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Cyclin A2 modulates kinetochore–microtubule attachment in meiosis II

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Introduction

Successful progression through meiosis is a crucial process for gamete development. In meiosis I (MI), homologous chromosomes are held together by chiasmata, whereas sister chromatids are coupled by the cohesin complex. The first meiotic division is initiated just before ovulation and is driven by the anaphase-promoting complex/cyclosome (APC/C)–mediated destruction of securin and cyclin B (Brunet and Maro, 2005; Homer et al., 2005; Peters, 2006; Holt and Jones, 2009). The loss of securin releases separase, which cleaves cohesin from chromosome arms, thereby allowing the resolution of chiasmata, separation of homologous chromosomes, and extrusion of the first polar body (Pb1; Nasmyth, 2002; Uhlmann, 2003; Kudo et al., 2005). This is rapidly followed by an increase in cyclin B–Cdk1 activity and formation of the second meiotic spindle, which remains stably arrested at meiosis II (MII) until fertilization breaks the Emi2-mediated inhibition of the APC/C (Marangos and Carroll, 2004; Ajduk et al., 2008). At fertilization (or parthenogenetic activation), an increase in Ca2+ leads to the destruction of Emi2, thereby releasing the brake on the APC/C and allowing destruction of securin and cyclin B (Rauh et al., 2005; Madgwick et al., 2006; Shoji et al., 2006). At exit from MII, separase removes cohesin from centromeric regions of sister chromatids, allowing sister separation (anaphase II) and exit from meiosis as marked by the extrusion of the second polar body (Pb2; Rattani et al., 2014).

Much attention has been focused on the role of cyclin B–Cdk1 on progression through both mitotic and meiotic cell divisions. In contrast, the role of cyclin A, the first cyclin ever cloned (Swenson et al., 1986), has received less attention. In higher vertebrates, there are two cyclin A isoforms: cyclin A1 and cyclin A2 (Minshull et al., 1990; Howe et al., 1995; Sweeney et al., 1996; Yang et al., 1997). Cyclin A1 is expressed almost exclusively in testes and is restricted to a role in male germ
cell meiosis (Liu et al., 1998), whereas cyclin A2 is widely expressed, and genetic deletion in mice results in peri-implantation lethality (Murphy et al., 1997). In invertebrates, Caenorhabditis elegans, and Drosophila melanogaster, there is a single isoform of cyclin A that plays important roles in controlling progression through mitosis (Kreutzer et al., 1995; Sigrist et al., 1995; Mc Cleland et al., 2009).

In mitotic cell cycles, cyclin A2 begins to accumulate in the S phase and continues to increase through the S and G2 phases before peaking in early prometaphase. At this point, within minutes of nuclear envelope breakdown (NEBD), cyclin A2 is destroyed by the APC/C (Girard et al., 1991; Erlandsson et al., 2000; den Elzen and Pines, 2001; Pesin and Orr-Weaver, 2007; Wolt husi et al., 2008). This prometaphase destruction occurs in the face of an active spindle assembly checkpoint (SAC; den Elzen and Pines, 2001; Geley et al., 2001; Wolt huesi et al., 2008) and leads to the clearance of cyclin A2 before cells reach the metaphase state (den Elzen and Pines, 2001). The established role for cyclin A2 in progression through the S and G2 phases of the cell cycle (Walker and Maller, 1991; Fur uno et al., 1999; Winston et al., 2000; Gong et al., 2007; Kalaszczynska et al., 2009) is consistent with its expression profile during this period. Similarly, inducing persistent expression of cyclin A2 through metaphase leads to disrupted microtubule–kinetochore interactions and delays entry into anaphase (Pines and Hunter, 1990, 1999; den Elzen and Pines, 2001), whereas depleting cyclin A2 leads to a significant increase in the fraction of cells displaying lagging/bridge chromosomes in anaphase (Godek et al., 2015; Kanakkanthara et al., 2016). Thus, cyclin A2 clearly has important roles in mitotic cell cycles, but the embryonic lethality of cyclin A2 knockout mice has restricted investigation into its role in female meiosis (Winston et al., 2000; Persson et al., 2005).

