCAK does not participate in any error correction mechanism at metaphase I before anaphase I in mouse oocytes, although it may do so at prometaphase I when chromosome congression takes place. Rather, MCAK persists at centromeres until telophase I, whereas AURKB disappears from centromeres after early anaphase I stage translocating to the spindle midzone, consistent with its localisation and function during cytokinesis in mitotic cells as part of the CPC (Ruchaud et al., 2007). The spatial separation of MCAK and AURKB at the transition from anaphase I to telophase I would render MCAK active to support chromosomes segregation at anaphase I, analogue to its proposed role in mitosis (Maney et al., 1998). The observation of overexpressed GFP-tagged MCAK in the midbody at the site of the cytokinesis furrow in an ongoing study suggests that, in fact, MCAK may have a function in depolymerising MTs of the central spindle but also such from lagging chromosomes (Wolfgang Klein, personal communication).

The contribution of MCAK to error correction may be less significant than previously thought (Kline-Smith et al., 2004; Knowlton et al., 2006) and MCAK appears not to be the primary error correction mechanism in mitosis (Lampson et al., 2004; Wordeman et al., 2007), since anaphase segregation errors are not corrected following loss of AURKB function, although

tension on AURKB-inhibited centromeres is reduced to control levels by excess centromeric MCAK (Wordeman et al., 2007). Whether contribution of MCAK to error correction in meiosis is similar to mitosis needs to be determined experimentally. During the course of mitosis a large number of merotelic attachments appear in cells (Cimini et al., 2003). So far, there is no data available on the frequency of merotelic attachments during meiosis I in mammalian oocytes. In fission yeast, AURKB is required for the faithful mono-orientation of sister chromatids in meiosis I, next to other regulatory proteins (Lee and Orr-Weaver, 2001; Cimini et al., 2003), to prevent merotelic attachment to both spindle poles (Hauf et al., 2007). In budding yeast, the two pairs of sister kinetochores on a bivalent attach to one MT each and one sister kinetochore may thus be inactivated, making merotelic attachment impossible. In fission yeast, however, both sister kinetochores are active in meiosis I and can attach to MTs (Hauf et al., 2007), similar to mouse kinetochores (Parra et al., 2004). This suggests that merotelic attachments may occur in mammalian oocytes just as in fission yeast. Interfering with AURKB regulation by selective drug targeting in mitosis results in an increase of merotelic connections and lagging of anaphase chromosomes (Cimini et al., 2006; Wordeman et al., 2007). I also observed anaphase lagging when the aurora kinase inhibitor ZM447439 was administered. The rise of hypoploidy after ZM exposure indicates that attachment defects were not fully resolved prior to anaphase I, despite a transient delay in the onset of anaphase I. From this it can be concluded that merotelic chromosomes are present during meiosis I in mammalian oocytes. Furthermore, it supports the notion that members of the CPC are involved in the correction of erroneous attachments both in mitosis and female meiosis. The phenotype of lagging anaphase chromosomes is also produced in mitosis following the disruption of MCAK function by RNAi or a dominant-negative approach (Maney et al., 1998; Kline-Smith et al., 2004; Ganem et al., 2005; Huang et al., 2007). In contrast, this study shows that MCAK depletion leads to an arrest of oocytes at meiosis I for an extended period of time (i.e. until 16h of culture), but also exhibited a dramtic delay in chromosome congression at meiosis I. For instance, at 10h most of the control oocytes injected with unspecific siRNA already progressed to anaphase I and the few ones at metaphase I had aligned chromosomes, whereas oocytes injected with specific siRNA has still unaligned chromosomes resembling prometaphase I stage. This suggests that MCAK uses depolymerase activity to destabilise faulty kinetochore-microtubule attachments prior to anaphase I in mammalian oocytes and is essential for chromosome congression. Although chromosomes may eventually assemble on the equtator during a prolonged meiosis I arrest, it might be that disturbed dynamics of microtubule polymerisation/depolymerisation in MCAK-depleted oocytes or subtile alterations in the spindle cause the prolongation of the SAC. Aurora kinase

A-mediated phosphorylation and MCAK activity have been implicated in formation and focusing of spindle poles in egg extracts of *Xenopus* (Zhang et al., 2009). The 'hairy' appearance of spindle poles in MCAK-depleted oocytes contrasts the smooth appearance of spindle poles in proteasome-inhibited meiosis I oocytes suggesting that polar MCAK might have a function in depolymerising astral MTs emanating from spindle poles. Whether and in which way this could influence cell cycle progression remains to be studied experimentally. An ongoing study overexpressing GFP-MCAK fusion protein by Wolfgang Klein in our group provides compelling evidence that 1. MCAK is associated with centrosomes at the spindle poles of meiosis I and II oocytes, 2. MCAK is present at sites of the midspindle where interpolar MTs may overlapp, and 3. MCAK is associated not only at the centromeres but also at sites of exchange/chiasmata. One can speculate that MCAK at sites of chiasmata prevents firm attachment of MTs to prevent precocious separation of chromatids and premature chiasma resolution, or is in some way actively involved during chromosome segregation at first anaphase such that MCAK depletion results in failures of chromosome separation although chromosomes have aligned at the equator. Again, further studies are required to elucidate whether and in which way MCAK contributes to chromosome disjunction and chiasma resolution. Strikingly, the inhibition of AURKB interferes with disjunction. It is discussed that relative localisation and cell cycle-dependent phosphorylation of substrates are important for normal chromosome congression and behaviour in mitosis while little is still known about meiosis-specific or conserved events.

