Susceptibility of mammalian oocytes to chromosome segregation errors with maternal aging

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Declaration

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Statement of Contribution of Others

Three publications (chapter 2, 3 and 4) are included for consideration in this thesis. The research higher degree candidate Yan Yun carried out all experiments except that Dr Simon Lane constructed the Venus-Ndc80 vector in chapter 4. The manuscripts in chapter 3 and 4 were initially written by Yan Yun with correction from all other co-authors. The manuscript in chapter 2 was drafted by Prof. Keith Jones. Dr Simon Lane wrote the software developed for live chromosome tracking and kinetochore analysis and prepared Figure 3E-H and Figure 5 in chapter 2. All other data analysis and figure preparation were performed by Yan Yun.

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Yun Y, Lane SI, Holt JE, Jones KT. N-terminal modification of Ndc80 can induce a spindle assembly checkpoint arrest in mouse oocytes. American Society for Cell Biology (ASCB), Philadelphia, United States (2014), **Poster presenation**

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Abstract

Advancing maternal age is a well-established risk factor associated with chromosome segregation errors in oocytes. In this thesis, I employed real-time high resolution imaging to examine the onset of aneuploidy and mechanisms regulating chromosome segregation in mouse oocytes. Specifically I determined 1) that although considerable cohesion loss occurs during MI, bivalent dynamics are not grossly affected, instead premature separation of dyads in MII was found to be the major segregation defect; 2) aged oocytes have decreased levels of SAC proteins on the kinetochores and possess both a lowered ability to maintain SAC arrest and re-establish bivalent biorientation following spindle disruption; 3) a genetic basis to aneuploidy susceptibility is likely to exist as suggested by the distinct aneuploidy phenotypes of two different mouse strains and 4) demonstrated that Ndc80 N-terminal modification was able to impose a robust SAC signalling, in doing so prevent chromosome mis-segregation in young mouse oocytes. Altogether this thesis directly examined the precise timing of chromosome segregation errors, and re-emphasized the role of a weakened SAC in maternal age-related aneuploidy.

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List of commonly used abbreviations

APC/C; Anaphase-Promoting Complex or Cyclosome

CPC; Chromosomal Passenger Complex

GVBD; Germinal Vesicle Breakdown

KT-MT; Kinetochore-Microtubule

MCC; Mitotic Checkpoint Complex

MetII; Metaphase II

MI; Meiosis I

MII; Meiosis II

NDJ; Non-disjunction

PBE; first Polar Body Extrusion

PSSC; Premature Separation of Sister Chromatids

SAC; Spindle Assembly Checkpoint

1 Introduction

1.1 Aims of this introduction

Meiosis is a highly specialized cell division process which allows maintenance of genetic diversity from one generation to the next in sexually reproducing species. Intriguingly, the female meiotic cell, the oocyte, is highly susceptible to chromosome mis-segregation during meiosis I resulting in embryonic aneuploidy, which is a leading cause of infertility, early miscarriage and birth defects in women. Moreover, it has been well established that advanced maternal age raises the incidence of aneuploidy in both human and mouse oocytes. Based on evidence from mouse models, several hypotheses have been proposed for age-related aneuploidy. The focus of this thesis is to investigate the mechanisms involved in control of chromosome segregation thereby providing a greater understanding of this age-associated phenomenon in mammalian oocytes.

This introduction will first provide an overview of the mammalian cell cycle machinery with respect to mitosis since it is the system in which the cell cycle has been best studied. The specialized nature of the first meiotic division in mammalian oocytes will then be examined, with a particular focus on homologous chromosome segregation; followed by an examination of current popular hypotheses on age-related aneuploidy.

1.2 Regulation of the cell cycle

In comparison to the prokaryotic cell, the eukaryotic cell cycle has evolved a complex process of division and duplication, known as 'mitosis'. It is the fundamental process of mitosis that allows for the development of a mature organism with diverse tissues and organs, from a single cell – zygote. In a diploid organism, the zygote is formed by the fusion of two haploid gametes – a sperm and an egg, which are in turn produced from a specialized form of cell division – meiosis. Mitosis and meiosis allow species to maintain genome stability and genetic diversity from one generation to the next (Lopez-Maury et al., 2008; Morgan, 2007).

In eukaryote cells, the key features of the mitotic cell cycle involve chromatin duplication, followed by sister chromatid segregation and cytokinesis. To ensure faithful chromosome segregation at each division, the cell cycle has evolved to consist of a series of biochemical reactions which regulate events in the correct timing and order (Morgan, 2007). The two key classes of regulatory molecules important here are the cyclins and cyclin-dependent kinases (CDKs), which constitute proteins that are well conserved among all eukaryotes (Hartwell and Weinert, 1989; Malumbres, 2011; Nurse, 2002). As the name suggests, CDKs are a family of protein kinases, which require cyclins as binding partners for activity. In general, CDKs remain at a constant level throughout the cell cycle, whilst changes in cyclins regulate the activity of these kinases. Phosphorylation events or direct inhibitor proteins are also involved in their regulation (Besson et al., 2008; Chymkowitch and Enserink, 2013; Wang et al., 2011a). The most essential CDK for cell cycle control is CDK1, whose predominant binding partner is cyclin B1 (Chymkowitch and Enserink, 2013; Wang et al., 2011a). A rise in CDK1 drives several major events of mitosis, including nuclear envelope breakdown and spindle assembly. On the contrary, its decline, which is driven by ubiquitin-mediated degradation of cyclin B1, causes mitotic exit (Enserink and Kolodner, 2010). Therefore proteasomal mediated degradation of cell cycle proteins represents a key regulatory mechanism for the cell cycle, and in

mammals the E3 ubiquitin ligase that controls both mitotic and meiotic progression is the Anaphase Promoting Complex/Cyclosome (APC/C), which will be the subject of the subsequent discussion.

1.3 The Anaphase-Promoting Complex or Cyclosome (APC/C)

The APC/C is a highly conserved, multi-subunit E3 ubiquitin (Ub) ligase that targets key regulatory substrates for proteolysis by the 26S proteasome (Barford, 2011a; Barford, 2011b; Peters, 2006; Pines, 2011). It is important to note that the APC/C itself shows little activity without binding its coactivators, of which two of the best studied are Cdc20 and Cdh1 (also known as Fzr1) (Dawson et al., 1995; Kimata et al., 2008; Schwab et al., 2001; Visintin et al., 1997). Both APC/C^{Cdc20} and APC/C^{Cdh1} mediate the degradation of a large range of substrates through recognizing their specific discrete motifs. The most well characterized motifs are the D-box (destruction box) (Glotzer et al., 1991) and the KEN-box (lysine-glutamate-asparagine box) (Pfleger et al., 2001), although some others have also been identified (Pines, 2011). The D-box is recognized by both APC/C^{Cdc20} and APC/C^{Cdh1}, while the KEN box is only recognized by APC/C^{Cdh1} (Pfleger and Kirschner, 2000). In addition phosphorylation is one of the important factors for regulating the APC/C activity. For example, Cdk1-dependent phosphorylation is stimulatory for APC/C^{Cdc20} due to its increased Cdc20 binding capability. However, Cdh1 phosphorylation by Cdk1 inhibits its association to the APC/C, thus resulting in reduced APC/C activity (Primorac and Musacchio, 2013). In mitosis, APC/C^{Cdc20} is active at the metaphase anaphase transition primarily due to high CDK1 activity; once entry into anaphase occurs, loss of CDK1 causes a switch to APC/C^{Cdh1} (Hagting et al., 2002; Jones, 2011; Kraft et al., 2003; Rudner and Murray, 2000). In so doing the APC/C temporally regulates the key proteins for destruction, thus allowing the cell cycle events to occur in the correct order.

As the name suggests, the APC/C is essential for promoting anaphase initiation, and this is ultimately achieved 1) by regulating CDK1 activity through cyclin B1 destruction and 2) through the resolution of the molecular ties that hold the chromosomes together, the cohesin complexes. The cohesin complexes effectively act as rings that encircle the chromosome arms. In order to break these ties, activity of the cysteine protease Separase is required, which targets and cleaves the

cohesin rings (Jallepalli et al., 2001). Prior to anaphase Separase is held inactive due to the binding of its chaperone protein –Securin, as well as its inhibitory phosphorylation by CDK1, and it has been shown that both of the pathways must be disrupted to achieve its activation (Chiang et al., 2011; Ciosk et al., 1998; Gorr et al., 2005; Huang et al., 2005; Stemmann et al., 2001). Upon activation at metaphase-anaphase transition, the APC/C targets both securin and cyclinB1 for destruction, thereby allowing activation of Separase, and so cohesin cleavage and chromosome segregation (**Figure 1.1**). Therefore, by regulating cyclin B1 (and thus CDK1 activity) and Securin, the APC/C ensures unidirectional, efficient mitotic exit (Jones, 2010; Pesin and Orr-Weaver, 2008; van Zon and Wolthuis, 2010). It is important to note here that in addition to the regulation of metaphase-anaphase transition, the APC/C is functional throughout the cell cycle.

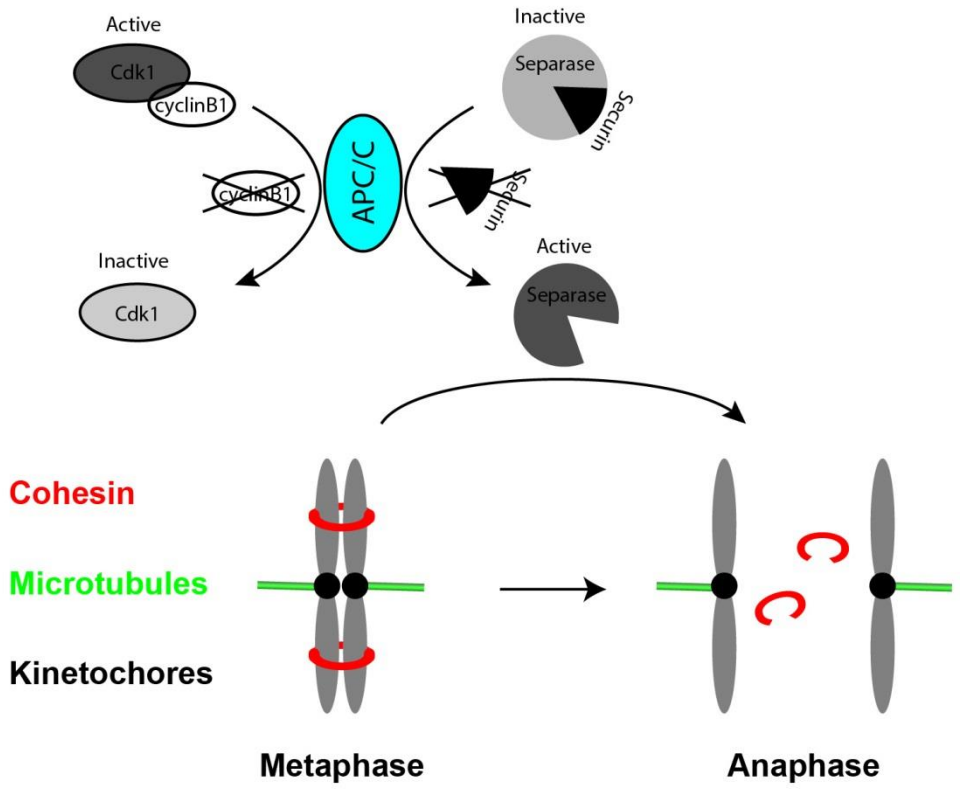

Figure 1.1 The APC/C ensures chromosome segregation. During the metaphase-anaphase transition, the APC/C targets both cyclinB1 and securin for destruction. CyclinB1 destruction results in inactivation of Cdk1, which together with securin degradation, lead to Separase activation. Active Separase then cleaves chromosome cohesin complex, followed by chromosome segregation and mitotic exit.

1.4 Kinetochore architecture and microtubule interaction

An essential and important prerequisite to APC/C mediated chromosome segregation is the stable and appropriate attachment of the chromatin to spindle microtubules. This is achieved through microtubule interaction with a structure known as the kinetochore, which consists of numerous protein complexes assembled on the chromosomes (Cheeseman and Desai, 2008; Cleveland et al., 2003). The inner kinetochore consists of a series of centromere proteins, such as CenpA and CenpC. The outer kinetochore, which connects the centromere-associated proteins to the plus ends of microtubules includes the KMN networks, composed of KNL1, Mis12 and Ndc80 complexes (Lampert and Westermann, 2011; Santaguida and Musacchio, 2009) (**Figure 1.2**).

Within the KMN network, the Ndc80 complex plays a particularly important role in the formation of microtubule-kinetochore attachments since it lies at the interface between the inner kinetochore and where spindle microtubule plus ends terminate (Cheeseman et al., 2004; DeLuca et al., 2005). This complex is highly conserved among species and composed of four components: Ndc80 (known as Hec1 in human), Nuf2, Spc24 and Spc25 (Ciferri et al., 2007; Ciferri et al., 2008; Wei et al., 2006; Wei et al., 2005). Structural analysis demonstrated that this four-protein complex forms an ~50 nm elongated molecule with globular domains that are separated by a α-helical coiled-coil shaft (Ciferri et al., 2008; Lampert and Westermann, 2011; Wei et al., 2006; Wei et al., 2005).

It has been well documented that Ndc80 complex plays an essential role in forming and stabilizing microtubule attachment. Depletion or mutation in any component of the complex causes misaligned chromosomes, disturbed spindle organization, reduced attachment stability and abnormal spindle checkpoint activity in both mitotic and meiotic cells (Ciferri et al., 2007; DeLuca et al., 2005; DeLuca et al., 2003; Janke et al., 2001; McCleland et al., 2003; McCleland et al., 2004; Sun, 2011; Sun and Kim, 2012; Sun et al., 2010; Sun et al., 2011; Tanaka and Desai, 2008; Xiong, 2011).

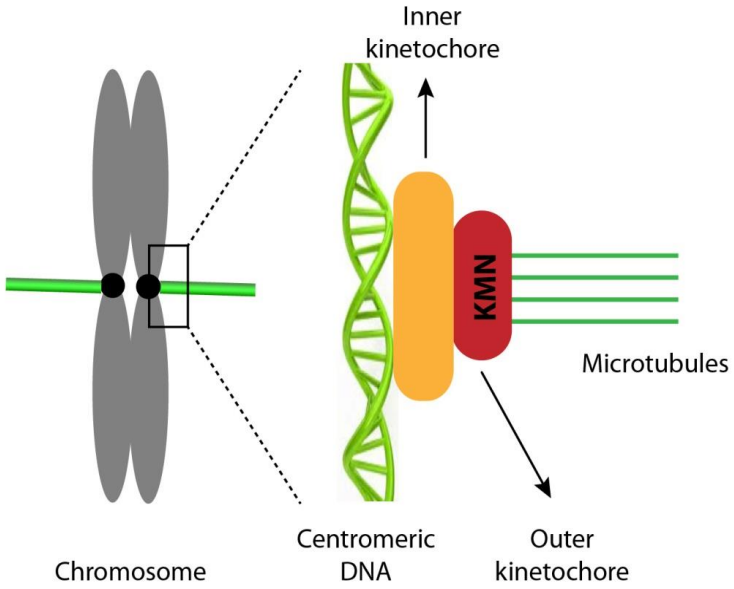

Figure 1.2 Schematic of kinetochore architecture. As the bridge between chromosome and microtubule, kinetochore is composed of inner kinetochore and outer kinetochore. Towards the microtubule plus end, the KMN forms attach¬ment sites for dynamic microtubules. On the other side, it is anchored to the chromosome centromere through interactions with multiple inner kinetochore complexes.

Further investigations revealed that N-terminal of Ndc80 is necessary for this complex to directly bind to microtubules, and the interaction is predominantly electrostatic (Ciferri et al., 2008; DeLuca et al., 2006; Guimaraes et al., 2008; Mattiuzzo et al., 2011; Miller et al., 2008; Tooley et al., 2011). Specifically, it involves actions of negative charges in microtubule C-terminal tails and positive charges in the N-terminal tail of Ndc80 as well as its calponin homology domain (CHD) (**Figure 1.3**). For example, either mutation to reduce the positive charge or deletion of Ndc80 N-terminal tail decreases or even abolishes its binding affinity to microtubules, rendering the protein unable to generate appropriate tension on sister kinetochores and congress chromosomes to the metaphase plate (Ciferri et al., 2008; DeLuca et al., 2006; Guimaraes et al., 2008; Miller et al., 2008). Moreover, the interaction of microtubules and kinetochore is regulated via Ndc80 N terminus phosphorylation by Aurora B kinase, and this error correction mechanism is essential for faith chromosome segregation (Ciferri et al., 2008; DeLuca et al., 2006; Lampert and Westermann, 2011). The kinase Aurora B will be discussed in section 1.5.

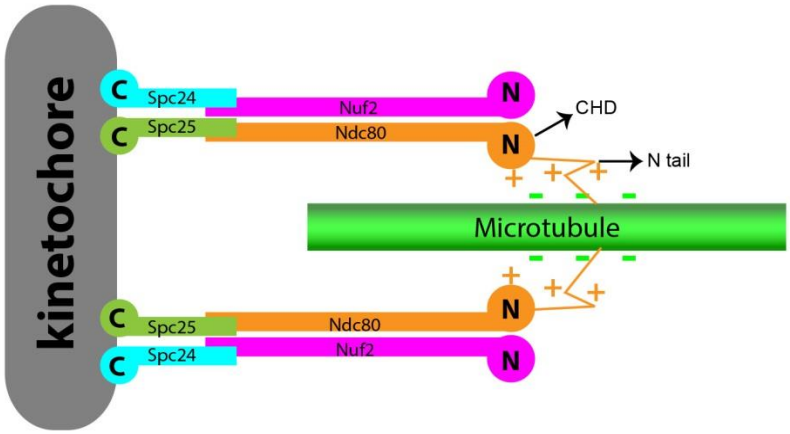

Figure 1.3 Model of interactions between Ndc80 complex and microtubules. Microtubule-Kinetochore binding is dependent on electrostatic interactions. Ndc80 complex is composed of two heterodimers: Ndc80-Nuf2 and Spc24-Spc25. With the former one, positive charges (+) in the N-terminal tail of Ndc80 and its CHD are essential to interact with negative charges (-) in microtubules, whereas in the opposite end, Spc24-Spc25 establishes the binding to the kinetochore.

1.5 The Spindle Assembly Checkpoint (SAC)

Correct microtubule-kinetochore interactions are essential to achieve faithful chromosome segregation and prevent aneuploidy. An important cell checkpoint that has evolved in eukaryotes to ensure the fidelity of this process, is the spindle assembly checkpoint (SAC) (Foley and Kapoor, 2013; Musacchio and Salmon, 2007). In mitosis this mechanism can sense unattached kinetochores or misaligned chromosomes, efficiently enough to detect one single kinetochore lacking microtubule occupancy (Rieder et al., 1995; Rieder et al., 1994), thus delaying anaphase initiation to give more time for kinetochore attachment or error correction (Musacchio and Salmon, 2007). The SAC is active during prometaphase, and able to generate a potent inhibitor of the APC/C by assembly on unoccupied kinetochores (Primorac and Musacchio, 2013). When all kinetochores are correctly attached and chromosome alignment at the metaphase plate is achieved, the SAC is satisfied and switched off. This results in activation of the APC/C, which targets cyclin B1 and Securin for destruction and allows chromosome segregation onset and mitotic exit (Clute and Pines, 1999; Musacchio and Salmon, 2007; Thornton and Toczyski, 2003).

The SAC was first characterized in *Saccharomyces cerevisiae*, where mutants of SAC proteins failed to impose an arrest in mitosis in response to spindle disruption. These proteins are conserved in all eukaryotes, and include mitotic-arrest deficient (Mad) family members Mad1, Mad2 and Mad3 (also known as BubR1 in humans), budding uninhibited by benzimidazole (Bub) family members Bub1 and Bub3, as well as kinase Mps1 and Aurora B (Foley and Kapoor, 2013; Hoyt et al., 1991; Li and Murray, 1991; Weiss and Winey, 1996).

It is generally accepted that the SAC signal originates from unattached kinetochores (Li and Nicklas, 1995; Musacchio and Salmon, 2007; Rieder et al., 1995), and integrity of kinetochores is required for the SAC protein recruitment (Lampert and Westermann, 2011). For example,

disruption of the Ndc80 complex reduced Mad1/Mad2 and Mps1 localization and caused faulty SAC function (Martin-Lluesma et al., 2002). Moreover, analysis on the network of dependencies for the SAC protein kinetochore localization revealed a conserved hierarchy among species. In fission yeast, a three-layered hierarchy was identified, with Ark1/Aurora B and Mph1/Mps1 on the top, followed by Bub1 and Bub3 in the middle, and Mad3/BubR1 and Mad1-Mad2 complex for the lowest level (**Figure 1.4**) (Heinrich et al., 2012). Specifically, Aurora B and Mps1 are required for the recruitment of all other SAC proteins to kinetochores. Bub3 depletion only impairs kinetochore enrichment of Mad1, Mad2 and Mad3, but has little effect on Aurora B and Mps1 (Heinrich et al., 2012). This hierarchy suggests that different levels of SAC components might act as elements of distinct pathways. Indeed, data have shown that kinetochores with microtubule occupancy but lack of tension were associated with Bub1 and BubR1 enrichment, but not Mad2; Mad2 responded and recruited to kinetochores when only the spindle was completely disrupted (Skoufias et al., 2001). Therefore, the SAC signalling is maintained and regulated in a complex way, with both spatial and temporal order.

Cdc20, one of the co-activators of the APC/C, is sequestered by a SAC complex termed the mitotic checkpoint complex (MCC) and so APC/C activation is prevented. The MCC is composed of three SAC components Mad2, BubR1, Bub3, as well as Cdc20 itself, and binds the APC/C to reduce its ubiquitin-ligase activity, thus protecting cyclin B1 and Securin from destruction (Clute and Pines, 1999; Foley and Kapoor, 2013; Musacchio and Salmon, 2007). The formation of MCC involves many of the SAC proteins, and one of the commonly accepted models is the ‘Mad2-template model’ (De Antoni et al., 2005; Foley and Kapoor, 2013; Luo et al., 2004; Musacchio and Salmon, 2007; Primorac and Musacchio, 2013). Specifically, Mad2 binds Mad1 to form the Mad1-C-Mad2 (closed Mad2) complex on unattached kinetochores, as the template, this complex then recruits O-Mad2 (open Mad2) to kinetochores, where O-Mad2 is turned into C-Mad2 by binding to Cdc20, in

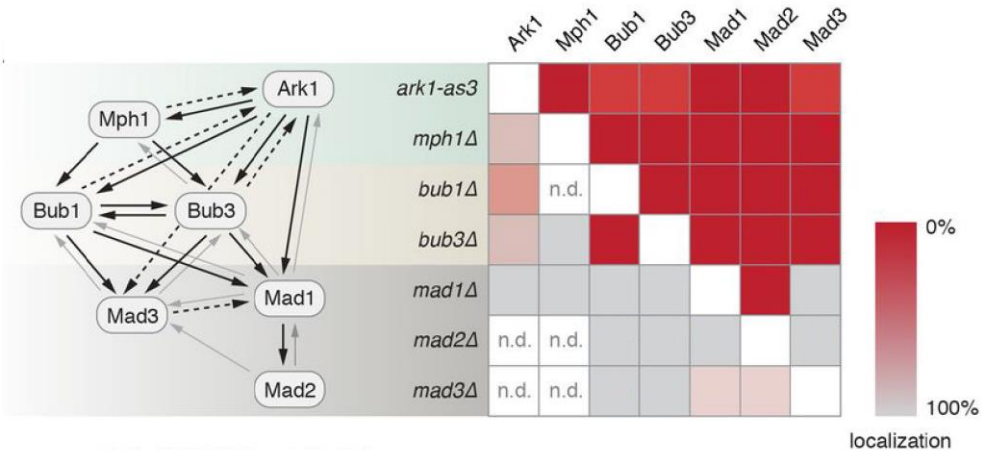

Figure 1.4 The hierarchy of SAC protein localization dependencies in fission yeast. Black lines with arrows indicate a dependency, dashed with a partial dependency and gray shows absence of dependency (For example, Mad1 kinetochore localisation is completely dependent on Ark1 localisation, while absence of Mad1 has no impact on Ark1 enrichment to kinetochores). The color from gray to red on the right shows kinetochore-localized SAC protein levels (high to low) after the individual SAC component is under depletion. n.d. indicates not determined (Heinrich et al., 2012). Note: Δ show mutant yeast stains devoid of indicated protein expression; loss of function study of Ark1 is based on kinase inhibitor.

doing so to create the basis of the MCC complex Cdc20-C-Mad2, which then binds BubR1-Bub3 to form the MCC.

In addition to the MCC, other SAC proteins including checkpoint kinases Mps1 and Aurora B, have recently been found to be essential for efficient MCC assembly and the SAC signal control (Herzog et al., 2009; Jia et al., 2011; Morrow et al., 2005; Tipton et al., 2013; Zich et al., 2012). This is perhaps not surprising given that both of them are required for proper recruitment of most of the other SAC proteins to kinetochores, as discussed in the above (Heinrich et al., 2012; Hewitt et al., 2010; Jelluma et al., 2010; Tighe et al., 2008; Zich et al., 2012). Mps1, a dual specificity kinase, is involved in several aspects of SAC control. In early mitosis its activity promotes the recruitment of Mad1-C-Mad2 to kinetochores (Hewitt et al., 2010; Maciejowski et al., 2010; Santaguida et al., 2010; Sliedrecht et al., 2010), while once in mitosis it is essential for O-Mad2 enrichment to the Mad2 template assembled on kinetochores (Hewitt et al., 2010). On the contrary, failed dissociation of active Mps1 from kinetochores retained active checkpoint at prometaphase, or reactivated spindle checkpoint during anaphase, suggesting its removal is a prerequisite for SAC silencing (Jelluma et al., 2010; Palframan et al., 2006). In addition, Mps1 has been shown to be indispensable in establishing sister-kinetochore bi-orientation and tension, thus contributing to the correction of attachment errors in a SAC-independent pathway (Maure et al., 2007; Petsalaki and Zachos, 2013), which shared the similar roles of Aurora B, jointly or independently (Jelluma et al., 2008; Saurin et al., 2011).

Aurora B, a member of the chromosomal passenger complex (CPC), is responsible for attachment error correction by phosphorylating kinetochore substrates in a tension-dependent mechanism (Kallio et al., 2002; Kelly and Funabiki, 2009; Liu et al., 2009). Specifically, for kinetochores lacking appropriate tension, centromere-localized Aurora B phosphorylates kinetochore substrates

and then destabilizes the erroneous microtubule attachments, since the phosphorylation events lower its binding affinity to microtubules, thus allowing for a new round of attachment attempt. Once sister kinetochores are under proper bi-orientation, the components at the outer kinetochores become physically stretched away from the inner centromere, where Aurora B is located. Meanwhile, phosphatase PP2A located at outer kinetochores maintains de-phosphorylating activities, which result in formation of stable end-on attachment to microtubules (Foley and Kapoor, 2013; Liu et al., 2009). In such a way Aurora B indirectly involves in the SAC, as its attachment and destabilization activity generates unattached kinetochores, which then activates the SAC (Pinsky et al., 2006). However, evidence also suggest a direct role of Aurora B in the SAC response, independently of error correction (Santaguida et al., 2011).

To summarize, although the network of SAC is extremely complex, great progress has been made in the last decade. Techniques such as molecular perturbation and crystal structure analysis will continue to provide insight into which other SAC components are involved and how they interact with each other. The mechanisms of how Mps1 and Aurora B function currently remain largely unknown, and these are to be investigated with respect to oocyte meiosis and are discussed in chapter 4 of this thesis.

1.6 Female meiosis – a specialized cell division unique to eggs

Whilst mitosis maintains growth and self-repair of multicellular organisms, the process of meiosis is necessary to allow genetic recombination and sexual reproduction (Baudat et al., 2013; Clift and Schuh, 2013; Hochwagen, 2008). One of the main differences between mitosis and meiosis is the way in which the chromosomes are segregated. Mitotic cells separate their sister chromatids once after DNA replication, whereas meiosis encapsulates one round of chromatin replication and two rounds of sequential chromosome segregation (Clift and Schuh, 2013; Hochwagen, 2008; Marston and Amon, 2004). Homologous chromosomes (also known as 'bivalents') are segregated in meiosis I (MI), while sister chromatid segregation occurs in meiosis II (MII), such that the number of chromosome in the resulting cells is reduced to half the original number, typically producing four haploid daughter cells. In male mammals, all of the four products will eventually become gametes – sperm. However in females, asymmetric division allows only one egg to be produced, with the smaller daughter cells (polar bodies) destined to degrade. This asymmetric division is developmentally essential because it allows an egg being able to retain plenty of nutrients such as mRNA and proteins and support the early embryo development after fertilization but prior to activation of the new embryonic genome (Brunet and Verlhac, 2011; Evans and Robinson, 2011; Fichelson and Huynh, 2007). It is important to note that the process of fertilization via fusion of an egg and a sperm, restores the chromosome number of offspring to diploid status.

1.6.1 Overview of meiosis

A unique feature of oocyte meiosis is the two distinct periods of arrest during development and maturation – prophase I arrest and metaphase II arrest (Jones, 2011). It has been documented that primordial germ cells replicate chromatin and subsequently establish cohesin in pre-meiotic S-phase of embryonic life (Revenkova et al., 2010; Watanabe et al., 2001), followed by a long prophase

arrest. The arrest lasts for several months in mice, or up to many decades in humans. In the reproductively mature adult, cohorts of these oocytes are then induced to resume division due to the cyclic surges in Luteinizing Hormone (LH) following puberty. With the stimulation of such hormone surge, oocytes undergo Germinal Vesicle Breakdown (GVBD) within hours, during which time chromatin condenses into individualized chromosomes. The subsequent events of prometaphase I, metaphase I and anaphase I take place in approximately 10 hours in mouse, followed by the second arrest at metaphase II without an intervening S-phase (**Figure 1.5**). This arrest is not released until the fertilization by a sperm, which results in calcium oscillations that initiate the second meiotic division (Carroll, 2001; Jones, 2011; Miao and Williams, 2012). The detailed events of MI will be discussed in the subsequent sections, which are the content involved in this thesis.

1.6.2 Primordial germ cells (PGC)

In mammals, all of the early embryonic cells produced by the first several divisions of a fertilized egg are totipotent, and they are capable to give rise to all types of cells, including germ cells (Hillman et al., 1972; Mitalipov and Wolf, 2009; Saiz and Plusa, 2013). For example, in both mice and humans, after the fusion of an egg and a sperm to be a zygote, all cells up to at least 4-cell stage of embryogenesis seem to have totipotency. However, once reaching a 16-cell morula stage, these totipotent cells initiate the differentiation into either the blastocyst's inner cell mass or the outer trophoblasts (Mitalipov and Wolf, 2009; Suwinska et al., 2008; Tarkowski et al., 2010). Following implantation and differentiation of the embryo, a small group of cells from the epiblast is induced by their neighbours to become primordial germ cells. These cells perform series of mitotic proliferation and migrate through the hindgut to their final destination – genital ridges (developing gonads) (McLaren, 2000; McLaren, 2003; Molyneaux and Wylie, 2004). After another rapid mitotic

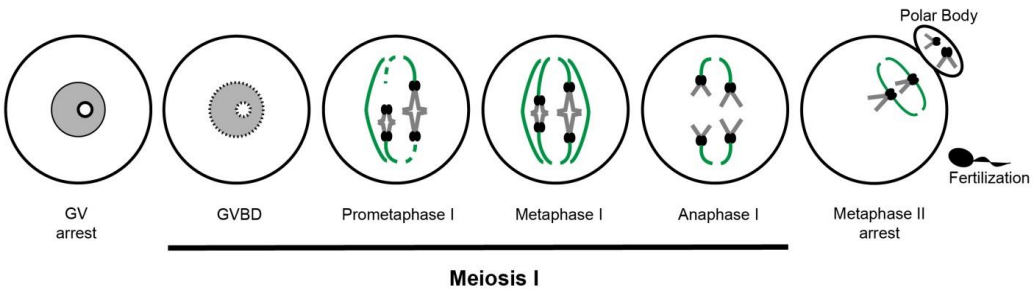

Figure 1.5 The stages of female meiosis in mouse showing major chromosome behaviors and two meiotic arrests.

Black circles represent kinetochores, grey for chromosomes and microtubules are in green.

proliferation, the undifferentiated gonads will develop into either a testis or an ovary, in which SRY gene localized on the Y chromosome plays an important role (Brennan and Capel, 2004; Edson et al., 2009). Specifically, it is believed that SRY actively induces testis development, while in the absence of SRY the ovary passively develops. In females, the germ cells are now called oogonia and develop in clusters. Meiosis is initiated in the oogonia at approximately 13.5 day post coitum (pdc) in mice and during the 3rd month in humans, and are then referred to as oocytes (Monk and McLaren, 1981; Pepling, 2006).

1.6.3 Cohesin establishment and bivalent formation

During the cell cycle, accurate chromosome segregation requires that the pair of newly synthesized sister chromatids are physically connected, and this linkage is regulated by a multi-subunit structure – the cohesion complex, which was first identified in *Saccharomyces* and *Xenopus* (Guacci et al., 1997; Losada et al., 1998; Michaelis et al., 1997). The cohesin complex in meiosis is composed of 4 core subunits: structural maintenance of chromosomes member SMC3 and germ cell specific components SMC1β (Revenkova et al., 2001), Rec8 (Parisi et al., 1999; Watanabe and Nurse, 1999) and STAG3 (Pezzi et al., 2000; Prieto et al., 2001).

Several key stages are included in the meiotic prophase: leptotene, zygotene, pachytene and diplotene. Upon entering leptotene, homologous chromosomes, with one coming from each parent, begin to condense and then align in pairs via DNA double stranded breaks (DSBs) in many species, including humans (Gerton and Hawley, 2005; Tesse et al., 2003). Zygotene stage is featured with the homologous chromosome alignment and synapsis, which is facilitated by the formation of synaptonemal complex (SC) (Kleckner, 2006; Page and Hawley, 2004). At pachytene SC assembly is completed and this is also the stage when chromosomal crossover is established. Specifically,

non-sister chromatids of homologous chromosomes exchange their homologous segments, in doing so form chiasmata at the sites (Page and Hawley, 2004). However, the crossovers and chiasmata are not visible until next stage – diplotene, in which the SC gradually dissolves, and the chiasmata physically link homologous chromosome pairs (also known as 'bivalents') until they are resolved in anaphase I (Lynn et al., 2007; Zickler and Kleckner, 1999).

1.6.4 Follicle growth and meiotic resumption

In the mammalian adult female, oocytes grow and gain meiotic competence within follicles, in which the oocyte-granulosa cell interaction plays essential roles (Shomper et al., 2014). The major stages classified during folliculogenesis include: primordial follicle, primary follicle, secondary follicle, antral follicle and preovulatory follicle (**Figure 1.6**). In female mice, upon arriving at the genital ridge, the primordial germ cells perform mitotic proliferation and form germline cysts, which are highly conserved among species during evolution (de Cuevas et al., 1997; Pepling et al., 1999; Pepling and Spradling, 1998). In the cysts, the germ cells initiate meiosis and subsequently oocytes begin to arrest in the diplotene stage. Following a breakdown in the germline cysts after birth, primordial follicles are formed and are composed of individual oocytes with surrounding pre-granulosa cells. The population of primordial follicles serves as an oocyte pool, which defines female reproductive life span (Pepling, 2006); although this idea is challenged by lines of evidence showing the existence of female germline stem cells in postnatal mammalian ovaries, which even produce developmental-competent oocytes (Niikura et al., 2009; Tilly et al., 2009; Zou et al., 2009). Follicle recruitment involves the activation of primordial follicles into primary follicles and subsequent cyclic recruitment for ovulation after puberty (McGee and Hsueh, 2000). Primary follicle formation is marked by morphological changes in granulosa cells to a cuboidal shaper, and when multiple layers of these somatic cells are present around the the oocyte, it is known as a

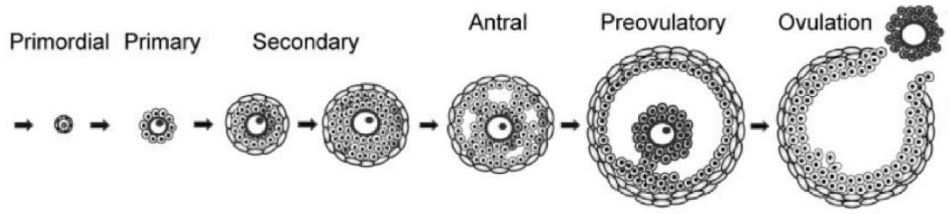

Figure 1.6 Schematic of the major stages of mouse folliculogenesis. Primordial follicles form 1–2 d after birth, in which oocytes remain arrested. With follicle recruitment, primary follicles develop which is characterized with morphological change in granulosa cells, followed by secondary follicle development containing multiple layers of granulosa cells around the oocyte. Further growth to form an antral follicle is dependent on gonadotrophic hormones. Following series of hormone actions, selected antral follicles eventually reach preovulatory stage, then are ovulated for fertilization (cited from (Edson et al., 2009)).

secondary or preantral follicle. It appears that these early stages of follicle development are not dependent on gonadotropin stimulation. The transition to antral folliculogenesis is marked by the formation of fluid-filled spaces, which eventually form an antral cavity. From this stage onwards, gonadotropins become essential and regulate the formation of a few selected antral follicles that reach preovulatory stage, followed by ovulation when the oocyte is ready for fertilization by sperm (Edson et al., 2009).

Acquisition of meiotic competence is associated with synthesis and activity of CDK1 (Kanatsu-Shinohara et al., 2000). CDK1, a serine-threonine kinase, is a key regulator of the G2/M transition in both mitosis and meiosis (Mehlmann, 2005). However, during prophase I arrest this kinase is kept in inactive by multiple pathways (Lane, 2012). Firstly, low CDK1 activity is maintained by its inhibitory phosphorylation on Thr14 and Tyr15. In general, high levels of cyclic adenosine monophosphate (cAMP) within the oocyte are responsible for maintaining CDK1 phosphorylation state. Specifically, elevated cAMP results in active protein kinase A (PKA), which then activates Wee1B kinase which is responsible for CDK1 phosphorylation and at the same time, inactivates Cdc25B phosphatase that would otherwise reverse Wee1B mediated inhibition (Han and Conti, 2006). Secondly, accumulation of cyclin B1 is prevented by the APC/C. It is well established that Cdh1, a coactivator of the APC/C, is indispensable for maintenance of low levels of cyclin B1 and mouse oocyte GV arrest (Holt et al., 2011; Reis et al., 2006). Contrary to the GV arrest, reentry to meiosis involves in a decrease of cAMP levels, so leading to PKA inactivation and subsequent Cdc25B activation and dephosphorylation of CDK1, followed by CDK1 kinase activation (Edson et al., 2009). In addition, cyclin B1 accumulation in the nucleus is also essential for the resumption (Ledan et al., 2001).