Cyclin A2 is expressed in ovarian and ovulated oocytes at the level of mRNA and protein (Winston et al., 2000; Fuchimoto et al., 2001; Persson et al., 2005; Wolgemuth, 2011; Touati et al., 2012). The functional role of cyclin A2 in meiosis has recently been investigated by injecting mouse oocytes with anti–cyclin A2 antibodies at different stages of meiosis to acutely inhibit cyclin A2 function. The effects include inhibition of the G2–M transition as indicated by delayed germlinal vesicle breakdown (GVBD), and in MI oocytes there is a dramatic failure to separate sister chromatids at the time of egg activation (Touati et al., 2012). In this study, we used the ZP3–Cre–LoxP system to specifically delete the cyclin A2 gene from growing oocytes (Fig. S1; Lewandoski et al., 1997; Lan et al., 2004). We report that cyclin A2–deficient oocytes progress through the G2–M transition and MI unhindered; however, nearly 40% of the oocytes show disrupted MI spindles, an increase in merotelic attachments, and a dramatic increase in lagging chromosomes on exit from MI. Our data reveal that cyclin A2 plays an essential role specifically in MI and in the oocyte to embryo transition.

Results

Cyclin A2 is necessary for normal MI spindle formation

Similar numbers of oocytes were ovulated in hormone-primed cyclin A2fl/fl and cyclin A2−− females (Fig. 1 a). To test our strategy for deleting cyclin A2 from oocytes, Western blotting was performed on oocytes from cyclin A2−− and cyclin A2fl/fl mice as controls. The Zp3 Cre was highly effective at reducing cyclin A2 levels to <3% of controls (Fig. S1, c and d).

Having established that cyclin A2 was effectively depleted, we used immunofluorescence and Hoechst labeling to examine the meiotic status of ovulated oocytes. This revealed that 37% of 71 ovulated MI-stage cyclin A2−− oocytes showed errors in MI spindle formation compared with <3% of cyclin A2fl/fl oocytes (Fig. 1 b). The abnormalities seen in cyclin A2−− oocytes included multipolar spindles and spindles with chromatin detached from the metaphase plate; some oocytes showed microtubule (MT) asters throughout the cytoplasm (Fig. 1 c). To examine the specificity of these observations, we microinjected cyclin A2 mRNA into germinal vesicle (GV)–stage cyclin A2−− oocytes before in vitro maturation. The expression of cyclin A2 during maturation resulted in an increase in cyclin A2 protein in MI-stage oocytes (Fig. S2 c) and a concomitant decrease in spindle abnormalities (Fig. S2 a). The abnormalities in the MI spindle of cyclin A2−− oocytes suggest that cyclin A2 plays a critical role in the formation of the MI spindle—a process that has received much less attention than formation of the MI spindle.

To examine when the MI spindle abnormalities first appeared, we used 4D live-cell confocal microscopy to image MI spindle formation in oocytes expressing EB1-GFP to visualize MTs and Hoechst 33342 stain to label DNA. Imaging began 8.5–9 h after release from 3-isobutyl-1-methylxanthine (IBMX; Fig. S3 and Videos 1, 2, and 3). ~1 h before extrusion of the first polar body, and continued through the interkinetic period, defined herein as the period from polar body formation to arrest at MI. In control oocytes, the interkinetic DNA remained clumped together, and bipolarity of the MI spindle was achieved in all oocytes within 20 min of the first polar body extrusion. Thereafter, the MI spindle increased in length, with chromosomes gradually becoming individualized but never staying from the spindle midzone. In contrast, in all cyclin A2−− oocytes examined, bipolarity was never established within 20 min and instead was achieved 40–60 min after formation of the first polar body. Once bipolarity was established, the chromosomes migrated from the midzone and were distributed over the developing spindle. These observations are consistent with the abnormal spindles identified in ovulated MI oocytes and suggest that the ability to form a bipolar MI spindle and to retain balanced forces on the chromosomes is compromised in the absence of cyclin A2.

Cyclin A2 is not required for MI

We next asked whether the MI spindle phenotypes had their origins in tMI. Comparison of timing of GVBD and polar body formation between cyclin A2−− oocytes and cyclin A2fl/fl oocytes revealed no significant difference in timing of GVBD, polar body formation, and entry into MI (Fig. 2, a and b). To closely examine the first meiotic spindle, the origin of the most aneuploidy (Hassold and Hunt, 2001), MI-stage oocytes were fixed and spindle organization was compared. Remarkably, no discernible differences were detected in either the size of the spindles or the alignment of the chromosomes (Fig. 2, c and d). Furthermore, the use of cold treatment and kinetochore and MT labeling to reveal kinetochore-attached MTs (Shomer et al., 2014) revealed that 77% of kinetochore pairs in cyclin A2−− oocytes showed correct polar MT attachment compared with 79.1% of control cyclin A2fl/fl oocytes (Fig. 2, e and f). The normality of MI in a cyclin A2−null background was further
supported by the finding that no aneuploidy was seen in the chromosome spreads of cyclin A2−/− MII-stage oocytes (Fig. S4). These findings show that cyclin A2 is dispensable for formation of a functional first meiotic spindle and has no observable impact on the timing or fidelity of meiotic progression in MI.