5.8 MCAK is involved in satisfying the SAC in meiosis I

Disrupting MCAK function in cultured mitotic cells using dominant negative mutant expression (Maney et al., 1998; Kline-Smith et al., 2004) or RNAi (Ganem et al., 2005) has minimal effects on bipolar spindle assembly or chromosome movement, whereas depletion of MCAK in HeLa cells delayed the timing of anaphase onset (Huang et al., 2007). This study demonstrates that RNAi-mediated knockdown of MCAK in mouse oocytes induces a much delayed congression of chromosomes at the spindle equator and a prolonged meiosis I arrest but does not prevent eventual chromosome alignment on a bipolar meiosis I spindle. The presence of SAC proteins Mad2 and BubR1 on kinetochores led me to the conclusion that MCAK could be involved in SAC activity. I next reasoned that inactivation of the SAC with RNAi would reverse the arrest if it was dependent on the SAC. Indeed, double depletion of MCAK and Mad2 led to meiosis II progression of mouse oocytes. While the SAC does not

monitor relative localisation of chromosomes on the spindle, e.g. displacement due to merotelic attachments when there is sufficient tension and saturation by MTs at kinetochores, alterations in microtubule dynamics may cause prolongation of the SAC in that it disturbs equilibrium between depolymerisation and polymersation within the spindle and at different sites (e.g. minus or plus-ends of MTs; kinetochore fibres or astral MTs at poles). This equilibrium is influenced in specific ways by the activities of kinesin-microtubule depolymerases (Desai et al., 1999; Howard and Hyman, 2007). Provided, MCAK has an essential role in contributing to spindle dynamics and microtubule turnover/stability at prometaphase I/metaphase I in oocytes, it is therefore conceivable that reduced activity of MCAK by knockdown of expression is causal to the first meiotic arrest. While many tasks have been attributed to this mitotic kinesin, ranging from spindle assembly and maintenance (Walczak et al., 1996; Kline-Smith and Walczak, 2002) to chromosome positioning and segregation (Maney et al., 1998; Walczak et al., 2002; Kline-Smith et al., 2004), a role of MCAK in SAC function, particularly during meiosis, has not been demonstrated yet.

Among the motor proteins involved in chromosome segregation, dynein and dynein light chain has been attributed a role in SAC function (Wojcik et al., 2001; Howell et al., 2001; Sivaram et al., 2009). It is targeted to kinetochores by a complex of proteins including Rod, ZW10 and Zwilch (RZZ). Dynein actively moves off kinetochores and along MTs during chromosome congression (Wojcik et al., 2001; Howell et al., 2001). Furthermore, mutations in *Drosophila* dynein disrupted function and blocked the removal of both kinetochore dynein and checkpoint proteins (Wojcik et al., 2001). Moreover, dynein mutants experienced a checkpoint-induced delay at the metaphase-anaphase transition in both the presence and absence of colchicine. Participation of dynein in checkpoint inactivation has also been demonstrated in mouse oocytes transporting Mad proteins from kinetochores to spindle poles (Zhang et al., 2007a). Therefore, dynein has a key role in the SAC that is independent of the role of proteins known to maintain an active checkpoint. Most recently, it was shown that a Cdk1-phosphorylated form dynein light chain 1 is essential for transport of Mad2/1 and ZW10 but not BubR1 off the kinetochore, consistent with checkpoint silencing (Sivaram et al., 2009). In keeping with existing models of checkpoint activation, it follows that an active checkpoint functions only when assembled at the kinetochore, whereas checkpoint inactivation is by dynein-mediated removal of the Rod-ZW10-complex, Mad2, and BubR1 from correctly attached kinetochores (Wojcik et al., 2001; Howell et al., 2001). Inhibition or depletion of the motor protein dynein produced a similar phenotype in metazoan cells arresting at metaphase with correctly aligned chromosomes and high levels of kinetochore-bound SAC proteins (Howell et al., 2001; Yang et al., 2007).

Comparing the phenotypes between cells resulting from either directly mutating or depleting dynein or one of its interacting partners with the depletion phenotype of MCAK in mouse oocytes suggests that both motor proteins occupy overlapping, yet also distinct roles in SAC function. Both motor proteins are involved in the inactivation of the checkpoint following correct alignment of chromosomes at metaphase. Whereas dynein mediates removal of checkpoint proteins from kinetochores in a mechanical and direct way leading to checkpoint inactivation in both mitotic and meiotic cells (Wojcik et al., 2001; Howell et al., 2001), MCAK's role with respect to SAC regulation is indirect, particularly with respect to meiosis. MCAK participates in a number of important cellular processes, which are also monitored by the SAC. Particularly in mitosis, the destabilisation of improper kinetochore-microtubule attachments requires AURKB and the depolymerising activity of MCAK to prevent segregation defects at anaphase (Kline-Smith et al., 2004; Knowlton et al., 2006). This indirectly influences SAC activity by creating unattached kinetochores halting cell cycle progression until bipolarity is obtained (Pinsky et al., 2006). Once bipolarity is established tension becomes important for SAC inactivation (Nicklas, 1997; Nicklas et al., 2001). The microtubule-kinetochore connection is normally destabilised at low kinetochore tension and stabilised by high tension between bi-oriented sister kinetochores (Nicklas, 1997; Nicklas et al., 2001). Characteristically, kinetochores of sister chromatids face opposite poles and are bi-oriented (amphitelic) in mitosis. As chromosomes bi-orient at metaphase and sisters are both translocating to opposite spindle poles, the mean interkinetochore distance increases. If sister kinetochores attach to MTs from the same pole in mitosis (syntelic attachment), not enough tension is detected as measured by a shorter mean interkinetochore distance. Unlike mitosis, sister kinetochores of each homologue attach to MTs from the same pole (monopolar) and are mono-oriented in meiosis I while the two homologues face opposite poles (bi-orientation). Previous studies have assessed the level of tension following the depletion of MCAK in mitosis (Kline-Smith et al., 2004; Ganem and Compton, 2004; Wordeman et al., 2007). Whereas ectopically increasing the level of MCAK activity on centromeres decreases sister centromere tension, although not to the point that the SAC is triggered, decreased levels of MCAK on centromeres substantially increase tension across sister centromeres (Wordeman et al., 2007). Two other studies say that the depletion of MCAK has no effect on tension (Ganem et al., 2005) or decreases tension (Kline-Smith et al., 2004). Wordeman and co-workers (2007) believe that this discrepancy may be caused by the inclusion of non-bioriented metaphase in the data pool. The global increase in tension in MCAK-depleted cells appears to be partly caused by increased time spent in a state in which both sisters of a single sister centromere pair are simultaneously engaged with MTs and attempting to move poleward at