1.6.5 Acentrosomal spindle assembly during early prometaphase

In the hours following meiotic resumption, the oocyte initiates the building of a bipolar spindle capable of segregating homologs. In mammalian somatic cells, a structure known as the centrosome is the origin of assembling microtubules (Tanenbaum and Medema, 2010). However, oocytes lack centrosomes, and bipolar spindle assembly is initiated from multiple microtubule organizing centres (MTOC) from a cytoplasmic microtubule network in prophase I (Carabatsos et al., 2000; Gueth-Hallonet et al., 1993; Schatten et al., 1986; Schuh and Ellenberg, 2007). Using cell-free Xenopus egg extracts, it was demonstrated that chromatin can act as origins for bipolar spindle assembly (Heald et al., 1996). But other studies showed that even in the absence of chromatin bipolar spindles can still assemble, despite with lower efficiency (Brunet et al., 1998; Yang et al., 2007). In mouse oocytes, over 80 MTOCs form de novo and are distributed throughout the ooplasm. Following GVBD these MTOCs congress at the centre of the oocyte and interact with each other. As meiosis I progresses, a ball of microtubules is formed, and MTOCs are ejected from the microtubule ball. The transformation into a bipolar spindle is achieved through a series of clustering events, during which Kinesin-5 is essential for both spindle bipolarization and subsequent elongation (Schuh and Ellenberg, 2007). During this process, the homologous chromosomes interact with microtubules via the kinetochores, and gradually establish stable connections as they align at the metaphase plate in preparation for segregation.

1.7 Homologous chromosome segregation: an error-prone process in oocytes

1.7.1 Unique features of homologous chromosome segregation

A requirement specific to meiosis is the need for sister kinetochores to mono-orientate and form attachments with microtubules emanating from the same spindle pole (Holt and Jones, 2009; Homer, 2011). Specifically, one bivalent is composed of four chromatids with each one containing a kinetochore. In meiosis I sister kinetochores act as one functional unit and are attached to microtubules in a 'side-by-side' conformation, in doing so the pair of sister chromatids is moved to the same spindle pole, while in meiosis II and mitosis 'back-to-back' arrangement is required for sister chromatid separation to the two opposite directions (**Figure 1.7**). In addition, it is notable that when the cohesin along chromosome arms is cleaved at anaphase I, so allowing for chiasmata resolution and homologous segregation, the centromeric cohesin must be retained to tether the sister chromatids together until anaphase II. An integral component of this stepwise loss of cohesion is shugoshin-dependent protection of centromeric cohesin (Clift and Marston, 2011; Holt and Jones, 2009). Specifically, in MI chromosome arm cohesin is phosphorylated but centromeric cohesin is not due to shugoshin co-localization, which works together with PP2A to maintain its dephosphorylation state, thus escaping from Separase-mediated cleavage at anaphase I (**Figure 1.8**) (Clift and Marston, 2011; Holt and Jones, 2009; Homer, 2011; Ishiguro et al., 2010; Lee et al., 2008). However, it has been reported that either redistribution of shugoshin (Gomez et al., 2007) or recruitment of an inhibitor (Chambon et al., 2013) mediates deprotection of centromeric cohesin and trigger sister chromatid segregation in meiosis II.

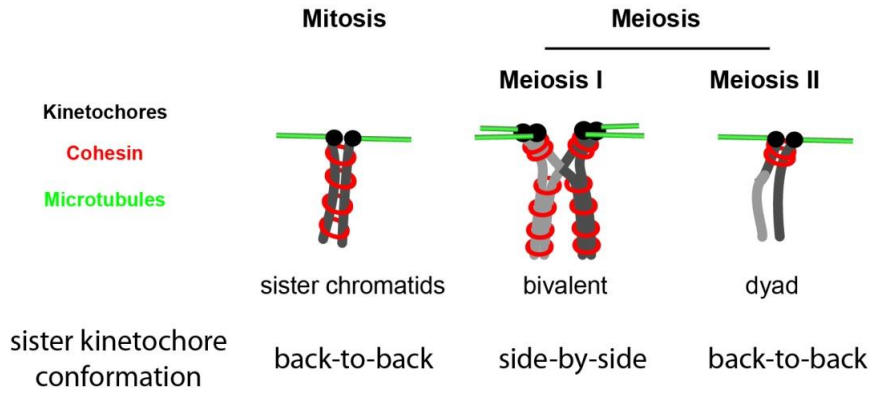

Figure 1.7 Sister kinetochore arrangements for microtubule attachment in mitosis and meiosis. In both mitosis and meiosis, sister chromatids are held together by cohesin complex. In mitosis, sister kinetochores are arranged in a 'back-to-back' pattern with each attached by microtubules from opposite spindle poles. However, in meiosis I, a pair of sister kinetochores are attached in a 'side-by-side' conformation to the same spindle pole, in so doing ensure the faithful segregation of homologous chromosomes. The 'back-to-back' pattern is resumed in meiosis II, similar to mitosis (Modified from (Holt and Jones, 2009)).

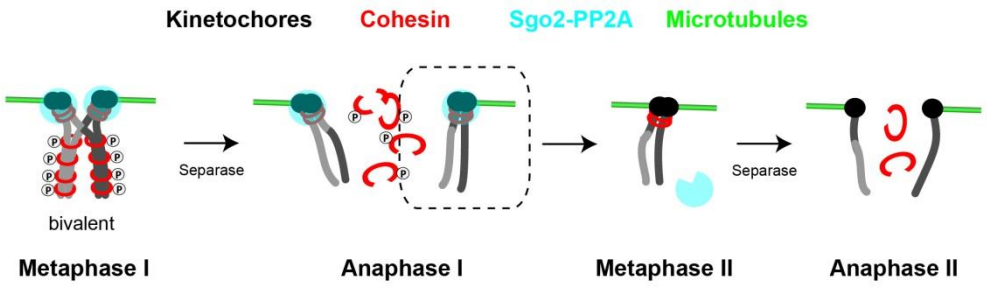

Figure 1.8 The stepwise loss of cohesin in meiosis I and II. A bivalent is depicted as dark and light grey here. Prior to anaphase I onset, phosphorylated cohesin along chromosome arms is cleaved by Separase, so allowing resolution of chiasmata and homologous chromosome segregation, whereas centromeric cohesin is retained due to Sgo2-PP2A dependent protection, which maintains its dephosphorylation state. In meiosis II, Sgo2-PP2A is off from functional sites, leading to cleavage of centromeric cohesin by Separase, followed by sister chromatid segregation at anaphase II.

1.7.2 Error-prone process of homologous chromosome segregation in oocytes

Meiotic errors in either the metaphase II egg or the fertilizing sperm can result in disastrous consequences for the newly formed zygote. It is well known that embryonic aneuploidy is a major cause of infertility, early miscarriage and birth defects such as Down's Syndrome (Hassold et al., 2007; Hassold and Hunt, 2001; Jones and Lane, 2013; Lamb and Hassold, 2004; Nagaoka et al., 2012). In the past century, with the great advancement of in vitro fertilization techniques, much data has been collected concerning oocyte and embryo aneuploidy in humans. This led to the finding that aneuploidies in early embryo are more likely to originate from egg rather than sperm, and more specifically, from homologous chromosome mis-segregation in meiosis I (Hassold et al., 2007; Hassold and Hunt, 2001; Jones and Lane, 2013; Nagaoka et al., 2012). Two types of chromosomal mis-segregation in mammalian oocytes are commonly observed: 1) non-disjunction (NDJ) of homologous chromosome and 2) precocious separation of sister chromatids (PSSC) (**Figure 1.9**). NDJ leads to the presence or absence of an extra whole univalent in either metaphase II egg or its polar body, thus inevitably producing an aneuploid egg; while PSSC is featured by the existence of single sister chromatids prior to anaphase II, and these eggs are prone to produce aneuploid embryos upon fertilization because these single chromatids would be probably segregated randomly at anaphase (Chiang et al., 2012; Kuliev et al., 2011).

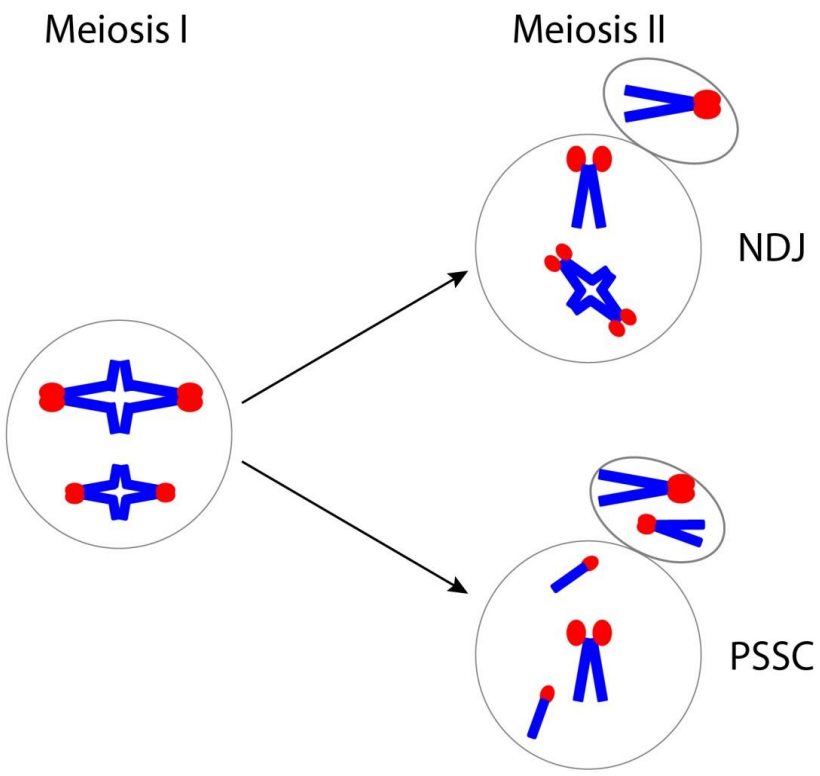

Figure 1.9 Schematic of chromosome mis-segregation in oocytes: NDJ vs PSSC.
An MI oocyte containing 2 bivalents is depicted, with chromatin in blue and kinetochore in red. NDJ of homologous chromosome results in a missing or an extra sister chromatid pair in the resulting egg, whereas PSSC is marked by the presence of single chromatids prior to anaphase II.

1.8 The SAC in mammalian oocytes

As discussed in section 1.5, the SAC network has been largely investigated in mitosis (Foley and Kapoor, 2013; Musacchio and Salmon, 2007), however, whether this mechanism exists in mammalian oocytes has been strongly debated over the last three decades, with the hypothesis that lack of the SAC might be a contributor for high incidence of aneuploidy in human eggs. In around 2000s, this hypothesis seems to be confirmed due to the observations on oocytes from 'XO' mice which possess only a single X chromosome (Hodges et al., 2001; LeMaire-Adkins and Hunt, 2000; LeMaire-Adkins et al., 1997). In this situation, it is expected that these oocytes would delay anaphase onset or arrest in MI if a similar SAC mechanism is present as it is in mitosis, since the univalent might incur incorrect microtubule attachments and inappropriate tension establishment. Surprisingly, no delay in timing of anaphase initiation is observed, with either equational segregation of the X-chromosome or intact segregation into the egg or polar body.

However, using a $Sycp3^{-/-}$ mouse oocyte model in which abnormal karyotypes occur at both MI and MII, it was shown that achiasmatic chromosomes (also known as univalents) are bi-oriented in a mitotic manner and are able to evade the functional SAC in meiosis I oocytes (Kouznetsova et al., 2007). Moreover, recent approaches such as molecular perturbation and specific kinase inhibition on mouse oocytes all appear to show that the SAC is active and essential to delay the anaphase onset, thereby preventing eggs from becoming aneuploidy (Sun and Kim, 2012). Moreover, the mechanism is found to be well conserved from mitosis to oocyte meiosis, with the same SAC components involved (Homer et al., 2009; Homer et al., 2005; Jones and Lane, 2013; Sun and Kim, 2012; Zhang et al., 2005), although recent work demonstrated a lack of spindle checkpoint in Xenopus oocytes (Shao et al., 2013). Specifically, all of the SAC proteins, such as Mad1/Mad2 (Homer et al., 2005; Kallio et al., 2000; Zhang et al., 2005; Zhang et al., 2004), Bub1, Bub3, BubR1

(Brunet et al., 2003; McGuinness et al., 2009; Wei et al., 2010; Yin et al., 2006), Mps1 (Hached et al., 2011) and Aurora B/C (Lane et al., 2010; Schindler et al., 2012; Sharif et al., 2010; Yang et al., 2010), have been identified in mammalian oocytes and are recruited to unattached kinetochores for initial function, and any function perturbation of these proteins accelerates the timing of anaphase and raise aneuploidy rate in the resulting eggs (Fan, 2010; Jones and Lane, 2013; Polanski, 2013; Sun and Kim, 2012).

Having established that the SAC is active in mammalian oocytes, a further question is raised: why are chromosomes in oocytes prone to mis-segregation, since that one single unattached kinetochore in mitosis is able to impose a robust SAC signal to delay the anaphase onset (Rieder et al., 1995; Rieder et al., 1994)? These are coupled by the recent work showing that bivalent segregation can be triggered, when a critical number of chromosomes, but not all, are biorientated and aligned at the metaphase plate in mouse oocytes (Gui and Homer, 2012; Kolano et al., 2012; Lane et al., 2012; Nagaoka et al., 2011; Sebestova et al., 2012). Therefore the SAC is not as stringent in mouse oocytes as it is in somatic cells.

This difference in the SAC response might be partly from innate nature of the homologous chromosomes, which require a mono-orientated attachment of sister kinetochores, since a univalent in mouse oocytes can undergo either intact or equational segregation without imposing a SAC arrest (Hodges et al., 2001; LeMaire-Adkins and Hunt, 2000; Nagaoka et al., 2011). The alternative possibility is that a threshold density of anaphase-wait signal is required to prevent the APC/C activation, so delaying bivalent segregation. For example, the SAC signal density raised from one unattached kinetochore in somatic cells is above the threshold, so is able to delay the anaphase onset (Foley and Kapoor, 2013; Musacchio and Salmon, 2007); however, due to the big volume of mammalian oocytes, the same threshold might only be achieved by 2 unattached bivalents (equal to

8 sister kinetochores) in oocytes (Hoffmann et al., 2011; Nagaoka et al., 2011). The fact that Xenopus oocytes are much larger in volume, and completely lack SAC control would seem to support this hypothesis (Shao et al., 2013). This model is attractive given that recent studies showing that the checkpoint control is graded rather than binary on-off switch, so less recruited SAC proteins on unattached kinetochores produce weaker anaphase-waiting signal, this would then delay anaphase onset less efficiently (Collin et al., 2013; Dick and Gerlich, 2013; Heinrich et al., 2013; Subramanian and Kapoor, 2013).

1.9 Chromosome mis-segregation in oocytes with maternal age

As already mentioned above, chromosome mis-segregation in mammalian oocytes is common, and it is generally accepted that the segregation errors predominantly arise during meiosis I. These eggs, if fertilized with a sperm, generate aneuploid embryos, causing early pregnancy loss and increased infertility, since most of the aneuploidies are lethal; even if survive to term, they are associated with birth defects, such as trisomy 21, 18 and 13 (Down, Edward and Patau syndromes respectively) (Jones and Lane, 2012; Jones and Lane, 2013; Nagaoka et al., 2012). Advanced maternal age has been well documented as one of the most important risk factors, both in humans and mice (Chiang et al., 2010; Handyside, 2012; Jones, 2008; Jones and Lane, 2013; Lister et al., 2010; Nagaoka et al., 2012).

The association between increasing maternal age and trisomy was first described nearly century ago, and later confirmed as an important etiological factor associated with human genetic disorders (Hassold and Hunt, 2009; Penrose, 1951; Penrose, 1954; Penrose, 2009). Specifically, in a clinically recognized pregnancy this incidence remains low in young women, at 2-3%, which increases to over 30% for women in their forties. Studies have established that the increased aneuploidies observed in early embryos primarily originate from oocytes rather than sperm, because chromosomal mis-segregation is dramatically increased with age in human oocytes, reaching a level of 60% or more (Fragouli et al., 2011; Griffin, 1996; Kuliev et al., 2011); However, in sperm the errors are generally structural abnormalities involved, rather than numerical changes (Lu et al., 2012; Templado et al., 2011a; Templado et al., 2011b). In addition, the fact that a donated oocyte from young women, if fertilized in vitro and transferred to an aged mother, improves implantation and pregnancy rates (Faddy et al., 2011), suggests there is no gross malfunctioning of the uterus with age, and places poor oocyte quality to the peak as increased infertility in older females (Navot et al., 1991).

Many hypotheses regarding age-related aneuploidy in human eggs have been proposed, in which mostly in terms of altered expression or/and localization in key proteins (Jessberger, 2012; Jones and Lane, 2012; Nagaoka et al., 2012; Selesniemi et al., 2011), however, none of them alone so far can explain the full provenance of this phenomenon. Indeed, accumulating evidence suggested that oocyte aneuploidy is more likely to be multifactorial, with diverse events involved from both intrinsic and extrinsic causes of oocytes (Jones and Lane, 2012; Jones and Lane, 2013; Wang et al., 2011b). These causes might individually lead to chromosomal mis-segregation, but more likely to act together to contribute to age-related aneuploidy. The following section will discuss the current key hypothesis concerning age-related aneuploidy, and the scientific questions that remain unanswered.

1.9.1 Centromeric cohesin deterioration

The cohesin complex, responsible for holding sister chromatids together from replication until segregation, is established in pre-meiotic S-phase of mitosis (section 1.6.3) (Uhlmann and Nasmyth, 1998). Furthermore, the chromatin-bound cohesin is stable once established in S phase until anaphase onset (Gerlich et al., 2006), during which it seems there is no or very little turnover of its components (Haering et al., 2004). These mechanisms are highly conserved in meiosis (Revenkova et al., 2010; Watanabe and Nurse, 1999; Watanabe et al., 2001), however, mammalian oocytes initiate meiosis during fetal development, but then arrest at prophase I before birth until puberty. Therefore cohesin may exist for decades in a woman's oocytes, and may be susceptible to damage over time, such as environmental insults. It has been well documented that Separase is responsible for cleaving the kleisin subunit of cohesin, so allowing chromosome segregation at anaphase onset (section 1.3) (Hauf et al., 2001; Nasmyth, 2011; Uhlmann, 2001; Uhlmann, 2003), and it seems that

cohesin in oocytes of aged mouse is more susceptible to premature activation of Separase (Chiang et al., 2011). Therefore, this protease must be precisely regulated during the long prophase arrest. Indeed, Separase activation is controlled by two independent mechanisms: securin binding and inhibitory phosphorylation by CDK1, and both of the pathways must be disrupted to achieve its activation (Chiang et al., 2011; Ciosk et al., 1998; Gorr et al., 2005; Huang et al., 2005; Stemmann et al., 2001). However, whether leaky Separase activity prior to anaphase exists remains unknown (Jessberger, 2012). If yes even with a quite low level, this would cause continual loss of chromosome cohesin over several decades, and eventually lead to aneuploidy when it is below the threshold level in human eggs.

Recent data suggested centromeric cohesin deterioration to be the leading hypothesis for age-related aneuploidy in oocytes (Chiang et al., 2010; Duncan et al., 2012; Lister et al., 2010; Merriman et al., 2012), and has been reviewed extensively (Chiang et al., 2012; Jessberger, 2012; Jones and Lane, 2013; Kurahashi et al., 2012; Nagaoka et al., 2012; Wang et al., 2011b). In two independent works using natural ageing mouse models, the levels of chromosome-associated Rec8, a germ cell specific cohesin component, are dramatically reduced, and consequently lead to destabilization of chiasmata and loss of the centromere cohesion, measured by the sister kinetochore distance in both MI and MII oocytes with age (Chiang et al., 2010; Lister et al., 2010). Moreover, cohesion loss is coupled with depletion of the centromeric cohesin protector Sgo2 (Lister et al., 2010). Importantly, as previously discussed, mouse oocytes appear to have little or even no turnover of the cohesin complex once S phase is complete (Revenkova et al., 2010; Tachibana-Konwalski et al., 2010). Therefore centromeric cohesin loss, based on mouse models, may well explain age-related chromosomal mis-segregation patterns, and consistent with the prevalent observations of single chromatids in aged human metaphase II eggs (Fragouli et al., 2011; Gabriel et al., 2011; Handyside et al., 2012; Kuliev et al., 2011).

However, it seems that the cohesin complex loaded onto the chromosomal arms and centromeres during S phase is in excess, because only ~10% of chromosome-associated Rec8 detected in a 9- or 12- month oocyte (compared to that in a 3-month oocyte) seems sufficient to maintain its bivalent integrity and prevent it from being aneuploid, suggesting a threshold amount of Rec8 is necessary to prevent chromosome mis-segregations (Chiang et al., 2010). It is worth noting that as Rec8 decreases, the interkinetochore distances accordingly increase (Chiang et al., 2010). This increase might allow greater flexibility of a sister kinetochore pair and malfunction it as one function unit, which would then increase the incidence of microtubule attachment errors, so allowing bivalent non-alignments and then NDJ or lagging chromosomes at anaphase. In addition, even in the oocytes from very old mice, chromosome-associated Rec8 is not detectable and the metII eggs possess prevalent of single chromatids, however, these single chromatids or univalents are not commonly observed in MI, evidenced from fixation data on single selected timepoint (Lister et al., 2010). Important questions that therefore need to be answered are: when exactly during oocyte meiosis, does premature separation occur; how are single chromatids are produced; how the dynamics of aged bivalents are affected due to the cohesion loss; how the univalents, if premature separated from bivalents, undergo segregation at anaphase, intactly or equationally? All of these concerns will be addressed in chapter 2 of the thesis.

1.9.2 Age-associated SAC malfunction

As discussed in section 1.8, the SAC appears to present and functional in mammalian oocytes, since its disruption can accelerate meiosis progression and increase aneuploidy rates (Jones and Lane, 2013; Sun and Kim, 2012). However, it is less effective when compared to that in mitotic cells, because recent evidence demonstrated that not all bivalents need to be biorientated for the SAC silencing in mouse oocytes (Gui and Homer, 2012; Kolano et al., 2012; Lane et al., 2012; Nagaoka

et al., 2011; Sebestova et al., 2012). It has been shown that aged oocytes possess more chromosome misalignments and anaphase defects (Chiang et al., 2010; Lister et al., 2010), which are generally caused by incorrect microtubule attachments (Cane et al., 2013; Thompson and Compton, 2011). It is possible that these attachment errors, if produced in a young oocyte, are sufficient to raise a robust SAC signal which is above the threshold, so delaying anaphase initiation and allowing more time for attachment error correction; however, aged oocytes might be more permissive of these errors due to its reduced SAC signal, and the inevitable consequence would be increased aneuploidy in the resulting metII eggs. Indeed, reduced expression and altered localization of the SAC components such as Mad2 and Aurora B have been observed to be associated with aged oocytes, including humans (Baker et al., 2004; Luciano et al., 2013; Pan et al., 2008; Steuerwald et al., 2001; Steuerwald et al., 2007). Specifically, levels of the SAC transcripts such as Mad2 and BubR1 in oocytes decrease with maternal aging, in both mice and humans (Pan et al., 2008; Riris et al., 2014; Steuerwald et al., 2001; Steuerwald et al., 2007). Moreover, in aged bovine oocytes Aurora B failed to properly localize to the chromosomes, and this is associated with reduced ovarian reserve and increased aneuploidy (Luciano et al., 2013). All of these data suggest that SAC malfunction may in some way contribute to the maternal ageing phenomenon.

However, by using the natural ageing mouse, two independent works demonstrated that it seems to be no aging defect in the SAC activity (Duncan et al., 2009; Lister et al., 2010). Specifically, measurements of either the length of MI, which would be expected to be shorter with defective SAC, or the ability of metaphase arrest when challenged with nocodazole, are similar between young and aged oocytes. Moreover, no correlation between early chromosome segregation and aneuploidy in individual aged oocytes was observed (Duncan et al., 2009). The global transcript analysis even showed levels of Aurora B were up-regulated in aged mouse oocytes (Pan et al., 2008), which might be reflective of the requirements of increased microtubule attachment error

correction. However, levels of the SAC proteins have never been examined so far in oocytes from natural ageing mouse. It is possible that in aged oocytes the total expression levels of these proteins are significantly reduced; or the total level remains the same but aged bivalents possess reduced ability to recruit or retain them for proper localization. It is also possible that aged oocytes do possess reduced SAC function, but extent of the reduction is not so dramatic as to compromise SAC function in the ways that have been measured so far (Duncan et al., 2009; Lister et al., 2010). All of these possibilities will be investigated and discussed in chapter 3 of the thesis.

1.9.3 Other causes

Besides centromeric cohesin loss and SAC malfunction, several other hypotheses have also been proposed to explain age-associated aneuploidy, including improper recombination in early meiosis and mitochondrial dysfunction (Bentov and Casper, 2013; Chiang et al., 2012; Eichenlaub-Ritter, 2012; Jessberger, 2012; Kurahashi et al., 2012; Vialard et al., 2011; Wang et al., 2011b). Meiotic recombination of homologous chromosomes occurring in prophase I during fetal life is essential for formation of crossovers, which then hold the newly formed bivalents together (Amunugama and Fishel, 2012; Kleckner, 2006; Kohl and Sekelsky, 2013; Yanowitz, 2010). In the 'two-hit' hypothesis accounting for chromosome segregation error-prone in oocytes (Bolcun-Filas et al., 2007; Lamb et al., 1996; Warren and Gorringe, 2006), the number and distribution of the established chiasmata are thought to affect the accuracy of subsequent bivalent segregation (Hassold et al., 2007; Lamb et al., 1997; Lamb et al., 1996; Lamb et al., 2005a). However, whether impaired meiotic recombination contributes to age-related aneuploidy remains unclear (Lamb et al., 2005b; Sherman et al., 2006). It is possible that the influences of improper recombination might compound other age-associated effects (ie cohesion loss), making oocytes more susceptible to segregation errors.

Mitochondrial function is necessary for oocyte maturation, fertilization and embryo development, and poor oocyte quality has been shown to be associated with mitochondrial dysfunction (El Shourbagy et al., 2006; May-Panloup et al., 2007; Van Blerkom, 2008). Accumulating evidence reveals decreased ATP levels, reduced mtDNA and mitochondria as well as aberrant mitochondrial structure are associated with aged oocytes (Iwata et al., 2011; Kushnir et al., 2012; Selesniemi et al., 2011). Other environmental risk factors, such as a high fat diet or smoking in humans and exposure to environmental pollutions (such as Bisphenol A) might also negatively impact oocytes during the long reproductive lifespan (Hunt et al., 2003; Jennings et al., 2011; Luzzo et al., 2012; Selesniemi et al., 2011; Zenzes et al., 1995).

1.10 Recent advances in high-resolution CLSM imaging in eggs

Increased age-related phenomenon, such as chromosome NDJ and pre-division, in both human and mouse oocytes has been well established (Handyside, 2012; Jones, 2008; Jones and Lane, 2013; Nagaoka et al., 2012), however, as discussed in the previous sections it remains unknown how and when exactly these segregation errors occur. For example, it is possible that univalents formed from premature separation of bivalents are common in aged oocytes due to cohesin deterioration, and these univalents might undergo either intact or equational segregation at anaphase, so causing aneuploid eggs. It is also possible that reduced cohesion has an adverse effect on bivalent congression or tension establishment in MI, so leading to mis-segregation. Real-time imaging of chromosomes in live oocytes would be the most effective way to examine these questions. Indeed, using this technique, several studies in the last 5 years have shown that aging does not lead to a deterioration in the SAC function (Duncan et al., 2009; Lister et al., 2010), but does cause common bivalent misalignment and anaphase defects (Chiang et al., 2010; Lister et al., 2010; Sebestova et al., 2012). However, with only chromosome labelling (with fluorescently tagged Histone 2B) neither confocal nor epi-fluorescent microscopy analysis could catalogue the detailed dynamics of individual bivalents. It is also impossible to accurately track the process of pre-division without knowing the associated kinetochore location and therefore there has been a need to develop a method of tracking chromosome and kinetochore movement concurrently, in this field of oocyte biology.

Kinetochore labelling for live-cell imaging requires a candidate protein that has the following feature: 1) its exogenous expression does not severely affect chromosome dynamics and segregation; and 2) its localization must be highly centromere-specific to minimize background noises. Both CenpA and CenpC were recently used in independent studies to label kinetochores in live cells, and real-time tracking of chromosomes and their kinetochores revealed previously

unrecognized features of kinetochore dynamics in both somatic cells and mammalian oocytes (Kitajima et al., 2011; Magidson et al., 2011). These similar systems with high-resolution imaging led the way to more quantitative analyses of homologues chromosome segregation errors, which helped shed light on the provenance of age-related mis-segregation in human eggs (Baumann, 2011; Musacchio, 2011). Using these live cell imaging approaches, the focus of this thesis will be to examine how dynamics of aged bivalents and their segregation are affected; to investigate whether defective SAC control and poor microtubule error correction contributes aged-related aneuploidy; as well as to explore how checkpoint pathways function prevent an egg from becoming aneuploid.

1.11 Thesis aims

Aim 1: Current evidence suggests centromeric cohesin deterioration is the leading cause of age-related aneuploidy in mammalian oocytes. However, it remains to be established how chromosome dynamics in aged oocytes are affected by cohesion loss, and exactly how and when the single chromatids are actually produced during meiosis. These questions are examined in my publication '**Premature dyad separation in meiosis II is the major segregation error with maternal age in mouse oocytes**', included here as chapter 2.

Aim 2: Despite current support for an age-related cohesin loss model, oocyte aneuploidy is likely to be multifactorial. Indeed, studies have established an association between increased aneuploidy with age and reduced SAC transcripts, both in mouse and human oocytes. However, other studies demonstrated that the SAC function is equally active in aged oocytes. The debate whether defective SAC is a risk factor of age-related phenomenon will be addressed in my publication '**Reduced ability to recover from spindle disruption and loss of kinetochore spindle assembly checkpoint proteins in oocytes from aged mice**', included here as chapter 3.

Aim 3: The Ndc80 protein, a component of the outer kinetochore has been shown to be essential in regulating microtubule kinetochore interactions, and its interruption causes faulty SAC function in mitosis. However, whether this function is conserved in mammalian oocytes remains largely unknown. Here by expressing Ndc80 with a modified N-terminal, I have observed a robust spindle checkpoint signalling in mouse oocytes. The mechanisms behind how this modification affects the dynamics of bivalents and why it keeps the checkpoint active are investigated in the publication '**Ndc80 N-terminal modification imposes a robust SAC signalling in mouse oocytes**', included here as chapter 4.

2 Premature dyad separation in meiosis II is the major segregation error with maternal age in mouse oocytes

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RESEARCH ARTICLE

Premature dyad separation in meiosis II is the major segregation error with maternal age in mouse oocytes

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ABSTRACT

As women get older their oocytes become susceptible to chromosome mis-segregation. This generates aneuploid embryos, leading to increased infertility and birth defects. Here we examined the provenance of aneuploidy by tracking chromosomes and their kinetochores in oocytes from young and aged mice. Changes consistent with chromosome cohesion deterioration were found with age, including increased interkinetochore distance and loss of the centromeric protector of cohesion SGO2 in metaphase II arrested (metII) eggs, as well as a rise in the number of weakly attached bivalents in meiosis I (MI) and lagging chromosomes at anaphase I. However, there were no MI errors in congression or biorientation. Instead, premature separation of dyads in meiosis II was the major segregation defect in aged eggs and these were associated with very low levels of SGO2. These data show that although considerable cohesion loss occurs during MI, its consequences are observed during meiosis II, when centromeric cohesion is needed to maintain dyad integrity.

KEY WORDS: Imaging, Chromosomes, Meiosis, Mouse, Oocyte

INTRODUCTION

In oocytes, chromosome mis-segregation is common and generates aneuploid embryos, so causing lowered implantation rates, pregnancy loss and birth defects (Nagaoka et al., 2012; Jones and Lane, 2013). It is generally thought that segregation errors predominantly arise during the first of the two meiotic divisions, in the hours preceding ovulation. This first meiotic division (MI) is physiologically triggered hormonally but is also induced when fully grown oocytes from Graafian follicles are released from their maturation-inhibitory ovarian environment, and so can be studied in detail through *in vitro* culture (Solc et al., 2010; Li and Albertini, 2013). The division at MI is unique in that it results in the segregation of homologous chromosomes ('bivalents'); the oocyte extrudes its first polar body, undergoes a brief period of interkinesis, and assembles a second meiotic spindle before arresting at metaphase (metII) of the second meiotic division (MII) (Chiang et al., 2012; Holt et al., 2013). The fertilizing sperm triggers completion of MII, in which the chromosomes ('dyads' or 'sister chromatids') remaining in the ooplasm are segregated; an event that is the same as the separation of sister chromatids in mitosis.

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Increased maternal age raises the incidence of aneuploidy, both in human and mouse oocytes (Jones, 2008; Chiang et al., 2010; Lister et al., 2010). The association of aneuploidy with maternal age has been explained mechanistically in a number of ways, but mostly in terms of losses in key proteins, deterioration in metabolic processes and gains in oxidative damage (Nagaoka et al., 2011; Selesniemi et al., 2011; Jessberger, 2012; Jones and Lane, 2012). One attractive hypothesis is that aging bivalents within germinal vesicle (GV) stage oocytes lose both the proteins that are responsible for maintaining cohesion (Tachibana-Konwalski et al., 2010; Chiang et al., 2010; Duncan et al., 2012; Merriman et al., 2012) and the proteins needed to protect centromeric cohesion, such as SGO2 (SGOL2 – Mouse Genome Informatics) (Lee et al., 2008; Lister et al., 2010). Such loss could cause aneuploidy by promoting the premature separation of bivalents into either univalents (i.e. sister chromatid pairs) or single chromatids. Indeed, single chromatids have been observed in both aged human and mouse metII eggs (Angell, 1997; Chiang et al., 2010; Lister et al., 2010; Kuliev et al., 2011; Merriman et al., 2012). The process that generates them has been called 'premature separation of sister chromatids' (PSSC) or 'pre-division'; however, it remains unknown when and how they are created.

Live-cell tracking of chromosomes would be the most appropriate technique to answer the question of when PSSC occurred in aged oocytes. Previously, live-cell imaging approaches have been used to demonstrate that aging does not lead to a deterioration in the spindle assembly checkpoint (SAC) (Duncan et al., 2009; Lister et al., 2010), the pathway responsible for inhibiting the anaphase-promoting complex (APC) ahead of bivalent biorientation. Also, live imaging has shown that misalignment and anaphase defects are more common in aged oocytes (Chiang et al., 2010; Lister et al., 2010). However, with these previous experiments using only chromosomal histone labelling, it was not possible to follow any detailed dynamics of individual bivalents. The process of PSSC, in the absence of knowing kinetochore location, cannot be accurately determined.

It would be helpful if techniques were employed to track individual bivalents during the entire period of meiotic maturation. This can be accurately done by labelling the kinetochores of bivalents and tracking their movements from the time of GV breakdown until metII arrest. Recently real-time tracking of chromosomes has been performed on oocytes from young mice during maturation (Kitajima et al., 2011). This previous work showed that bivalents are error-prone in establishing correct attachment to spindle microtubules, with on average three rounds of microtubule error correction preceding stable biorientation. This puts a different emphasis on the causes of aneuploidy: that the SAC may not be responsive to small errors in microtubule-kinetochore attachment. In fact, a number of recent studies have all established that the APC becomes active, and anaphase triggered, even though chromosome alignment and attachment errors persist (Nagaoka et al., 2011; Gui and Homer, 2012; Kolano et al., 2012; Lane et al., 2012; Sebestova et al., 2012).

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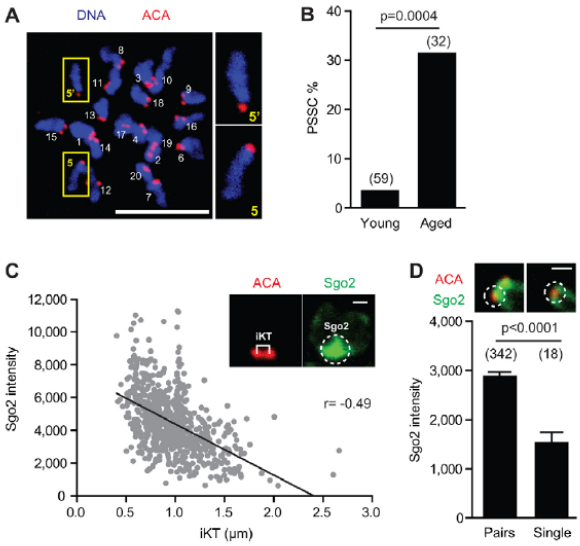

Fig. 1. Loss of chromosome cohesion in aged eggs correlates with less SGO2. (A) Nineteen sister chromatid pairs (dyads) and two single chromatids (boxed, 5 and 5′; inset, zoomed) in an aged metII mouse egg. Numbering done to aid counting. (B) PSSC rate in young versus aged fixed eggs (Fisher's exact test). (C) Scatter plot demonstrating association of reduced centromeric SGO2 intensity with increasing iKT distance (measured as shown in inset, Spearman correlation). (D) Centromeric SGO2 intensity (mean and s.e.m.) at one kinetochore of a sister chromatid pair or single chromatid, with representative images shown on top (Mann-Whitney U). Scale bars: 1 μm in C,D; 10 μm in A. ACA, anti-centromeric antibodies.

Here we have employed a real-time kinetochore-tracking approach to study the relative importance of cohesion and congression defects in the segregation of bivalents from aged mice. By tracking, we have managed to reduce the intensity of imaging to such an extent that we can follow the movements of individual bivalents with a temporal resolution of 2 minutes over a 12- to 15-hour time window. This has afforded us a level of detail that has not previously been used to study chromosome dynamics in aged eggs.

RESULTS

Balanced pre-division in the eggs of aged mice generates single chromatids

To explore the association of aneuploidy in oocytes with maternal age, we compared 1-month-old (young) and >12-month-old (aged) mice. GV-stage mouse oocytes were *in vitro* matured for 13-14 hours, by which time they had extruded their first polar body, and the resulting mature eggs arrested at metII. No procedures were performed on these oocytes following collection and during subsequent maturation. The dyads in these eggs were *in situ* spread with monastrol, to observe their morphology with Hoechst dye and their kinetochores with anti-centromeric antibodies (ACA). We counted a low, 3% incidence of single chromatids in eggs from young mice, which increased to 31% with maternal age (Fig. 1A,B; supplementary material Fig. S1A).