Cyclin A2 protein levels increase in MII oocytes

The role of cyclin A2 specifically during MII suggests that the dynamics of cyclin A2 expression may be different from those seen in mitosis, where it is destroyed at the prometaphase–metaphase border (Di Fiore and Pines, 2010; Kabache and Compton, 2013). To examine the expression profile of cyclin A2 during meiosis, oocytes were examined at G2/prophase arrest (GV), in early prometaphase 2 h after release from arrest (GVBD), in metaphase of MI (MI) 8 h after release, and after superovulation (MII). Western blot analysis showed that the cyclin A2 level was stable in early prometaphase and then declined by ∼30% in the MI stage. From MI to MII, the level of cyclin A2 increased approximately threefold and was double that seen in the GV and GVBD stages (Fig. 3, a and b).

To examine the mechanism underpinning the increase in cyclin A2 protein between MI and MII, we examined the amount of polysome-associated cyclin A2 transcript at different stages of meiosis and compared it to that of securin. The data reveal that there is a threefold increase in polysome-associated cyclin A2 mRNA in MII oocytes compared with the GV or MI stages (Fig. 3 c, left). In contrast, polysome-associated securin remains constant throughout all stages (Fig. 3 c, right). Together, these data suggest that cyclin A2 translation is up-regulated late in meiosis, reaching a maximum in MII-stage oocytes—an expression pattern consistent with the observed MII phenotype.

Cyclin A2−/− oocytes have an increased rate of lagging chromosomes during MII exit

The abnormalities in MII spindles observed in the present study and previous observations indicating that inhibition of cyclin A2 prevents sister separation (Touati et al., 2012) strongly suggest that cyclin A2−/− oocytes may fail to exit from MII. Therefore, we examined the ability of cyclin A2−/− oocytes to undergo the second meiotic division in response to a brief exposure to 7% ethanol, a routine approach to triggering exit from MII. Time-lapse confocal imaging of oocytes undergoing MII exit revealed that similar proportions of control and cyclin A2−/− oocytes underwent sister chromatid separation (96.2% vs. 89.4%, respectively) and that the time to initiation of anaphase was similar in cyclin A2−/− and control oocytes (17.1 ± 1.5 and 15.6 ± 1.1 min, respectively; Fig. 4 b). Polar body extrusion was reduced in cyclin A2−/− oocytes (96.2% and 56.7%, respectively), but even in these oocytes, 31.7% underwent sister separation and were arrested with aberrant anaphase and telophase structures (Fig. 4 a). The most dramatic phenotype was revealed on close examination of anaphase in oocytes that extruded the polar body. The vast majority of cyclin A2−/− oocytes (75.4%) showed lagging chromosomes during anaphase compared with controls (16%; Fig. 4 a). Together, these data confirm that cyclin A2 is dispensable for sister separation and instead plays a major role in spindle and chromosome organization during progression into and out of MII.

A high frequency of lateral and merotelic attachments during MII in cyclin A2−/− oocytes

Merotelic attachments are a feature of MII spindle formation (Kouznetsova et al., 2014), and the origin of lagging chromo-
somes in mitosis is attributed to merotelic attachments (Cimini et al., 2001; Thompson and Compton, 2011).

To investigate the role of cyclin A2 in the formation of kinetochore–microtubule (kt–mt) attachments in the MII spindle, we analyzed the attachment types at 12.5 h (early MII) and 14.5 h (mature MII) in control and cyclin A2−/− oocytes (Fig. 5).