the same time. In a normal somatic cell, paired chromosomes oscillate until they are bi-oriented and aligned at the metaphase plate (Skibbens et al., 1993; Rieder and Salmon, 1994). Upon chromosome alignment at metaphase MCAK is required to suppress the oscillations since antisense and dominant-negative experiments with a motorless mutation of MCAK resulted in lagging chromosomes in anaphase suggesting that these lagging chromosomes might be caused by continued oscillations of separated chromatids at anaphase (Maney et al., 1998). Schuh et al. (2007) recently found in mouse oocytes that, once bi-oriented, chromosomes also perform slow oscillatory movements, but, unlike in somatic cells, MCAK activity might not be required to suppress oscillations of bivalent chromosomes. Therefore, I did not observe a disassociation of active MCAK from AURKB at metaphase I, in contrast to mitosis, where MCAK switches its affinity from centromeres to kinetochores once bipolar attachment is established (Andrews et al., 2004). Concomitantly, MCAK activity increases to suppress oscillations. The decrease in centromeric tension following depletion of centromere-bound MCAK in Ptk2 cells suggested that the depolymerase activity of MCAK is needed to generate tension across centromeres (Kline-Smith et al., 2004). This role of MCAK as a "tension generator" seems tempting in mouse oocytes. The presence of BubR1 and Mad2 in meiosis I-arrested MCAK-depleted oocytes indicates a lack of tension. In contrast to mitotic cells, where loss of Mad2 staining was correlated with initial MT attachment, loss of Mad2 staining in meiotic cells appeared to be tension-dependent (Yu et al., 1999). While sister kinetochores of each homologue attach to MTs from the same pole (monopolar) and are mono-oriented in meiosis I, the pulling force exerted by MTs from opposite spindle poles does not create tension across sister centromeres. Instead, chiasmata holding bivalent chromosomes together not only ensure their alignment on the meiosis I spindle but also generate the needed tension until the onset of anaphase I. Immunostaining with CREST antibody on spread meiosis I oocytes depicts the closely associated sister kinetochores of each homologue as a single unit (Figure 4.11B'), indicating tension-less kinetochores, in contrast to spread metaphase II oocytes, where each kinetochore of a sister chromatid shows a distinct centromere signal within a visible distance to each other, reminiscent of mitotic centromeres (Figure 4.10E'). This would explain why I did not observe a redistribution of MCAK at metaphase I centromeres, consistent with results in spermatogenesis (Parra et al., 2006). Even though the establishment of bipolarity appeared not to be disturbed in MCAK-depleted oocytes, spindle poles were less focused than in control meiosis I oocytes, indicating that MCAK may have a role at the spindle poles. It was recently demonstrated in *in vitro* frog egg extract that spindle bipolarity requires MCAK activity and localisation to spindle poles through Aurora kinase A-mediated phosphorylation (Zhang et al., 2008). Depleting MCAK in

the presence of monastrol, which creates monopolar spindles through inhibition of Eg5 (Kapoor et al., 2000), affects pole focusing. Whereas MCAK is not needed for MT flux, the kinesin Kif2A is found at spindle poles and implicated in regulating flux (Ganem et al., 2005), but is also in a functional relationship with MCAK (Ganem and Compton, 2004). If MCAK activity at the poles is more critical in the overall process of pole organisation and flux, it could adversely regulate kinetochore activity and tension (Maddox et al., 2003). It was not possible to recognise MCAK at spindle poles by antibodies in fixed and permeabilised oocytes, because epitopes are likely concealed by interactions with other proteins and/or masking of epitopes by close proximity to other proteins of the spindle. It became possible for the first time to recognise MCAK in living oocytes by expression of a GFP-tagged MCAK fusion protein confirming the presence and enrichment of MCAK at centromeres in meiosis I and II, but also making MCAK visible at the spindle poles (Wolfgang Klein, personal communication). This suggests that MCAK has a unique and important role in mediating integrity of spindle poles in acentriolar spindle of mammalian oocytes. Furthermore, I imagine that proper MCAK function at centromeres and poles is used to generate tension at meiotic centromeres in meiosis I, which contributes to satisfying the SAC.

5.9 Meiosis-specific versus oocyte-specific functions of MCAK and its possible involvement in sexual dimorphism in chromosome segregation

MCAK distribution has recently been extensively studied in male meiosis but not in oogenesis. In first meiosis of spermatocytes INCENP and AURKB load first on chromocentres prior to nuclear envelope breakdown, followed by Shugosin-like 2 (SGOL2) protein and MCAK accumulation at centromeres at late diakinesis/early diplotene (Parra et al., 2009). The immunofluorescent analysis of maturing oocytes with MCAK-specific antibodies in the present study suggests that the protein is initially present along chromosome arms in female meiosis I, and especially enriched in the region of sister chromatid cohesion rather than on centromeric heterochromatin and centromeres as in spermatogenesis. This might relate to the specific requirements for acentriolar spindle formation in oocytes, in which a gradient of TPX-2 at chromosomes is involved in initiation of spindle formation (Brunet et al., 2008). It is feasible that chromosome-bound MCAK has a role to prevent stable attachment of MTs to sister chromosome arms to prevent their untimely separation and chiasma resolution rather than to the centromeres at this early stage of meiotic resumption.

Another major function of MCAK might be associated with focusing of spindle poles (Zhang et al., 2008; De et al., 2008), an activity which appears regulated primarily by AURKA-mediated phosphorylation of MCAK at early M-phase (Zhang et al., 2008).