PSSC can result in either balanced pre-division, in which the two sisters of a pair remain in the egg, or unbalanced, in which only a single chromatid can be observed. If PSSC is after cytokinesis at completion of MI, then only balanced pre-division is possible, because the single chromatids so generated cannot be partitioned between the oocyte and already-extruded polar body. However, if PSSC is before cytokinesis, then either balanced or unbalanced pre-division can occur, dependent on the direction of the segregation, into either the polar body or the oocyte (supplementary material Fig. S1B). In human eggs, the ratio of balanced to unbalanced pre-division is 5:1 (Angell, 1991; Angell et al., 1991; Angell, 1997). Consistent with this here, for the ~30% of aged mouse metII eggs showing PSSC, all pre-division gave even numbers of chromatids. As such eggs had either two or four single chromatids, and contained a total of 40 kinetochores (supplementary material Fig. S1C). The lack of any unbalanced pre-division is suggestive of an error after cytokinesis; however, the use of only metII eggs precludes the accurate timing of such an event.

Loss of chromosome cohesion in aged eggs correlates with less SGO2

The *in situ* spreads of aged metII eggs additionally demonstrated both a reduction in levels of SGO2 and also an increase in the distance between sister kinetochores [interkinetochore (iKT) distance], a measurement that would be expected to increase when dyads lose their cohesion (supplementary material Fig. S2A-C) (Lister et al., 2010; Duncan et al., 2012; Merriman et al., 2012). The iKT measurement can be taken as an inverse measure of the cohesive ties holding sister chromatids together, with larger measurements equating to less cohesion. Indeed, here we observed an inverse correlation between SGO2 levels and iKT distance (Fig. 1C). As such, those dyads with the largest iKT distance tended to have reduced SGO2. It was also found that SGO2 intensity on kinetochores of single chromatids in metII eggs was about half that on single kinetochores from dyads (Fig. 1D), suggesting that chromosomes that had developed a segregation error possessed less SGO2. In summary, the measurements show a strong association between an age-related loss in sister chromatid cohesion and reduced SGO2 in metII eggs.

Weakly attached bivalents are common in aged oocytes

In order to understand the influence of maternal age on meiotic chromosomes more fully, we moved to examine maturing oocytes by fixing them during meiosis I. In metaphase I (metI) oocytes from aged mice, bivalents appearing to be weakly held together, as assessed by the lack of Hoechst staining between the two sister

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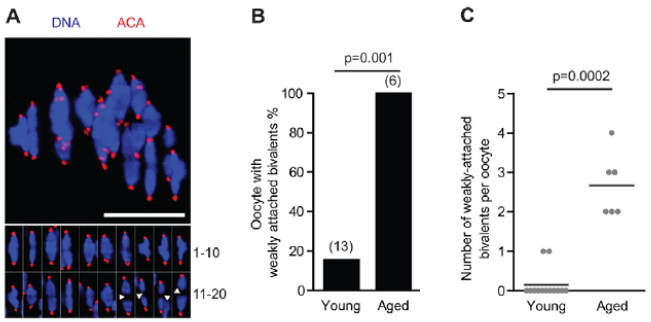

Fig. 2. Weakly attached bivalents in aged oocytes. (A) Four weakly attached bivalents in an aged oocyte, marked by arrowheads (inset). (B,C) Percentage of oocytes with weakly attached bivalents (B) and the number present per oocyte (C; the horizontal line indicates the mean). Scale bar: 10 µm. ACA, anti-centromeric antibodies. (B) Fisher's exact test; (C) Mann-Whitney U.

chromatid pairs, were commonly observed (Fig. 2A; supplementary material Fig. S3A-C). Such bivalents were found in just 15% (n=13) of young, but in all (n=6) aged oocytes (Fig. 2B). Additionally, young oocytes contained a maximum of one weakly attached bivalent, whereas aged oocytes had a mean of 2.7 (Fig. 2C). Furthermore, these were associated with an increased kinetochore separation, defined as the distance separating the two sister kinetochore pairs within a bivalent (supplementary material Fig. S3A,D). One possibility is that these bivalents could be prone to undergo premature separation into univalents in MI. However, we had no direct observation of the separation, and therefore refer to them as weakly attached bivalents. This was a more accurate description, because we noted that these affected bivalents were congressed and under tension, rather than randomly distributed on the meiotic spindle, which would have been anticipated if association was lacking. Therefore, we conclude that some cohesive ties were maintained, albeit at a weak level. However, it was possible that ties could eventually be lost at later time points in MI, so generating univalents.

Real-time 4D chromosome imaging by confocal tracking algorithm

We employed a technique for labelling and tracking both bivalents and kinetochores by 4D confocal microscopy, using histone 2B-mCherry and EGFP-CENPC, respectively (Fig. 3A). This follows the same approach used recently to explore bivalent dynamics in young oocytes (Kitajima et al., 2011). By this technique, which tracked the movements of chromosomes frame by frame, we managed to limit our x-y imaging to a 30×30 µm square – the area occupied by all the bivalents in a meiotic spindle. In the absence of such tracking, when the x-y area was increased to 80×80 µm, to accommodate the oocyte circumference, most oocytes arrested in MI and so did not complete meiotic maturation (not shown). Therefore, tracking was essential in order for oocytes to be imaged continuously over a period of 12 hours.

Initially, the effects of this 4D imaging protocol were examined on oocytes with respect to their ability to complete MI and the chromosome complement at metII. Bivalents underwent normal segregation during MI when imaged every 2 minutes over several hours (supplementary material Movie 1); and no significant differences were observed in either MI completion (70-90% for all conditions), or premature separation of sister chromatids (supplementary material Fig. S1D). As such it is concluded that the imaging protocol had no significant impact on the faithful segregation of chromosomes.

Real-time tracking of bivalents in aged oocytes would be informative for three reasons: (1) to determine whether the process of bivalent congression and establishment of tension necessary for faithful segregation is affected by age; (2) to examine the fate of the bivalents classified as weakly attached in fixed metI oocytes; (3) to determine the origin of the balanced pre-division/PSSC also observed on fixed cells, such balanced pre-division possibly being derived from those weakly attached bivalents in MI. The first reason, relating to the measurement of congression and tension establishment, is especially relevant to oocytes given recent reports showing that the SAC becomes satisfied, and the APC activated, when the initial interactions of microtubules with kinetochores are established, rather than when they are correct and bivalents are under tension (Nagaoka et al., 2011; Gui and Homer, 2012; Kolano et al., 2012; Lane et al., 2012). With age, there may be a reduction in the ability of bivalents to establish correct attachments to both spindle poles, and given APC activation would be unaffected by such events, chromosome segregation would still occur but with greater errors. This idea can be pursued by tracking kinetochores and measuring the tension developing across each bivalent – here tension gives a readout of amphitelic attachment. It is important to note that tension can also result from incorrect, merotelic attachment (Cimini et al., 2003); however, these would be observed at anaphase onset as lagging chromosomes (Thompson and Compton, 2011).

Bivalent congression is not affected by maternal age

Bivalent congression was measured in histone 2B-mCherry and EGFP-CENPC expressing oocytes, by tracking chromosome dynamics over several hours leading up to anaphase in both young and aged oocytes (Fig. 3B). Previously we established that bivalent nonalignment was most accurately defined when displacement was >4 µm from the spindle equator (Fig. 3C) (Lane et al., 2012). As such here, congression of all bivalents was achieved at least 3 hours ahead of anaphase independent of age (Fig. 3D). This suggests there was no gross malfunctioning of bivalent congression in aged oocytes.

To examine the dynamics of bivalents in more detail, with the possibility that subtler differences may be found, three measurements were performed on each bivalent over a 5-hour window, up to 30 minutes before anaphase onset (Fig. 3E). Firstly, we measured the distance between the two sister kinetochore pairs within a bivalent, defined here as 'kinetochore separation'. This increases as tension develops across the bivalent owing to microtubule pulling forces from opposing spindle poles. The second measurement is the distance that separates the bivalent from the

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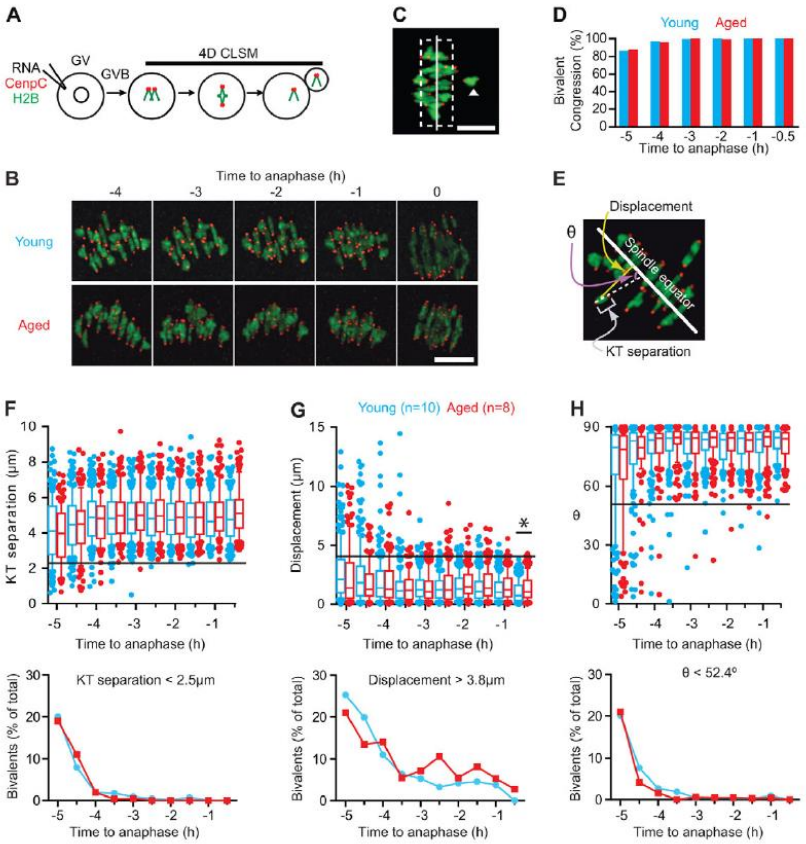

Fig. 3. Congression and establishment of tension for bivalents are not affected by age. (A,B) Method used for chromatin and kinetochore labelling in live oocytes (A), and the resulting bivalent images at selected time points in young and aged oocytes (B). (C) A meiotic spindle, with one bivalent (arrowhead), classified as nonaligned because it is >4 μm (dashed line) from the spindle equator (solid line). (D) Percentage of congressed bivalents in young (n=10) and aged (n=8) oocytes at the times indicated. (E-H) Three parameters used on bivalents and their kinetochores (E); the separation distance between the two pairs of sister kinetochores (F, top), the displacement of the midpoint of the bivalent from the equator (G, top), and the bivalent axis of orientation (θ) relative to the spindle equator (H, top). Horizontal lines indicate minimum (F,H) or maximum (G) measurements made on young oocytes at the −0.5 hour time point; bivalents outside the lines were considered 'at risk' of mis-segregation. The percentage of bivalents found to be 'at risk' for each time point (F-H, bottom). Box plots, whiskers at 10-90 centiles; the asterisk indicates P<0.05 (Kruskal-Wallis). Scale bar: 10 μm in B,C.

spindle equator, defined as 'bivalent displacement', which reduces to a minimum level once congression is achieved. The third measurement is the angle of orientation that the axis of the bivalent makes in relation to the spindle equator. In our calculation, this angle (θ) tends towards 90°, as bivalents align perpendicular to the spindle equator. Kinetochore separation, bivalent displacement and θ could be measured for each bivalent in all oocytes and at every time point.

During the period of observation, bivalents were under tension, with a mean kinetochore separation of ~5 μm (Fig. 3F); they were aligned on the spindle equator, having a mean displacement of <2 μm (Fig. 3G); and were essentially parallel with the spindle poles, subtending a >80° angle with respect to the spindle equator (Fig. 3H). No significant differences were observed between the two age groups at any time point within 5 hours of anaphase, with the single exception of greater bivalent displacement in aged oocytes at 30 minutes before anaphase (Fig. 3G). The box plots made for each measurement help identity outlying bivalents with 'at risk' characteristics for mis-segregation (KT separation <2.5 μm; displacement >3.8 μm; and θ <52.4°). The proportion of such bivalents has its maximal value at ~20-25% 5 hours before anaphase, but decreases with time owing to establishment of congression and biorientation (Fig. 3F-H). Using these 'at-risk' measures, no differences were observed between young and aged oocytes. Therefore, in response to the first question we conclude that the process of congression and establishment of tension across the bivalent is not affected by age.

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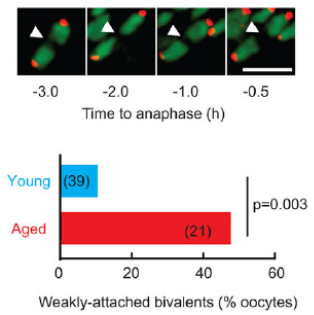

Fig. 4. Weakly attached bivalents in aged oocytes maintain association in MI. The two sister chromatid pair halves of a weakly attached bivalent maintained their association during MI (arrowheads) and were more prevalent in aged oocytes (Fisher's exact test). Scale bar: 5 µm.

Weakly attached bivalents retained integrity during MI

Weakly attached bivalents, with no apparent H2B-mCherry signal between the two sister chromatid pairs, were more frequent in live aged oocytes (Fig. 4), confirming earlier observations on fixed cells. Such bivalents did not, however, undergo premature separation, but instead maintained the appearance of biorientation (Fig. 4). Although premature separation of a bivalent into two univalents was not observed, 2 from 26 aged oocytes contained univalents in the 5-hour assessment period (supplementary material Fig. S4A). By contrast, univalents were never found in young oocytes (n=39). Of the two aged oocytes, kinetochore tracking was only possible in one. Univalents have previously been reported to undergo biorientation in MI, and so divide equationally (Kouznetsova et al., 2007), an event that would have accounted for the balanced PSSC observed in the metII eggs. However, in this oocyte we found that neither univalent was biorientated. Specifically, one univalent that was positioned near the spindle pole separated into two single chromatids at anaphase, both of which remained in the oocyte. The other univalent also separated into two single chromatids, both of which partitioned into the polar body after lagging for several minutes at the spindle equator (supplementary material Fig. S4B).

Synchrony of bivalent separation at the metaphase-anaphase transition is independent of age

The presence of weakly attached bivalents and even univalents in aged MI oocytes is consistent with a loss of cohesion, most likely during prophase arrest. To understand the provenance of single chromatids in metII eggs, and to better study the fate of weakly attached bivalents, we went on to study the separation of bivalents at the metaphase-anaphase transition in more detail. Excepting those two aged oocytes in which univalents were already present, 20 bivalents were observed at every time point before anaphase onset, and all were biorientated on the metaphase plate (Fig. 5A,B). The precise timing of separation of each bivalent (relative to when the mean kinetochore separation first increases, i.e. Fig. 5A,B, time 0) revealed that most weakly attached bivalents separated 2-4 minutes earlier (Fig. 5B, green line; supplementary material Fig. S3E,F). However, this did not lead to mis-segregation of the two sister chromatid pairs (supplementary material Fig. S3F). In conclusion, early separation of bivalents that appeared either normally or weakly attached was a feature of aged oocytes, possibly indicative of reduced cohesion, but was not itself responsible for age-related PSSC. Indeed, for all bivalents segregation was a highly synchronous event, lasting 8-10 minutes, with >80% separating in a 4-minute window regardless of oocyte age (Fig. 5C,D).

Lagging chromosomes at anaphase onset in aged oocytes

Despite the synchrony in the timing of bivalent segregation, we nonetheless observed lagging chromosomes. These were defined as persisting for a period of at least 30 minutes following anaphase onset, and they were significantly more common in aged than young oocytes (>40%, n=23 versus <5%, n=29; Fig. 6A,B), and 64% (n=11) of these produced aneuploid metII eggs. However, lagging chromosomes were not derived from weakly attached bivalents, because 82% (n=11) of oocytes containing lagging chromosomes previously had no weakly attached bivalents, and conversely 86% (n=14) of oocytes that had weakly attached bivalents had no lagging chromosomes at anaphase.

Single chromatids are generated in aged MII eggs following interkinesis

Finally, we addressed the third of our questions asked of live cell tracking: when following anaphase I were single chromatids generated? They must arise either simultaneously with the MI division, such that their segregation is at the same time as sister chromatid pairs, or alternatively be produced when the metII spindle is forming.

Following polar body extrusion a period of interkinesis separates MI from MII, and this lasts between 1.5 and 2 hours, ending with the formation of the metII spindle (Madgwick et al., 2006). Decondensation of chromatin, probably caused by a loss of CDK1 activity (Kubiak et al., 1992), begins within several minutes of anaphase onset and disrupts both CENPC and H2B fluorescent protein signals, making it impossible to resolve individual kinetochores (Fig. 7A; supplementary material Movie 2).

In the period following anaphase but preceding decondensation, separation of sister chromatids in aged oocytes was not observed to occur, with the single exception of the one containing two univalents (supplementary material Fig. S4B). However, in aged oocytes during the subsequent metII arrest, 92% (n=24/26) had single chromatids, as opposed to a rate in young mice of 4.5% (n=1/22; Fig. 7B). Despite the lost resolution during interkinesis, we could observe the premature separation of a sister chromatid pair in 46% of those aged eggs, at 133±24 minutes after anaphase onset (n=11/24; Fig. 7C,D). This period was shortly after the recondensation of CENPC and H2B fluorescent protein signals, and during the assembly of the metII spindle (Madgwick et al., 2006).

In the remaining 13 oocytes, the exact timing of the sister chromatid separation could not be determined, and so may have occurred at any point from anaphase I onwards. It remains possible that sister chromatids that have lost cohesion nonetheless travel together to the same pole and cannot be distinguished in the brief period of poleward movement that precedes decondensation. However, for those where sister separation was observed in MII, the newly formed single chromatids oscillated about the spindle equator, presumably because they have only a single kinetochore that fails to establish simultaneous attachment to both spindle poles (Fig. 7E; supplementary material Movie 2). Such movement is consistent with the interaction of the kinetochore with microtubules from one pole continually being destabilized, only to be replaced by attachment to the other pole.

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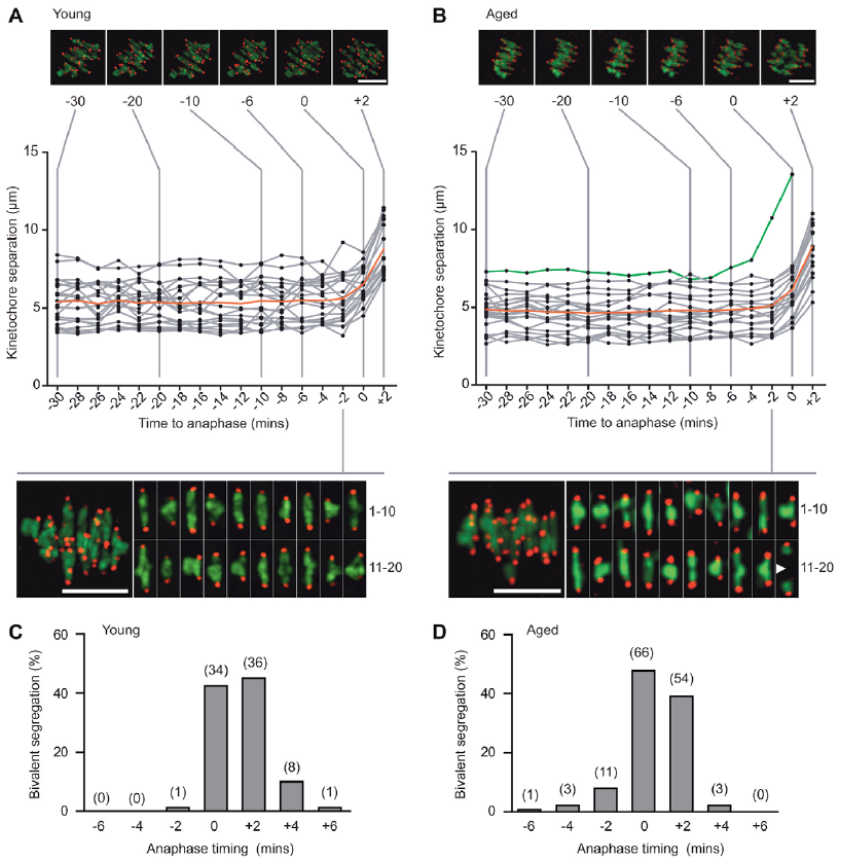

Fig. 5. In aged oocytes anaphase onset is synchronous. (A,B) Kinetochore separation in a representative young (A) or aged (B) oocyte plotted against time to anaphase onset. Separation is measured as the distance between the two sister kinetochore pairs. Above, selected time points showing a *z*-projection of bivalents. Below, bivalents in the last frame before anaphase onset. Red lines show the mean value; grey lines show individual values. The green line in B shows tracking of a weakly attached bivalent performing 'premature but successful separation' at −4 minutes, marked by the arrowhead in the inset. (C,D) Timing of individual bivalent segregation in young (C) or aged (D) oocytes, relative to the time of the initial increase in mean kinetochore separation (n=12, 4 from young and 8 from aged; 91% counting efficiency of bivalents). In parenthesis, bivalent number. Scale bars: 10 μm.

DISCUSSION

Here we employed a chromosome-tracking approach to examine bivalent dynamics in oocytes from aged mice. An algorithm allowed the imaging area to be reduced to that containing only the meiotic spindle, and real-time tracking allowed imaging to follow the movement of the spindle from the centre to the oocyte cortex during MI. This protocol allowed us to perform 4D spindle imaging with a temporal resolution of 2 minutes continuously for a period of greater than 10 hours, without any noticeable loss in rates of meiotic maturation. By contrast, with conventional imaging of the entire oocyte, completion of MI was severely compromised. This is the first imaging-based study on oocytes from aged mice to be able to track chromosomes throughout the period of MI, with high temporal resolution, from the time of meiotic resumption until metII arrest. Such long-term confocal imaging with kinetochore tracking has only been applied previously to oocytes from young mice (Kitajima et al., 2011), whereas in aged mice, imaging has been limited to the period of anaphase (Chiang et al., 2010) or based on epi-fluorescence analysis, which lacks resolution in the *z*-axis and cannot reliably track individual bivalents or their kinetochores (Lister et al., 2010). In so doing we have been able to catalogue the movements of bivalents and kinetochores in a way not previously performed, and by tracking establish the effects of maternal aging on chromosome dynamics in MI through to metII arrest.

We observed no defects in bivalent congression with age, and conclude that deterioration in the dynamics of spindle assembly is probably not a cause of maternal age-related aneuploidy. However, it remains significant for the aetiology of aneuploidy that during

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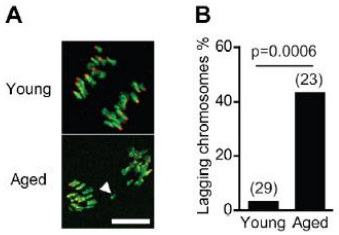

Fig. 6. Lagging chromosomes in aged oocytes. (A) Representative images of anaphase in a young and an aged oocyte; the arrowhead shows lagging chromosome. (B) Higher incidence of oocytes with lagging chromosomes at anaphase in aged mice (Fisher's exact test). The number of oocytes is indicated in parentheses. Scale bar: 10 µm.

assembly, the initial attachments of microtubules to bivalents are often incorrect and yet, despite this, satisfy the SAC and lead to APC activity (Kitajima et al., 2011; Nagaoka et al., 2011; Gui and Homer, 2012; Kolano et al., 2012; Lane et al., 2012). Such a system would be prone to segregation errors if these attachments failed to get corrected.

It is likely that with age, microtubule-kinetochore attachment errors increase. This is inferred by the raised numbers of lagging chromosomes during anaphase in aged oocytes. Their presence failed to alter segregation dynamics, implying that they have no influence on the activity of the SAC. Lagging chromosomes are most likely generated by increased incorrect microtubule attachment, which would have been promoted by reduced cohesion, a conclusion supported by the finding of univalents, weakly attached bivalents, raised interkinetochore distance, and reduced SGO2 on kinetochores in aged oocytes seen in this study. It may be that lowered chromosome cohesion, measured here and in other aging mouse and human oocyte studies (Chiang et al., 2010; Lister et al., 2010; Duncan et al., 2012; Merriman et al., 2012), allows greater flexibility within a sister kinetochore pair, so promoting incorrect attachment. Indeed, the importance of rigidity in bivalent structure for their faithful segregation at MI, in this case caused through recombination, has previously been established in yeasts (Lacefield and Murray, 2007; Sakuno et al., 2011). The lagging chromosomes, if analogous to mitosis, are likely to be generated from merotelic kinetochore-microtubule attachment, in which microtubules from both spindle poles attach to one of the sister kinetochore pairs. Merotelic bivalents would, if the same as sister chromatids, align normally on the metaphase plate (Cimini et al., 2003; Cimini et al., 2004; Thompson and Compton, 2011), and as such have gone undetected in our previous analysis of bivalent congression and tension. Indeed, increased kinetochore stretch, although often assumed to equate to amphitelic attachment, is appreciated to be consistent with such attachment but does not guarantee it.

Live imaging has helped resolve the fate of the weakly attached bivalents. The two sister chromatid pairs that constitute such a bivalent typically separated 2-4 minutes earlier; however, their segregation was always faithful. They were also not responsible for

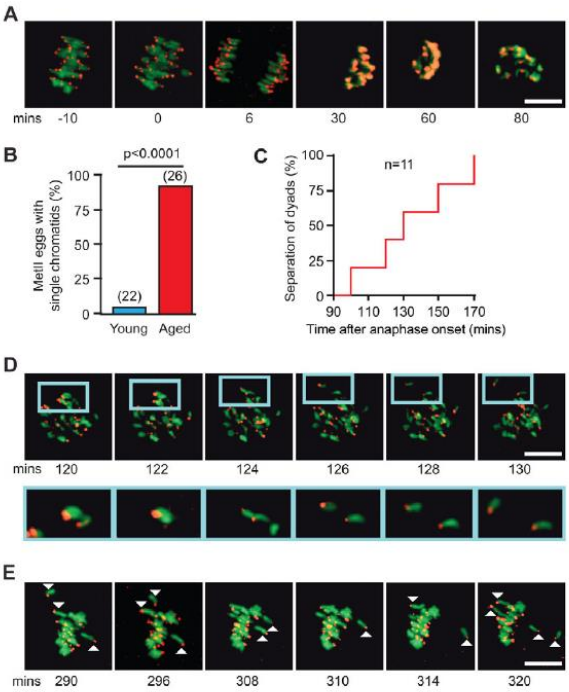

Fig. 7. Generation of single chromatids in aged eggs during MII. (A) Loss and then gain of the H2B chromatin and CENPC kinetochore signals associated with MI exit, interkinesis and establishment of a metII spindle. (B) Percentage of metII eggs with single chromatids is higher in aged oocytes (Fisher's exact test). (C) Timing of separation of a dyad into single chromatids in oocytes from aged mice. (D) Premature separation of a sister chromatid pair (dyad) following recondensation, boxed and expanded in inset. (E) Oscillatory behaviour of single chromatids during metII arrest. Representative of 26 aged oocytes examined. All times are relative to anaphase onset. Scale bars: 10 µm.

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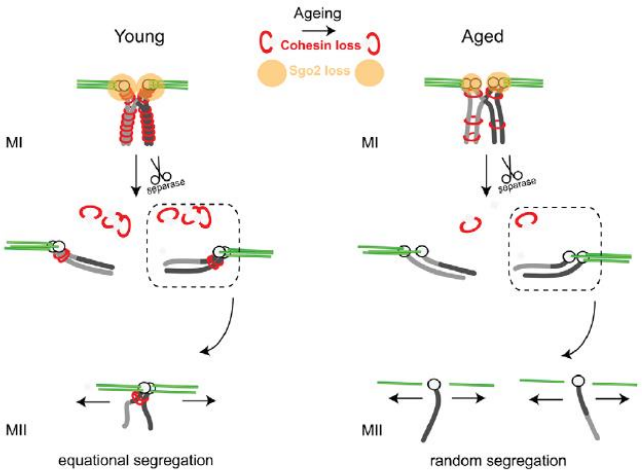

Fig. 8. Model for the generation of single chromatids following maternal aging. The effect of maternal aging is depicted on a bivalent (dark and light grey) during meiosis I (MI) and entry into meiosis II (MII). Aging is associated with a loss of cohesin and SGO2, which results in centromeric cohesion loss at anaphase. However, the two single chromatids co-segregate normally because they are attached to the same pole. When attachment of microtubules (green) is established following exit from interkinesis, the two single chromatids no longer are able to maintain their cohesion, and attach independently to microtubules. They are therefore subject to random segregation in MII, as opposed to the normal equational segregation, and consequently are liable to generate aneuploid embryos.

generating the lagging chromosomes at anaphase. Although not appearing detrimental in this age of mouse, it is likely, however, that they are a manifestation of a weakening of chromosome cohesion. This would only worsen with further maternal aging, and probably predispose the egg to aneuploidy by prematurely generating univalents or single chromatids. In this regard it would be interesting for future studies to determine how univalents behave in oocytes from aged mice, and such a study is possible using models with obligatory univalents (Nagaoka et al., 2011). It may be that the fate of the division of the univalent in MI is affected by maternal age.

Despite the above observations in MI, the main defect with age was the premature separation of dyads during metII arrest, revealed from the live-cell imaging. In about half of oocytes the event of sister chromatid separation was captured during imaging, and occurred 2 hours after anaphase I, as the metII spindle was assembling. Such visible separation was probably as a result of newly established tension across the dyad as the spindle microtubules assemble and attach to kinetochores. In the remaining half the event could not be captured, but likely times when this occurred are during the recondensation event itself, which may also be associated with microtubule attachment, and when resolution of the separation not possible, or alternatively during the anaphase I poleward movement of sister chromatids. In all instances the molecular reason for the premature separation is likely to be the same: cohesion loss associated with MI occurs at the centromere to a sufficient degree that sisters are no longer tethered together (Fig. 8).

The increase of pre-division from 31% observed on fixed eggs to 92% on live cells are probably from the exogenous proteins or/and illumination, although the same procedure had no obvious impact on the faithful chromosome segregation tested on the young oocytes. These findings highlight the selective nature of the present study into maternal aging: in assessing one mouse type (CD1), at one time point (12 months) using one type of procedure (*in vitro* maturation performed without supporting cumulus cells and often following imaging). Further aging studies are needed in order to determine the relative importance sometimes attributed to hormone concentrations, metabolic status and environmental factors (Jones and Lane, 2012) – all of which may preferentially affect oocytes from aged mice. Our observations suggest that aged oocytes are more sensitive to extrinsic factors, which would lend support to a multifactorial mechanism of age-related aneuploidy. The resulting single chromatids contained the least SGO2, supporting a model in which the primary cause of increased aneuploidy with maternal age is a loss in centromeric chromosome cohesion (Chiang et al., 2010; Lister et al., 2010; Tachibana-Konwalski et al., 2010; Chiang et al., 2011; Jessberger, 2012). Indeed, it has been demonstrated directly in mouse oocytes that centromeric cohesion is more vulnerable to the actions of separase (Espl1 – Mouse Genome Informatics) than arm cohesion (Chiang et al., 2011), and because of an age-related loss in SGO2 this vulnerability would only be increased.

Our observations are consistent with human studies that have shown a prevalence of pre-division in eggs from older women (Angell, 1997; Kuliev et al., 2011), but suggest that they result from errors that manifest themselves in MII, despite having origins in MI. This conclusion agrees with recent work on human oocytes, which has extensively screened both polar bodies and suggested that a large component of the maternal age effect is due to segregation errors arising in MII (Fragouli et al., 2011). The present work highlights that biopsy of the first polar body alone, which would have been normal in most aged oocytes here, may not be an effective screening method for aneuploidy. Therefore, one important clinically relevant consequence of this study is that maternal aging can result in oocytes that have a minimally defective first meiotic division, have a normal chromosomal complement to their first polar body, and yet contain a latent chromosome defect of separated sister chromatids at metII that could generate aneuploid embryos following fertilization.

MATERIALS AND METHODS

Materials

All chemicals were from Sigma-Aldrich (Australia), unless stated otherwise.

Oocyte collection and culture

Mice were used in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and were approved by the University of Newcastle Animal Care and Ethics Committee. For the aging study, 1-month- or >12-month-old Swiss CD-1 outbred females were used (Animal Resources Centre, Perth, Australia and Charles River Breeding Laboratories, Kingston, NY, USA). These mice were housed in pathogen-

free facilities. All mice were hormonally primed with intraperitoneal injection of 10 IU pregnant mares' serum gonadotrophin (Intervet, Australia). After 44-48 hours, GV-stage oocytes were collected from the ovary and mechanically stripped of surrounding cumulus cells. Only oocytes with an integral cumulus were used. A range of 10-30 oocytes for young, and 1-5 oocytes for old, were collected per mouse. Denuded oocytes were handled in M2 media containing 2.5 µM milrinone at 37°C for a period between 1 to 3 hours. Meiotic maturation was stimulated by milrinone washout, and all further culture was under mineral oil at 37°C.

Preparation of RNA and microinjection

H2B-mCherry, subcloned into a pMDL vector, which is optimized for expression in mammalian oocytes (Madgwick et al., 2006) and EGFP-CENPC, contained within a pGEM vector, were linearized via digestion with *Kpn*I and *Swa*I respectively. 5′-capped cRNAs were synthesized using either T3 (H2B) or T7 (CENPC) mMessage mMachine (Ambion) according to the manufacturer's instructions. cRNA (1500 ng/µl) was dissolved in nuclease-free water and stored at −80°C. Microinjection was performed as previously described using fabricated micropipettes that were inserted into the oocyte using the negative capacitance facility of an electrophysiological amplifier (Madgwick et al., 2006). cRNA was injected at a pipette concentration of 500 ng/µl for EGFP-CENPC and 200 ng/µl for H2B-mCherry. A measured 0.1–0.3% of oocyte volume injection was given using a timed pressure injection facility of a Pneumatic PicoPump (World Precision Instruments, Stevenage, UK).

Immunofluorescence

Oocytes were fixed and permeabilized in 2% formaldehyde in PHEM buffer (60 mM PIPES, 25 mM HEPES, 25 mM EGTA, 4 mM $MgSO_4$) with 0.5% Triton X-100 and 1 µM taxol. Blocking was performed in 7% normal goat serum in phosphate-buffered saline (PBS) with 0.1% Tween 20. Primary antibodies were diluted in PBS with 3% bovine serum albumin (BSA)/0.1% Tween 20 with overnight incubation at 4°C: SGO2, gift of Y. Watanabe (Lee et al., 2008), or anti-ACA (CREST; 90C1058, Cortex Biochem, CA, USA). Secondary antibodies were Alexa-633 (SGO2) or Alexa-555 (ACA) conjugated (Invitrogen). Oocytes were counterstained with Hoechst (20 µg/ml) before mounting in Citifluor (Citifluor, UK).

Confocal time-lapse imaging

4D imaging was performed using an Olympus FV1000 confocal laser scanning microscope (CLSM) equipped with a 60×/1.2 NA UPLSAPO oil immersion objective lens. The CLSM was housed in a 37°C temperature-controlled environment. Chromosomes were imaged using 15-18 *z*-sections, with *z*-resolution of 2.0 µm. The *x*-*y* area was set to 320×320 pixels (~30×30 µm) and tracking was performed in real time using ImageJ (NIH) macros that were written to maintain chromosomes within the centre of field of view. The minimum time between consecutive frames was 2 minutes. The imaging period was between 0 to 15 hours after GV breakdown.

In situ chromosome analysis

MetII eggs were incubated in M2 media containing 200 µM monastrol for 1 hour, a process that spreads chromosomes sufficiently for analysis (Lane et al., 2010). Following incubation, eggs were assessed for aneuploidy by kinetochore and chromatin imaging using either EGFP-CENPC and H2B-mCherry; or anti-ACA antibodies and Hoechst.

Image processing and analysis

To improve the kinetochore signals in live oocytes, the images were processed using ImageJ software, by the subtraction of a ten-pixel Gaussian blur from a two-pixel Gaussian blur, as suggested (Kitajima et al., 2011). The positions of the kinetochores for each bivalent and at every time point were logged using semi-automated ImageJ macros. Algorithms then trialled 1,000,000 spindle orientations to establish the best fit with the bivalent kinetochore positions in 3D. A second-best-fit algorithm then fixed the position of the spindle equator. Finally, for each bivalent the angle of intersect with the equator was determined, as well as its distance from the equator and the separation of the two kinetochore pairs. SGO2 fluorescence calculation was performed as previously described (Lane et al., 2012) with modification. Specifically, SGO2 intensity between two sister kinetochores with a pair in metII eggs was made by measuring mean fluorescence within a 2 µm diameter circle centred on the midpoint joining the two kinetochores. For single chromatids in metII eggs, only a 1 µm diameter circle was required, and when comparison with sister chromatid pairs, measurement was against a 1 µm diameter circle centred on one of the kinetochores of the pair.

Statistical analysis and replicate numbers

Dichotomous data were analysed using Fisher's exact test. For multiple comparisons we employed Kruskal–Wallis ANOVA, with Dunn's post-test. All of other means analysis was performed using Mann–Whitney test. Data were processed using GraphPad Prism 5, with $P<0.05$ set for significance. At least three separate aged mice were tested from at least two independent experiments. All data are pooled.

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Competing interests

The authors declare no competing financial interests.