In control oocytes at 12.5 h, the vast majority of kinetochores (78%) had already formed polar attachments, whereas 11% remained unattached, 7% were merotelic (Fig. 5 a, inset 1), and 4% had lateral connections. This contrasted with cyclin A2−/− oocytes, in which only just over 10% of kinetochores had polar attachments, with the vast majority showing lateral connections (79.4%; Fig. 5 a, insets 2, 3, and 4). The remaining kinetochores were largely unattached, with less than 1% being merotelic at this time point. By 14.5 h, most of the control oocytes showed a “mature” spindle phenotype, with nearly all kinetochores showing polar attachments (95%; Fig. 5 c, inset 5) and the remainder showing merotelic or lateral attachments. However, in cyclin A2−/− oocytes, the transition to polar attachments was significantly delayed such that only 59.4% of kinetochores showed polar attachments.

Figure 2. Cyclin A2 is not necessary for progression through MI. (a) Rates of GVBD in cyclin A2fl/fl (n = 102) and cyclin A2−/− (n = 106) groups. P > 0.05, by t test. Data is mean and SEM from four replicate experiments. (b) Rates of polar body extrusion (PBE) in cyclin A2fl/fl (n = 92) and cyclin A2−/− (n = 82) groups at different time points. P > 0.1, by t test. Data is mean and SEM from three replicate experiments. (c) MI oocytes from cyclin A2fl/fl and cyclin A2−/− mice showed apparently normal spindle structures confirmed in d by analysis of spindle length (top left), spindle width (top right), and chromosome alignment (bottom). No differences were detected between cyclin A2fl/fl (n = 82) and cyclin A2−/− (n = 91) groups. Error bars represent SEM. Bars, 10 µm. P > 0.1, by t test. (e) kt–mt attachments in cold-treated MI-stage oocytes from cyclin A2fl/fl and cyclin A2−/− mice. One example of each attachment type observed in MI-stage oocytes is shown. Bars, 10 µm. (f) Analysis of attachment types in oocytes from cyclin A2−/− (1,019 attachments from 27 oocytes) and cyclin A2fl/fl (839 attachments from 22 oocytes) mice. Colored surroundings of each box correspond to the colors in the quantification of attachment types in each bar. Arrows indicate the kinetochore pair with the described attachment. Bars, 2 µm. P > 0.1 by χ² test.
polar attachments (Fig. 5 d), whereas there was a dramatic increase in merotelic attachments (23.9%: Fig. 5 c, inset 7), and 16.6% of attachments remained lateral (Fig. 5 c, insets 6 and 8). Interestingly, all the chromosomes in the “mini-spindles” seen in oocytes showed lateral attachments (Fig. 5 c, insets 3 and 4; and Fig. 5 c, inset 8). Cyclin A2 therefore plays a critical role in the transition of an “immature” MII spindle, as shown in a are representative of three replicate Western blots. Cyclin A2 increases at the MII stage, whereas securin is low. (b) Densitometric analysis of cyclin A2 levels. Data are mean and SEM from three individual experiments analyzed by t test. (c) Association of cyclin A2 (left) and securin (right) mRNA to polysomes at the GV, MI, and MII stages. Data are expressed as relative intensities. Data are mean and SEM from three individual experiments by t test. *, P < 0.05; **, P < 0.01.

Discussion

We used a genetic model to delete cyclin A2 from growing oocytes. Uniquely for metaphase in any cell type thus far studied, mammalian oocytes stabilize cyclin A2 during metaphase II, where it plays a critical role in spindle formation and in the prevention of merotelic attachments and lagging chromosomes during anaphase.

In all cells studied to date, cyclin A2 is destroyed in prometaphase so that its levels are greatly reduced during metaphase. In oocytes, we confirmed that cyclin A2 is destroyed during prometaphase I and remains low during progression through MI, but we were surprised to find that cyclin A2 was present in MII oocytes at levels significantly higher than those seen in prophase-arrested, GV-stage oocytes. The increase in cyclin A2 in MII oocytes appears to be a result of persistent translation as suggested by association with polysomes. Its accumulation may be further enhanced by inhibition of the APC/C by Emi2 during prometaphase II, a pathway that ensures that cyclin B levels can increase rapidly, thereby driving entry into MI (Suzuki et al., 2010; Sako et al., 2014). The presence of cyclin A2 during MI may therefore, in part, be a by-product of the mechanisms invoked to maintain a stable MI arrest as the oocyte awaits fertilization.