A distinct role of MCAK specific to female meiosis can also be postulated from the differences in MCAK distribution between oocytes and spermatocytes at the transition from meiosis I to meiosis II. While this transition is characterized by a brief interphase in male meiosis before spermatocytes progress to prometaphase II, oocytes transit continuously without interphase from telophase I to prometaphase II, and retain condensed chromosomes. Unlike spermatocytes, oocytes divide unequally to form one small first polar body and a large oocyte. Accordingly, the sequence of acquisition of centromeric proteins to centromeres at meiosis II follows the pattern in meiosis I in male meiosis from meiotic interphase (with intact nucleus) to metaphase II. AURKB transits to the interpolar spindle in spermatogenesis while MCAK disappears from centromeres at telophase I. I show here that MCAK remains continuously enriched at centromeres from prometaphase I to telophase I up to prometaphase II and metaphase II in mouse oocytes, while AURKB translocates to the mid-spindle at anaphase to telophase I and is absent from centromeres briefly at this stage but immediately before completion of cytokinesis associates again with chromosomes and later, with centromeres (Vogt et al., 2009). Thus, analysis of dynamic distribution of MCAK suggests that it has distinct functions in male and female meiosis and that the temporal availability of the depolymerase could contribute to synchronize events in spindle formation, chromosome congression, anaphase progression and cytokinesis in mammalian female meiosis.

The differences in localisation and activity of MCAK, in particular the observation of overexpressed GFP-tagged MCAK in the midbody at the site of the cytokinesis furrow (Wolfgang Klein, personal communication), might also contribute to the sexual dimorphism in processing dicentric chromosomes or lagging chromosomes from recombinant paracentric inversions in male and female meiosis. Unlike in male meiosis, segregation of a dicentric chromatid thus frequently results not in breakage, stretching, or loss, but instead in precocious separation of the sister centromeres of at least one homologue in oocytes and embryos (Koehler et al., 2002). This could relate to depolymerisation of MTs attached to the second centromere of lagging chromosomes by interpolarly and centromerically localised MCAK in late telophase I while chromosomes are more likely to break without centromeric and interpolar MCAK at telophase I in spermatogenesis.

5.10 Regulation of the SAC during meiosis I in mammalian oocytes: consequences of altered expression with respect to age and aneuploidy

The detection and correction of MT attachment errors requires members of the SAC and CPC in mitosis and meiosis (Figure 5.2). Mad2 is a key component of the SAC in mammalian oocytes because it halts anaphase onset in response to unattached or improperly attached kinetochores and in this way protects the cell from aneuploidy. Mad2 accumulates at unattached kinetochores of bivalent chromosomes generating a "wait-anaphase" signal by blocking APC/C activity until all chromosomes have bi-oriented. Whereas the core SAC proteins, like Mad2, are required to signal the presence of unattached kinetochores, Aurora kinase B (AURKB) as part of the CPC detects the presence of non-bipolarly attached, particularly merotelic, kinetochores. The CPC influences SAC activity indirectly through destabilisation of erroneous attachments and creation of unattached kinetochores. Detachment of faulty MTs from kinetochores is not primarily carried out by the depolymerase activity of MCAK, which associates with centromeres at the metaphase I-to-anaphase I transition placing MCAK in the vicinity of AURKB. Rather, MCAK is involved in satisfying the SAC during meiosis I, possibly by contributing to the necessary tension force upon bipolar alignment and normal chromosome segregation at anaphase I.

Several studies in human and mouse oocytes from aged females have lead to the concept that alterations in gene expression contribute to the high risk for errors in chromosome segregation with advanced maternal age (Eichenlaub-Ritter and Boll, 1989; Steuerwald et al., 2001; Hamatani et al., 2004; Pan et al., 2008). In particular, such studies suggest that genes involved in spindle formation, checkpoint control, and protein stability may be altered in abundance (Steuerwald et al., 2001; Hamatani et al., 2004; Pan et al., 2008), while transient loss of cohesion between sister chromatids might contribute to untimely chiasma resolution (Angell, 1991; Hodges et al., 2005). This study suggests that critical reductions in proteins regulating spindle dynamics, chromosome behaviour and checkpoint control, such as MCAK and Mad2, are predisposing to first and second meiotic errors. Moreover, the multiple functions of AURKB in regulation of cell cycle control, spindle and chromosome segregation predict that overexpression as well as knockdown and loss of kinase activity by chemical inhibition cause disturbances in activity of its substrates, like MCAK. Together with their altered expression this can induce spindle aberrations, changes in chromatin formation and chromosome behaviour, which in a synergistic way compromise faithful chromosome segregation in mammalian oocytes predisposing aged oocytes to aneuploidy that may lead to trisomies, like

Down Syndrome, after fertilization and contribute to implantation failure and spontaneous abortion.