Author contributions

The experiments were performed and figures prepared by Y.Y. S.I.R.L. wrote the software developed for live chromosome tracking and kinetochore analysis. Y.Y. and S.I.R.L. performed all data analysis. K.T.J. instigated the study, supervised the work and was the lead author in writing of the manuscript, on which all authors contributed.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.100206/-/DC1

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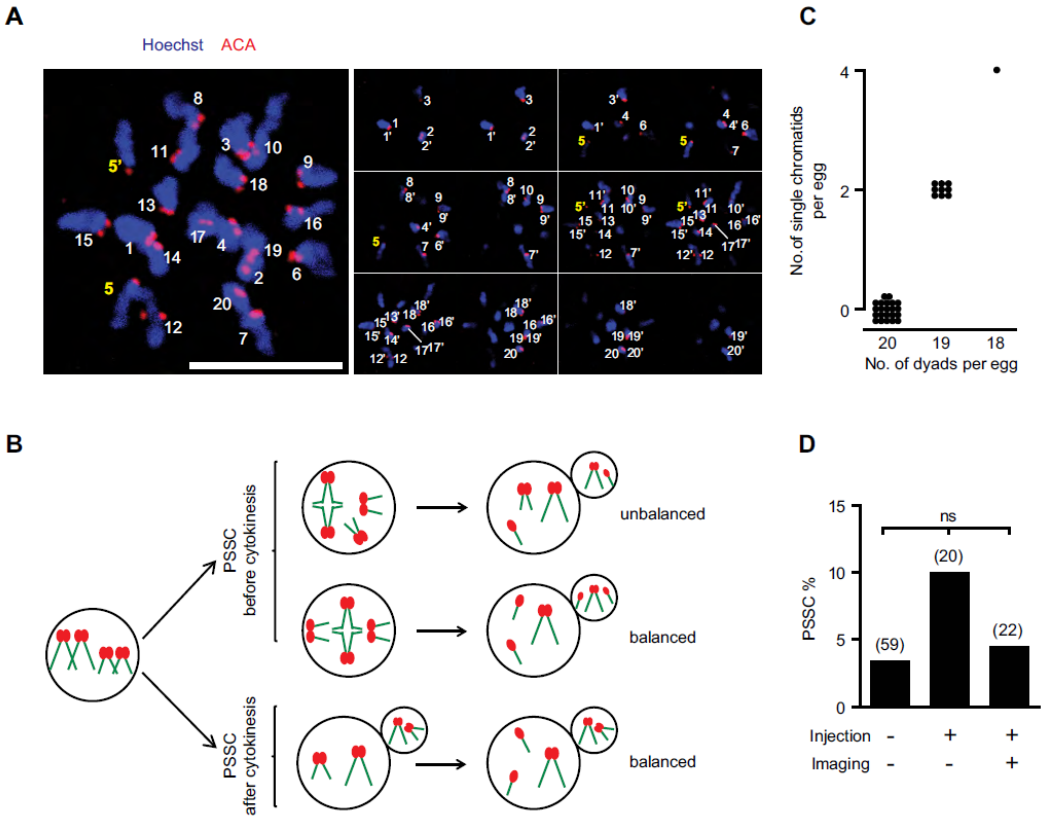


Fig. S1. PSSC in aged oocytes. (A) The left image is a maximal intensity z-projection of chromosomes (blue; Hoechst) and their kinetochores (red; anti-ACA); as shown in Fig 1A. Sister chromatid pairs are labeled in white and two single chromatids are labeled in yellow. Right images are sister chromatid pairs/single chromatids taken from individual z-sections labeled with corresponding numbers. Note that the kinetochores of chromosomes 2 and 19 overlap in the maximum intensity projection, but are readily resolved in the individual z-sections. Scale bar represents 10 μm. (B) Schematic showing balanced and unbalanced pre-division. If sister chromatid separation happens before cytokinesis this can result in either balanced or unbalanced division, but only balanced pre-division can ever be observed if premature separation happens after cytokinesis. (C) The number of single chromatids and dyads observed per egg, showing only balanced pre-division from aged mice. (D) PSSC rate in metII eggs associated with injection and imaging during in vitro maturation. Neither causes a significant increase in PSSC.

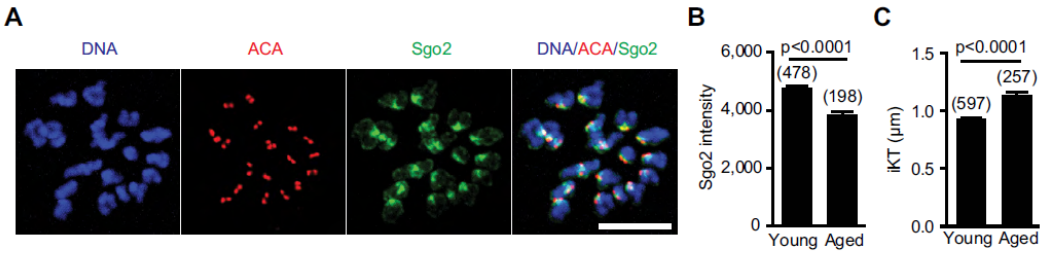


Fig. S2. SGO2 intensity and iKT distance measurements in young and aged metII eggs. (A) Chromosomes from a metII egg with SGO2 and kinetochore (ACA) immunostaining. Scale bar represents 10 μm. (B) Comparison of centromeric SGO2 levels between young and aged metII eggs, showing significantly less SGO2 with age. (C) iKT distance measurements, made between the kinetochores of the sister chromatid pair, in young and aged eggs, showing greater distance with age. (B,C) shown are mean and s.e.m.

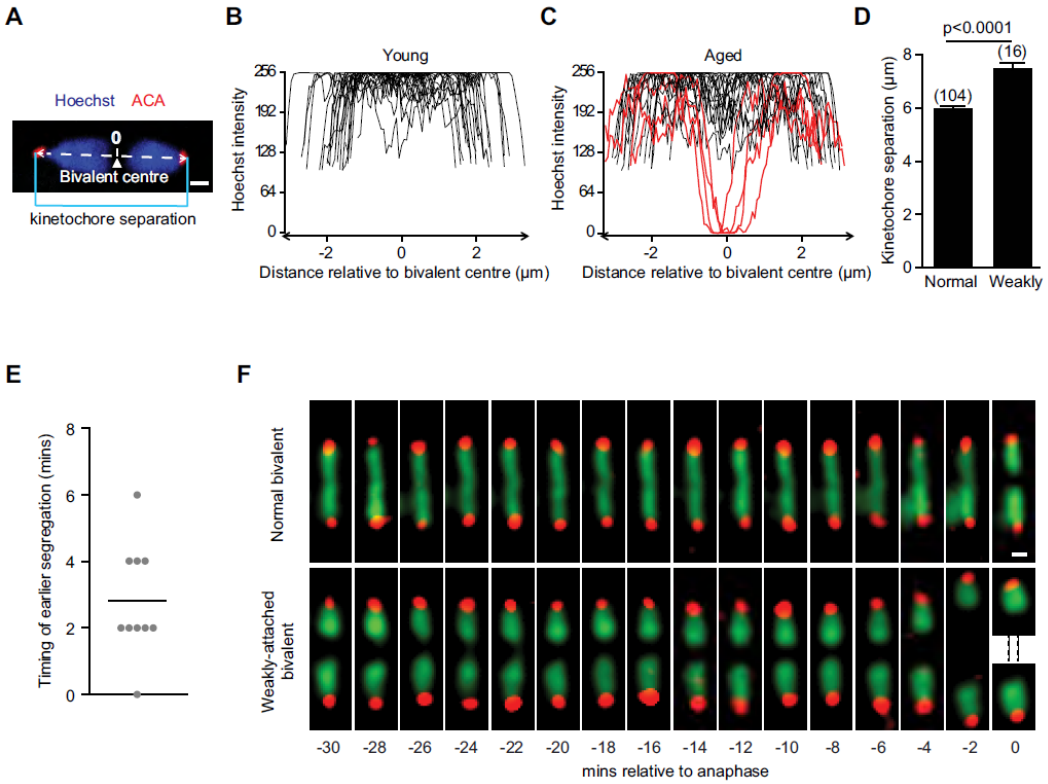


Fig. S3. Weakly-attached bivalent measurement and premature segregation. (A) Measurement of Hoechst intensity and kinetochore separation for individual bivalents from fixed oocytes. Intensity score was calculated along a line joining the two sister kinetochore pairs within the bivalent. Kinetochore separation is the distance between the two pairs of sister kinetochores. (B, C) Measurement score of Hoechst intensity from 20 bivalents of a young (B) and aged (C) oocyte. Red lines in (C) indicate four weakly-attached bivalents, as shown in Fig 3A. (D) Kinetochore separation in weakly-attached bivalents is significantly increased (mean and s.e.m.). Number of bivalents indicated in parenthesis. (E) Segregation timing of weakly-attached bivalents (10 bivalents from 5 aged oocytes) relative to their normally-attached siblings from the same oocyte. Horizontal line represents the mean. (F) Representative images of a normal and weakly-attached bivalent tracked during the last 30 minutes before anaphase onset. The weakly-attached bivalent showed earlier (4 minutes) segregation. (A, F) Scale bar represents 1 μm.

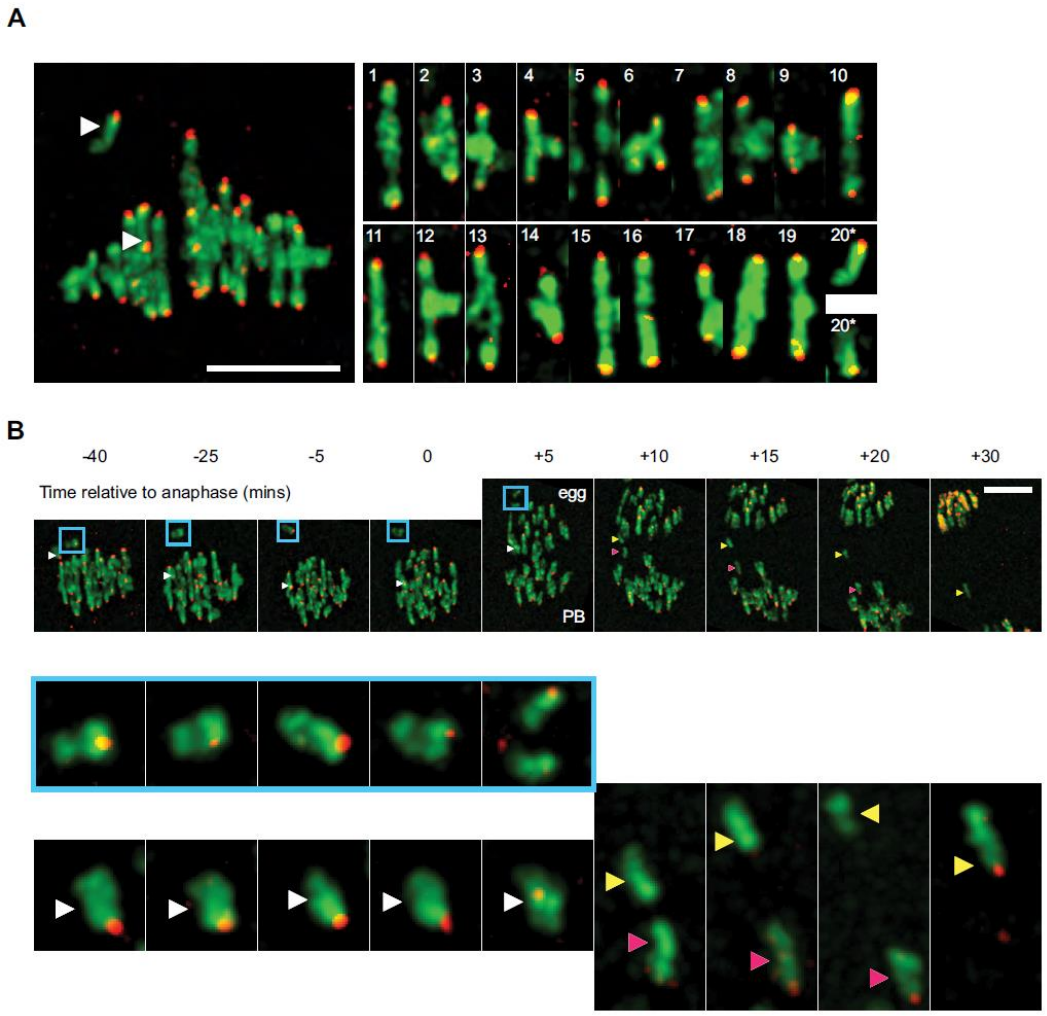
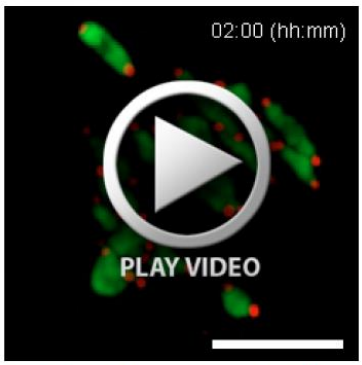
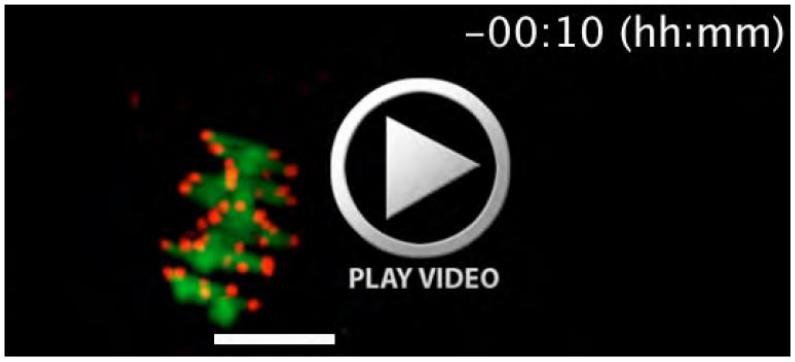


Fig. S4. The fate of univalents at anaphase I in an aged oocyte. (A) Left image shows a maximum intensity z-projection of an aged oocyte containing two univalents (arrowheads) 3 hours before anaphase onset. Right images show individual z-sections of the bivalents (1-19) and two univalents (20*). (B) The fate of the two univalents at anaphase shown in a maximum intensity z-projection (top row) and in enlarged, single z-sections (bottom two rows) at the times indicated, relative to anaphase onset. One univalent (blue box) separates into two single chromatids, which both partition into the oocyte. The other univalent (unboxed, white arrowhead), separates into two single chromatids (pink and yellow arrowheads), one of which (yellow) lags at the spindle equator leading to co-segregation into the polar body. (A,B) Scale bar represents 5 μm.



Movie 1. Oocyte with chromosome and kinetochore labeling undergoing faithful segregation at anaphase. Elapsed time given from GVB. Kinetochores (EGFP-CenpC) in red, chromosomes (H2B-mCherry) in green. Scale bar represents 10 μm.



Movie 2. Generation of single chromatids following chromosome recondensation during Movie 2. Elapsed time given from anaphase-onset. Kinetochores in red, chromatin in green. Some of the images from this movie are displayed in Fig. 7. Following anaphase onset, chromatin is observed to decondense (0:08-1:06). Chromatin in the first polar body (PB1) is not tracked and so is lost from the field of view at 0:50. Chromosome recondensation (1:06-1:38) is followed by separation of a dyad (arrowheads) at 2:04, and the resulting single chromatids oscillate about the spindle equator. *White box*, showing the separation event, is enlarged on right hand side of video (2x magnification). Scale bar represents 10 μm.

3 Reduced ability to recover from spindle disruption and loss of kinetochore spindle assembly checkpoint proteins in oocytes from aged mice

REPORT

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Reduced ability to recover from spindle disruption and loss of kinetochore spindle assembly checkpoint proteins in oocytes from aged mice

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Keywords: spindle assembly checkpoint, oocyte, aging, cell cycle, aneuploidy, error correction, meiosis

Abbreviations: GVBD, germinal vesicle breakdown; KT-MT, kinetochore-microtubule; MetII, metaphase II; MI, meiosis I; MII, meiosis II; PBE, first polar body extrusion; SAC, spindle assembly checkpoint

Currently, maternal aging in women, based on mouse models, is thought to raise oocyte aneuploidy rates, because chromosome cohesion deteriorates during prophase arrest, and Sgo2, a protector of centromeric cohesion, is lost. Here we show that the most common mouse strain, C57Bl6/J, is resistant to maternal aging, showing little increase in aneuploidy or Sgo2 loss. Instead it demonstrates significant kinetochore-associated loss in the spindle assembly checkpoint protein Mad2 and phosphorylated Aurora C, which is involved in microtubule–kinetochore error correction. Their loss affects the fidelity of bivalent segregation but only when spindle organization is impaired during oocyte maturation. These findings have an impact clinically regarding the handling of human oocytes ex vivo during assisted reproductive techniques and suggest there is a genetic basis to aneuploidy susceptibility.

Introduction

Aneuploidy is a major cause of infertility, early miscarriage, and birth defects.[1-3] It is primarily brought about by the mis-segregation of chromosomes during the 2 meiotic divisions of the oocyte (meiosis I, MI; meiosis II, MII), and advanced maternal age is an important risk factor. The leading hypothesis to account for the maternal age effect, based on studies in mice that replicate this phenomenon, posits that aneuploidy is derived from chromosome cohesion loss during the long period of prophase I arrest, which is unique to mammalian oocytes and precedes the first of the 2 meiotic divisions.[4,5] This is based on measurements of cohesion loss in oocytes from aged females and the inability of prophase I oocytes to replace cohesin components during the aging period.[6-11]

The spindle assembly checkpoint (SAC) is a near universal pathway that acts to prevent aneuploidy and involves members of the Mad and Bub families, Mps1 and Aurora B kinase.[12,13] The SAC is active during prometaphase, and by assembly on unoccupied kinetochores, it generates a potent inhibitor of the anaphase-promoting complex.[14] The SAC is satisfied and switched off when all kinetochores engage with spindle microtubules, and the kinetochore–microtubule (KT-MT) interaction causes chromosome alignment (biorientation) at the metaphase plate as a consequence of equal and opposite pulling forces from both poles. The SAC is certainly active in mouse oocytes, as its loss can raise aneuploidy rates considerably.[1,15-18] However, the SAC is not as stringent in mouse oocytes as it is in somatic cells,[19-23] and in human and mouse oocytes, aging is associated with a reduction in levels of SAC components such as Mad2.[24-27] Although these data offer credence for a SAC basis to the maternal aging phenomenon, there are other studies demonstrating the SAC is active in aged oocytes.[7,28] First, oocytes of aged and young mice seem to share the same ability to arrest in response to nocodazole-induced spindle damage. Second, the length of MI, which would be much shorter in the absence of the SAC, is observed to be no different in aged oocytes, and, further, there is no association between aneuploid eggs and the time taken to transit through MI, as there should be if the SAC were being bypassed, resulting in chromosome mis-segregation.[28]

Given recent work establishing the SAC is not so stringent in MI,[19-23] and that the establishment of correct KT-MT for chromosomes (bivalents) is very poor,[29] we decided to reexamine changes in the SAC with age. We used C57Bl6/J mice, because it is the standard laboratory mouse used in genome sequencing.

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Results

C57Bl6/J oocytes have cohesion loss but no change in Sgo2 with maternal age

First, to examine if there is any age-related phenomenon in this mouse stain, chromosome spreads were performed on metaphase II (metII) oocytes from young and old mice following in vitro maturation. This was measured by rates of first polar body extrusion (PBE) and occurred at high rates independent of maternal age (**Fig. 1A**). Using this method, in a healthy normal metII oocyte, one would expect to observe 20 pairs of sister chromatids (dyads), which have been produced following the reductional division in MI, and this was the case in the vast majority of oocytes, with only a small number (n = 4/109) not containing 20 dyads (**Fig. S1A**). Unsurprisingly given its low incidence, there was no statistical significant rise in aneuploidy rate with maternal age, although it was modestly higher in aged mice (**Fig. 1B**). This suggests that this strain is in some way resistant to a maternal age effect when compared with other aged mice, which can have rates of between 20–60% at comparable or even younger ages.[6,10,23,25,30]

A loss in chromosome cohesion is thought to be a factor important in maternal age effect segregation errors in oocytes.[6-8,10] Therefore, we examined if the lack of any pronounced maternal aging on aneuploidy was due to a resistance or tolerance to such cohesion deterioration. The kinetochore separation (interkinetochore or iKT separation) within dyads of metII oocytes were calculated; this distance tends to increase as the cohesion holding the dyad together diminishes. In agreement with others,[6,7,10] we observed iKT increases in oocytes from aged mice (**Fig. S1B**). This suggests that in the C57Bl6/J strain, like other mice examined, and also in women,[9] there is a general loss in chromosome cohesion, although in this particular strain it is not associated with an increase in aneuploidy to the same level observed elsewhere.

In addition to a loss in chromosome cohesion with maternal age, levels of centromeric Sgo2 have also been measured to decrease.[7,31] So for example, we recorded a very significant drop in Sgo2 in Swiss mice at >12 mo as compared with 1 mo, which was associated with an increased aneuploidy rate with age.[31] As suggested in the first study showing this association,[7] such a decline is likely an important factor in the mis-segregation of bivalents during aging, because Sgo2 protects centromeric cohesin from separase-mediated degradation during MI. Its continued presence is therefore essential to maintain dyad integrity until MII.[32,33] Any premature Sgo2 loss in MI would make centromeric cohesin vulnerable to separase-mediated cleavage, so generating single chromatids, which have been observed in other aging mouse strains.[6,7,31] However, single chromatids were not found in the C57Bl6/J strain employed here, therefore we would predict no such Sgo2 drop if there was any causal relationship. Kinetochore-associated Sgo2 levels were measured by immunofluorescence from in situ chromosome of oocytes in MI (**Fig. 1C**), and in agreement with our prediction, no loss in Sgo2 staining occurred (**Fig. 1D**; **Fig. S1C**). However, when the same procedure was repeated on MI oocytes from a different mouse stain (Swiss CD-1), we found a significant reduction of centromere-associated Sgo2 with maternal age (**Fig. S1D**). These opposing observations on aneuploidy and centromeric Sgo2 localization between the 2 mouse stains suggest that the retention of this centromeric protector in aged C57Bl6/J mouse oocytes would account for the lack of observed single chromatids and supports a genetic basis to the age-related phenomenon.

Reduced Mad2, phospho-Aurora kinase, and ACA associated with aged bivalents

Although with maternal age there was no change in centromeric Sgo2, there was a rise in iKT separation, suggesting some relaxation in the cohesive forces holding chromosomes together. One possible interpretation for this is that aging results in a loss in the binding or retention of some chromosome-associated proteins but not others. To examine this idea further, we measured for changes, with respect to maternal age, in levels of 2 proteins: Mad2 and phosphorylated Aurora C (pAurora C). Aurora C is a substitute of Aurora B during meiosis,[34-37] and its active form pAurora C is thought to be involved in destabilizing erroneous KT–MT attachment, as such contributing to the fidelity of bivalent division.[34,38-40]

One further reason for examining Mad2 is that previous studies have shown that levels of this transcript, like those of other SAC components, are lower in oocytes of both aged mice and humans.[24,25] However, there appears to be no aging defect in SAC activity,[7,28] as measured either by the length of MI, which would be predicted to be shorter in a situation of reduced SAC, or in the ability to arrest in response to a nocodazole challenge. It is therefore thought that the SAC does not reduce in functionality in aged oocytes.

We assessed Mad2 on kinetochores at 3 h after resumption of MI, marked by germinal vesicle breakdown (GVBD), when the SAC is active,[22] and found a significant ~30% reduction in mean levels in oocytes from aged mice (**Fig. 2A**). This reduction was apparent even when we attempted to maximally stimulate and re-engage the SAC activity by a 2-h, 10-μM nocodazole treatment at 5–7 h after GVBD (**Fig. 2B**), a later MI timepoint normally associated with low SAC activity. This procedure causes complete dissolution of all microtubule fibers and a re-engagement of Mad2 with all unoccupied kinetochores.[22]

When the same immunofluorescence analysis was performed for centromere-associated pAurora C, we similarly observed a significant fall in aged oocytes to a level about half that in young mice (**Fig. 2C**). This analysis was performed at 5 h after GVBD, a timepoint that would correspond to when error correction is active and needed.[22,29]

These data imply that with age, the centromere-kinetochore of a bivalent possesses reduced ability to recruit or retain both Mad2 and pAurora C. Indeed, this phenomenon of reduced levels may be more general, given that immunostaining with anti-centromeric antibody (ACA), which detects centromeric protein epitopes, was also found to be 61% lower in aged oocytes when compared with young (**Fig. 2D**). These data taken together suggest that many protein factors associated with the kinetochore and centromere may decline with age, although, as demonstrated with Sgo2, this decline is not universal.

Aged oocytes have a reduced ability to arrest with low-dose nocodazole

Reduced levels of key proteins in aged oocytes involved in the SAC at the kinetochore of the bivalent, such as Mad2 and pAurora C, may have an impact on their ability to undergo SAC activation and microtubule error correction during MI. This is notable given a recent study showing that the SAC response is likely to be graded rather than a binary on–off switch, and its strength depends on the amount of Mad2 recruited to kinetochores.[41] In principle, a reduction in either Mad2 or pAurora C, could influence the fidelity of bivalent segregation at the end of MI. This is supported by the observations that when individual SAC proteins are reduced or knocked-out in oocytes, rates of aneuploidy in the resulting eggs are raised,[1,15,17,42] and when young oocytes are cultured with the Aurora kinase inhibitor ZM447439, a significant reduction in kinetochore pAurora C (Fig. S2A) and a rise in aneuploidy at MI completion are both measured (Fig. S2B).[40]

Despite the potential of reduced SAC activity being present in aged oocytes, it seemed unlikely that the SAC was actually nonfunctional, because there was no increase in aneuploidy with age (Fig. 1B) to a similar level achieved following loss of individual SAC proteins,[15,17,42] and the duration of MI was not shortened (Fig. 3A). This lack of aging effect on duration is consistent with previous reports[7,28,43] but would have occurred with an ineffectual SAC, because premature prometaphase APC activity shortens MI timing.[17,44,45]

One possibility not explored so far is that maternal aging may affect SAC activity, due to a lowering of Mad2 and pAurora C, but the reduction is not so dramatic as to compromise SAC function in the ways that have been measured previously. Oocytes proceed through MI with unattached and/or incorrectly attached bivalents[19-23] that from equivalent mitotic chromosome studies are predicted to activate a checkpoint, and yet complete microtubule depolymerization leads to a meiotic arrest.[17,45] These seemingly conflicting observations are resolved by the proposition that a small number of attachment errors are tolerated by the SAC, because it may not generate a sufficiently strong "wait-anaphase" signal, whereas when many bivalents are affected, the SAC is no longer weak and can act as a checkpoint. On this basis, we thought differences between young and aged oocytes may be uncovered if a weaker stimulus for the SAC was applied; one which is below the level induced by complete microtubule depolymerization. Under such circumstances it may be that a 25–50% fall in Mad2 and pAurora C on kinetochores becomes functionally significant.

To identify appropriate doses of nocodazole, oocytes from young mice were first exposed to a concentration series, and their ability to undergo PBE was assessed (Fig. 3B). We observed a very narrow dose range, over which the effects of nocodazole transitioned from being negligible to causing a near complete block to PBE. At a dose of 100 nM or less, oocytes underwent PBE at rates of >80%, similar to no additions, while at doses of 300 nM, oocytes remained arrested in MI (Fig. 3B). It was only at 200 nM that a partial effect could be observed, with 18% of oocytes undergoing PBE (Fig. 3B). Intriguingly, when oocytes from old mice were challenged to this dose, a significantly greater percentage—nearly double—failed to arrest, and went on to extrude their polar body (Fig. 3C). At the higher dose of 400 nM nocodazole, meiotic arrest was observed at ~90% in both young and aged mouse oocytes (Fig. 3D). Therefore, these observations show that with advancing maternal age the SAC responds less efficiently to low-level spindle disruption.

Aged oocytes have increased rates of aneuploidy following spindle disruption

Mad2 is recruited to unoccupied kinetochores to activate the SAC,[15] and initial KT–MT attachments in mouse oocytes are predominantly incorrect.[29] Therefore, 400 nM nocodazole treatment was used to examine for reduced SAC function in aged mouse oocytes as well as to examine its ability to repair erroneous KT–MT attachments, a process dependent on Aurora kinase.[38,46,47] Our approach was to dissolve completely existing KT–MT attachments mid-way through MI, and then measure

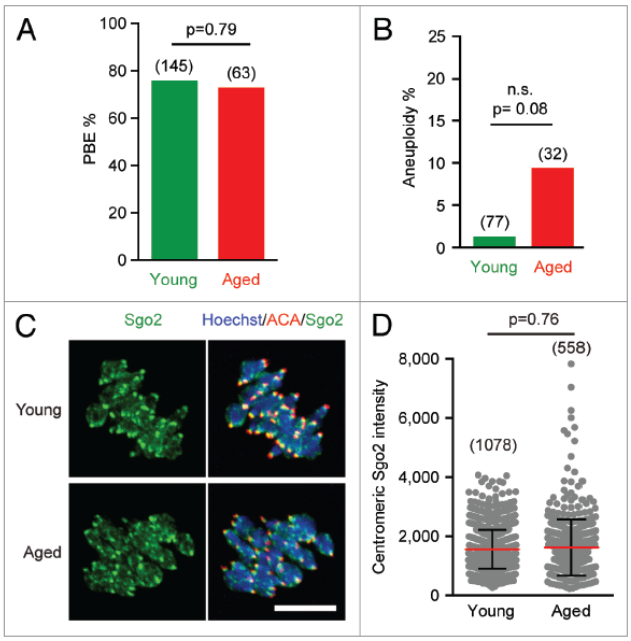

Figure 1. Maternal aging in C57Bl6/J oocytes does not significantly raise aneuploidy rates or lead to Sgo2 loss. (**A**) Polar body extrusion rates in oocytes from young vs. aged mice, showing no significant difference (Fisher test). (**B**) Percentage of aneuploid metII eggs following MI completion, showing no significant increase with age (Fisher test). (**C**) Sgo2 immunostaining in young and aged oocytes, 5 h after GVBD. Scale bars represent 10 µm. (**D**) Centromeric Sgo2 intensity is unchanged with age (mean ± s.d; Mann–Whitney U test; 27 young vs. 16 aged oocytes). (**A and B**) in parenthesis, number of oocytes examined; (**D**) in parenthesis, number of centromeres examined.

the extent to which the oocyte can establish new correct attachments by assessing for rates of aneuploidy in the resulting metII egg. If erroneous KT–MT attachments persist at anaphase onset, then this would result in a higher aneuploidy rate. Adding 400 nM nocodazole for a 2-h window, between 5–7 h after GVBD, is a dose sufficient to completely interrupt all existing microtubule fibers, but following its washout leads to new meiotic spindle formation. This procedure did not affect either the rate or the timing of polar body extrusion between young and aged oocytes (**Fig. 3E and F**), because >90% of oocytes underwent PBE at ~200 min after nocodazole washout. The similarities here in timing are consistent with our hypothesis that complete disruption of microtubules generates a sufficiently strong signal to fully activate the SAC, independent of maternal age, and so produce an effective "wait-anaphase" signal. Interestingly, however, when we analyzed the chromosome content of the resulting metII eggs, we observed a significantly higher, 3.5-fold, increase in the rate of aneuploidy with age (**Fig. 3G**). The majority of these aneuploidies (82%, n = 17) were in the form of one extra or missing dyad, which may have been due to a non-disjunction event in the bivalent at MI. Therefore, consistent with a recent report showing an association between KT–MT attachment and chromosome mis-segregation in aged oocytes,[43] our data suggest that the processes associated with the establishment of correct KT–MT attachment following spindle disruption are in some way impaired with age.

Prevalence of non-aligned chromosomes accounting for the increased rate of aneuploidy

The increased aneuploidy observed following nocodazole treatment may have been caused by one of a number of factors, including an inability to repair incorrect KT–MT attachments that form following drug washout. To explore the possibilities more fully, it would be informative to visualize bivalents in real time on the meiotic spindle. Therefore, oocytes expressing EGFP-Map4 and H2B-mCherry, to label microtubules and chromosomes respectively, were imaged by 4D confocal microscopy following nocodazole washout until anaphase onset (**Fig. 4A; Videos S1 and 2**). Two hours before anaphase, many oocytes, independent of maternal age, possessed non-aligned bivalents—up to 9 per oocyte (**Fig. 4B**). However, even at this timepoint, the extent of bivalent non-alignment was significantly greater in the aged oocytes. Tracking chromosomes in young oocyte in the 2 h leading up to anaphase showed that the majority of non-aligned bivalents eventually achieved biorientation at anaphase onset (**Fig. 4B and C; Video S1**). In contrast, in aged oocytes bivalent non-alignment often persisted, such that they had significantly greater numbers at all timepoints measured (**Fig. 4B; Video S2**). At anaphase onset, very few young oocytes had non-aligned

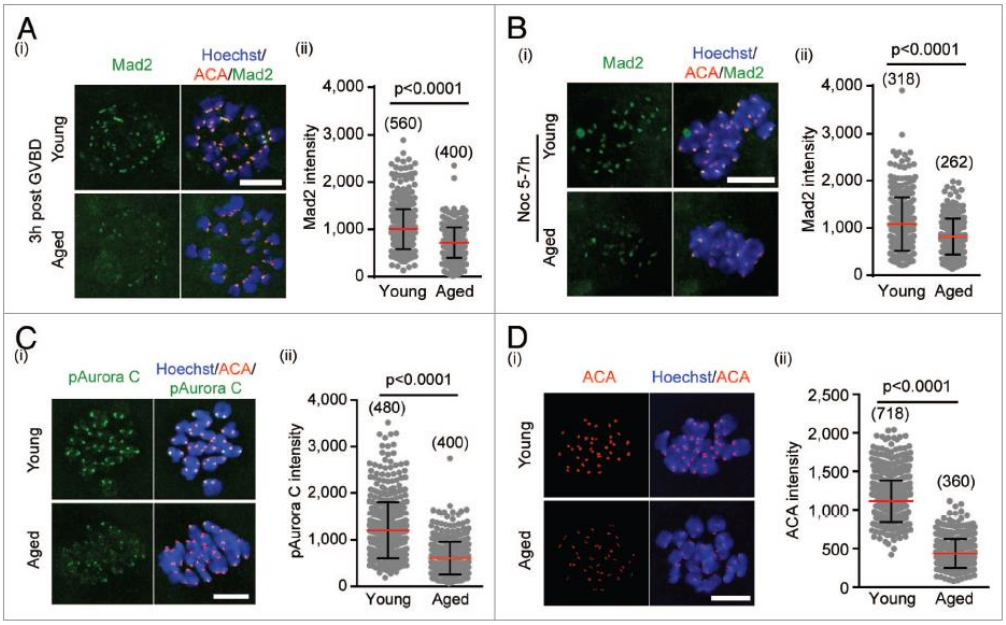

Figure 2. Centromere-associated proteins are lost with age. (**A**) Kinetochore Mad2 immunostaining (i), and its quantification (ii), in oocytes at 3 h after GVBD (14 young vs. 10 aged oocytes). (**B**) As for (**A**) but in oocytes at 5–7 h and following nocodazole addition (13 young vs. 9 aged oocytes). Aged oocytes showed lower Mad2, independent of nocodazole addition. (**C**) pAurora C immunostaining (i), and its quantification (ii) at 5 h (12 young vs 10 aged oocytes). (**D**) ACA immunostaining (i) and its quantification (ii) at 5 h (18 young vs 9 aged oocytes). pAurora C (**C**) and ACA (**D**) were both significantly decreased in aged oocytes. (**A–D;** ii) mean ± s.d. (Mann–Whitney *U* test). Scale bars represent 10 μm. (**A–D**; ii) in parenthesis, number of centromeres examined.

bivalents, whereas aged oocytes on average had 1–2, with a maximum of 6 (Fig. 4B and C).

Discussion

The incidence of aneuploidy in both mouse and human oocytes increases with maternal age.[1,2,4,5,48,49] However, here we observed that C57Bl6/J mice, at 17–19 months old, are largely resistant to this maternal-age related aneuploidy when compared with other strains. Sgo2 protects centromeric cohesion from degradation in MI,[32,33] and there is evidence that loss of Sgo2 during the uniquely long meiotic arrest of mammalian oocytes may account for aneuploidy by promoting cohesion loss.[7,31] In keeping with this hypothesis, and so explaining the resistance of C57Bl6/J mice to maternal-aging related aneuploidy, we observed little Sgo2 loss in oocytes in this strain. We did, however, observe maternal age-related losses in the SAC proteins Mad2 and pAurora C,[35] as well as a drop in ACA staining that may reflect a more widespread loss in kinetochore–centromere proteins. In an unperturbed MI division such reductions in SAC proteins appeared to have little impact, but they did correlate with a lowered ability of aged oocytes to maintain arrest with nocodazole. Furthermore, aged oocytes were far less able to establish bivalent biorientation following spindle disruption. We hypothesize that these effects are due to reduced Mad2 and pAurora C, because they are known to influence these processes of SAC arrest and error correction.

Loss of kinetochore-associated proteins with maternal age

It is intriguing that oocytes from the mouse strain C57Bl6/J exhibited resistance to maternal age-related aneuploidy, since we and others have found a much higher incidence in other strains.[6,7,10,23,25,31] In some strains, rates of aneuploidy up to 60% have been reported, which is equivalent to that seen in women.[10,50,51] Such differences suggest there may well be a genetic susceptibility to age-related aneuploidy that initially could be investigated by interrogating gene expression profiles. However, the differential loss of kinetochore proteins Sgo2, Mad2, pAurora C, and ACA observed in our study provides an important starting point in understanding this phenomenon. It would be interesting to examine why Sgo2 protein is much lower in some aged strains than others. Furthermore, it needs to be determined why maternal aging in the strain used here can affect levels of ACA, Mad2, and pAurora C but leave Sgo2 unperturbed.

Shugoshins (Sgo1 and Sgo2) are a hub for regulating chromosome segregation and appear to have multiple functions.[52,53]

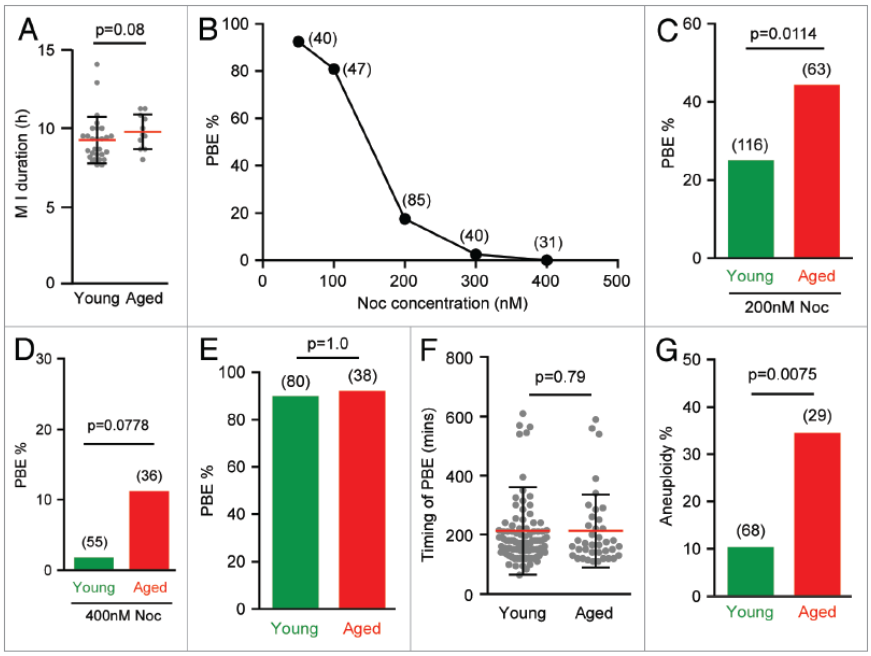

Figure 3. Aged oocytes have reduced sensitivity to nocodazole and lowered microtubule error correction. (**A**) MI duration was similar between 2 age groups during in vitro maturation (mean ± s.d; Mann–Whitney *U* test). (**B**) Dose response of young oocytes to nocodazole, added at 5 h after GVBD. (**C**) Aged oocytes had a significantly higher PBE rate with 200 nM nocodazole treatment (Fisher test). (**D**) No significant difference was observed on PBE rate with age following 400 nM nocodazole treatment (Fisher test). (**E and F**) PBE rates (**E**; Fisher test) and timing (**F**; mean ± s.d; Mann–Whitney *U* test) were similar in young or aged oocytes after a brief, 400 nM nocodazole challenge at 5–7 h post GVBD. (**G**) Aneuploidy examined in resulting metII eggs showed a significantly higher increase in aged eggs after such challenge (Fisher test). (**B–E** and **G**) in parenthesis, number of oocytes examined.