The prometaphase destruction of cyclin A2 in mitosis is thought to be important because persistent high levels of cyclin A2 can cause continuous chromosome movements and a delay in entry into anaphase (den Elzen and Pines, 2001), presumably because of its role in destabilizing kt–mt interactions (Kabche and Compton, 2013; Godek et al., 2015). It is not clear why the MII spindle tolerates cyclin A2 during metaphase and mitotic spindles do not, but the acentriolar nature of meiotic spindles is one obvious difference. More importantly, MT stability and spindle function are directly regulated by high levels of MAPK cumulation may be further enhanced by inhibition of the APC/C (Kabche and Compton, 2013; Perry and Verlhac, 2008).

Kinetochore microtubules (kMTs) have a longer half-life in cyclin A2−/− oocytes

MT dynamics are critical for error correction and the resolution of merotelic attachments (Thompson and Compton, 2011). Remarkably, cyclin A2 has recently been shown to promote MT turnover in bipolar prometaphase spindles, thereby allowing kt–mt attachment error correction until just before metaphase (Kabche and Compton, 2013). To examine whether MT stability in cyclin A2−/− oocytes may contribute to abnormal kt–mt attachments and lagging chromosomes, we adapted an assay for measuring MT stability for use in MII oocytes (FitzHarris, 2009). To measure MT stability, oocytes were injected with mRNA-encoding photoactivatable GFP (PAGFP)–α-tubulin and matured to the MII stage. The spindle was located using a small amount of co-injected X-rhodamine–labeled tubulin protein, and a line of PAGFP–α-tubulin was photoactivated close to the MII chromosomes by brief illumination with the 405-nm laser (Fig. 6 a). The decay of the PAGFP fluorescence provides a measure of MT stability (Zhai et al., 1995). In mitotic cells, this decay in MTs comprises two components with distinct half-lives: (a) a rapid turnover (short half-life) in the population of non-kMTs and (b) a more stable (longer half-life) component reflecting the kMTs (Zhai et al., 1995; Kabche and Compton, 2013; Fig. 6). Similarly, we found that the fluorescence decay of the PAGFP in both cyclin A2+oocytes and cyclin A2−/− oocytes fits a double exponential curve (Fig. 6 b). We found that treatment of oocytes with Taxol stabilizes both populations of MTs, confirming that this assay can measure changes in MT stability (Fig. 6). The half-life of non-kMTs is in the vicinity of 20–30 s, whereas that of kMTs is 2.5 ± 0.5 min in cyclin A2+ oocytes and 3.9 ± 0.5 min in cyclin A2−/− oocytes (Fig. 6 c). This 56% increase in MT stability strongly supports the idea that at least some of the effects of cyclin A2 on erroneous attachments are mediated via MT stability during MII spindle formation.

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interkinesis period during MII spindle formation (analogous to prometaphase) that loss of cyclin A2 first manifested in spindle defects, including a loss of spindle organization and dispersal of chromosomes over the developing spindle. Closer examination of kt–mt interactions during this period shows that between early metaphase II (12.5 h after human chorionic gonadotropin [hCG]) and "mature" metaphase II (14.5 h), there is a dramatic transition from lateral to polar kt–mt attachments, and this process is disrupted in the absence of cyclin A2. The resultant increase in merotelic and lateral attachments in cyclin A2<sup>−/−</sup> has dramatic consequences for MII exit, leading to a three- to four-fold increase in lagging chromosomes.

The underlying explanation for these defects in spindle and kt–mt interactions is unclear, but the recent finding that cyclin A2 destruction is necessary for switching from unstable to stable kt–mt attachments at the prometaphase–metaphase transition in mitosis is a likely contributing factor (Kabeche and Compton, 2013). In this system, knockdown of cyclin A2 causes premature kt–mt stability in prometaphase and an increase in lagging chromosomes on metaphase exit, similar to the phenotype observed in oocytes. We found in MII spindles of cyclin A2<sup>−/−</sup> oocytes that there is an increase in kt–mt stability that may explain the problems seen in the transition from lateral to polar attachments and the associated failure to correct merotelic attachments.

The demonstrated role of cyclin A2 in MII contrasts dramatically with the failure to detect any impact of cyclin A2 ablation on MI. The timing of the G2–M transition and completion of MI is unaffected in cyclin A2–deficient oocytes, as are the size and shape of the MI spindle, the chromosome alignment, the frequency of kt–mt attachments, and the frequency of aneuploidy. How can this disparity between the roles of cyclin A2 in MI and MII be explained? It may be that MI has additional or more effective approaches to maintaining Aurora kinase–mediated kt–mt instability during prometaphase I, as indicated by the reduced level of phosphorylation seen on Aurora substrates in MII compared with MI (Yoshida et al., 2015). Additionally, it may simply be that the protracted nature of prometaphase I, which lasts ∼7 h compared with ∼1 h in prometaphase II, allows sufficient time to build a spindle and correct any errors of kt–mt attachment.