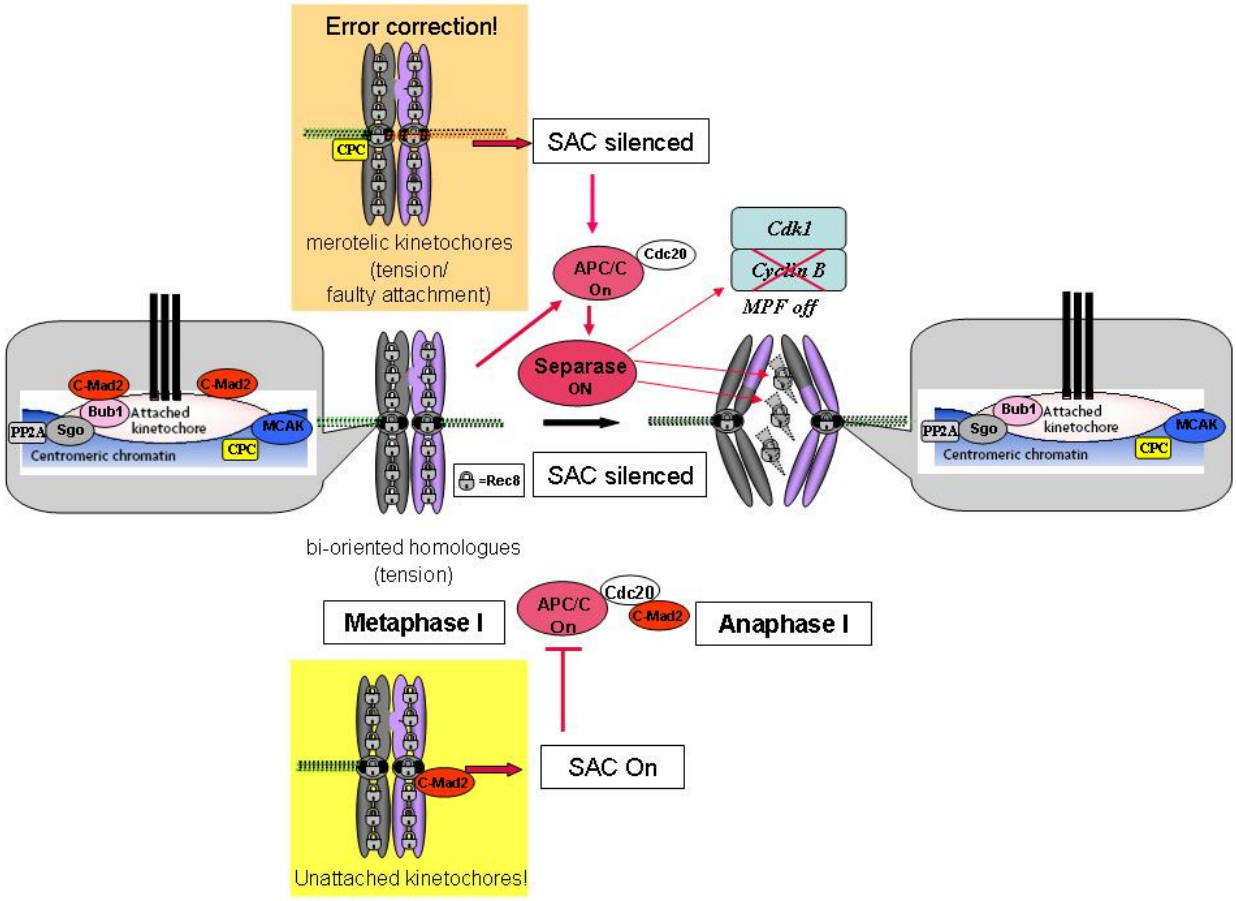


Figure 5.2 Regulation of the SAC at the metaphase I-anaphase I transition in mammalian oocytes. See text for details.

6 Materials and Methods

6.1 Chemicals, enzymes, and materials

All chemicals were purchased from Sigma-Aldrich Chemical Company (Deisenhofen, Germany), Carl Roth (Karlsruhe, Germany), and Serva (Heidelberg, Germany). Enzymes were obtained from Sigma-Aldrich, Roche Diagnostics (Mannheim, Germany), and Promega (Madison, USA). All dishes for oocyte culture were from Nunc (Langenselbold, Germany).

6.2 Animals and culture of mouse oocytes

Outbred MF1 mice originally obtained from Harlan Winkelmann (Borchen, Germany) or the Jackson laboratory (Bar Habor, USA) were bred at the university animal facility under a twelve hour light/dark cycle with water and feed (Harlan Teklad Global 2019) *at libitum*. Ovaries were isolated from young females (6-12 weeks) at diestrous of the natural cycle (determined by vaginal smear analysis) and placed in a Petri dish with warm M2 (+) medium with 14 mg/ml bovine serum albumin (BSA; Sigma). This M2 (+) medium is more suitable for oocyte isolation, because the viscosity of the M2 (+) medium due to the higher concentration of BSA is similar to that of the follicular fluid. Oocytes with an intact germinal vesicle (GV) were collected from large antral follicles by puncturing them with a fine needle (Terumo, Belgium), then isolated from surrounding cumulus cells with a finely drawn glass capillary (Clark Electromedical Instruments, GL 150T-10) and transferred to a four-well dish with M2 medium (Sigma-Aldrich) containing 4 mg/ml BSA, which was placed in an ungassed incubator under high humidity at 37°C for up to 16h.

6.3 Microinjection

For microinjection experiments, oocytes were maintained at the GV stage in M2 medium supplemented with 10µM cilostamid (Sigma-Aldrich), a specific phosphodiesterase-3 inhibitor. Cilostamid prevents the drop in cAMP (cyclic adenosine monophosphate) and inactivation of protein kinase A (PKA) that is required for spontaneous resumption of maturation. They were microinjected using a semi-automatic injector with micromanipulators (Eppendorf, Hamburg, Germany) mounted to a Zeiss Axiovert 35 microscope (Jena, Germany). Holding pipettes and microinjection capillaries (Type Pronucleus 1.2) were obtained from BioMedical Instruments (Zöllnitz, Germany). About 1 µl of solution was micropipetted (Eppendorf, Hamburg, Germany) into capillary tubes. GV-staged oocytes were immobilised using a holding pipette and the tip of the microinjection capillary was introduced

across the zona pellucida and oolemma into the ooplasm. A pressure pulse was applied to microinject a solution equivalent to ~10% of the total oocyte volume. Oocytes were released into cilostamid-free M2 medium after 6 hours following RNAi injection to undergo meiotic maturation.

6.4 Inhibitor treatment and checkpoint activation

For inhibition of AURKB activity, 1.5 µM ZM 447439 (ZM inhibitor; Tocris, UK) or solvent (DMSO, Sigma-Aldrich) was supplemented to M2 medium. This low concentration of the ZM inhibitor preferentially inhibits Aurora kinase B (Vogt et al., 2009). The proteasome was inhibited by exposure to 5µM MG132 (Sigma-Aldrich) to inhibit progression of oocytes to anaphase I although oocytes should possess active APC/C and have been released from the spindle attachment checkpoint (SAC). For meiotic arrest due to SAC activation, GV-staged oocytes were cultured in M2 medium with 100 nM nocodazole (Sigma-Aldrich) for 14-16 hours, as previously shown (Shen et al., 2005). Control oocytes were placed in medium with solvent (DMSO).