Recently in mouse oocytes Sgo2 was shown to be necessary for holding sister chromatids together during the division at MI, facilitating bivalent bi-orientation and alignment, and regulating the SAC through its recruitment or interactions with PP2A, MCAK or Mad2 on kinetochores.[54] However, it seems that only heterochromatin protein 1 (HP1) and the SAC protein Bub1 affect Shugoshin recruitment to kinetochores.[55-58] Therefore, one possibility may be that Sgo2 association with bivalents is not so readily impaired by maternal aging in C57Bl6/J as other chromosome-associated proteins. In contrast, the requirements for pAurora C and Mad2 to attach to kinetochores may be reliant on multiple proteins, whose assembly may be more easily perturbed in the process of aging. Mad2 recruitment appears to be dependent on kinetochore integrity,[59] other SAC proteins, such as Mad1, Bub1, and Mps1,[60,61] as well as the chromosomal passenger complex (CPC).[62] Similarly, Aurora C, if like Aurora B, is dependent on the assembly of the CPC[63] and haspin kinase activity.[64,65] Or an alternative would be that Aurora C itself is

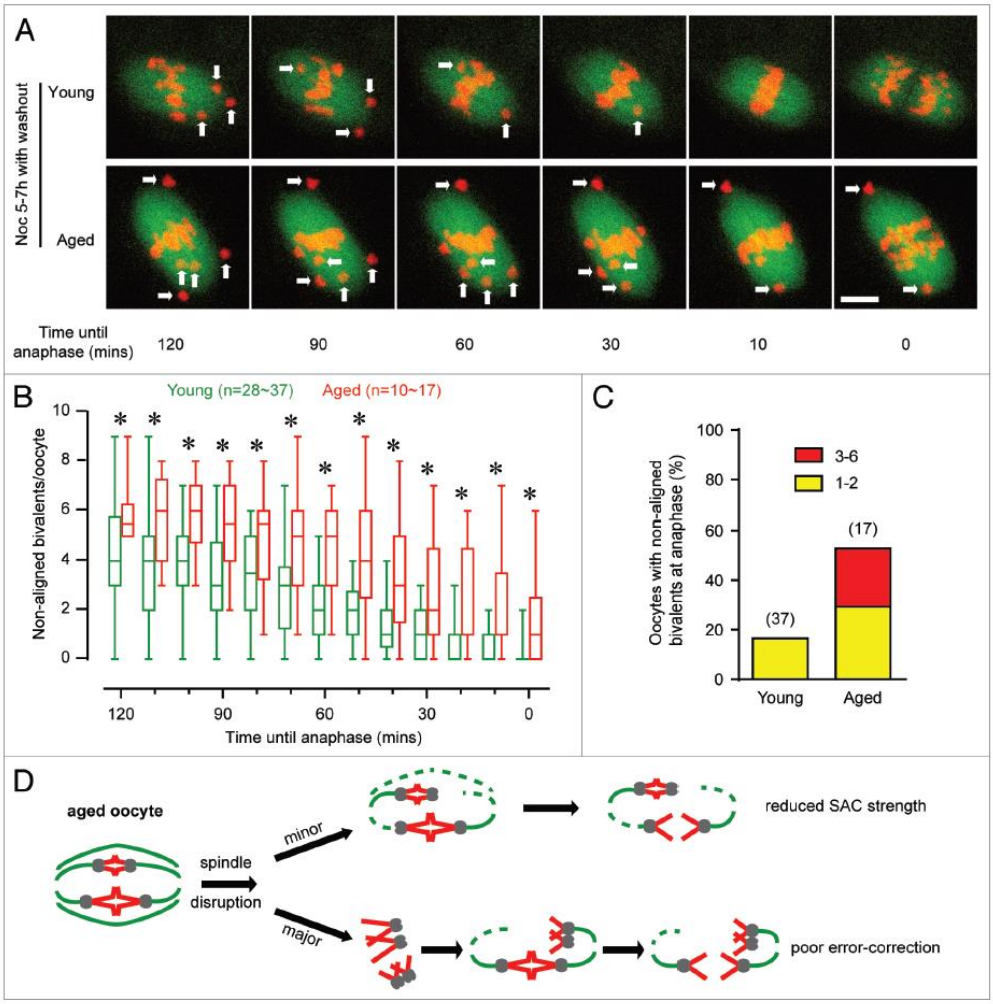

Figure 4. Non-aligned bivalents fail to biorientate and mis-segregate at anaphase after spindle disruption. (**A**) Following a 2 h nocodazole incubation and washout in MI, non-aligned bivalents at different time points were examined from young and aged oocytes, marked with arrows. Spindle is labeled in green and chromosomes in red. Movies for both are available (**Videos S1 and 2**). Scale bar represents 10 μm. (**B**) Mean number of non-aligned bivalents per oocyte in the period leading up to anaphase (whiskers are min and max). Asterisks indicate significant differences (Sidak ANOVA test). (**C**) Percentage of oocytes with either 1 or 2, or 3 or more, non-aligned bivalents at anaphase onset. (**D**) Schematic showing reduced SAC strength and poor error correction in oocytes from aged mice. (**B and C**) in parenthesis, number of oocytes examined.

normally recruited to kinetochores, but its activation mechanism may get perturbed in the process of aging, leading to the diminution of pAurora C. In support of a hypothesis where multiple proteins may determine Mad2 and pAurora C recruitment, it has been previously reported that the transcript of INCENP, a component of the CPC, is reduced in oocytes of aged mice.[25] It remains to be determined whether other proteins are altered with maternal aging and how these changes affect the fidelity of bivalent segregation in oocytes.

Reduced SAC with maternal age

A functional SAC is essential for faithful chromosome segregation in both mitosis and meiosis,[12,13] and interruption of SAC components in mouse oocytes, such as Mad2 or Aurora C, accelerates meiotic progression and increases aneuploidy rates.[1,15] In fact we have recently observed that SAC proteins have functions that last the entire period of MI, not just the canonical period of prometaphase, and are essential in slowing down the time taken to complete MI—a phenomenon that promotes bivalent biorientation and reduces mis-segregation.[66] However, whether a defective SAC is involved in aged mouse oocytes remains elusive. We have observed that with increased maternal age, the bivalent has a reduced ability to recruit or retain Mad2 and pAurora C proteins. This may reflect a general decline in whole-oocyte levels of these proteins, something that, with respect to Mad2, is supported by lowered transcript levels and is observed in both mice and humans.[24,25,27] Considering levels of staining for ACA were also lower, we suggest their loss may mark a general decline in proteins associated with the centromere, kinetochore, and possibly all heterochromatin—proteins that likely include cohesins, a suggestion supported by the measured increases in iKT distances with age. It is interesting to note there might be some association between reduced SAC proteins and cohesion loss with maternal age, which is supported by a recent report showing that cohesin deterioration may compromise SAC function by impairing sister kinetochore biorientation and its SAC signal production in mouse oocytes.[67]

Despite a 25–50% fall in Mad2 and pAurora C on kinetochores that might indicate a reduced SAC activity in aged oocytes, it is highly unlikely that the SAC is completely non-functional. This is because MI duration was not shortened with age, a finding consistent with previous studies.[7,28,43] An alternative conclusion is that aged oocytes possess reduced SAC functionality, but the extent of the reduction is not so large as to compromise SAC function in ways that have been measured so far. This appeared to be the case, because using a weaker stimulus for the SAC, a low dose of nocodazole, it was observed that the ability of the checkpoint to provide a brake on anaphase was compromised and, consequently, more oocytes completed MI and extruded a polar body (Fig. 4D, top pathway).

A failure of chromosomes to faithfully achieve biorientation is recognized as a contributing factor in their resulting mis-segregation both in somatic cells[68] and aged oocytes.[43] Consistent with this, we observed a much lower rate of bivalent biorientation in aged oocytes following brief spindle disruption, which consequently led to mis-segregation at completion of MI (Fig. 4D, lower pathway). It is interesting to note that in another strain of mouse this lower rate of biorientation was seen,[43] but occurred normally in MI without any drug additions. So the same phenomenon appears to be observed in 2 different mouse stains, but in the present study it is less pronounced—requiring drug intervention for it to be uncovered. We hypothesize that the present findings are due to our observed 50% reduction in pAurora C, because this kinase has been reported to be the predominant isoform regulating meiotic progression and is known to be responsible for repairing erroneous KT–MT attachments in mouse oocytes.[34,35,38] Recently it has been demonstrated that Sgo2 inhibits Aurora B/C activity at kinetochores, and that this inhibition may be required for chromosome bi-orientation in MI oocytes.[54] However, we have observed that following spindle disruption at 5–7 h, young oocytes go on to complete MI with only ~10% aneuploidy rates, suggesting an active error correction is functioning during MI. Furthermore, our finding that Sgo2 levels do not decrease with maternal age suggests that this protein is not the major determinant for the observed changes in pAurora C kinase activity.

Mouse strain choice is a highly relevant factor in studying aneuploidy

One important conclusion from this study is that it supports a genetic basis to maternal age-related aneuploidy, because some mouse strains share the human phenomenon and others do not.[6,7,10,31] Inroads to this genetic susceptibility are tractable and can be achieved within just a few years, since mRNA sequencing techniques on different strains are now feasible at the level of the single oocyte.[69]

We conclude it is important to appreciate that there appears differences between strains of mice that may make universal conclusions about the way oocytes behave with respect to maternal aging difficult to reach. So, for example, some strains show aging differences in bivalent biorientation,[43] while others do not;[31] some show loss in Sgo2,[7,31] while others do not (present study). These appear to reflect true strain differences rather than discrepant results between laboratories.

Materials and Methods

Oocyte collection and RNA microinjection

Mice were used in accordance with ethics approved by the University of Newcastle Animal Care and Ethics Committee. For the aging study, either 1-mo- and >12-mo-old Swiss CD-1 outbred females or 1 mo and 17–19 mo C57Bl6/J ex-breeders were used. GV stage oocytes were collected from young or aged mice without hormone injection. A range of 20–30 oocytes for young, and 0–5 oocytes for old, were collected per mouse. Oocytes were handled in M2 media containing 2.5 μM milrinone (Sigma-Aldrich) under mineral oil at 37 °C, and maturation was stimulated by milrinone washout, timed relative to extrusion of the first polar body.[70] Microinjections were performed on the heated stage of a Nikon TE300 inverted microscope as previously described.[71] H2B-mCherry and EGFP-MAP4 (EUROSCARF, p30518) cRNAs were injected at a pipette concentration of 200 and 500 ng/μl respectively, with a measured 0.1–0.3% of oocyte volume.

Immunofluorescence

Oocytes were fixed and permeabilized in 2% formaldehyde in PHEM buffer with 0.5% TritonX-100 as described previously.[31] For metII eggs, monastrol was used to spread chromosomes for aneuploidy analysis before fixation.[40] Blocking was performed in 7% normal goat serum in PBS with 0.1% Tween-20. Primary antibodies were diluted in PBS with 3% BSA / 0.1% Tween-20 with overnight incubation at 4 °C: Sgo2, Mad2, phospho-Aurora C, or ACA. Secondary antibodies were Alexa-633 or Alexa-555 conjugated (Invitrogen). Oocytes were counterstained with Hoechst (20 μg/ml) prior to mounting in Citifluor (Citifluor Ltd). For intensity comparison experiments, young and aged mouse oocytes were collected and processed with the identical procedure, and then images were captured on the same day with the same confocal settings.

Confocal imaging and image analysis

An Olympus FV1000 confocal microscope was used on both fixed and live cells.[22,31] For quantitative analysis of kinetochore Sgo2, Mad2, and pAurora C, oocytes were double stained with ACA, performed on the same day, and z-stacks of 1 μm z-resolution were acquired using identical settings. All images were analyzed using ImageJ (NIH, Bethesda), and fluorescence calculation was performed as previously described with modifications[31,72] (**Fig. S1C**). Specifically, the single z-plane with greatest ACA was set to 0, then a z-projection with 3 successive planes (–1; 0; +1 μm) was acquired. All ACA and centromere-associated proteins fluorescence was captured by this z-sectioning. Kinetochore fluorescence intensity, a 2 μm diameter circle (red line), centered on the ACA (red), was background subtracted. The background was taken as the lowest value measured from 8 surrounding circles of the same size (B1-B8, dotted white line). For time-lapse imaging, oocytes were housed in a 37 °C temperature controlled environment, and ImageJ macros were used to track and maintain chromosomes within the center of field of view.[31] Images were captured every 5 min, with z-resolution of 3.0 μm. In the EGFP-Map4- and H2B-mCherry-expressing oocytes, metaphase plate was defined as a drawn line, where >10 bivalents congressed, and non-aligned chromosomes were those clearly away from this line in all 3 dimensions.

Statistical analysis

Dichotomous data were analyzed using Fisher test. For multiple comparisons we employed Sidak ANOVA test. All of other means analysis was performed using Mann–Whitney *U* test. Data were processed using GraphPad Prism 6, with $P < 0.05$ set for significance. For most of the comparisons, more than 10 aged mice were tested from at least 2 independent experiments. All data are pooled.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Author Contributions

All authors helped devise the study. The experiments were performed and figures prepared by Y.Y., supervised by K.T.J. and J.E.H. S.I.R.L. wrote the software for live cell imaging and kinetochore analysis. S.I.R.L., J.A.M., and E.A.M. provided critical assistance and advice during performing the whole project. The manuscript was written by Y.Y. and K.T.J., with input from all authors.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cc/article/28897

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Supplemental Material to:

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Reduced ability to recover from spindle disruption and loss of kinetochore spindle assembly checkpoint proteins in oocytes from aged mice

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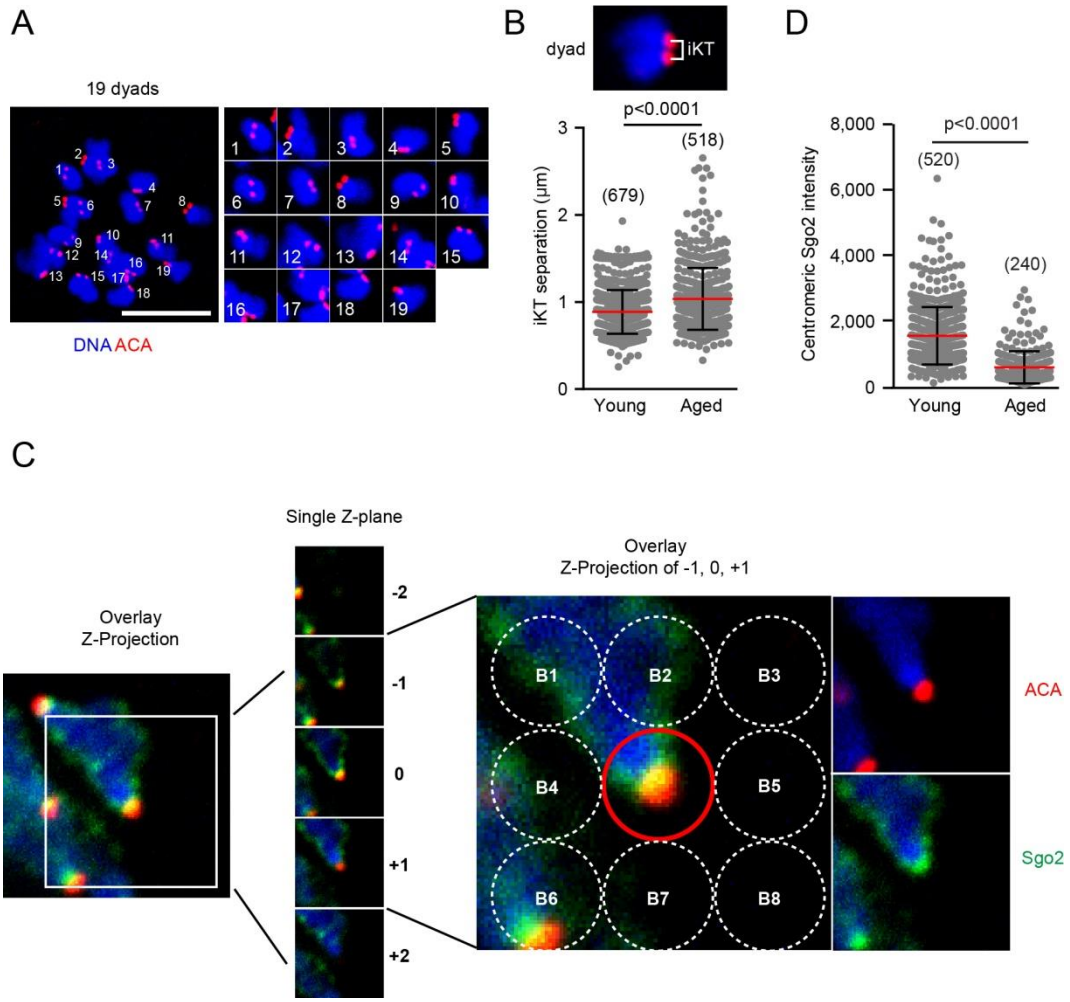

Fig. S1. Analysis of interkinetochore distance (iKT) separation in metII eggs and measurement of centromeric Sgo2 intensity in MI oocytes. (A) A chromosome spread of a metII egg that is hypoploid. Left image, maximal intensity z-projection of chromosomes and their kinetochores (ACA staining). Dyads (sister chromatid pairs) are labeled in white. Right images, individual 19 dyads taken from single z-planes. Scale bar represents 10 µm. **(B)** iKT separation, measured between the kinetochores of a dyad, showed greater distance in oocytes from aged mice (mean ± s.d; Mann Whitney U test; 34 young vs 26 aged eggs). **(C)** Measurement of centromere-associated protein intensity. For quantitative analysis of kinetochore Sgo2 (as shown here), Mad2

and pAurora C, oocytes were ACA counterstained, and images captured with 1.0 μm z-resolution. The single z-plane with greatest ACA was set to 0, then a z-projection with three successive planes (-1; 0; +1 μm) acquired. All ACA and centromere-associated proteins fluorescence was captured by this z-sectioning. Kinetochore fluorescence intensity, a 2μm diameter circle (red line), centered on the ACA (red), was background subtracted. The background was taken as the lowest value measured from 8 surrounding circles of the same size (B1-B8, dotted white line). **(D)** In a different mouse stain oocytes (Swiss CD-1 outbred), centromeric Sgo2 intensity is significantly reduced with age (mean ± s.d; Mann-Whitney U test; 13 young vs 6 aged oocytes). B and D in parenthesis, number of dyad pairs and centromeres examined respectively.

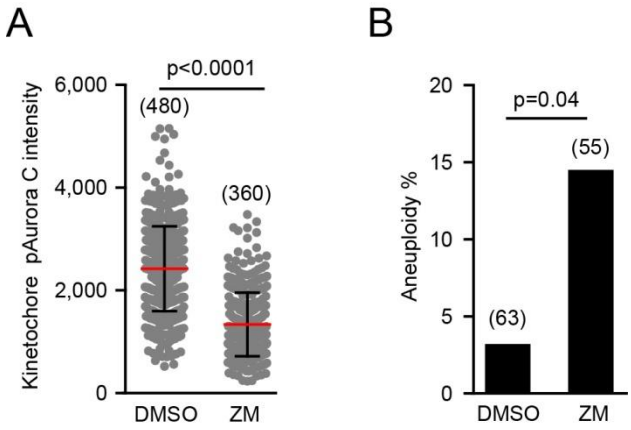

Fig. S2. ZM447439 reduced kinetochore bound pAurora C and raised aneuploidy rates. (A) Kinetochore associated **p**Aurora C intensity measured in oocytes, 2 h after GVBD, treated with either 10 µM ZM447439 or DMSO vehicle for 3 h. The Aurora kinase inhibitor significantly reduced kinetochore-bound pAurora C (mean ± s.d; Mann Whitney U test; 12 DMSO vs 9 ZM treated oocytes). **(B)** Aneuploidy rates in metII eggs, treated with either 10 µM ZM447439 or DMSO during MI. Rates of aneuploid eggs were significantly increased with Aurora kinase inhibition (Fisher's test). A and B in parenthesis, number of centromeres and oocytes examined respectively.

Video 1. Following spindle disruption an oocyte in which bivalent congression precedes anaphase. In this oocyte, from a young mouse, previously treated with 400 nM nocodazole, at 5 h after GVBD for 2 h, bivalent congression is observed to be complete 15 mins before anaphase-onset. The meiotic spindle is labeled with EGFP-MAP4 and the bivalents with H2B-mCherry. Time stamp: minutes remaining until anaphase-onset. Scale bar represents 10 μm.

Video 2. Following spindle disruption an oocyte in which bivalent congression is not complete at anaphase onset. In this oocyte, from an aged mouse, previously treated with 400 nM nocodazole, at 5 h after GVBD for 2 h, bivalent congression is observed not be complete in any frame before anaphase-onset. The meiotic spindle is labeled with EGFP-MAP4 and the bivalents with H2B-mCherry. Time stamp: minutes remaining until anaphase-onset. Scale bar represents 10 μm.

4 Ndc80 N-terminal modification imposes robust SAC signalling in mouse oocyte

Ndc80 N-terminal modification imposes robust SAC signalling in mouse oocytes

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Summary

Correct microtubule-kinetochore (MT-KT) interactions are essential to achieve faithful chromosome segregation and prevent aneuploidy. An important cell checkpoint that has evolved in eukaryotes to ensure the fidelity of this process, is the spindle assembly checkpoint (SAC) which delays anaphase-onset until all kinetochores are correctly attached to the spindle. A key component of the MT-KT interface is Ndc80 (also known as Hec1 in humans), which is essential for maintaining kinetochore integrity and stability during mitosis. However its function in mammalian oocytes, which are prone to aneuploidy, is largely unknown. Here we found that N-terminal modification of Ndc80 efficiently blocked first polar body extrusion (PBE) in mouse oocytes. Further investigations showed that N-terminal modified Ndc80 expression increased erroneous MT-KT attachments. These errors had no obvious effect on bivalent congression, but did induce robust SAC signaling since an increase in kinetochore-bound Mad2 was detected, and Mps1 or Aurora kinase inhibition rescued PBE rates. Our data show a highly conserved role for Ndc80 in establishing MT-KT attachments and regulating the SAC in mouse oocytes.

Introduction

Mammalian oocytes are often regarding as being susceptible to undergo chromosome (bivalent) mis-segregation in meiosis I (MI), generating aneuploid eggs and embryos. However at least in mice, oocytes possess a Spindle Assembly Checkpoint (SAC), which contributes to preventing aneuploidy by inhibiting the Anaphase-Promoting Complex (APC) (Jones and Lane, 2013; Sun and Kim, 2012). Non-aligned bivalents do not stall or stop MI (Gui and Homer, 2012; Kolano et al., 2012; Lane et al., 2012; Nagaoka et al., 2011; Sebestova et al., 2012), suggesting that correct microtubule-kinetochore (MT-KT) attachment is not a pre-requisite for SAC satisfaction and so APC activity. Instead it has been proposed that oocytes possess a weak SAC compared to somatic

cells, and indeed APC and SAC activities have been discovered to co-exist in oocytes during MI (Lane and Jones, 2014) - probably because the SAC signal from a few kinetochores is insufficiently amplified to inhibit all APC activity.

At the MT-KT interface is the Ndc80 complex, comprising of the proteins Ndc80/Hec1, Nuf2, Spc24 and Spc25 (Cheeseman et al., 2004; Ciferri et al., 2007; DeLuca et al., 2005). Disruption of the Ndc80 complex in somatic cells causes misaligned chromosomes, disturbed spindle organization, and reduced attachment stability (Ciferri et al., 2007; DeLuca et al., 2005; Janke et al., 2001; McCleland et al., 2003). Furthermore, disruption of the Ndc80 complex reduces Mps1 and Mad1/Mad2 localization and causes abnormal SAC function (DeLuca et al., 2003; Kline-Smith et al., 2005; Martin-Lluesma et al., 2002; Meraldi et al., 2004). The N-terminal region of Ndc80 is especially important for microtubule binding; structural and functional studies have revealed the key roles of Ndc80 N-terminus in regulating dynamic behavior of MT-KT interaction (Ciferri et al., 2008; DeLuca et al., 2006; Guimaraes et al., 2008; Mattiuzzo et al., 2011; Miller et al., 2008; Tooley et al., 2011).

In mouse oocytes, reduced levels of Ndc80 or Spc25 causes severe chromosome misalignment and abrogated SAC signaling (Sun et al., 2010; Sun et al., 2011). Therefore, despite the fact that MI is unique in having fused sister kinetochores that co-segregate, it seems likely that the Ndc80 complex serves a meiotic function that parallels its mitotic one. However, at least one novel meiotic function has been described for Ndc80 in its ability to stabilize cyclin B2 (Gui and Homer, 2013).

In the current study we examined the effects of Ndc80 overexpression in mouse oocytes- in order to uncover more about how this protein functions in mammalian meiosis. A recent similar approach in mitotic cells found that stable clones with overexpression of either C-terminal Ndc80 or untagged Ndc80 construct were never established, possibly because its cellular expression levels are tightly regulated, and any interruption may severely affect cell growth; while overexpression of an N-terminal tagged construct caused multipolar spindles due to the centriole splitting (Mattiuzzo et al., 2011). Here we find different effects of Ndc80 in mouse oocytes, and these findings uncover more insight into how the SAC functions in mammalian oocytes.

Results and Discussion

N-terminus modification of Ndc80 causes increased attachment errors and inhibits meiosis I completion.

Structural and functional studies have revealed the key roles of Ndc80 N-terminus in regulating dynamic behavior of MT-KT interaction in mitosis (Ciferri et al., 2008; DeLuca et al., 2006; Guimaraes et al., 2008; Mattiuzzo et al., 2011; Miller et al., 2008; Tooley et al., 2011). In mammalian oocytes, recent studies demonstrated that siRNA or antibody injection of either Ndc80 or Spc25 caused severe chromosome misalignment and abrogated SAC signaling (Sun et al., 2010; Sun et al., 2011). To further investigate the role of Ndc80 in mouse oocytes, vectors expressing untagged Ndc80 (Ndc80) or Venus-tagged Ndc80 at the N-terminus (Ndc80-N) or C-terminus (Ndc80-C) were constructed. As shown in **Fig. 1A**, following cRNA injection of oocytes with Ndc80-N and Ndc80-C, fused proteins were specifically localised on the centromeres, consistent with its function as a kinetochore component. Ndc80-C injected oocytes went on to extrude polar bodies at a rate of over 65%, however, Ndc80-N expression inhibited polar body extrusion (PBE) to a rate of ~10% **(Fig. 1B)**. We hypothesize that this effect is primarily due to Ndc80 N-terminus

modification rather than its exogenous over-expression, because both Ndc80 and Ndc80-C expression caused only a slight drop in PBE, when compared to expression of a non-kinetochore construct Histone 2B (H2B) (**Fig. 1B)**; although the other possibility could be that over-expression of exogenous Ndc80 in some way down-regulated its endogenous expression. This observation is not surprising since the N-terminus of Ndc80 binds microtubules directly in mitosis (Ciferri et al., 2008; DeLuca et al., 2006; Guimaraes et al., 2008; Miller et al., 2008). Therefore we predict that blocking this domain by the Venus tag , if analogous to mitosis, may impair MT-KT interaction and the spindle checkpoint pathway.

We next sought to examine whether the modification to the N-terminus of Ndc80 affects microtubule attachment configuration, since stable end-on attachments are a prerequisite for subsequent accurate chromosome segregation and mitotic exit (Foley and Kapoor, 2013; Tanaka and Desai, 2008). Oocytes were treated with calcium to destabilize non-kinetochore associated microtubules, followed by immunostaining with α-tubulin and anti-centromeric antibodies (ACA) at 7-hour post germinal vesicle breakdown (GVBD). This time corresponds to ~1 hour before anaphase in oocytes, when they have achieved a state of metaphase with all chromosomes aligned, and when APC-mediated cyclin B1 and securin degradation is approaching completion (Lane et al., 2012). In total, we were able to accurately assess the status of K-fibers from over 98% of sister kinetochore pairs (4 control vs. 9 Ndc80-N oocytes) and observed that all kinetochores from both groups possessed microtubule attachments. However merotelic attachments, in which microtubules from both spindle poles attach to one of the sister kinetochore pairs, were found in control oocytes at a rate of 7.6%, which was significantly higher at 17.7% in the Ndc80-N group **(Fig. 1C)**. It is important to note here that initial establishment of microtubule attachments in mouse oocytes is predominantly incorrect, and each bivalent undergoes an average of 3 rounds of error correction to

achieve stable biorientation during the long meiosis I (Kitajima et al., 2011). The observation of no vacant kinetochores in Ndc80-N oocytes indicates that initial MT-KT interaction may not be affected by this modification. However, it has been well documented that phosphorylation of Ndc80 N-terminus by Aurora B kinase is essential for the process of error correction in mitotic cells (Campbell and Desai, 2013; Lampson and Cheeseman, 2011; Liu et al., 2009). Therefore, we predict that N-terminus modification of Ndc80 may interfere with Aurora B-dependent phosphorylation and in doing so, impair the ability to repair incorrect microtubule attachments.

Metaphase-anaphase transition failure in Ndc80-N oocytes is independent of bivalent congression and tension establishment.

Having identified that N-terminus modification of Ndc80 increased the prevalence of merotelic attachments, we next sought to examine whether these erroneous attachments affected bivalent congression and tension establishment, since these kinetochore/bivalent dynamics are associated with SAC function (Foley and Kapoor, 2013; Lara-Gonzalez et al., 2012). We employed an approach established recently for labeling and tracking both bivalents and their kinetochores by 4D confocal microscopy, using H2B-mCherry and EGFP-CenpC respectively (Kitajima et al., 2011). Bivalent dynamics were analyzed at 2-8 hours post GVBD, which encompassed the periods of prometaphase through to initiation of anaphase in the majority of control oocytes **(Fig. 2A; Movies 1 and 2)**. Non-alignment was defined as described previously (Lane et al., 2012) as a bivalent >4 μm away from the spindle equator. Using this threshold, we found no obvious differences in bivalent congression, with over 97% of bivalents located on the spindle equator at 5-8 hours in both groups **(Fig. 2B)**.

Kinetochore separation, defined here as the distance apart of the two sister kinetochore pairs within a bivalent, can be regarded as a readout of tension; since this distance increases as the tension across the bivalents is established, due to the pulling forces from microtubules attached to opposing spindle poles. During the period of observation, tension on bivalents was gradually established between 2-5 hours and then maintained a mean kinetochore separation of ~5μm until anaphase onset, at which time control, but not Ndc80 oocytes, experienced a significant increase due to bivalent segregation **(Fig. 2C)**. The lack of impact of Ndc80 N-terminal modification upon bivalent congression and tension in oocytes is in agreement with previous studies in mitotic systems which have shown that merotelic attachments are responsible for tension across sister chromosomes and aligns them on the metaphase plate (Cimini et al., 2004; Cimini et al., 2003; Thompson and Compton, 2011). It is notable that sister kinetochores act as one functional unit and are attached to microtubules in a 'side-by-side' conformation in meiosis I, rather than 'back-to-back' arrangement which is the case in mitosis (Holt and Jones, 2009; Homer, 2011). In a previous study investigating aneuploidy in oocytes from aged mice, we similarly observed an increased incidence of merotelic attachments measured from lagging chromosomes at anaphase, without any gross errors in congression and tension establishment across the bivalents in meiosis I (Yun et al., 2014b). Therefore, our data showed that increased merotelic attachments in Ndc80-N oocytes failed to cause significant changes in bivalent congression and tension formation to inhibit metaphase-anaphase transition.

Ndc80 N-terminus modification inhibits APC/C activity and imposes SAC signaling

Anaphase initiation is achieved by APC/C mediated degradation of both cyclin B1 and securin which regulates CDK1 activity and the resolution of the cohesin complexes respectively (Jones, 2010; Pesin and Orr-Weaver, 2008; Primorac and Musacchio, 2013). Separase is responsible for

cleavage of the cohesin rings, however due to the binding of its chaperone protein – securin, it is kept inactive prior to anaphase. To examine whether inhibition of APC/C activity in Ndc80-N injected oocyte accounts for the failure of bivalents to segregate, we employed securin-mCherry cRNA co-injection and tracked its degradation. In control oocytes expressing Venus-H2B (H2B-N) securin was degraded reaching minimal fluorescence around the time of PBE, 8-9 hours post GVBD **(Fig. 3A)**. However such degradation was not observed in Ndc80-N/securin-mCherry oocytes, rather securin showed a gradual accumulation **(Fig. 3B)**. Therefore we conclude that failure of bivalent segregation in Ndc80 N-terminus modified oocytes may result from the inhibition of the APC/C activity.

We therefore next sought to investigate whether the SAC pathway is activated, since an active SAC is able to generate a potent inhibitor of the APC/C – MCC, by sensing kinetochore attachment or tension (Lara-Gonzalez et al., 2012; Primorac and Musacchio, 2013). Moreover, a recent study demonstrated that the strength of SAC response depends on the amount of Mad2 recruited to kinetochores (Collin et al., 2013). Therefore we measured kinetochore bound Mad2 levels by immunofluorescence in oocytes at 8 hours post GVBD (**Fig. 4A**). At this timepoint the SAC in mouse oocytes is inactive, allowing for the activation of metaphase-anaphase transition, so the kinetochores should possess low levels of, or completely lack Mad2 (Gui and Homer, 2012; Kitajima et al., 2011; Lane et al., 2012). This was true of control oocytes, however Ndc80-N oocytes retained ~40% more Mad2 on kinetochores (**Fig. 4B**). We predict that such an increase in kinetochore-associated Mad2 is sufficient to maintain a robust SAC function in oocytes. Consistent with our findings, it has been previously shown that high levels of kinetochore Mad2 are not essential for keeping an active SAC signaling in mitotic cells (Martin-Lluesma et al., 2002).

One possibility for the increased kinetochore retention of Mad2 levels in Ndc80-N oocytes is that pathways responsible for Mad2 dissociation from kinetochores in meiosis I are impaired. To examine for this possibility, we employed the same Mad2 immunostaining procedure at two earlier timepoints. We found a ~50% drop in kinetochore Mad2 signal from 3.5 hours to 6 hours post GVBD with a concomitant accumulation of Mad2 at sites consistent with spindle poles, as previously reported (Holt et al., 2012; Maiato et al., 2004; Zhang et al., 2007), while Mad2 was recruited to kinetochores at a higher level when challenged with spindle poison – nocodazole (**Fig. 4A, C**). These data suggest that the process of Mad2 transfer from kinetochores to the spindle poles may not be grossly affected with N-terminus modification of Ndc80. However, it is important to note that a ~50% decrease in kinetochore Mad2 observed here is somewhat less than that seen **(Fig. 4C)** in other studies, which showed over 70% decline at similarly selected timepoints in mouse oocytes (Gui and Homer, 2012; Lane et al., 2012). Therefore, our results showed that Ndc80-N expression have no severe effects on kinetochore Mad2 dynamics in Meiosis I, however the slightly compromised pathways involving removal of Mad2 from kinetochores might contribute to elevated Mad2 kinetochore localization and active checkpoint signaling in late Meiosis I.

Abolishing Aurora kinase or Mps1 activity efficiently rescues the SAC-imposed arrest in Ndc80-N oocytes.

To further confirm SAC activation in response to Ndc80-N we next inhibited the kinases, Aurora B/C and Mps1, both of which appear to be essential for an active checkpoint signalling in mouse oocytes (Hached et al., 2011; Lane et al., 2010; Sharif et al., 2010). Moreover, these kinases appear to be master regulators of the SAC that lie upstream of other SAC components, such as Mad2 (Heinrich et al., 2012). One further reason for examining Aurora B/C and Mps1 in this context is that both are known to be responsible for microtubule attachment error correction through

phosphorylating kinetochore substrates in a tension-dependent mechanism in mitosis (Liu et al., 2009; Maure et al., 2007). In live Ndc80-N oocytes, we did observe the established tension across bivalents measured by kinetochore separation (**Fig. 2C**); however, we cannot guarantee that this measurement equally reflects intrakinetochore stretch, which has recently been shown to be required for kinetochore phosphorylation and SAC regulation (Maresca and Salmon, 2009; Uchida et al., 2009). In addition, a previous study showed that removal of Mps1 from kinetochores is a prerequisite for mitotic checkpoint silencing, and failed dissociation of active Mps1 retained active checkpoint although chromosome alignment and interkinetochore tension were unaffected (Jelluma et al., 2010). Therefore, one possibility is that increased MT-KT attachment errors, due to Ndc80 N-terminus modification, cause enhanced Aurora B/C and Mps1 activities on kinetochores, which maintains a robust SAC. In this case we predicted that failure of the metaphase-anaphase transition in Ndc80-N oocytes might be rescued if Aurora B/C and Mps1 activities are inhibited.

To test the hypothesis, we used ZM447439 (ZM) and Reversine (Rev), which specifically inhibit Aurora B/C and Mps1 kinases respectively (Lane et al., 2010; Lane and Jones, 2014). Ndc80-N oocytes were treated with inhibitors at 6-hour post GVBD, a timepoint at which the majority of Mad2 has left kinetochores in mouse oocytes (**Fig. 4C**) (Gui and Homer, 2012; Kitajima et al., 2011; Lane et al., 2012). In agreement with our prediction, Aurora B/C or Mps1 inhibition rescued Ndc80-N induced metaphase arrest, leading to anaphase initiation within 2 hours of treatment (**Fig. 5A**). Moreover, there were no differences found in the timing of anaphase onset when compared to control H2B oocytes after treatment with either inhibitor (**Fig. 5B**), and correspondingly PBE rates were both significantly rescued (**Fig. 5C**). To further confirm that bivalent segregation and meiosis I completion in Ndc80-N oocytes could be triggered by checkpoint signaling silencing and subsequent APC/C activation, we monitored securin degradation with Mps1 inhibition. Indeed we

found that securin fluorescence in Ndc80-N oocytes began to decrease immediately following Mps1 inhibition and eventually reached minimum within ~1 hour, which was similar to the degradation profile in H2B oocytes (**Fig. 5D**). These securin degradation data were consistent with the observations on timing of anaphase onset following Rev treatment (**Fig. 5A and B**). It is notable that inactivation of either Aurora B/C or Mps1 seems sufficient to trigger checkpoint silencing in oocytes with Ndc80 N-terminus modification (**Fig. 5A-C**). This is in agreement with studies that identified that these kinases coordinate with each other in kinetochore recruitment and activation (Saurin et al., 2011; van der Waal et al., 2012), and eventually correct microtubule connections to prevent chromosome segregation errors in other cell types (Meyer et al., 2013). Our data here suggested a highly conserved mechanism of SAC signalling among eukaryotic cells, including mammalian oocytes.

Conclusion remarks

In summary, expression of an N-terminus modified Ndc80 in mouse oocytes causes increased microtubule merotelic attachments. However, these attachment errors seem to have no gross effects on bivalent dynamics, because we found that chromosome congression was not disrupted and tension across the bivalents was established. Nevertheless a robust checkpoint signaling was imposed, since both inhibited securin degradation and raised levels of kinetochore-bound Mad2 were detected in Ndc80-N oocytes. We hypothesize SAC activation in this context may originate from persistence of Aurora B/C or Mps1 kinase activity at metaphase I, both of which are involved in attachment error correction in mitotic cells (Liu et al., 2009; Maure et al., 2007; Petsalaki and Zachos, 2013), because abolishing either of the kinases efficiently rescued metaphase-anaphase transition and meiosis I completion. An alternative explanation could be that Ndc80-N expression recruits and stabilizes Mad2 at the kinetochore, whose retention maintains active SAC; in this case

either Aurora B/C or Mps1 inhibition would silent the checkpoint, because both kinases play essential roles in the upstream of SAC hierarchy (as shown in Fig 1.4). These data taken together show the conserved role of Ndc80 N-terminus in mammalian oocytes and emphasize the dual function of Aurora B/C and Mps1 kinases, which couple attachment error correction and spindle checkpoint cooperatively.

Methods

Animals and oocyte culture

F1 hybrid mice (C57Bl6 females × CBA males) were used in accordance with ethics approved by the University of Newcastle Animal Care and Ethics Committee. GV stage oocytes were collected from mice hormonally primed with intraperitoneal injection of 10 IU pregnant mares' serum gonadotrophin (Intervet, Australia), and handled in M2 media containing 2.5 μM milrinone at 37°C. Meiotic maturation was stimulated by milrinone washout, and all culture was under mineral oil at 37°C.

cRNA production and microinjection

Ndc80 cDNA was prepared from mouse oocytes using the Smart PCR cDNA synthesis kit (Clontech Laboratories) according to the manufacturer's instructions, and cloned into Destination vector using the Chordate Gateway system (Invitrogen). 5'-capped cRNAs were synthesized using either T3 or T7 mMessage mMachine (Ambion, USA), and dissolved in nuclease-free water. cRNA microinjection was performed as previously described (Holt et al., 2012; Holt et al., 2011; Yun et al., 2014b) at a pipette concentration of ~200 ng/μl for Ndc80, 500 ng/μl for Securin, 100 ng/μl for H2B and 500 ng/μl for CenpC. A measured 0.1–0.3% of oocyte volume injection was given on the

heated stage of a Nikon TE3000 inverted microscope, using a timed pressure injection facility of a Pneumatic PicoPump (World Precision Instruments, Stevenage, UK).

Immunofluorescence

Oocytes were fixed and permeabilised in formaldehyde as described previously (Yun et al., 2014a; Yun et al., 2014b). For k-fiber staining, oocytes were treated with Ca^{2+} buffer for 2 minutes [100 mM Pipes (pH 7.0), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1% Triton X-100] before fixation (Lane et al., 2012). Blocking was performed in 7% normal goat serum in PBS with 0.1% Tween-20. Primary antibodies were diluted in PBS with 3%BSA/0.1%Tween-20 with overnight incubation at 4°C: α-tubulin (1:400, Invitrogen), ACA (1:400, Cortex Biochem) and Mad2 (1:1000, a kind gift from Dr R.H. Chen). Secondary antibodies were Alexa 633- or 555-conjugated (Invitrogen). Oocytes were counterstained with Hoechst (20 μg/ml) prior to mounting in Citifluor (Citifluor Ltd, UK). For the comparison experiments, all groups were processed with the same antibody stock dilution, and images captured on the same day with the same confocal settings.

Imaging and analysis

Images were captured using a Nikon TE3000 inverted microscope fitted with heated stage (For Securin imaging; Roper Scientific, Trenton, NJ) or an Olympus FV1000 confocal microscope (For all other imaging). All images were analysed using ImageJ (NIH, Bethesda, USA) and fluorescence calculation was performed as previously described (Yun et al., 2014a). For confocal time lapse imaging, in-house ImageJ macros were used to track chromosome movements with z-resolution of 2.0μm, at 5-minute intervals. To improve the kinetochore signals for localisation in live oocytes, the images were processed using Image J software, by the subtraction of a ten-pixel Gaussian blur from a two-pixel Gaussian blur, and the positions for individual bivalents at each timepoint were acquired using house-made macros, as previously described (Lane and Jones, 2014; Yun et al., 2014b).

Statistical analysis

Dichotomous data were analyzed using Fisher's test. For multiple comparisons we employed Kruskal-Wallis ANOVA, with Dunn's post-test. All other mean analysis was performed using either Mann-Whitney or student t-test using $P<0.05$ for significance (ns, $P\geq0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.0001$).

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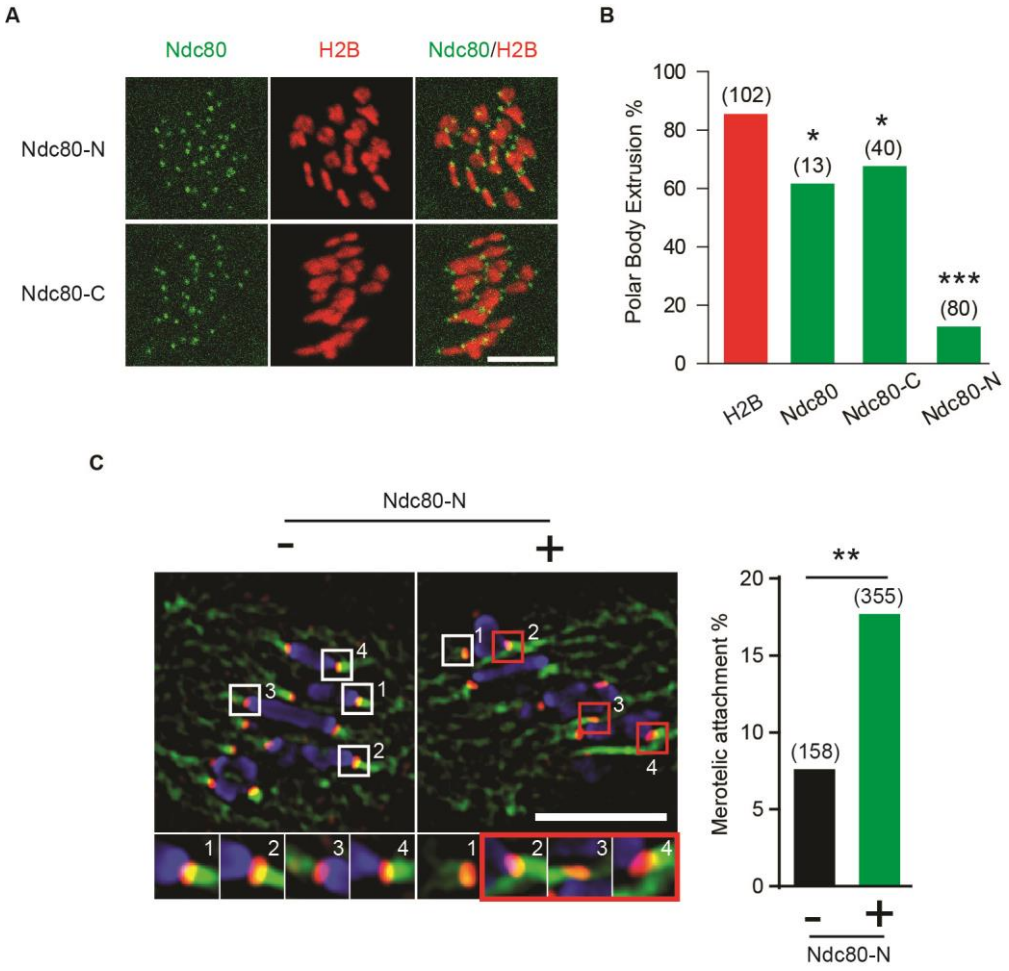

Fig. 1 N-terminus modification of Ndc80 does not affect its kinetochore localization, but causes failure of PBE and microtubule attachment errors in oocytes. (A) Kinetochore localization of both Ndc80 N- and Ndc80 C-terminal Venus tags in live oocytes following cRNA microinjection. **(B)** The majority of Ndc80-N expressing oocytes failed to undergo meiosis I completion (Fisher's test; all compared to H2B group). **(C)** Repreventative oocytes with K-fibre immunostaining at 7h post GVBD (left). Significantly increased numbers of merotelic attachments (red box) were detected in the Ndc80-N injected oocyte group (centre, right; Fisher's test). B in parenthesis, number of oocytes examined; C in parenthesis, number of kinetochore pairs examined. (A, C) Scale bars represent 10µm.

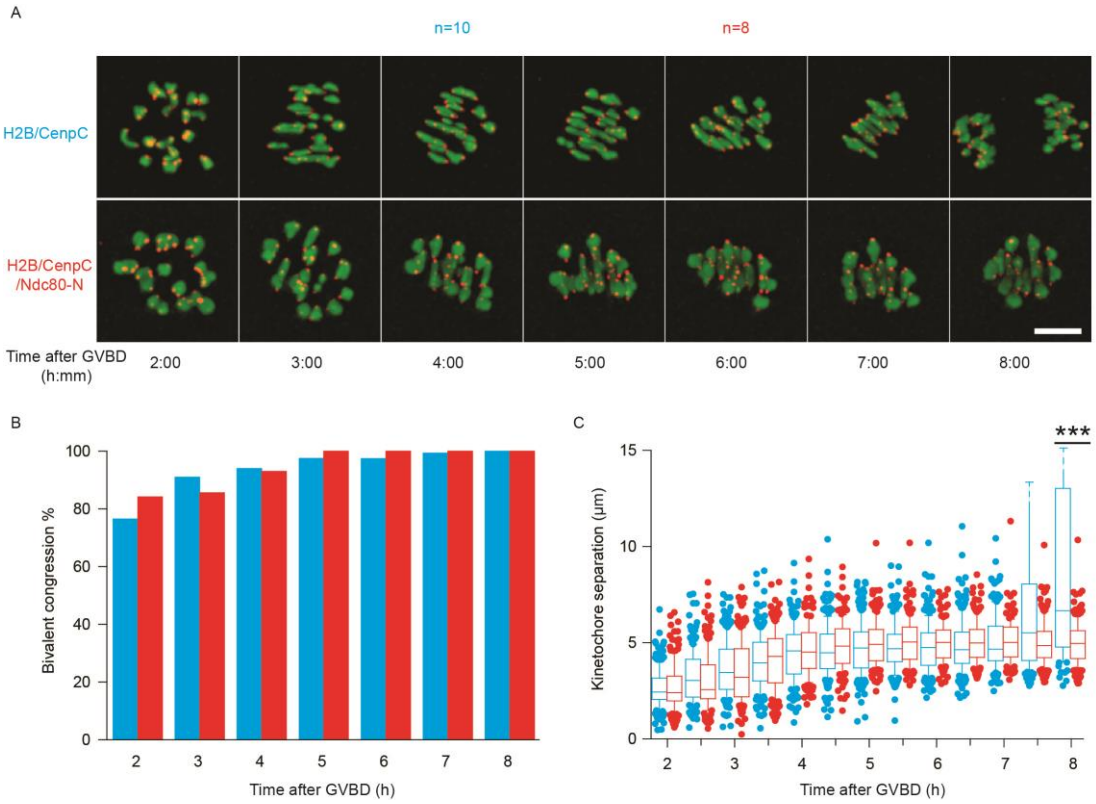

Fig. 2 Bivalent congression and tension were unaffected by Ndc80-N. (A) H2B-mCherry (Green) and EGFP-CenpC (Red) were used for chromatin and kinetochore labelling, respectively. Shown are selected timepoints from control (H2B/CenpC, top) and Ndc80-N (H2B/CenpC/Ndc80-N, bottom) oocytes. Scale bar represents 10µm. **(B,C)** No significant differences were observed in bivalent congression (B) and kinetochore separation (distance between the two pairs of sister kinetochores within a bivalent; C); Box plots, whiskers at 10-90 centiles; Asterisks indicate $P<0.01$ (Kruskal-Wallis ANOVA, with Dunn's post-test).

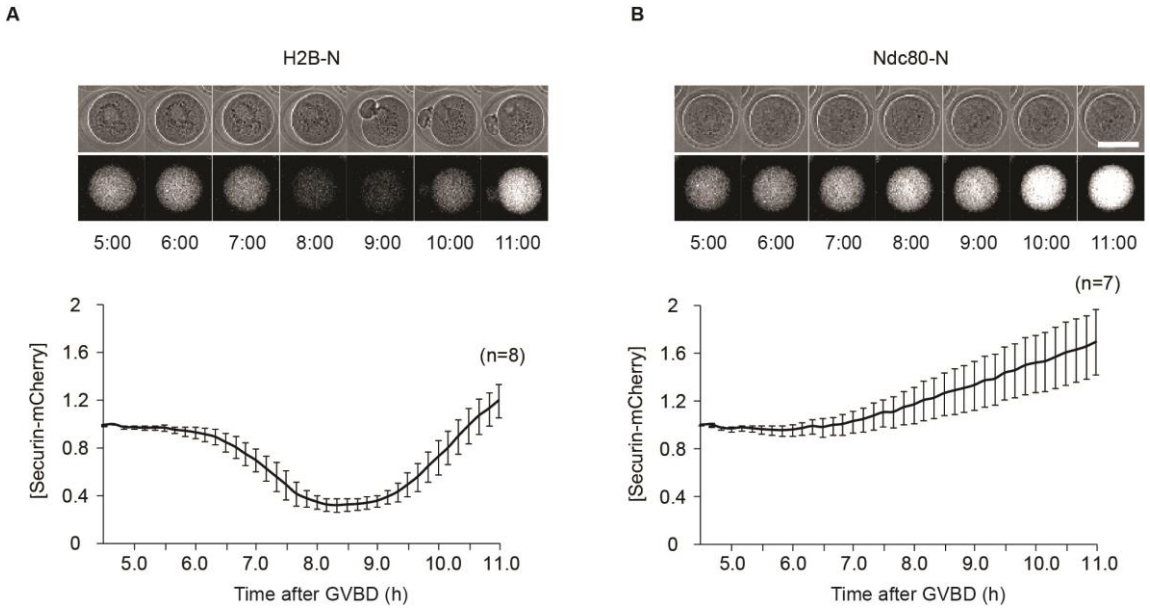

Fig. 3 Ndc80-N modification inhibits APC/C activity. Securin-mCherry degradation profile in oocytes expressing H2B-N (A) and Ndc80-N (B), showing inhibition of the APC/C activity by Ndc80-N modification. Representative timepoints shown in the top panels (Brightfield and Fluorescence). (A, B) in parenthesis, number of oocytes examined. Scale bar represent 50µm.

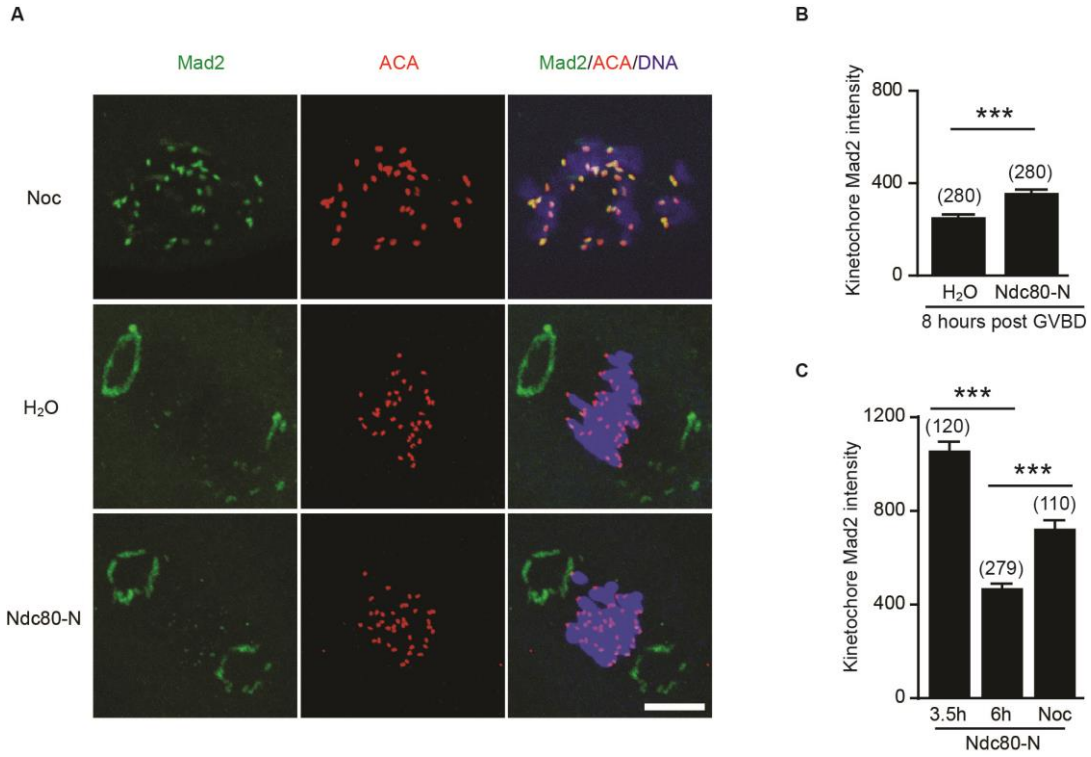

Fig. 4 Significant increase in kinetochore Mad2 signal in Ndc80-N oocytes. (**A**) Mad2 and ACA immunostaining was performed on oocytes following nocodazole treatment, Ndc80-N cRNA or H_2O injection. Scale bar represents 10µm. (**B)** Ndc80-N oocytes possessed higher kinetochore Mad2 intensity at 8h post GVBD (mean ± sem; Mann-Whitney test). **(C)** ~50% of Mad2 was lost from kinetochores in Ndc80-N oocytes at 6h post GVBD, when compared to 3.5h (mean ± sem; Mann-Whitney test). (B, C) in parenthesis, number of centromeres examined.

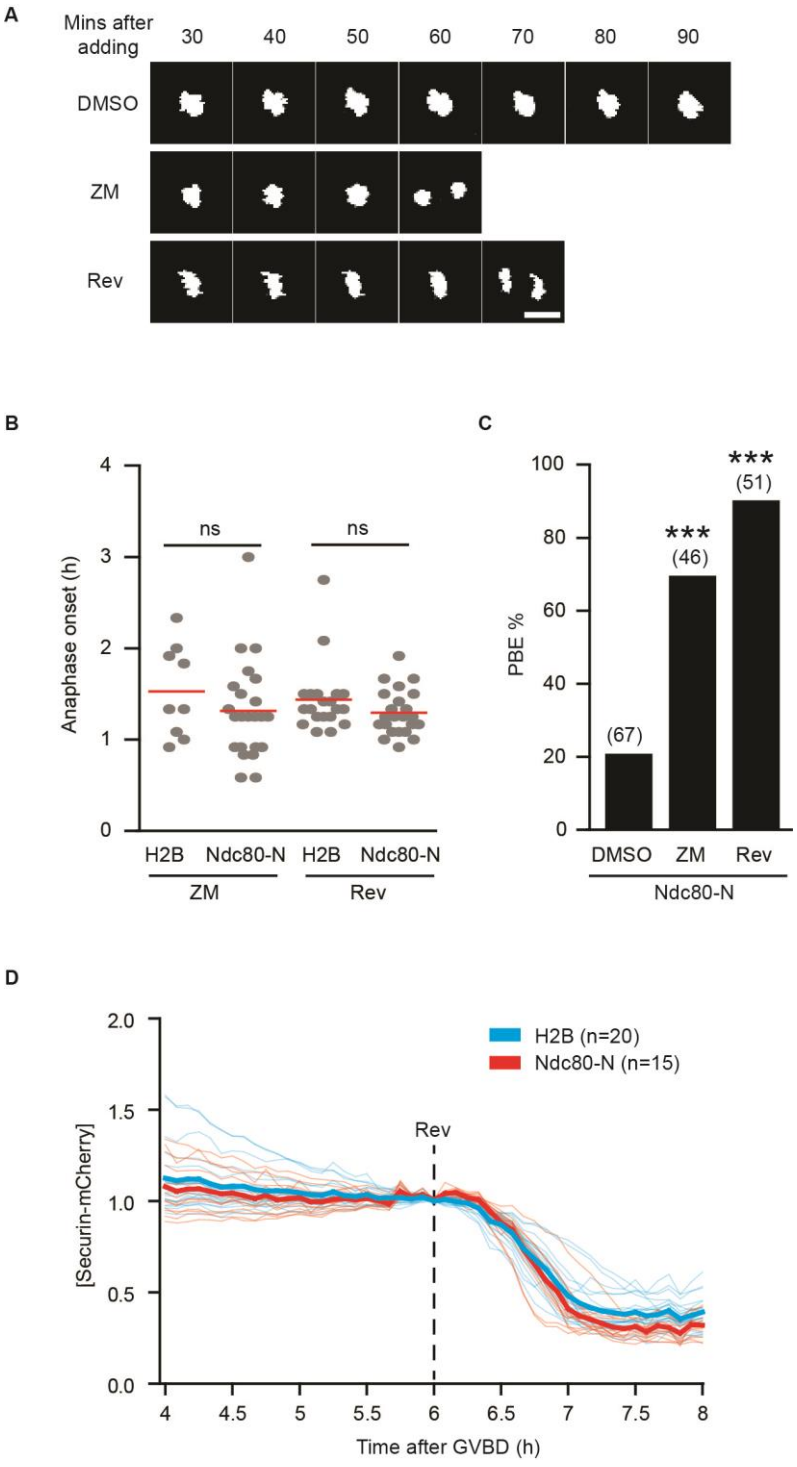

Fig. 5 Abolishing Aurora or Mps1 kinase efficiently overrides Ndc80-N mediated SAC arrest. **(A)** Ndc80-N oocytes were treated with DMSO, ZM447439 or Reversine at 6h after GVBD. Anaphase was induced 1-2h after treatment with either ZM or Reversine. Scale bar represents 20µm. (**B)** No significant differences were observed in the time of anaphase onset between Ndc80-N and H2B expressing oocytes, following either ZM or Reversine treatment (red line, mean; Mann-

Whitney test). **(C)** Both ZM and Reversine treatment significantly rescued PBE in Ndc80-N oocytes (Fisher's test; both compared to DMSO group). **(D)** Inhibition of Mps1 induced similar Securin degradation profiles in both control and Ndc80-N oocytes. (C, D) in parenthesis, number of oocytes examined.

Supplementary movie 1 Control oocytes with chromosome and kinetochore labelling underwent bivalent segregation at anaphase. Elapsed time given from GVBD (00:00). Kinetochore (EGFP-CenpC) in red, chromosome (H2B-mCherry) in green. Some of the images from this movie are displayed in Fig. 2A. Anaphase onset occurred at 07:50. Scale bar represents 10 μm.

Supplementary movie 2 Ndc80-N oocytes with chromosome and kinetochore labelling maintained arrested in MI. Elapsed time given from GVBD (00:00). Kinetochore in red, chromosome in green. Some of the images from this movie are presented in Fig. 2A. Failure of bivalent segregation was observed even until 13:20. Scale bar represents 10 μm.

5. General discussion

5.1 Bivalent non-disjunction vs sister chromatid pre-division

Chromosome mis-segregation can cause aneuploidy in the resulting daughter cells, which contain an abnormal number of chromosomes. In somatic cells aneuploidy is commonly associated with tumorigenesis; while in germ cells it is a common cause of embryo loss and birth defects (Fragouli et al., 2013; Lee, 2014). In humans the majority of aneuploidies result in termination of the developing fetus, although trisomies such as 21, 18 and 13 can survive to term (Driscoll and Gross, 2009). Studies of chromosome segregation have been widely examined in diverse meiotic systems, including *Xenopus* egg extracts, *grasshopper* spermatocytes and *C. elegans* oocytes (Dumont et al., 2010; Houghtaling et al., 2009; Rebollo and Arana, 1995; Valdeolmillos et al., 2007). In *C. elegans* oocytes, chromosome segregation is regulated by two steps: kinetochores are required to orient chromosomes, but then chromosome separation during anaphase is kinetochore-independent (Dumont et al., 2010). In *Xenopus* oocytes, it has been shown that activation of the APC/C and degradation of cyclin B is not required for its Meiosis I completion (Jones, 2011; Taieb et al., 2001). Moreover, recent data showed that meiosis I in *Xenopus* oocytes seems not error prone, although they are lack of spindle assembly checkpoint control (Liu et al., 2014; Shao et al., 2013). However, oocytes from a commonly used study model – mouse, follow several characters as in human eggs. For example, both are shown to be error-prone in meiosis I, despite the SAC control is present (Hassold and Hunt, 2001; Kitajima et al., 2011; Nagaoka et al., 2011; Sun and Kim, 2012). Moreover, mouse oocytes also display similar age-associated aneuploidy phenomenon, as observed in humans (Jones and Lane, 2013). Therefore, using mouse oocytes the focus how chromosome segregation is regulated will compose the following discussion section of this thesis.

Previous studies have demonstrated that the incidence of chromosome segregation errors in both mouse and human oocytes increases with maternal age (Chiang et al., 2012; Jessberger, 2012; Jones and Lane, 2013; Nagaoka et al., 2012; Wang et al., 2011b). This thesis examined fundamental molecular processes involved in chromosome segregation using the mouse model in order to gain insight into how such age-associated aneuploidy arises. Interestingly, the aneuploidy rates in oocytes from aged mice of different strains varies from 20-60% suggesting that this age-related phenomenon has a genetic basis, consistent with recent studies showing predisposition of PSSC in mouse oocytes is dependent on genetic background (Chiang et al., 2010; Danylevska et al., 2014; Merriman et al., 2012; Pan et al., 2008; Sebestova et al., 2012; Selesniemi et al., 2011). Furthermore some strains show prevalence of NDJ with predominantly hypoploid or hyperploid metII eggs (Merriman et al., 2012; Pan et al., 2008; Sebestova et al., 2012; Selesniemi et al., 2011); some possess mostly individualized sister chromatids in metII eggs, representative of PSSC (Lister et al., 2010; Yun et al., 2014b), whilst others demonstrate both NDJ and PSSC (Chiang et al., 2010; Shomper et al., 2014),as shown in **Table 5.1**.

It is important to note here that the variable percentages of aneuploidy/pre-division in aged metaphase II eggs might be a consequence of the diverse mouse strains and mouse ages used in the studies. Such an effect is emphasized by a recent study showing that frequency of sister chromatid pre-division is affected by genetic background, by directly comparing three commonly used mouse strains – CD1, C3H/HeJ and C57BL/6. For example, the prevalence of prematurely separated sister chromatids is significantly higher in oocytes from C3H/HeJ and CD-1 mouse stains, at 19% (n=174) and 11.6% (n=242) respectively, than in C57BL/6 oocytes at a rate of only 3.7% (n=215) (Danylevska et al., 2014). Another possibility that might account for the variability in aneuploidy/pre-division rates, could be that methods used for chromosome counting by different

Table 5.1: Summary for percentage of aneuploidy and pre-division in mouse oocytes with maternal age

	Mouse strain	**Used technique**	**Mouse age**	**Aneuploidy or Pre-division**	**Cited literature**
Aneuploidy (numerical chromosomal aberration)	C57BL/6	Monastrol spread with chromosome and kinetochore labelling	17-19 month	Aneuploidy 9% (n=32)	(Yun et al., 2014a)
	CD1	Monastrol spread with chromosome and kinetochore labelling	19–25 month	Aneuploidy 42.5% (n=47)	(Sebestova et al., 2012)
	B6D2F1	Chromosome spreads with Giemsa staining	17-18 month	Hyperploidy 25% (n=44)	(Pan et al., 2008)
Pre-division	CD1	Monastrol spread with chromosome and kinetochore labelling	>12 month	Pre-division 31% (n=32)	(Yun et al., 2014b)
	C57BL/Icrfa^{t}	Chromosome spread with DAPI only or DAPI and ACA staining	14 month	Pre-division 92% (n=14) **H2b-RFP injected**	(Lister et al., 2010)
Both Aneuploidy and Pre-division	B6D2F1 male * C57BL/6 female	Chromosome spread with DAPI staining	12 month	Hyperploidy 5% Hypoploidy 18% Pre-division 7% (n=18–23)	(Selesniemi et al., 2011)
	B6D2F1/J	Monastrol spread with chromosome and kinetochore labelling	16-19 month	Aneuploidy 25% Pre-division 36% (n=36) **H2B-GFP injected**	(Chiang et al., 2010)
	MF1 mice	Monastrol spread with chromosome and kinetochore labelling	15-17 month	Aneuploidy 33.3% Pre-division 66.6% (n=24)	(Shomper et al., 2014)

laboratories varied. Moreover, exogenous protein expression may have also impacted chromosome segregation behaviour of in vitro matured oocytes. This is notable because H2B RNA injection has routinely been employed to label chromosomes for live cell studies, however, there were no studies demonstrating whether exogenous expression of chromosome associated proteins has an impact. In fact, by comparing a dose-concentration series of H2B RNA injections, I found that high level of exogenous H2B expression was disastrous for the resulting metaphase II eggs, in which single chromatids are prevalent (data not shown). It is understandable why this effect has received little attention, because majority of chromosome labelling studies focus on meiosis I, but as I have shown (Chapter 2) sister chromatid pre-division may only occur during meiosis II. Therefore, together such confounding factors make it difficult to reach universal conclusions with respect to maternal age effects.

Here in chapter 2 I confirmed that PSSC was feature of aged oocytes from the Swiss CD1 strain, whilst using the same technique, in chapter 3 I demonstrated that in fact C57Bl6/J mouse oocytes were relatively resistant to age-related aneuploidy, unless exposed to spindle perturbation, after which NDJ was the predominant outcome (Yun et al., 2014a). These strain-dependent differences allowed me to examine specific aspects of chromosome segregation in oocytes in order to understand how aneuploidy arises., One of the screened candidates was Sgo2, which was significantly declined in aged CD1 mouse oocytes where single chromatids are common, but was unchanged in aged C57BL/6 oocytes that are resistant to sister chromatid pre-division. These differences will be fully discussed in section 5.2.

5.2 Cohesin deterioration in oocytes with maternal age

Single chromatids are commonly found in the oocytes of both aged human and some strains of mice, and based on evidence primarily from mouse models, the leading hypothesis is that chromosome cohesin deteriorates with age (Angell, 1997; Chiang et al., 2010; Duncan et al., 2012; Fragouli et al., 2011; Gabriel et al., 2011; Handyside et al., 2012; Kuliev et al., 2011; Lister et al., 2010; Shomper et al., 2014; Yun et al., 2014b). Both cohesion protein Rec8 and Sgo2 - a protector of centromeric cohesion - are reduced with maternal age (Chiang et al., 2010; Lister et al., 2010); moreover, mammalian oocytes appear to lack a mechanism for cohesin complex replacement once established during fetal life (Tachibana-Konwalski et al., 2010). However, it has been unclear when and how these single chromatids are created during the 2 meiotic divisions, and this became the focus of the studies outlined in chapter 2.

To follow the detailed dynamics of individual chromosomes and to track the process of PSSC in aged oocytes, I employed a real-time kinetochore tracking approach with kinetochore and chromosome labelled simultaneously (Kitajima et al., 2011). Using this advanced technique, I found that rare univalents were observed in meiosis I of aged CD1 mouse oocytes, although the percentage of weakly-attached bivalents was raised, indicating at least some chromosomal cohesin loss might have occurred. These observations are consistent with a recent study showing that aged CD1 mouse oocytes contained elevated numbers of closely positioned univalents without visible chiasmata, which is more accurately defined as ‘weakly-attached bivalents’ here, because live cell imaging data demonstrated that these bivalents are not prematurely separated, but maintain association until anaphase onset (Sebestova et al., 2012; Yun et al., 2014b). Moreover, by applying detailed quantitative analysis on bivalent dynamics, my data examined no gross effects on bivalent congression and tension establishment with maternal age. This is consistent with findings from a

previous report showing that chromosome congression and migration to the oocyte cortex around anaphase I occurred normally in aged oocytes (Lister et al., 2010). However, some other studies examined severe chromosome misalignment in meiosis I from aged oocytes (Lister et al., 2010; Sebestova et al., 2012; Shomper et al., 2014). One possibility that could explain the disparity with my results, is that my studies utilised exogenous CenpC expression for labelling kinetochores, which could potentially stimulate microtubule-kinetochore interaction and thus improve alignment status in aged oocytes, but more data is required to confirm this. Nevertheless, consistent with previous data, lagging chromosomes at anaphase I were observed in aged CD1 oocytes, which are thought to be from merotelic attachments to kinetochores, because these can lead to normal chromosome congression to spindle equation, however would display anaphase defects as lagging chromosomes (Chiang et al., 2010; Lister et al., 2010; Sebestova et al., 2012; Shomper et al., 2014; Thompson and Compton, 2011; Yun et al., 2014b).

The high resolution imaging revealed that the main defect with age in Swiss CD1 mice, PSSC, occurred during metII arrest, at ~2 hours after anaphase onset when the metII spindle was assembling (**Fig. 8 in chapter 2**). The important clinically-relevant consequence of this study is that maternal aging may cause minimally defective chromosome segregation in MI, so producing a normal chromosomal complement in the first polar bodies; however, the metII egg may in fact contain prematurely separated single chromatids, which could produce aneuploid embryos following fertilization. Our findings challenge the use of first polar body biopsy alone, as a robust aneuploidy screening technique for embryo implantation during IVF.

It is important to note that initial cohesion complex proteins appear to be loaded onto chromosomes in excess, since even when Rec8 levels in moderately aged oocytes are ~10% of

those detected in young mice, the integrity of chromosome pairs is maintained (Chiang et al., 2010). Based on this, one possibility is that oocytes with chromosome-associated cohesion below this threshold level would produce prematurely separated sister chromatids; alternatively reduced Sgo2 with age could aggravate premature separase-mediated cleavage of centromeric cohesin, as its continued presence is essential to maintain dyad integrity until MII (Lee et al., 2008; Llano et al., 2008). Previous studies and the work presented here in chapter 2 demonstrated that chromosome cohesion loss and Sgo2 depletion occurred together, and these depletions were consistent with the prevalence of prematurely separated sister chromatids in metII eggs (Lister et al., 2010; Yun et al., 2014b). However, as shown in chapter 3, aged C57Bl6/J mouse oocytes possessed normal levels of centromeric Sgo2, yet an increased iKT distance, suggestive of some cohesion loss. One possibility could be that chromosome cohesin deterioration during long prophase I arrest and prometaphase I is independent of Sgo2/PP2A pathways, which may only function and protect its Separase-mediated cleavage at anaphase I. Alternatively, centromere localization of PP2A or its activity is impaired, thus causing premature loss of cohesin even with intact Sgo2 binding. Either way, the unchanged centromeric Sgo2 examined in aged C57BL6/J mouse oocytes would be expected to prevent centromere cohesin from degradation at anaphase I, and this is consistent with my observation that rare single chromatids were found in metII eggs (Yun et al., 2014a). Taken together, I concluded that differential retention of centromeric Sgo2 in aged oocytes may determine whether an oocyte will undergo PSSC, and provides an important starting point in understanding the phenomenon.

5.3 Defective checkpoint control and error correction with age

As discussed above, loss of chromosome cohesin and Sgo2 contributes to age-related chromosome segregation errors, particularly to bivalent pre-division (Chiang et al., 2010; Chiang et al., 2012; Jessberger, 2012; Lister et al., 2010). However, mammalian oocyte aneuploidy is more likely to be multifactorial and implicate other key chromosome related proteins (Jones and Lane, 2012; Selesniemi et al., 2011; Shomper et al., 2014; Wang et al., 2011b). In chapter 3, I set out to examine the impact of ageing on other chromosome associated proteins, including SAC proteins (Homer et al., 2005; McGuinness et al., 2009; Sun and Kim, 2012). My findings revealed that in fact ageing affects the levels of SAC proteins present at kinetochores. This would seem to agree with previous studies showing a reduction of the SAC components at the transcript level in both aged human and mouse oocytes (Baker et al., 2004; Pan et al., 2008; Riris et al., 2014; Steuerwald et al., 2001; Steuerwald et al., 2007). However, further investigation is required to determine whether the kinetochore reduction of SAC proteins is due to impaired recruitment or total level reduction. This is a challenge because the number of aged oocytes available for experimentation is a limiting factor but emerging micro-proteomics techniques may enable protein level detection using a small number of eggs, which should provide more information on this question. Current evidence suggests that total SAC protein reduction is the most likely underlying factor, because their transcripts have been reported to be reduced with age, as discussed above, and moreover a recent study showed an age-related reduction in levels of the SAC protein, BubR1, in human eggs consistent with their transcript decline (Riris et al., 2014). Besides total SAC protein decline, it is also possible that kinetochore integrity is altered with age such that protein retention is impaired. This hypothesis is supported by my finding that aged centromeres may have reduced ability to retain their protein components, because I found 61% lower ACA signal when compared to young oocytes.