6.5 Knockdown of expression by siRNA

siRNA duplexes were synthesized by *in vitro* transcription using the Silencer siRNA Construction Kit according to the manufacture's protocol (Ambion, USA) targeting the following genes:

*Mm*Mad2L1	5' -AAAGTATCTCAATAATGTGGT- 3'
(GenBank accession no. NM 019499)	(nt position 234-255)
*Mm*Kif2C (MCAK)	5' -AAGGAGATGGAGAAAATGAAG- 3'
(GenBank accession no. NM 134471)	(nt position 108-129)

These sequences were selected by scanning the length of the two genes using the siRNA Target Finder tool (Ambion, Austin, TX, USA).

Manufacture of the ds siRNA required that the two DNA oligonucleotide templates for each targeted gene were synthesized (Sigma-Genosys, Haverhill, UK). The following gene-specific template sequences were successfully used:

siRNA Mad2 sense	5'-AATGTCTAATCACTGAGCGAACCTGTCTC-3'
siRNA Mad2 antisense	5'-AATTCGCTCAGTGATTAGACACCTGTCTC-3'
siRNA MCAK sense	5'-AATATTCCTGTGCTCGCTTTACCTGTCTC-3'
siRNA MCAK antisense	5'-AATAAAGCGAGCACAGGAATACCTGTCTC-3'

The unrelated non-specific template sequences were designed as followed:

siRNA Mad2 sense	5'-AATGTCTAATCCATGACGGAACCTGTCTC-3'
siRNA Mad2 antisense	5'-AATTCCGTCATGGATTAGACACCTGTCTC-3'
siRNA MCAK sense	5'-AAATTCTCGTTTCGCTGTTCACCTGTCTC-3'
siRNA MCAK antisense	5'-AATGAACAGCGAAACGAGAATCCTGTCTC-3'

Each 29-nt DNA template was compromised of 21-nts encoding either the Mad2- or MCAK-targeting siRNA and a 8-nt sequence complementary to a T7 promotor primer. DNA templates were hybridised for 5 min at 70°C to a T7 promotor Primer supplied by the kit consisting of a T7 promotor sequence and an 8-nt sequence complimentary to the 8-nts within the siRNA transcription templates. The 3' ends of the hybridised oligonucleotides were then extended using the Klenow fragment of DNA polymerase for 30 min at 37°C to create ds templates for siRNA *in vitro* transcription. Following T7 promotor-driven *in vitro* transcription for 2 hr at 37°C, the resulting RNA transcripts were hybridised overnight at 37°C to produce dsRNA now consisting of 5' terminal leader sequences, a 19-nt target specific dsRNA, and 3' terminal UUs. The DNA templates were removed by incubation at 37°C for 2 hr with a DNase and a single-strand specific RNase which does not cleave UU residues thereby producing a siRNA with UU dinucleotide overhangs. siRNAs were eluted into 100 µl nuclease-free water to produce stock solutions of 400-800 µg/ml as quantified by absorbance at 260 nm. siRNA products were analysed by electrophoresis using a 1,2% formaldehyde-agarose gel.

For microinjection, stock solutions were not diluted, when a single knockdown (Mad2 or MCAK) was performed. For double-knockdown experiments (Mad2 and MCAK), the siRNA with the higher stock solution concentration was diluted to the concentration of the siRNA with the lower stock solution concentration. The concentrations of siRNAs used in this study were in the range between 20 and 60 µM, which are higher than the siRNA concentrations utilized by Kim et al. (2002) and lower than the concentrations of dsRNA utilized by Wianny and Zernicka-Goetz (2000) in knockdown experiments with mouse oocytes.

6.6 Quantitative real-time RT-PCR

Total RNA was isolated from 15-20 mouse oocytes using the NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany). 20-30 ng/µl of template RNA was directly used for real time RT-PCR. 20 µg of glycogen (Roche Diagnostics, Mannheim, Germany) was added as carrier. The real time RT-PCR reaction was carried out in a one-step procedure by using the

Roche LightCycler (Roche Diagnostics, Germany) and the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol with selected primers (Table 6.1). The following RT-PCR protocol was used: reverse transcription (50°C for 20 min), initial activation step for HotStarTaq DNA Polymerase (95°C for 15 min), amplification and quantification repeated 55 times (PCR program: 94°C for 15 sec, 58°C for 45 sec , 72°C for 60 sec) and a melting curve program. The specificity of RT-PCR products were analysed by gel electrophoresis. Quantification was by LightCycler Software Version 3.5.3. Statistical analysis for quantitative analysis was performed by group-wise comparison based on PCR efficiencies and the mean crossing point deviation between sample and control group using Relative Expression Software Tool (Pfaffl et al., 2002). Expression ratio of Mad2 and MCAK mRNA levels from cells depleted with specific siRNAs relative to cells depleted with negative control siRNAs were calculated by REST software and converted to percentage of gene knockdown as described by Lim et al. (2007). MCAK and Mad2 levels were normalized to ß-actin expression levels. Experiments were repeated and analysed three times.

Table 6.1 RT-PCR primer sequences

RT-PCR Primers	
Mad2	5'-GCATTTTGTATCAGCGTGGCAT-3'
Mad2	5'-GGCTTTCTGGGACTTTTCTCTACG-3'
MCAK	5'-TCCGGAATGGAGTCGCTTCAC-3'
MCAK	5'-GTCGACTTTGATCATCCGGGC-3'
ß-actin	5'-TGCGTGACATCAAAGAGAAG-3'
ß-actin	5'-GATGCCACAGGATTCCATA-3'

6.7 Immunofluorescence

For analysis of centromere and kinetochore proteins at various stages of maturation oocytes were gently spread and fixed to retain antigenicity of centromeric proteins as previously described (Hodges and Hunt, 2002). In short, the zona pellucida was removed mechanically after brief exposure of oocytes to 7mg/ml pronase (Roche Diagnostics, Mannheim, Germany). For fixation, oocytes were transferred to a clean microscope slide, which had been dipped in a solution of 1% paraformaldehyde in destilled H_2O (pH 9.2) containing 0.15% Triton X-100 and 3 mM dithiotreitol. The slide was allowed to dry slowly in a humid chamber for several hours before being washed in 0.4% Photoflo (Kodak) in destilled H_20. For indirect

immunoflourescence, spreads were briefly rinsed with PBS and blocked by PBS containing 1% BSA, 0,2% powdered milk, 2% normal goat serum, and 0.1 M glycine for 30 min. A sheep anti-MCAK antibody (a gift from Linda Wordeman, University of Washington, Seattle, WA), mouse anti-Mad2 (a gift from Beth Weaver, UCSD, La Jolla, CA) and mouse anti-BubR1 (a gift from Stephen Taylor, University of Manchester, UK) were used (1:50; 60 minutes). Following two washes in PBS, secondary antibodies were added to slides: an anti-sheep FITC-conjugated (Sigma-Aldrich), an anti-rabbit FITC-conjugated (Sigma-Aldrich) or an anti-mouse FITC-conjugated antibody (Sigma-Aldrich), respectively (1:50, 1 hour). Amplification of signal was by rabbit anti-fluorescein and goat anti-rabbit Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA). Aurora kinase B (AURKB) antibody (Transduction Laboratories, Lexington, KY, USA) labelling (1:50; 60 minutes) was localised after labelling with an anti-mouse TRITC-conjugated antibody (Sigma-Aldrich). Human CREST serum (HCT-100, Immunovision, Luxembourg) followed by anti-human IgG TRITC conjugate (Sigma) was used to localise the centromere domain (1:50). Trimethylation of K9 in centromeric histone H3 (Abcam, UK) was also analysed as marker of condensation/epigenetic state of centromeric heterochromatin by a specific antibody (rabbit anti-H3K9 trimethyl) in spread control and ZM-treated oocytes at 1:100 dilution followed by anti-rabbit FITC-conjugated antibody. Finally, chromosomes were stained with DAPI (Sigma-Aldrich) and mounted with a coverslip using 0.2% DABCO (Sigma-Aldrich) in 20% glycerol.

To quantify statistically the level of protein depletion following RNAi, images of five to ten spreads from each group were obtained by defined setting of the CCD camera and software. Subsequently, staining intensity was determined from the mean gray values of CREST, MCAK, and Mad2 foci, respectively, using ImageJ software version 1.38s (National Institutes of Health, United States; http://rsb.info.nih.gov/ij). The mean gray values of MCAK and Mad2 were compared relative to CREST, whose staining was unaffected by RNAi, in each group to determine the magnitude of reduction in staining intensity as percentage following specific RNAi. Background for gray values of MCAK was determined in spreads stained by CREST/anti-human TRITC antibody and anti-sheep FITC antibody, omitting MCAK first antibody reaction.

For analysis of chromosome congression *in vivo*, oocytes were cultured in M2 medium containing 0.5 µg/ml BisBenzimide H 33342 (Hoechst 33342; Sigma-Aldrich) for 15 min at 37°C.

Extraction and fixation of oocytes for spindle immunofluorescence was done as previously described (Eichenlaub-Ritter and Betzendahl, 1995). In short, the zona pellucida was removed mechanically after brief exposure to pronase. Oocytes were then extracted in a pre-warmed

microtubule-stabilizing solution containing glycerol, Triton X-100 and EGTA for 45–60 min at 37 °C (25% (v/v) glycerol, 2% Triton, 50mM KCl, 0.5mM $MgCl_2$, 25mM HEPES, 20 µM phenyl-methyl-sulfonyl-fluoride (PMSF), 5mM EGTA, pH 6.75). Oocytes were attached to a slide coated with poly-l-lysine (Sigma) and fixed for 8 min in 100% methanol at -20 °C. After rinsing with PBS labelling was with a monoclonal mouse anti-alpha-tubulin (Sigma-Aldrich; 1:400; 60 min, 37°C), followed by polyclonal anti-mouse FITC-conjugated antibody (1:60). Chromosomes were stained with 10 µg/ml DAPI or 1 µg/ml propidium iodide (Sigma-Aldrich). For confocal microscopy oocytes were also fixed according to a procedure by Messinger and Albertini (1991). In short, oocytes were fixed in a pre-warmed microtubule-stabilizing buffer (0.1 M Pipes, pH 6.9, 5 mM $MgCl_2.H_2O$, 2.5 mM EGTA) containing 2.0% formaldehyde, 0.5% Triton X-100, 1µM taxol, 10 units ml^{-1} aprotinin and 50% deuterium oxide for 20 minutes at 37°C, followed by blocking (PBS, 1% BSA, 0,2% powdered milk, 2% normal goat serum, 0.1 M glycine and 0.01% Triton X-100). Fixed oocytes were stored at 4°C in blocking solution until processed for indirect immunofluorescence. MTs of the spindles were labelled by a monoclonal mouse anti-alpha-tubulin antibody (1:200; 60 min, 37 °C). Second antibody was an anti-mouse FITC-conjugated antibody (1:50). Chromosomes were stained with propidium iodide and oocytes were mounted on poly-l-lysine coated slides with DABCO.