However, decreased presence of SAC proteins does not cause complete loss of checkpoint function since MI duration was not shortened, a finding consistent with previous studies (Duncan et al., 2009; Lister et al., 2010). Moreover, the other two studies concluded that the SAC function is not impaired in oocytes with age or its defection may not contribute to age-related aneuploidy. The conclusions were mainly based on the following data: 1) oocytes of aged and young mice share the same ability to arrest in response to nocodazole-induced spindle disruption; 2) the length of MI duration, which would be expected much shorter in the absence of the SAC, has no difference in aged oocytes; 3) no association between the time taken to transit through MI and aneuploidy eggs is observed, since it should be if the SAC were being bypassed, resulting in chromosome mis-segregation. However, these studies used only a single dose of nocodazole, which was continuously present throughout the whole maturation process (Duncan et al., 2009; Lister et al., 2010). One possibility not explored to date, could be that maternal aging does affect SAC activity, but the effect is not dramatic enough to compromise its function in ways that have been measured previously. Therefore, differences may be uncovered if a weaker stimulus for the SAC was applied. To test this idea, series doses of nocodazole were examined at metaphase I (5 hours after GVBD), a stage in which the SAC activity is believed to be low (Gui and Homer, 2012; Kitajima et al., 2011; Lane et al., 2012). With 200 nM nocodazole treatment, over 80% of young oocytes have a reactivated SAC activity and arrested in MI, while nearly half of aged oocytes failed to arrest, and extruded polar bodies. Based on the findings that mild spindle disruption in aged oocytes caused a reduction in their ability to maintain SAC arrest and chromosomes were less able to establish bivalent biorientation following a major spindle disruption (Fig. 4D in chapter 3), I conclude that SAC defect is subtle. This is clinically relevant because it suggests that environment factors may aggravate a slightly defective SAC in aged human oocytes during IVM procedure, leading to aneuploid outcomes

It is important to note that the gradual cohesion loss in the process of ageing may in some way affect SAC signaling, because decreased centromere cohesion may allow greater kinetochore pair flexibility and impair the ability of sister chromatid kinetochores within a bivalent to act as a single, functional unit for microtubule interaction. This could lead to incorrect attachment, which may then trigger the SAC pathway. Indeed a recent study showed that cohesion deterioration may compromise the SAC function by impairing sister kinetochore biorientation and its SAC signal production in mouse oocytes (Tachibana-Konwalski et al., 2013). More directly, another recent report demonstrated that loss of chromosome cohesion in oocytes from older females caused altered kinetochore geometry in MI, which is associated with increased merotelic attachments from microtubules (Shomper et al., 2014).

5.4 MT-KT interaction and SAC signalling

As stated in the previous section, I concluded that defective SAC function contributes to age-related aneuploidy in mouse oocytes. However, how the SAC function is regulated in mammalian oocytes remains to be further investigated. To gain insight into this mechanism, I investigated the Ndc80 protein, which has been extensively studied in somatic cells and is regarded to regulate MT-KT interaction and SAC signaling (Ciferri et al., 2008; DeLuca et al., 2006; Guimaraes et al., 2008; Kline-Smith et al., 2005; Mattiuzzo et al., 2011; Meraldi et al., 2004; Miller et al., 2008; Tooley et al., 2011). Here I found that N-terminus modified Ndc80 expression in mouse oocytes caused increased microtubule merotelic attachments, which agrees with the data revealed in somatic cells and suggests a conserved function on MT-KT interaction. However, these attachment errors seem to have no gross effects on bivalent congression. This is not surprising because no vacant kinetochores (completely lacking microtubule attachment) were identified, and merotelic attachments, in which microtubules from both spindle poles attach to one of the sister kinetochore pairs, if analogous to mitosis, are expected to be sufficient to align bivalents at the metaphase plate and establish tension. Such attachments would be expected to generate lagging chromosomes at anaphase, which was indeed the case for the aged mouse oocytes in my study, and that of others (Shomper et al., 2014; Thompson and Compton, 2011; Yun et al., 2014b). However, the raised robust SAC prevents anaphase initiation in Ndc80-N expressing oocytes. In a physiological situation, somatic cells /oocytes provide a mechanism for microtubule attachment error correction, which is dependent on the spatial proximity of Aurora B/C to the outer kinetochore components; therefore kinetochores with erroneous microtubule attachment would be detached, allowing for new round of attachment establishment (Campbell and Desai, 2013; Lampson and Cheeseman, 2011; Liu et al., 2009). However in Ndc801-N oocytes, N-terminus modification may interfere with Aurora B-dependent phosphorylation and in doing so, impair the ability to repair incorrect microtubule attachments.

These finds are consistent with a previous study showing that overexpression of another component of Ndc80 complex – Spc25 – caused higher accumulation of cyclin B1 in mouse oocytes (Sun et al., 2010). However it seems effects of the Ndc80 complex disturbance on the SAC function in mitotic cells are controversial, and one of these interesting studies showing that an active spindle checkpoint was raised with partial depletion of Nuf2/Ndc80, while complete depletion led to inactive SAC in Hela cells (Kline-Smith et al., 2005; Meraldi et al., 2004). These data suggest some different mechanisms in which Ndc80 complex works in oocytes. Indeed previous studies have shown a spindle localization of Ndc80 and its ability to stabilize cyclin B2 in mouse oocytes (Gui and Homer, 2013; Sun et al., 2011). Here my findings provide insights into understanding how kinetochore component couples MT-KT interaction with spindle checkpoint pathway. However, in an extensively prolonged prometaphase I of mammalian oocytes, more data on how conserved the pathway is in which spindle checkpoint responses to microtubule kinetochore interactions are needed, and these will shed light on better understanding the susceptibility of aneuploidy in human eggs.

5.5 Further directions and clinical implication

My data explain why it is difficult to reach universal conclusions regarding the way oocytes behave with respect to maternal ageing. Firstly, the evidence presented here strongly suggests that the age-related aneuploidy phenomenon in mammalian oocytes is multifactorial, in which centromere cohesion loss, defective SAC function and others, all contributing (**Fig. 5**). These factors might act individually, but are more likely to act together to cause aneuploidy in oocytes. Secondly, our findings suggest that a genetic susceptibility to age-related aneuploidy in mammalian oocytes may be uncovered initially by interrogating differential gene expression profiles. If any gene expression changes are found to be associated with aneuploidy, these candidates could potentially be used as effective screening markers for aneuploidy prior to IVF embryo transplantation. This is attractive because recent studies showed that polar bodies might reflect its sibling oocyte on the transcript profile (Jiao and Woodruff, 2013; Reich et al., 2011).

Much insight has been gained into the susceptibility of mouse oocytes to aneuploidy with age in the last 5 years (Chiang et al., 2010; Chiang et al., 2011; Danylevska et al., 2014; Duncan et al., 2009; Lister et al., 2010; Merriman et al., 2012; Sebestova et al., 2012; Selesniemi et al., 2011; Shomper et al., 2014; Yun et al., 2014a; Yun et al., 2014b), however it seems that mouse strains used in different laboratories have diverse effects on chromosome segregation type and rate as discussed above. This raises a serious question: how much similarity is there between mouse and human oocytes with regard to mechanism of aneuploidy? To understand this question more data from human eggs must be sought. Moreover, more powerful techniques involving both live cell imaging and quantitative detection of at the molecular level, is required due to the limited resource of human eggs. Attractively, recent studies have established methods for DNA and RNA sequencing on single cells, and these have been applied to screen embryo aneuploidy and even detect single-

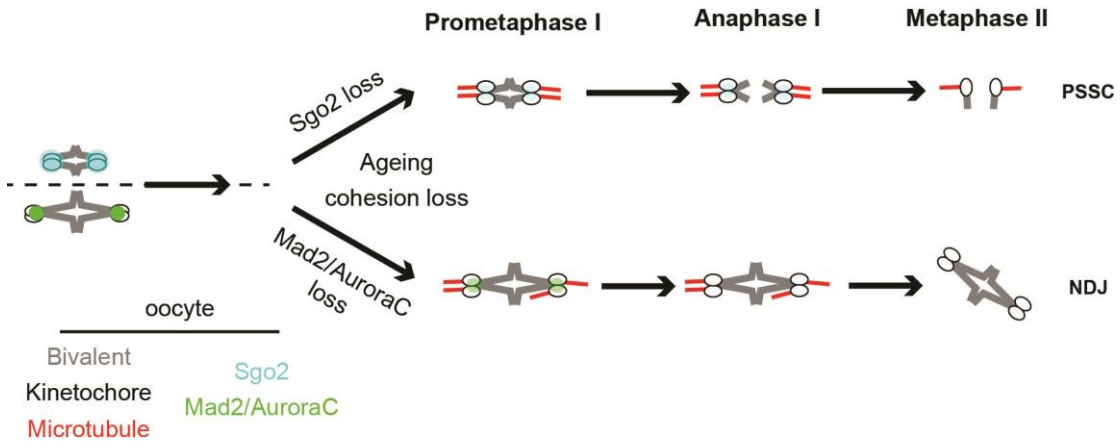

Figure 5 Schematic showing both cohesion loss and defective SAC contribute to chromosome mis-segregation of mammalian oocytes following maternal ageing. The effects of maternal ageing are depicted on two bivalents (grey; **above**: cohesion loss and sgo2 reduction; **below**: defective SAC). Cohesion loss has been extensively proved to be associated with chromosome segregation errors in mammalian oocytes with age. However, prevalent sister chromatid pre-division occurs when only centromeric Sgo2 (Cyan) is simultaneously reduced. Specifically, despite of cohesion loss during Meiosis I, the sister chromatid pairs co-segregate normally to the same pole at anaphase I. When attachment of microtubules (red) is established in Meiosis II, the two sister chromatids no longer are able to maintain their cohesion and perform premature separation. On the other hand, reduced SAC proteins (green) on the kinetochore in aged oocytes lead to less efficient microtubule error correction and lowered ability to maintain SAC arrest, so producing higher rate of aneuploidy metII eggs.

gene disorders in IVF (Hou et al., 2013; Smallwood et al., 2014; Xue et al., 2013; Yan et al., 2013). These techniques will be promising for IVF field because they provide powerful tools to screen aneuploidies, to diagnose genetic diseases and to determine quality of oocytes and embryos. In addition, one recent exciting report demonstrated that SAC component BubR1 expression is significantly reduced in older female eggs, at both transcript and protein levels (Riris et al., 2014). Overall, such techniques are expected to make inroads into understanding the susceptibility of human oocytes to chromosome mis-segregation, thereby improving reproductive diagnostics and assisted reproductive therapies.

Appendix

I. Additional publication

Timing of anaphase-promoting complex activation in mouse oocytes is predicted by microtubule-kinetochore attachment but not by bivalent alignment or tension

My contribution to this publication includes:

Simon Lane and Yan Yun developed the high resolution real-time imaging with chromosome and kinetochore labelled simultaneously. Yan Yun produced EGFP-CenpC mRNA and imaged the oocytes used in supplementary movie 5.

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Timing of anaphase-promoting complex activation in mouse oocytes is predicted by microtubule-kinetochore attachment but not by bivalent alignment or tension

Simon I. R. Lane, Yan Yun and Keith T. Jones*

SUMMARY

Homologous chromosome segregation errors during meiosis I are common and generate aneuploid embryos. Here, we provide a reason for this susceptibility to mis-segregation by live cell imaging of mouse oocytes. Our results show that stable kinetochore-microtubule attachments form in mid-prometaphase, 3-4 hours before anaphase. This coincided with the loss of Mad2 from kinetochores and with the start of anaphase-promoting complex/cyclosome (APC/C)-mediated cyclin B1 destruction. Therefore, the spindle assembly checkpoint (SAC) ceased to inhibit the APC/C from mid-prometaphase. This timing did not coincide with bivalent congression in one-third of all oocytes examined. Non-aligned bivalents were weakly positive for Mad2, under less tension than congressed bivalents and, by live-cell imaging, appeared to be in the process of establishing correct bi-orientation. The time from when the APC/C became active until anaphase onset was affected by the rate of loss of CDK1 activity, rather than by these non-aligned bivalents, which occasionally persisted until anaphase, resulting in homolog non-disjunction. We conclude that, in oocytes, a few erroneous attachments of bivalent kinetochores to microtubules do not generate a sufficient SAC 'wait anaphase' signal to inhibit the APC/C.

KEY WORDS: Cell cycle, Oocyte, Spindle assembly checkpoint, Mouse

INTRODUCTION

In mammals, the first meiotic division is hormonally triggered in the hours preceding ovulation, and results in the segregation of homologous chromosomes (bivalents). Sister chromatids, which remain attached in meiosis I, are then only segregated in meiosis II, a division therefore resembling mitosis. A notable feature of the meiotic segregation of bivalents in women is that it is a particularly error-prone event, leading to aneuploid embryos (Hassold and Hunt, 2009; Jones, 2008).

Aneuploidy is regarded as a driver of abnormal cell function and tumorigenicity, and dividing cells have multiple checkpoints that act to prevent chromosome mis-segregation (Holland and Cleveland, 2009; Williams and Amon, 2009). During mitosis, correct segregation is achieved through the mitotic checkpoint/spindle-assembly checkpoint (SAC), which can delay anaphase until amphitelic microtubule-kinetochore attachments are complete (Khodjakov and Pines, 2010; Musacchio and Salmon, 2007). Prior to this, the kinetochores produce a diffusible 'wait-anaphase' signal that sequesters Cdc20, an activator of the anaphase-promoting complex/cyclosome (APC/C), which is responsible for the degradation of key substrates at the metaphase-anaphase transition (Peters, 2006; Yu, 2007). This stabilizes the APC/C substrates cyclin B1 and securin, thereby maintaining CDK1 activity and inhibiting separase (Peters, 2006). The signal is regarded to be exquisitively sensitive, with a single unoccupied kinetochore enough to prevent anaphase (Rieder et al., 1995).

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There is currently much interest in what satisfies the SAC. In mitosis, microtubule attachment to kinetochores per se, the resulting tension across the kinetochore generated by stretching or the tension across the centromere of amphitelically attached sister kinetochores have all been suggested to nullify the signal that keeps the APC/C inactive (Khodjakov and Pines, 2010; Maresca and Salmon, 2010; Santaguida and Musacchio, 2009). It is still unclear whether the prevalence of aneuploidy in mammalian oocytes reflects a defect in the SAC during meiosis. Evidence that a univalent X chromosome can divide reductionally during meiosis I in mice suggested that mammalian oocytes might not possess a SAC (LeMaire-Adkins et al., 1997) but more recent studies have all shown mouse oocytes respond to SAC protein knockdown or loss as do somatic cells, with an increase in aneuploidy (Hached et al., 2011; Homer et al., 2005; Li et al., 2009; McGuinness et al., 2009; Wei et al., 2010; Yin et al., 2006). There is evidence in both mice and fission yeast that univalents in meiosis I could evade the SAC by establishing bi-orientation and so bypass the normal requirement to mono-orientate (Kouznetsova et al., 2007; Sakuno et al., 2011). However, in a Mlh1 knockout mouse, in which many univalents are generated in meiosis I, these appear not to bi-orientate (Nagaoka et al., 2011), although they do generate a ~2 hour delay in polar body extrusion. It is not currently known whether under such circumstances the SAC becomes engaged but fails to fully arrest the oocyte, analogous to the process of 'mitotic slippage' in somatic cells (Brito and Rieder, 2006).

What remains to be investigated, therefore, are the factors that contribute to SAC silencing and APC/C activation during the normal course of female meiosis I. Here, we have focused in detail on the timing of the loss of the SAC protein Mad2 from kinetochores in mouse oocytes, providing through such observations, an account of why segregation errors arise in oocytes and how the timing of meiosis I is controlled.

MATERIALS AND METHODS

Materials

All chemicals were from Sigma-Aldrich (Australia), unless stated otherwise. Stocks of nocodazole (40 μM), roscovitine (100 mM) and flavopiridol (5 mM) were stored in DMSO and used at a minimum dilution of 0.1%.

Animals and oocyte culture

F1 hybrid mice (C57Bl6 females × CBA males) were used in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and were approved by the University of Newcastle Animal Care and Ethics Committee. Oocytes were collected from mice injected with 10 IU equine serum gonadotropin (Invitrogen, New Zealand) in M2 media as described previously (Reis et al., 2006). For longer term culture, oocytes were washed into MEM (Gibco) with 20% FCS at 37°C in 5% CO_2 (Holt et al., 2011). Milrinone (1 mM) was added to maintain prophase I arrest (Holt et al., 2010). For maturation studies, oocytes were synchronized by incubation in milrinone for 2 hours followed by its rapid washout. Only oocytes that underwent NEB 30-60 minutes after milrinone removal were used.

cRNA manufacture

cRNA was transcribed in vitro from purified, linear dsDNA template using a mMessage T7 RNA kit (Ambion) or a T3 RNA polymerase kit (Promega). cRNA was suspended in nuclease-free water and its concentration determined by photospectrometry or gel electrophoresis.

Microinjection and live cell imaging

cRNA microinjections were performed in M2 media on the stage of an inverted TE3000 Nikon microscope using a 37°C heated chamber and Narishige micromanipulators (Madgwick et al., 2006). Pressure injections to achieve a 0.2-3% oocyte volume were performed as described previously (Gorr et al., 2006). cRNAs were injected with pipette tip concentrations of ~500 ng/μl. Images were captured by epifluorescence using a Nikon Biostation IM (20× objective) or a Nikon TE3000 microscope (20× objective and Roper Scientific, UK, CCD camera), or by confocal scanning laser microscopy on an Olympus FV1000 (60× objective).

Immunofluorescence

Oocytes were fixed in 2% formaldehyde in PHEM buffer (60 mM Pipes, 25 mM HEPES, 25 mM EGTA, 4 mM $MgSO_4$) containing 0.5% Triton X-100 and 1 μM taxol. For analysis of k-fibers, some oocytes were incubated in pre-cooled M2 media on ice for 10 minutes before fixation or treated with a high Ca^{2+} permeabilizing buffer for 2 minutes [100 mM Pipes (pH 7.0), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1% Triton X-100]. Oocytes were then fixed, washed and further permeabilized in PBS containing 0.5% Triton X100, then blocked in 7% normal goat serum with PBS-Tween. Immunofluorescence was performed using antibodies for tubulin (1:400, A11126, Invitrogen, Australia), CREST (1:400, 90C-CS1058, Bioclone Australia) and Mad2 (1:1000, a kind gift from Dr R. H. Chen, Taipei, Taiwan). Antibody solutions were supplemented with 7% goat serum and 0.2% Tween 20, and incubated overnight at 4°C. Secondary antibodies were Alexa 633-, 555- and 488-conjugated (Invitrogen). Oocytes were briefly stained with Hoechst (20 mg/ml) before mounting on glass slides with Citifluor (Citifluor, UK). In Mad2/CREST fluorescence experiments or stable k-fiber experiments, different groups of oocytes were processed for immunofluorescence in parallel using the same antibody solutions.

Immunoblotting

Oocytes were briefly washed in PBS with 1% PVP, suspended in 1× sample buffer (Invitrogen) and run on a NuPage 10% gel (Invitrogen) according to the manufacturer's instructions. Immunoblotting (50 oocytes per lane) was performed using antibodies against securin (1:200, ab3305, Abcam, UK), cyclin B1 (1:500, ab72, Abcam), and actin (1:400, ab3280, Abcam) overnight at 4°C, using 3% BSA in PBST. ECL Plus (GE Healthcare, UK) was used for detection with an anti-mouse IgG (1:5000, P0447, DAKO, UK).

Confocal imaging and image analysis

An Olympus FV1000 fitted with a 60× objective was used on fixed cells for all confocal microscopy. *Z*-stacks were compiled with 0.5 μm intervals. For Mad2/ CREST or k-fiber analysis, scans were performed using identical settings and on the same day.

All images were analyzed using Metamorph and Metafluor software (Universal Imaging, PA, USA) or ImageJ (NIH, Bethesda, USA) and figures assembled with Adobe Illustrator CS4. Calculation of Mad2-YFP fluorescence at kinetochores was made by recording mean fluorescence intensity from a 1 μm diameter circle centered on the YFP signal associated with bivalents. This intensity reading was self-ratioed with respect to the start of the recording (3 hours post NEB). Calculation of Mad2 immunostaining at kinetochores was made by recording the mean fluorescence intensity of both Mad2 and CREST colocalized immunosignals, which were subtracted for background.

Statistical analysis

P values were calculated using an unpaired Student's *t*-test or by ANOVA, with Tukey's post-hoc analysis (Minitab16, PA, USA).

RESULTS

Timing of k-fiber formation and APC/C activation in mouse oocytes

Prophase I oocytes in dictyate arrest spontaneously undergo nuclear envelope breakdown (NEB) when disassociated from the ovary. The extrusion of the first polar body, which is an event that marks the completion of meiosis I, occurred at 8.1±0.7 hours (mean±s.d., n=100) post-NEB in the mouse strain used here. Cyclin B1 and securin degradation, however, began a few hours earlier, at ~5 hours post-NEB (supplementary material Fig. S1A-C). This same timing, at 7.9±0.4 hours, (n=26; supplementary material Fig. S1D,E) was also observed with exogenous cyclin B1, following 1.0 pg cRNA cyclin B1-GFP injection. This procedure generates 25-50 ng of protein hour^{-1} and approximates to 5-10% of endogenous cyclin B1 concentration (Madgwick et al., 2004).

These initial observations show that cyclin B1 and securin degradation begin about 3 hours before either anaphase or cytokinesis (polar body extrusion), which are parallel events in mouse oocytes (Verlhac et al., 2000). By comparison, the duration of cyclin B1 degradation in somatic cells is about 20 minutes (Clute and Pines, 1999; Gavet and Pines, 2010). Given that in both oocytes and somatic cells this APC/C substrate loss is Cdc20 dependent (Jin et al., 2010; Li et al., 2007; Reis et al., 2007), the data collectively point towards a period of meiotic APC/C activity that lasts 10 times longer, from APC/C^{cdc20} activation to cytokinesis, than it does in mitosis of most cultured cell lines.

To place this prolonged prometaphase period of cyclin B1 and securin degradation in context with major meiotic events, we first examined the integrity of the spindle at the time of APC/C activation. In mouse oocytes, lateral attachment of microtubules to kinetochores precedes more stable end-on k-fiber formation (Brunet et al., 1999), as it does in mitosis. End-on k-fibers, which can be assessed in cells following either cold-shock or calcium treatment (Amaro et al., 2010; DeLuca et al., 2006; Kitajima et al., 2011; Salmon and Segall, 1980; Toso et al., 2009; Weisenberg and Deery, 1981), were observed on the vast majority of kinetochores at 5 hours, but not 4 hours, post-NEB. This observation was made using either Ca^{2+} treatment or cold shock (Fig. 1A; supplementary material Fig. S2A,B), and stable k-fibers continued to develop during the remaining 3 hours of meiosis I, as assessed by cold-shock (supplementary material Fig. S2A).

Monotelic attachment of sister kinetochores ensures that homologous chromosomes rather than sister chromatids separate in meiosis I. At 5 hours post-NEB we could perform individual

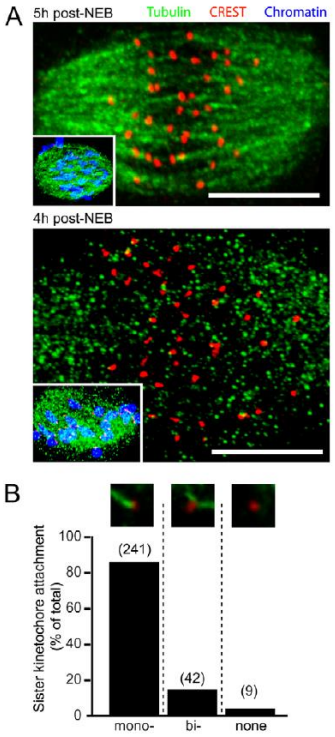

Fig. 1. Kinetochore occupancy by microtubule k-fibers at 5 hours after NEB. (**A**) Representative meiotic spindles from Ca^{2+}-treated oocytes at 4 hours (n=6 oocytes) and 5 hours (n=29 oocytes) after NEB. Insets, oocytes fixed at the same timepoint but under conditions that immunostain for all tubulin, not just k-fibers. Scale bars: 10 µm. (**B**) Sister kinetochore attachment status to k-fiber microtubules (mono-, mono-oriented; bi-, bi-oriented) at 5 hours after NEB. The number of sister kinetochores examined are given in parentheses.

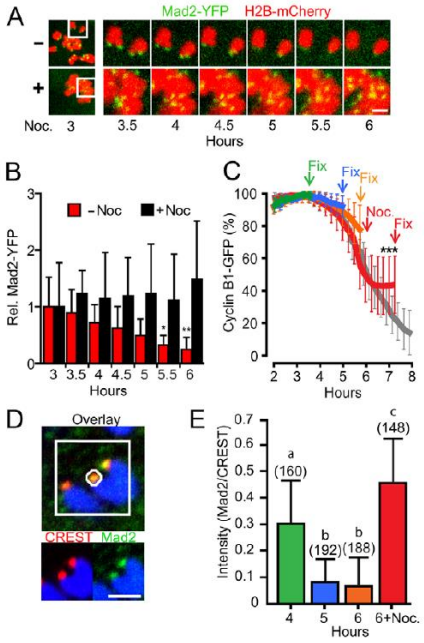

Fig. 2. Similar timing for loss of Mad2 from kinetochores and initiation of cyclin B1 degradation. (**A**) Representative timelapse of Mad2-YFP and H2B-mCherry fluorescence in live oocytes, with or without nocodazole (n=5 oocytes); insets at 3 hours are enlarged in subsequent timepoints. (**B**) Kinetochore Mad2-YFP intensity ratio, expressed relative to 3 hours, at times post-NEB with or without nocodazole (n=10-16 kinetochores per timepoint). *P<0.05, **P<0.01 (ANOVA, Tukey's) compared with the 3-hour timepoint. (**C**) Mean cyclin B1-GFP fluorescence levels in groups of oocytes, expressed as a percentage of maximum reached, which were fixed (except gray trace) or treated with nocodazole at the times indicated. *P<0.05 compared with control (gray). (**D**) Mad2 and CREST immunostaining of a representative bivalent of an oocyte at 4 hours post NEB. Inner circle, area of Mad2 and CREST fluorescence intensity measured following a background subtraction made in the outer square. (**E**) Mad2/CREST ratios, made on the oocyte groups from C, using the technique of D; groups with different letters (a, b, c) are significantly different from one another (P<0.001); the number of kinetochores analyzed are in parentheses. Scale bars: 2 µm in A,D. All error bars are s.d.

analysis on sister kinetochore pairs to determine their attachment status to k-fibers. In summary, over 90% (n=292/320, from eight oocytes) of sister kinetochore pairs were sufficiently resolved by confocal analysis to allow for accurate assessment. Of those sister kinetochore pairs that could be resolved, 97% (n=283/292) were associated with k-fibers (Fig. 1B), with a large majority of these (>80%) having monotelic attachment. These data suggest that when the APC/C commences its activation, the majority of sister kinetochore pairs have established end-on interactions with microtubules, although such attachment is not necessarily correct.

Mad2 loss from kinetochores coincides with APC/C activation

Given the seemingly synchronous appearance of stable k-fibers and initiation of APC/C activation by 5 hours post-NEB, we wanted to determine whether the SAC is also satisfied at this time. To assess SAC activity, live oocytes expressing H2B-mCherry and Mad2-YFP were imaged by 4D confocal microscopy during meiosis I. At 3-4 hours following NEB, Mad2-YFP was detected on all kinetochores, consistent with the observed lack of end-on microtubule attachment to kinetochores at this time (supplementary material Fig. S2) and the idea that this lack of attachment would lead to SAC activity (Kim et al., 2010; Kulukian et al., 2009). A significant drop in kinetochore Mad2-YFP was then observed during the following 2-3 hours, a process that was not due to photobleaching because the YFP signal was maintained for several hours when the experiment was repeated in the presence of nocodazole to destabilize microtubules (Fig. 2A,B).

We confirmed that there was a loss of endogenous Mad2 from kinetochores, coincident with APC/C activation, using oocytes injected with cyclin B1-GFP cRNA. Oocytes were fixed prior to

APC/C activation at 4 hours post-NEB (Fig. 2C, green) or after APC/C activation at 5 and 6 hours (Fig. 2C, blue and orange, respectively). Some oocytes at 6 hours were also incubated with nocodazole for 1 hour before fixation so as to inhibit the APC/C (Fig. 2C, red). All fixed cells were then immunolabeled for Mad2 and CREST, and the respective fluorescent signals on kinetochores were ratioed for each group (Fig. 2D). Kinetochores from oocytes fixed after the onset of APC/C activation were found to have significantly less associated Mad2 when compared with those fixed before cyclin B1 degradation onset or treated with nocodazole. Therefore, we could quantitate a significant loss in kinetochore-associated Mad2 between 4 and 5 hours post-NEB, coinciding with the timing of stable microtubule-kinetochore attachment and APC/C activation (Fig. 2E). We also confirmed that the same timing of loss of Mad2 from kinetochores was observed in the absence of exogenous cyclin B1, using fixed oocytes at specific timepoints during meiosis I (supplementary material Fig. S3).

APC/C activation is not associated with bivalent congression

The above data indicated that the overall level of SAC-induced APC/C inhibition was diminished at about 3 hours before anaphase onset and cytokinesis, as judged by the initiation of cyclin B1 degradation and the loss of Mad2 from kinetochores. Therefore, we wanted to investigate the extent to which bivalent congression was complete at this time. Maturing oocytes were monitored for APC/C activity by waiting for a steady loss in cyclin B1-Venus fluorescence following its cRNA injection at the prophase I stage. When such a decline was observed, further cyclin B1 imaging was stopped and the status of congression assessed using a Hoechst dye. Interestingly, in nearly half of all the oocytes examined (43%, n=42) we could readily observe chromosomes that were not positioned near the spindle equator (Fig. 3). These were confirmed as bivalents, rather than univalents, which may have been generated by premature reductional division of the bivalent (supplementary material Fig. S4).

Many non-aligned bivalents have attachment defects and can recruit Mad2

From the above it seems likely that once the APC/C has become active in prometaphase then the SAC is not sufficiently re-engaged to inhibit meiotic progression during the process of establishing bivalent bi-orientation. We decided to examine this by assessing Mad2 localization to the kinetochores of all bivalents at 5 hours post-NEB, a time when the APC/C is active in degrading cyclin B1. The bivalents from fixed oocytes, immunostained for Mad2, CREST and labeled for chromatin, were individually assessed for congression, as measured by the distance of their kinetochores from the spindle equator. Those bivalents having one or both sister kinetochores pairs within 4 μm of the spindle equator were considered to be congressed, and those with both kinetochores outside this distance were considered to be non-aligned (Fig. 4A). This distance from the spindle midzone meant that by our definition non-aligned bivalents were always clearly separated from all the other bivalents that had congressed. The mean distance of kinetochores from non-aligned bivalents from the spindle equator was 6.3±1.3 μm (n=52).

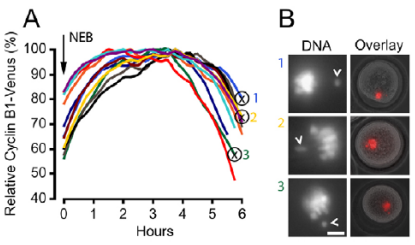

Fig. 3. Cyclin B1 degradation in the presence of non-aligned bivalents. (**A**) Representative cyclin B1-Venus levels in ten maturing oocytes (n=42), expressed as a percentage of the maximum fluorescence. Time 0 hours corresponds to NEB as indicated, and at the end of the recording oocytes were imaged for chromatin. This event is marked (cross inside a circle) for the three oocytes shown in B. (**B**) Chromatin and bright-field images of three numbered oocytes from A, at the times indicated. Arrowheads indicate non-aligned bivalents. Scale bar: 5 μm.

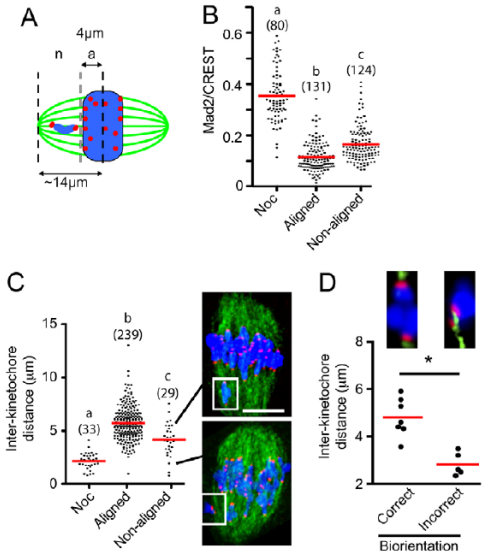

Fig. 4. Non-aligned bivalents have more Mad2 and are under less tension than those on the spindle equator. (**A**) Meiotic spindle schematic, with bivalents classified as aligned 'a', with kinetochores ≤4 μm from the spindle midzone, or non-aligned 'n' (>4 μm). (**B**) Mad2/CREST ratios in bivalents at 5 hours post-NEB that were either aligned or non-aligned, or following nocodazole addition. (**C**) Inter-kinetochore distances of bivalents that are aligned, non-aligned or from nocodazole-treated oocytes. Inter-kinetochore distance is defined as the separation of the two sister kinetochore pairs within a bivalent. Two examples of meiotic spindles are shown with a non-aligned bivalent and its corresponding inter-kinetochore distance (white box, line). Scale bar: 10 μm. (**D**) Inter-kinetochore distance in non-aligned bivalents (n=12). Bivalents were categorized as being bi-oriented (n=7), with the two pairs of sister kinetcohores being attached to opposite spindle poles, or non-bi-oriented (n=5). The non-bi-oriented bivalent shown has both sister pairs attached to the same pole. (B-D) Parentheses indicate number of kinetochores examined. (A,C,D) Chromatin is shown in blue; tubulin, green; kinetochores, red. (B-D) Groups with different letters (a,b,c) or marked by an asterisk are significantly different from one another (P<0.001); mean values are indicated by horizontal red lines.

We observed that Mad2 staining on kinetochores of non-aligned bivalents was significantly more intense than on those that were congressed (Fig. 4B, $P<0.001$, measurements on both pairs of sister kinetochores were used). However, the amount of Mad2 on these non-aligned bivalents remained significantly below the levels achieved following complete loss of microtubule attachment through nocodazole addition (Fig. 4B) and also below the Mad2 levels on kinetochores at 4 hours post-NEB (supplementary material Fig. S3), at which time no k-fibers can be observed (supplementary material Fig. S2).

The above data suggest that many non-aligned bivalents were associated with raised levels of Mad2, compared with bivalents at the spindle equator. If the attachment of non-aligned bivalents were correct, we would predict that microtubule stretch would develop tension across them, leading to greater separation of their two sister kinetochore pairs. For bivalents at or near the spindle equator and so classified as aligned, the mean separation of the two pairs of sister kinetochores was 5.7±1.6 µm (±s.d., $n=239$ pairs; Fig. 4C), significantly greater than that measured in non-aligned bivalents (4.2±1.6 µm; $n=29$; $P<0.001$; Fig. 4C). Although mean tension was weaker in non-aligned bivalents, it was not absent, given that nocodazole added to collapse all tension across the bivalents significantly reduced kinetochore separation still further (Fig. 4C).

To examine the k-fiber attachment of the two sister kinetochore pairs associated with each non-aligned bivalent, oocytes were immunostained for tubulin and CREST following Ca^{2+} treatment at 5 hours post-NEB. In total, 12 non-aligned bivalents were observed, and in just over half (58%, $n=7/12$), the bivalent was observed to be bi-oriented with monotelic attachment of each sister pair to opposite poles. In the remaining bivalents (42%, $n=5/12$) the attachment was incorrect, with either monotelic attachment of both sister pairs to the same pole ($n=1$), one kinetochore pair attaching to both poles ($n=1$), one kinetochore pair being vacant ($n=1$) or a combination of the above ($n=2$). As expected, the non-aligned bivalents that were classified as bi-oriented by k-fiber attachment and orientation had much greater stretch across them than those bivalents that were non-bi-oriented (Fig. 4D).

In summary, individual non-aligned bivalents can have characteristics that overlap with bivalents under tension on the spindle equator and those in which all microtubule attachment has been abolished with nocodazole. Their kinetochores have an intermediate level of Mad2 association and appear to be under less tension. Such observations are consistent with the non-aligned bivalents falling into one of two distinct categories. First, those that have failed to make correct attachments to microtubules at both spindle poles, a situation that generates unbalanced forces and bivalent movement towards the pole with greater attachment, thus generating the non-alignment in the first instance. Second, those non-aligned bivalents that have had their erroneous attachment corrected, becoming bi-oriented and as such are moving back towards the spindle equator. However, although this is the simplest interpretation of the above, and is consistent with recent reports tracking bivalent movements and error correction in meiosis I (Kitajima et al., 2011), here they are based on fixed oocytes only, and need further analysis in live cells.

Non-aligned bivalents do not inhibit the APC/C and can generate aneuploidy

So far, we had observed non-aligned bivalents in fixed oocytes. In order to understand more fully their provenance and fate, we expressed histone2B-mCherry (H2B-Cherry) and followed bivalent movements with high-temporal acquisition by 2D epifluorescence. All oocytes examined displayed rapid oscillations of bivalents about the spindle midzone that were estimated to last 1-3 minutes but which result in only very small displacements from the spindle equator (supplementary material Movie 1, left panel). They appear to be analogous, in terms of duration and displacement, to the oscillations of sister chromatids during mitosis that have been reported elsewhere (Jaqaman et al., 2010; Skibbens et al., 1993). However, in addition, many of the oocytes had non-aligned bivalents, which were readily identifiable as separate from the spindle equator and which persisted over a much longer timeframe, from tens of minutes to hours (Fig. 5A; supplementary material Movie 1, right panel).

We wondered whether the presence of non-aligned bivalents had any effect on the timing of anaphase. If non-aligned bivalents were able to generate a robust SAC checkpoint and so inhibit the APC/C, then one would predict that this timing would be delayed, in the same way observed with low doses of the spindle poison nocodozale (Wassmann et al., 2003). Comparing oocytes in which bivalents remained aligned from the period 4.5 hours post NEB onwards with those where non-aligned bivalents were evident for at least 1 hour, we observed no difference in the timing of polar body extrusion (Fig. 5A,B). The lack of any change in the timing of anaphase in those oocytes with persistently non-aligned bivalents suggests they have no influence on SAC activity. However, if this were so, we should not observe any difference in APC/C activity between the two groups. To test this, we measured the rate of cyclin B1-Venus degradation in oocytes co-expressing H2B-Cherry. As carried out previously, comparing oocytes in which bivalents remained aligned from the period 4.5 hours post NEB onwards with those where non-aligned bivalents were evident for at least 1 hour, we observed no difference in cyclin B1 degradation, measured by when it starts and stops, or by its maximal rate of loss (Fig. 5C,D).

We thought it important to examine the fate of non-aligned bivalent until the time of anaphase in oocytes co-expressing cyclin B1-GFP with H2B-Cherry. We observed that in ~70% of oocytes ($n=52$), bivalents were congressed at the time of APC/C-induced cyclin B1 loss and maintained this congression until anaphase (supplementary material Movie 2), but not in 30%, where non-aligned bivalents were observed to persist for extended times (Fig. 5E,F; supplementary material Movie 3). Therefore, we surmise that most of the non-aligned bivalents observed previously in fixed oocytes at 5 hours post-NEB (Fig. 4) are likely to be chromosomes that have not yet congressed at the spindle equator. However, at later meiotic times it was still possible to observe bivalents leave the metaphase plate, which would have been classified as congressed at earlier timepoints (Fig. 5E, arrowhead). Importantly, for the oocyte in the prevention of non-disjunction, the majority of these non-aligned bivalents did eventually congress at the metaphase plate in the time period before anaphase (Fig. 5F). However, in 2 out of the 51 oocytes examined (4%), bivalents persisted until anaphase onset and underwent non-disjunction (Fig. 5E; supplementary material Movie 4).

Finally, we examined the movement of the kinetochores of non-aligned bivalents in live oocytes ($n=9$) by injecting GV oocytes with CenpC-GFP and H2B-mCherry cRNA to confirm that non-aligned bivalents do indeed congress and become stably bi-orientated on the metaphase I spindle. Once bi-orientated, the bivalents moved towards the spindle equator and showed the same characteristics as all the other bivalents (supplementary material Movie 5). These live cell observations confirm the behavior interpreted from the fixed oocytes, in which non-aligned bivalents

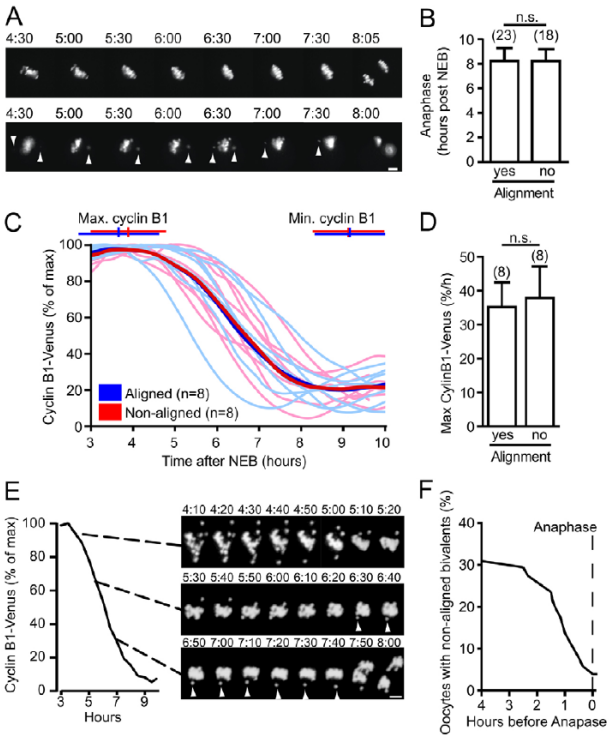

Fig. 5. Non-aligned bivalents fail to inhibit the APC/C and can undergo non-disjunction at anaphase. (**A**) Representative images of oocytes expressing H2B-mCherry, with either aligned bivalents present for the entire period of imaging (top) or non-aligned bivalents (bottom). (**B**) Timing of anaphase onset in oocytes with or without aligned bivalents. (**C**,**D**) Cyclin B1-Venus levels in oocytes with or without aligned bivalents, showing the time at which maximum and minimum cyclin B1 levels were reached. Alignment was observed by co-expression of H2B-mCherry, and the means of individual recordings are in bold (D). Mean maximal rate of cyclin B1 degradation in those oocytes from C. (**E**) Cyclin B1-Venus fluorescence and corresponding H2B-mCherry images, in an oocyte in meiosis I at the times indicated after NEB. Arrowheads indicate a non-aligned bivalent undergoing non-disjunction. Scale bar: 10 μm. (**F**) Timing of bivalent congression in oocytes measured against the time to anaphase-onset (n=78). (A-D) Persistent non-aligned bivalents were classified as being present for periods of 1 hour or greater. (B,D) Errors are s.d.; n.s., non-significant (P>0.05, t-test); numbers of oocytes analyzed are in parenthesis.

initially appear to be under less tension and move polewards, probably towards the pole with which it has established greater attachment. Correction of the erroneous attachment is then likely to occur, based on the observations that very few non-aligned bivalents do undergo non-disjunction, and the bi-oriented bivalent now under tension from both poles moves to the spindle equator.

CDK1 activity is important in the timing of anaphase following APC/C activation

What then dictates the timing of bivalent segregation in oocytes, given the APC/C is activated 3-4 hours ahead of anaphase? One possibility is that the gradual decline of cyclin B1 over 3-4 hours maintains a high CDK1 activity and this lengthy period permits sufficient time for bi-orientation of all bivalents. To examine this, the pan-CDK inhibitor roscovitine (Bain et al., 2007) was used at 100 μM, a minimally effective dose to inhibit CDK1 in mouse oocytes, as judged both by its ability to block NEB and through in vitro CDK1 kinase assays (supplementary material Fig. S5) (Deng and Shen, 2000).

Roscovitine was added to maturing oocytes expressing H2B-mCherry between 3 and 7 hours post-NEB, and the timing of anaphase recorded by timelapse imaging (Fig. 6A-C). The overall percentage of oocytes stimulated to undergo anaphase by Cdk inhibition increased as meiosis progressed. Thus, following roscovitine addition at 5 hours post-NEB, 87% of oocytes underwent anaphase, compared with fewer than 20% at 3 hours (Fig. 6B). The ability of a few oocytes to show anaphase movement in the absence of k-fibers at 3 hours would be consistent with the findings that at least in oocytes this anaphase pulling force can be provided by microtubules not associated with the kinetochore (Deng et al., 2009; Dumont et al., 2010). The large increase in the ability of oocytes to undergo anaphase when roscovitine is added between 3 and 4 hours (19.2 versus 71.4%, P<0.001) correlates well with the formation of stable microtubules between 4 and 5 hours after NEB, suggesting that this process is more efficient in the presence of k-fibers.

Anaphase onset occurred prematurely following roscovitine addition at all times tested, therefore decreased CDK1 activity may be an important step in the timing of meiotic exit (Fig. 6A,C; supplementary material Movie 6). At 7 hours post-NEB, the most meiotically advanced time assessed, anaphase was ~20 minutes after drug addition (Fig. 6C). However, at earlier meiotic times anaphase onset was much more delayed, up to 55 minutes. This same anaphase timing was also observed using flavopiridol at a dose of 5 μM (supplementary material Fig. S5) (Potapova et al., 2006), suggesting the delay is not specific to one CDK inhibitor. Instead, this delay is probably due to the need to recruit other pathways, such as those involving phosphatases, which are known to be needed for mitotic and meiotic exit (Schindler and Schultz, 2009; Schmitz et al., 2010). It was possible to restore the delay to anaphase after roscovitine addition in oocytes injected with a very high dose of cyclin B1-GFP cRNA (100 pg) 100-fold greater than

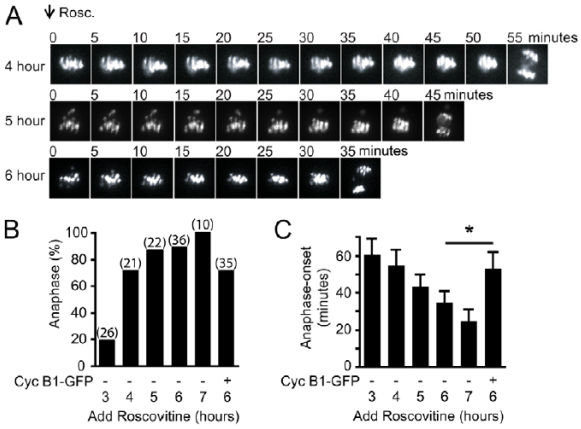

Fig. 6. Reduced CDK1 activity accelerates anaphase onset. (**A**) Representative H2B-mCherry fluorescence images every 5 minutes following 100 µM roscovitine addition to oocytes at the times indicated post-NEB. (**B**,**C**) Percentage of oocytes undergoing anaphase (B) and associated timing of anaphase onset measured in minutes after roscovitine addition (C), calculated from timelapses in A; '+', oocyte injected with 100 ng cyclin B1-GFP cRNA before NEB. (B) Numbers of oocytes analyzed in parentheses are the same for C. (C) *P<0.001, error bars are s.d.

used previously, and which masks any observable cyclin B1-GFP degradation (not shown) and would be expected to give a high level of CDK1 activity (Fig. 6C).

DISCUSSION

In mitosis, complete congression of all sister chromatids onto a metaphase plate is closely coupled with the initiation of cyclin B1 degradation (Clute and Pines, 1999). Even in chromosomally unstable cell lines, anaphase is not observed without first achieving full alignment (Thompson and Compton, 2008). The observations here were therefore surprising, that the initiation of APC/C activity, measured by cyclin B1 degradation, in at least one-third of all mouse oocytes occurred several hours before complete bivalent congression. Instead, we found that the cyclin B1 loss was more closely associated with the attachment of the vast majority of sister kinetochore pairs to microtubules. Strain differences do influence the timing of meiosis I and cyclin B1 degradation in mice (Ledan et al., 2001; Polanski et al., 1998), but in our F1 hybrids, 5 hours post-NEB was when loss of this APC/C substrate was first seen and when ~97% of all kinetochores were associated with k-fibers.

At the kinetochores, the attachment of microtubules 5 hours post-NEB led to the loss of the SAC protein Mad2. It is likely that this event in meiosis, as in mitosis, is responsible for APC/C activation. Unoccupied kinetochores are well known to recruit cytosolic Mad2, which is then thought to undergo conformational changes rendering it able to inhibit Cdc20 (De Antoni et al., 2005; Kim et al., 2010; Yu, 2006). It is not entirely clear how microtubule attachment causes Mad2 to be lost from kinetochores and the checkpoint silenced, but it does involve at least in part dynein motor-driven movement to the spindle poles and the Mad2-binding protein p31comet (Gassmann et al., 2010; Griffis et al., 2007; Yang et al., 2007).

The reason why congression of sister chromatids in mitosis but not bivalents in meiosis is an event coupled with APC/C activation may well be a reflection of the fact that building a spindle in the absence of centrosomes is less efficient and so takes longer to achieve (Hornick et al., 2011; Mahoney et al., 2006). Alternatively, it may be that achieving bi-orientation of bivalents through the monotelic attachment of sister kinetochore pairs is less efficient than the equivalent process with respect to sister chromatid bi-orientation in mitosis. However, independent of the reasoning for the delay in meiosis, in both instances kinetochore occupancy appears to be the major driver of APC/C activation. The observation that many bivalents are not congressed at the spindle equator, and that these bivalents have much shorter inter-sister kinetochore distances, suggest that they are not fully bi-oriented and under maximal tension. Such bi-orientation is achieved in meiosis I by monopolar attachment of each sister kinetochore pair to opposite spindle poles, and this ensures maximal tension across the bivalent.

The present data are therefore consistent with a model in which initial attachment of microtubules to sister kinetochores satisfies the SAC, as also suggested in mitosis (Khodjakov and Pines, 2010), but that these attachments may be erroneous and need to be repaired. Indeed, this repair may be very common in mouse oocytes, given that three rounds of microtubule error correction have been calculated to occur for each bivalent, measured from the time of NEB until polar body extrusion (Kitajima et al., 2011). It is likely, but remains to be firmly established, that Aurora kinases play a part in this process, as they do in mitosis, by destabilizing erroneous attachment that fail to generate adequate tension across the kinetochores (Lane et al., 2010; Liu et al., 2009; Welburn et al., 2010; Yang et al., 2010).

In mouse oocytes, the time following SAC satisfaction corresponded to a long, 3- to 4-hour, period of cyclin B1 degradation, a length that has been observed previously in other studies (Herbert et al., 2003; Homer et al., 2005; McGuinness et al., 2009). This timing, of anaphase-onset from the start of APC/C activation, is found here to be shortened by CDK1 inhibition, suggesting at least in part that the period is governed by CDK1 activity. Consistent with this, mouse oocytes appear to contain much greater amounts of cyclin B1 than cultured somatic cells, so much so that it is in a several-fold molar excess to its kinase binding partner CDK1 (Arooz et al., 2000; Kanatsu-Shinohara et al., 2000).

The non-aligned bivalents that persist at this time had Mad2 associated with their kinetochores, but levels were much weaker when compared with those kinetochores that had not established any microtubule attachment or when microtubules were stripped off kinetochores by nocodazole. A weak Mad2 association with

non-aligned bivalents would be in keeping with the finding that, in the majority of cases, the sister kinetochores have some attachment to microtubules, and suggests that Mad2 can remain on weakly attached mouse bivalents, as has been reported in insect spematocytes in meiosis I (Nicklas et al., 2001). However, it is clear that such a weak Mad2 signal is not sufficient to transduce a strong SAC-mediated APC/C inhibition, and it may be that, in oocytes, weak or absent attachment to only a few bivalents is insufficient to generate a 'wait-anaphase' signal.

We conclude that the present findings are highly relevant to the etiology of aneuploidy. The rate at which we observe non-aligned bivalents to undergo non-disjunction here at anaphase onset is compatible with the aneuploidy rate we previously reported for this strain by using chromosome spreading techniques (Lane et al., 2010). Given that our data are consistent with attachment being the primary driver of SAC satisfaction, then this explains the lack of a block to meiosis I when many univalents are present through genetic loss of the recombination gene *Mlh1* (Nagaoka et al., 2011). Importantly, it also helps explain why the most commonly observed cause of maternal meiosis I-derived Down Syndrome (trisomy 21), prevalent in about 40% of all cases, is associated with a lack of any recombination between the homologous chromosomes (Lamb et al., 1997; Lamb et al., 1996). In such a scenario, the univalents of chromosome 21 during meiosis I would satisfy the SAC by being able to form monopolar attachments. This is highly reminiscent of MUG cells in mitosis, which are generated by preventing DNA replication and so produce a M phase with unpaired single chromatids. In these cells, mitosis still occurs but in the absence of any tension being able to develop across sister kinetochores (O'Connell et al., 2008). Finally, our data are relevant to the rise in age-related aneuploidy observed both in human and mouse oocytes. Oocytes spend the majority of their lives arrested at the dictyate stage of prophase I, at which time they have little to no capacity to repair damaged cohesion molecules such as Rec8 and SMC1β (Revenkova et al., 2010; Tachibana-Konwalski et al., 2010). As such, with increasing maternal age the ties holding bivalents together become weaker (Chiang et al., 2010; Lister et al., 2010; Merriman et al., 2012). This decline in cohesion with age will probably exacerbate initial incorrect kinetochore attachments, which do not generate a strong 'wait-anaphase' signal, and so fail to inhibit the APC/C in oocytes and may go uncorrected by the time of anaphase-onset leading to mis-segregation.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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Supplemental Figures

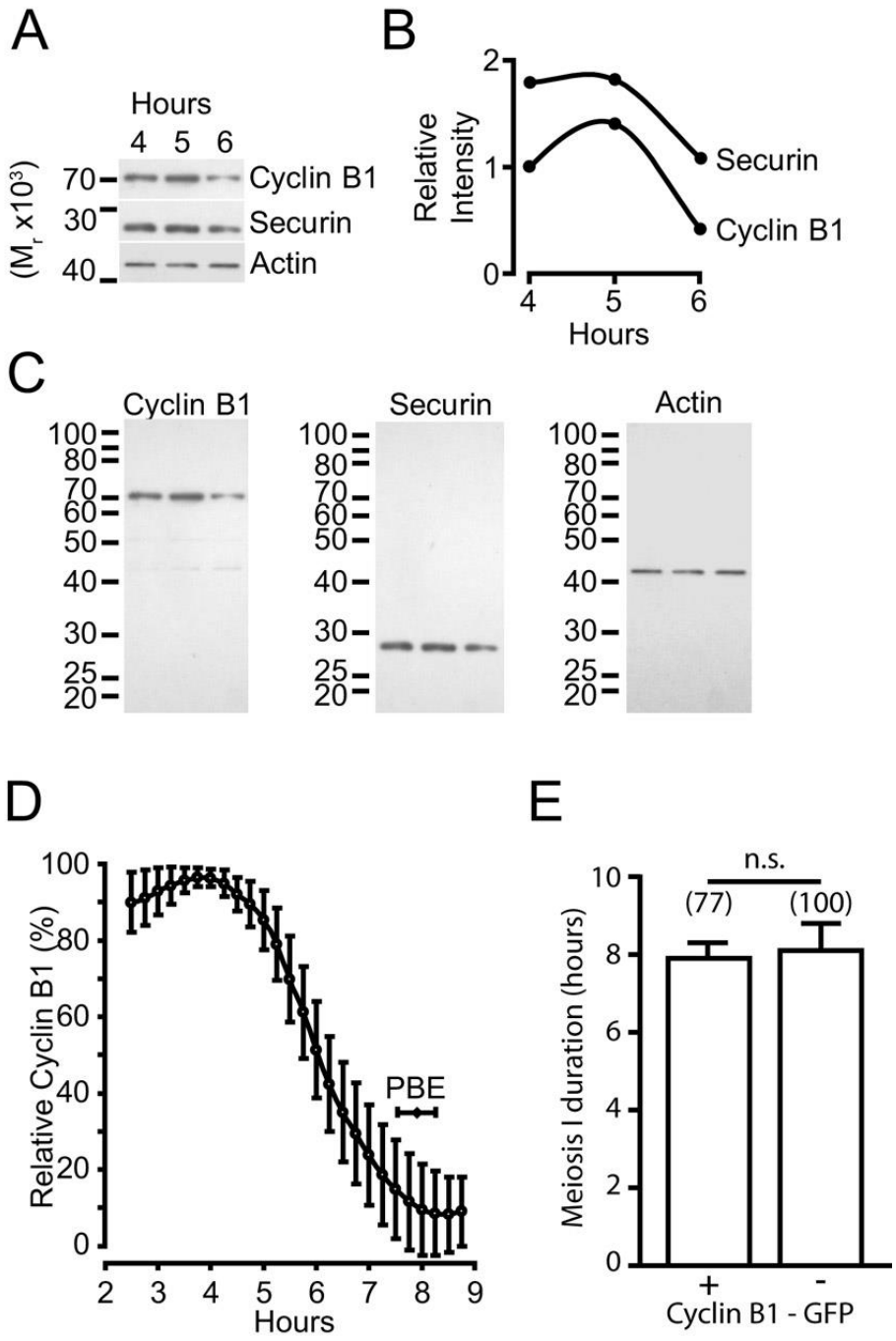

Fig. S1. Timing of cyclin B1 and securin degradation in oocytes. (**A**) Immunoblots for cyclin B1 and securin, with an actin loading control, in synchronized oocytes at the times indicated after NEB. (**B**) Densitometric analysis of the immunoblots blot shown in A. (**C**) Uncropped immunoblots from

A. (**D**) Mean cyclin B1-GFP intensity of normalized traces from oocytes during meiosis I (n=77). Error bars are s.d. The average timing of polar body extrusion with its associated s.d. is marked PBE. (**E**) Comparison of the duration of meiosis I (from NEB to polar body extrusion) in uninjected oocytes or those injected with cyclin B1-GFP at the prophase I stage. The mean timings are not significantly different (P>0.05, t-test). Parentheses indicate the number of oocytes analyzed.

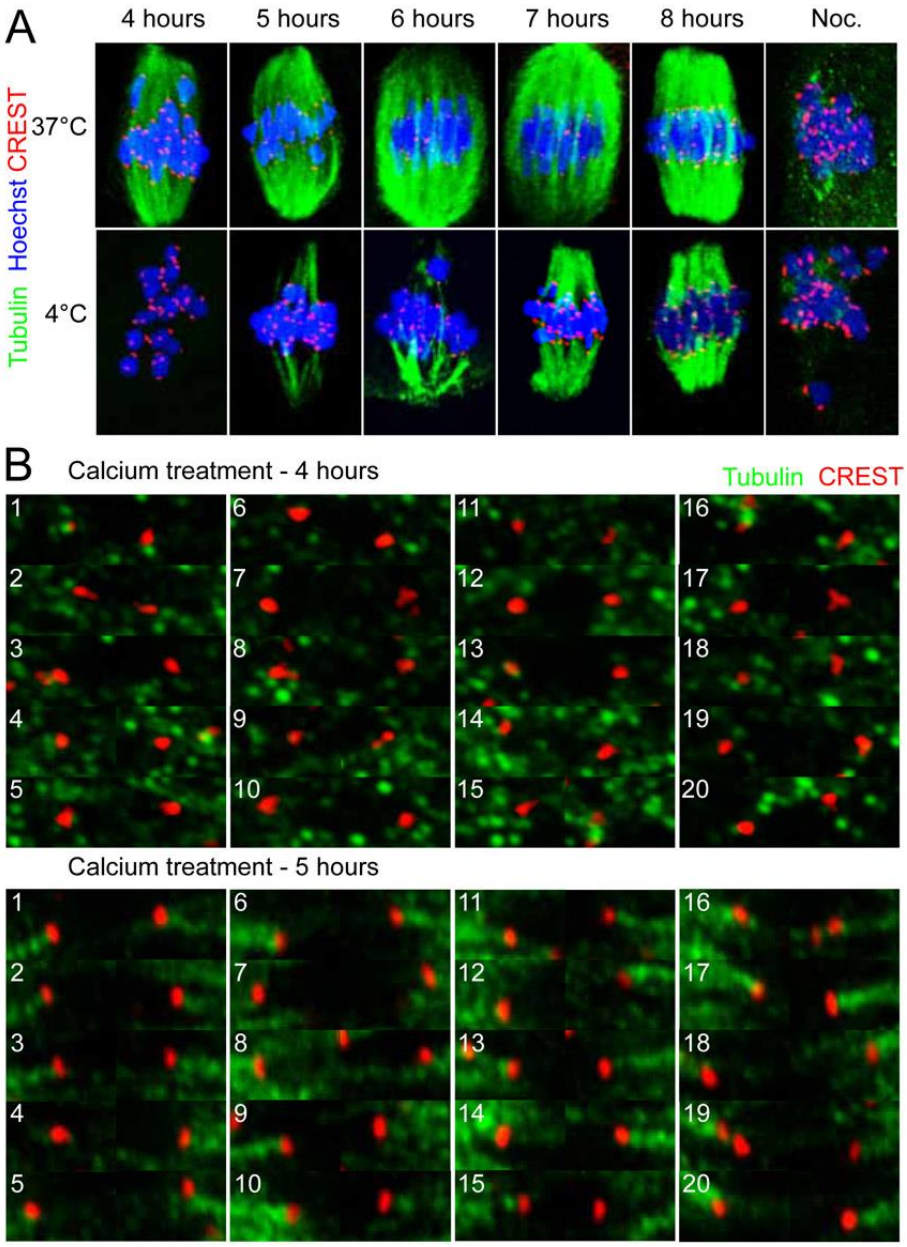

Fig. S2. Timing of k-fiber formation in oocytes. (**A**) Representative meiotic spindles from oocytes taken at the times indicated following NEB, or at 8 hours with nocodazole (Noc). The bottom row shows oocytes that had been cold shocked before fixation to visualize only k-fibers. K-fibers were first visible in oocytes by 5 hours post-NEB (*n*=5-8 oocytes per time point). (**B**) Images of individual kinetochores from the oocytes depicted in Fig. 1A following Ca^{2+} treatment at either 4 or 5 hours after NEB. Kinetochores belonging to the same bivalent are shown together and the 20

bivalent pairs are labeled. This pairing was carried out manually by looking for the association of the two sister kinetochore pairs to the chromatin of a single bivalent. To compose the figure, and because sister kinetochores appears in different *z*-sections, we have sometimes spliced images together.

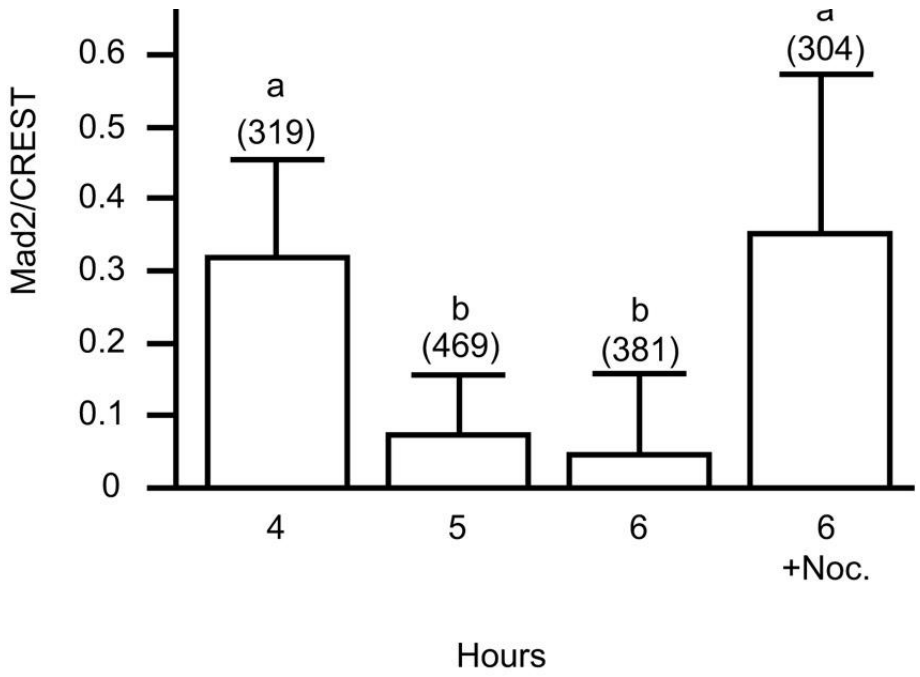

Fig. S3. Mad2 immunostaining of sister kinetochores at different time points in meiosis I. Mad2/CREST signal ratios were calculated on kinetochores of synchronized oocytes fixed at 4, 5 and 6 hours post-NEB, or following nocodazole incubation at 6 hours. The mean Mad2/CREST ratio with s.d. is shown for each group. Parentheses indicate the number of kinetochores analyzed; groups with different letters (a, b) are significantly different from one another (P<0.001).

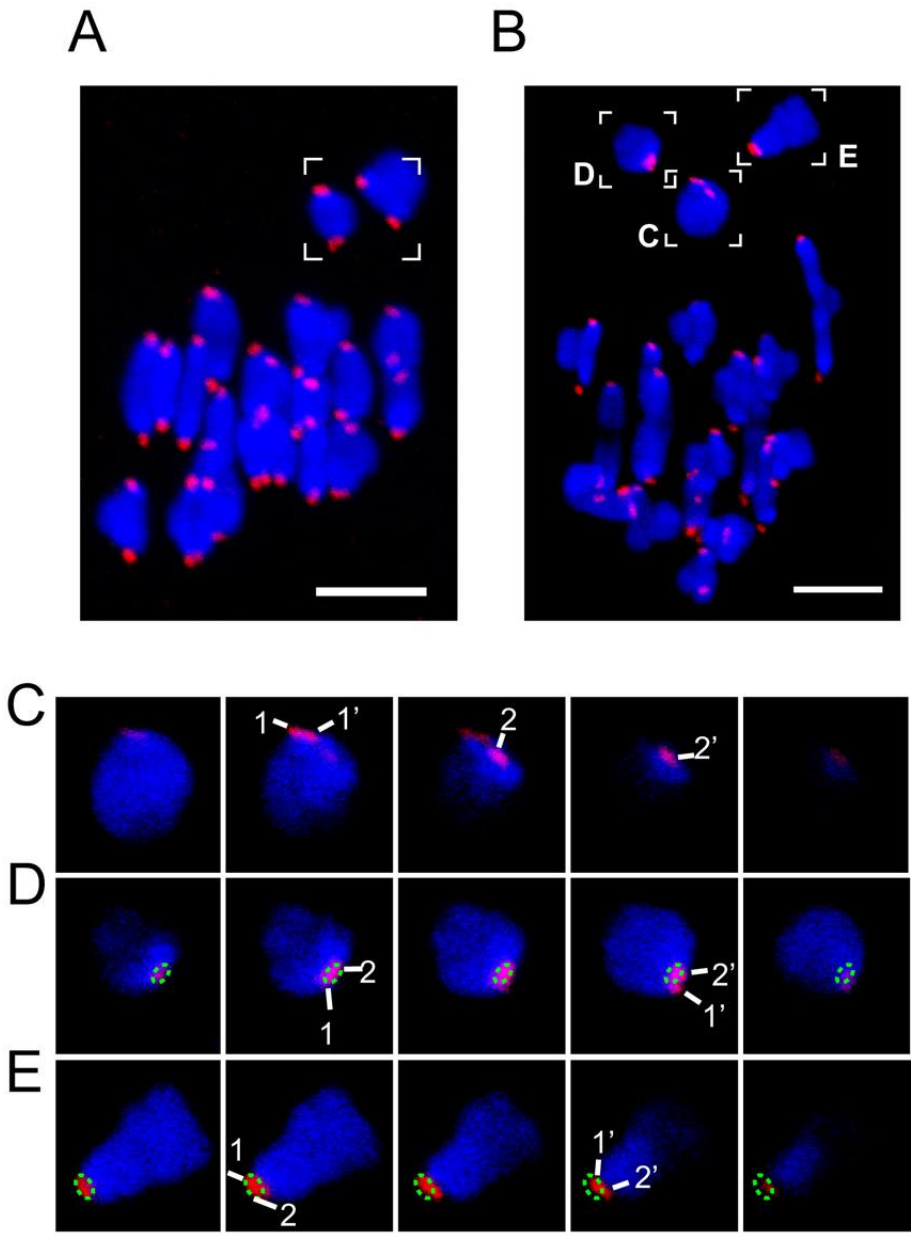

Fig. S4. The non-aligned chromosomes observed in meiosis I are bivalents. (**A**) Bivalent (blue; Hoechst) and kinetochore (red; CREST) staining in an oocyte at 5 hours post-NEB. Boxed; the non-aligned chromosomes can be classified as bivalents by morphology. (**B-E**) Bivalent and kinetochore staining in an oocyte at 5 hours post-NEB (B), with three non-aligned bivalents. The sister kinetochore pairs (labeled 1,1′ and 2,2′) of the three non-aligned bivalents are closely associated, but can be distinguished by serial confocal sections with 0.5 µm *z*-resolution (C-E). Scale bars: 5 µm in A,B.

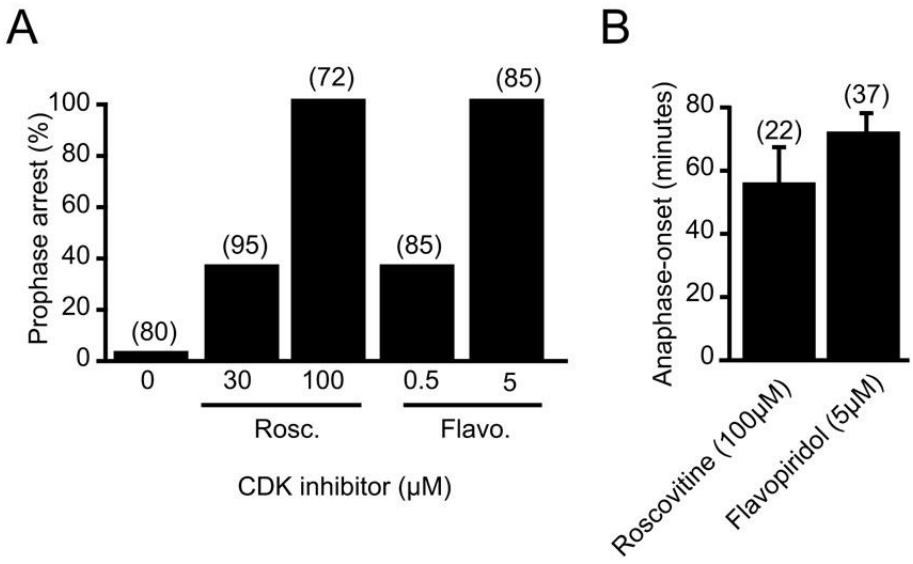

Fig. S5. The block to NEB and timing of anaphase using Cdk inhibitors. (**A**) The percentage of oocytes remaining prophase I arrested at 2 hours after washout of milrinone following culture with the Cdk inhibitors roscovitine or flavopiridol at the doses indicated. (**B**) In oocytes at 4 hours post-NEB, the timing of anaphase onset following culture with roscovitine or flavopiridol. Numbers of oocytes analyzed in parentheses.

Movies

Movie 1. Bivalent movements during meiosis I. Oocytes expressing histone 2B-mCherry cRNA were imaged by epifluorescence with a sampling frequency of 1 image/10 seconds. (Left) In this oocyte, high-frequency small movements of bivalents are readily observed, although this does not result in any displacement from the spindle midzone. (Right) In this oocyte, much longer lasting, larger bivalent displacements can be observed. In this oocyte, a bivalent moves towards the pole (06:00:00-06:10:00), reorientates (6:05:00-06:20:00) and returns to the spindle midzone by about 06:27:00. Time is hours:minutes:seconds post-NEB.

Movie 2. Cyclin B1 degradation in an oocyte with aligned bivalents. Histone 2B-mCherry (red) fluorescence and associated cyclin B1-GFP levels (green bar) in an oocyte during meiosis I. The oocyte had been injected with cyclin B1-GFP and histone 2B-mCherry during prophase I arrest. A confocal *z*-series for H2B-mCherry and associated cyclin B1-GFP fluorescence was acquired every 10 minutes. Cyclin B1-GFP fluorescence is represented on a bar scale as a percentage of the maximum fluorescence recorded during its meiotic division (here at 04:10 hours:minutes). All times are post-NEB.

Movie 3. Cyclin B1 degradation in the presence of non-aligned bivalents. Histone 2B-mCherry (red) fluorescence and associated cyclin B1-GFP levels (green bar) in an oocyte during meiosis I. The oocyte had been injected with cyclin B1-GFP and histone 2B-mCherry during prophase I arrest. A confocal *z*-series for H2B-mCherry and associated cyclin B1-GFP fluorescence was acquired every 10 minutes. Cyclin B1-GFP fluorescence is represented on a bar scale as a percentage of the maximum fluorescence recorded during its meiotic division (here at 04:10 hours:minutes). All times are post-NEB.

Movie 4. Cyclin B1 degradation in the presence of a bivalent that undergoes non-disjunction. Histone 2B-mCherry (red) fluorescence and associated cyclin B1-GFP levels (green bar) in an oocyte during meiosis I. In this oocyte a non-aligned bivalent (top right; 06:30 hours:minutes onwards) is observed to undergo non-disjunction. The method of recording and protocol is the same as for Movie 2.

Movie 5. Biorientation of a non-aligned bivalent. Histone 2B-mCherry (green) and CenpC-GFP (red) fluorescence in an oocyte during mid-prometaphase I. The oocyte was imaged by 3D confocal microscopy at 90-second intervals and the sequence displayed as a maximal intensity *z*-projection. The inter-kinetochore distance of the non-aligned bivalent (blue kinetochores) is shown by the moving bar (left) and is calculated in three dimensions. Scale bar: 10 μm.

Movie 6. Anaphase timing modified by roscovitine addition. Timing of anaphase onset in four oocytes following roscovitine addition. Oocytes are expressing histone 2B-mCherry, pseudocolored to distinguish the timing of 100 μM roscovitine addition, which was made at: 4 hours (blue), 5 hours (yellow), 6 hours (red) or at 6 hours in an oocyte injected with 100 pg cyclin B1-GFP cRNA at the GV stage (green). Meiotically more advanced oocytes underwent anaphase the quickest following roscovitine addition. Cyclin B1 overexpression delayed anaphase onset. Upper timestamps are elapsed times since NEB; lower timestamps are elapsed times since roscovitine addition.

II. M2 culture media

1) Weigh out the following components and dissolve into MiliQ H2O;

5.533g NaCl

0.356g KCl

0.162g KH2PO4

0.293g MgSO4.7H2O

0.349g NaHCO3

4.349g 60% Na Lactate (liquid)

0.036g Na Pyruvate

1.000g Glucose

0.010g Phenol Red

0.060g Penicillin

0.050g Streptomycin

0.252g CaCl2.2H2O

2) Dissolve 4.969g HEPES in 50ml MiliQ H2O, adjust to pH7.4 with 5M NaOH;

3) Add 2) to 1), then dissolve BSA gently with a final concentration of 0.4%;

4) Top up with MiliQ H2O to 1000ml in a volumetric flask;

5) Osmolarity measurement (should be 283-289 mOsmol);

6) Filter and aliquot into 50ml Falcon tubes, store in fridge and can be used up to 2 weeks.

III. Image J macro

This macro was originally developed by Dr Simon Lane with brief modification here. It is used for kinetochore protein intensity quantification in immunostained MI oocytes (For analysis details, please refer to supplementary figure **S1C** in chapter three). Two functions are defined by using the letter ‘s’ and ‘l’ keys for ‘Setup’ and ‘Log data’ respectively.

```
print("Press [s] to setup image");
print("Use the 'point tool' to select kinetochore")
print("Log data by pressing [l]");

macro "image setup [s]"
{
imagename = getTitle();
Stack.setDisplayMode("composite");
Stack.setChannel(1);
run("Blue");
Stack.setChannel(2);
run("Red");
Stack.setChannel(3);
run("Green");
run("Channels Tool... ");
Stack.setDisplayMode("composite");
Stack.setActiveChannels("111");
run("Point Tool...", "mark=0 auto-measure label selection=green");
setTool(7);
}

macro "log data [l]"
```

```
{
getVoxelSize(width, height, depth, unit);
calibration = width;
xx= getResult("X", nResults-1);
xx=xx*(1/calibration);
yy= getResult("Y", nResults-1);
yy=yy*(1/calibration);
zz= getResult("Slice", nResults-1);
homologn number = round((nResults/2)+0.1);
bgpos = newArray(-24, 0, 24);
cheeseX = newArray(0,0,0,1,1,1,2,2,2);
cheeseY = newArray(0,1,2,0,1,2,0,1,2);
bgx = -24;
bgy = -24;
Z1=zz-1; Z2=zz+1;
run("Z Project...", "start=Z1 stop=Z2 projection=[Sum Slices]");
makeOval(xx-12, yy-12, 24, 24);
Stack.setDisplayMode("color");
Stack.setChannel(2);
getStatistics(area, mean);
setResult("Channel 2", nResults-1, mean);
crest = mean;
Stack.setChannel(3);
getStatistics(area, mean);
setResult("Channel 3", nResults-1, mean);
kinetochoreprotein = mean;
ch2bg = 10000;
ch3bg = 10000;
for(i=0; i<9; i++)
```

```
{
bgx = cheeseX[i];
bgx = xx + bgpos[bgx];
bgy = cheeseY[i];
bgy = yy + bgpos[bgy];
makeOval(bgx-12,bgy-12,24,24);
Stack.setChannel(2);
getStatistics(area, mean);
if(mean<ch2bg){ch2bg=mean;}
Stack.setChannel(3);
getStatistics(area, mean);
if(mean<ch3bg){ch3bg=mean;}
}
setResult("Channel 2 bg", nResults-1, ch2bg);
setResult("Channel 3 bg", nResults-1, ch3bg);
setResult("Homolog", nResults-1, homolognumber);
close();
setFont("Arial", 8, "Bold");
setForegroundColor(255,255,255);
setBackgroundColor(255,255,255);
run("Overlay Options...", "stroke=yellow width=1 fill=yellow set");
makeOval(xx-2, yy-2, 4, 4);
run("Add Selection...");
run("Overlay Options...", "stroke=yellow width=1 fill=none");
run("Add Selection...");
makeText(homolognumber,xx+3,yy);
run("Add Selection...");
makeOval(0, 0, 1, 1);
}
```

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