6.8 Micoscopy and image acquisition

For fluorescence microscopy of chromosome spreads and spindle morphology, cells were viewed with a Zeiss Axiophot fluorescence microscope using a 100x Neofluar (1.30 NA) oil objective (Zeiss, Jena, Germany) and imaged with a sensitive coupled charge device camera (SensiCam, PCO CCD imaging, Kelheim, Germany), which was controlled using SensiControl software version 4.03. Exposure time of the camera and intensity values of black/white pixels were adjusted to defined settings manually prior to image recording. Spindles were also imaged on a confocal laser scanning microscope, the Leica TCS SP2 (Leica Microsystems, Heidelberg, Germany), using a 63x HCX PL APO (1.32 NA) oil objective. For all immunofluorescent images, Z-series optical sections were obtained at 0.5-0.6 µm steps and then 2D/3D-reconstructed with Leica Confocal software (Leica Microsystems, Heidelberg, Germany). Images were processed with Adobe Photoshop (Adobe Systems, Mountain View, CA, USA). Immunofluorescent images of chromosome spreads were analysed and processed using the ImageJ software Version 1.38s. Chromosome spreads were further analysed and processed using ImageJ software. Final images were processed with Adobe Photoshop 7.0 software (Adobe System Inc., San Jose, CA, USA).

For live imaging oocytes were analysed by polarisation microscopy (OCTAX EyeWear™ MX, kindly provided by MTG, Altdorf, Germany) by placing them into preheated drops of 10 µl M2 medium covered with mineral oil (Sigma-Aldrich) in a WillCo Wells BV dish with glass bottom (Ref. No.: GWSt-5040, Amsterdam, Netherlands) on a heated stage of a Nikon microscope equipped with 20 x objective lens and warm plate (MTG), appropriate filters and LCD liquid crystal optics and hardware for imaging and recording for qualitative and quantitative polarization microscopy. Time-lapse microscopy was performed by taking images at 2 min intervals from 420 min of maturation to 960 min (culture overnight) to assess time of transition from M-phase to anaphase I, cytokinesis and first polar body formation and spindle length non-invasively in living oocytes. For analysis of kinetics of polar body formation logarithmic plots and functions were generated for each experimental condition with Microsoft Excel software (Microsoft Corp, Redmond, WA, USA).

6.9 C-Banding for chromosomal analysis

All oocytes were spread and C-banded as previously described (Eichenlaub-Ritter and Boll, 1989). For chromosome spreading a protocol based on a modified Tarkovski method was employed (Tarkowski, 1966). In brief, oocytes were placed into 1% sodium citrate, and then individually fixed and spread in ice-cold methanol/acetic acid (3:1). Chromosomes were stained by C-banding according to standard methods (Tarkowski, 1966; Eichenlaub-Ritter and Boll, 1989). Meiotic status of oocytes resuming maturation was analysed by counting numbers of oocytes with bivalent chromosomes or metaphase II chromosomes (dyads), independent of ploidy. Hyperploid oocytes with > 20 metaphase II chromosomes or the respective numbers of chromatids or hypoploids containing 16 to 19 metaphase II chromosomes or the respective numbers of chromatids were determined.

6.10 Statistics

Statistical analysis was by chi square-test with Yates correction. Meiotic progression, nuclear maturation and chromosomal constitution were considered significant ($p<0.05$) in comparison between treated (RNAi, ZM447439, nocodazole) and control groups. Furthermore, spindle aberrations and failure in chromosome congression were considered significant ($p<0.005$) in comparison between treated (RNAi, ZM, nocodazole) and control groups.

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List of Abbreviations

APC/C:	anaphase promoting complex/cyclosome
ATP:	adenosine 5'-triphosphate
AURKA:	aurora kinase A
AURKB:	aurora kinase B
AURKC:	aurora kinase C
BSA:	bovine serum albumin
BUB:	budding uninhibited by benzimidazole
cAMP:	cyclic adenosine mono-phosphate
CDC20:	cell division cycle 20
CDK:	cyclin-dependent kinase
CENP:	centromere protein
CPC:	chromosomal passenger complex
CSF:	cytostatic factor
DABCO:	1.4 Diabicyclo [2.2.2] octane
DAPI:	4,6-diaminidino-2-phenylindole
DMSO:	Dimethylsulfoxid
DNA:	deoxyribonucleic acid
EMI1/2:	early mitotic inhibitor 1/2
FITC:	fluorescein isothiocyanate
GFP:	green fluorescent protein
GV:	germinal vesicle
GVBD:	germinal vesicle breakdown
INCENP:	inner centromere protein
ICIS:	inner centromere Kin-I stimulator
MI:	meiosis I
MII:	meiosis II
MAD:	mitotic arrest deficient
MAPK:	mitogen-activated protein kinase
MCAK:	mitotic centromere associated kinesin
2-ME:	2-Methoxyestradiol
MPF:	maturation promoting factor
MT:	microtubule

MTOC:	microtubule organising centre
mRNA:	messenger RNA
PP2A:	protein phosphatase 2A
PBS:	phosphate-buffered saline
PB:	polar body
PLK1:	polo-like kinase 1
PMS:	pregant mare serum
REST:	Relative Expression Software Tool
RNA:	ribonucleic acid
RNAi:	RNA interference
RT-PCR:	reverse transcription – polymerase chain reaction
RZZ:	Rough ZW10 Zwilch
SA1/2:	stromal antigen 1/2
SAC:	spindle assembly checkpoint
SCC:	sister chromatid cohesion
SGO1/2:	shugoshin 1/2
SGOL2:	shugoshin-like protein 2
siRNA:	small interfering ribonucleic acid
SMC:	structural maintenance of chromosomes
SYCP3:	synaptonemal complex protein 3
TPX-2:	targeting protein for the *Xenopus* kinesin xklp2
TRITC:	tetramethylrhodamine isothiocyanate
XKCM1:	*Xenoupus* kinesin catastrophe modulator-1

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Hiermit erkläre ich, Edgar-John Vogt, dass die vorliegende Dissertation von mir selbstständig und nur mit den hier aufgeführten Hilfsmitteln und Quellen angefertig wurde. Sämtliche Experimente sind von mir selbst ausgeführt worden, ausser wenn explixit auf dritte verwiesen wird.

Ich habe weder anderweitig versucht, eine Dissertation einzureichen oder eine Doktorprüfung durchzuführen, noch habe ich diese Dissertation oder Teile derselben einer anderen Prüfungskommission vorgelegt.

Bielefeld, den 20. Oktober 2009