Although anaphase II in cyclin A2<sup>−/−</sup> oocytes has a high frequency of lagging chromosomes, the ability of the oocytes to undergo sister separation is unhindered. This is to be expected because merotelic attachments are not detected by the SAC (Sluder et al., 1997; Cimini et al., 2001; Gui and Homer, 2012), and we could find no evidence of kinetochore-associated Mad2 in cyclin A2<sup>−/−</sup> oocytes (unpublished data). Inhibition of cyclin A2 using injection of antibodies has
previously indicated a role for cyclin A2 in progression into MI and in sister separation at MII (Touati et al., 2012), neither of which are consistent with the genetic model reported herein. The difference in approach is likely to explain the inconsistency between the two studies. Off-target effects of the antibodies may partly be to blame, but the acute inhibition afforded by antibody injection in wild-type MII oocytes may generate a different phenotype from that seen when oocytes develop in the absence of cyclin A2. In zebrafish and mice (Daude et al., 2012; Banerjee et al., 2013; Smart and Riley, 2013; Kok et al., 2015; Rossi et al., 2015; Wakayama et al., 2015), genetic ablation/mutation can lead to a different phenotype than when the protein is removed acutely via inhibition of translation (morpholinos). At least for some genes, it has been demonstrated that compensation occurs in the case of deleterious mutations but not in the case of more acute, morpholino-based gene knockdowns (Gopinathan et al., 2014). Cyclin E has been shown to compensate for cyclin A in fibroblasts (Winston et al., 2000), but such compensation does not occur in embryonic stem cells, and we found no evidence of cyclin E up-regulation in cyclin

Figure 5. Cyclin A2 is necessary to prevent a high rate of abnormal k–mt interactions in MII-stage oocytes. Representative images of k–mt attachments in cold-treated control and cyclin A2 knockout oocytes fixed at 12.5 h after hCG as oocytes reach MI (a) and at 14.5 h after hCG when oocytes have arrested at the mature MII stage. Arrow shows stabilized non-kMT. (c). (a and c, insets) Examples of different attachment types (5 shows normal polar attachment; 2 and 6 show lateral attachments; 3, 4, and 8 show examples of attachments in nonspindle chromosomes and microspindles; and 1 and 7 show merotelic attachments). Bars: (yellow) 10 µm; (orange) 2 µm. Analysis of the different attachment types at 12.5 h (b) and 14.5 h (d) in cyclin A2<sup>−/−</sup> oocytes (n = 11 and 19, respectively) and cyclin A2<sup>+/−</sup> oocytes (n = 14 and 16, respectively). Numbers in parentheses represent total number of attachments examined. Note the increase in lateral and merotelic attachments and the delay in establishing polar attachments in cyclin A2<sup>−/−</sup> oocytes. ***, P < 0.001. Data on attachment types were pooled from the indicated number of oocytes and analyzed using a χ² test.
A2<sup>−/−</sup> oocytes (unpublished data). The genetic approach adopted in this study revealed increases in merotelic attachments and lagging chromosomes in MII oocytes, but we could find no evidence to support a role for cyclin A2 in deprotection of cohesin and subsequent sister separation.

In conclusion, cyclin A2 plays an essential role in ensuring the faithful separation of chromosomes specifically during MII. The absence of an effect in MI defines a clear difference in the role and regulation of kt–mt attachments and error correction between the two meiotic divisions. Whereas in somatic cells, cyclin A2 destruction marks the switch from prometaphase to metaphase, no such switch occurs in MII oocytes, where inhibition of the APC/C for MII arrest also allows the accumulation of cyclin A2. The persistence of cyclin A2 during the prefertilization MII arrest may provide the oocyte with the flexibility in kt–mt attachments for improved error correction during the prolonged MII arrest. Finally, this role of cyclin A2 in ensuring the fidelity of MII has implications for the origins of infertility, where chromosome anomalies in MII are known to contribute to aneuploidy in early mammalian embryos (Hassold and Hunt, 2001).

### Materials and methods

#### Mouse strains and genotyping

Homozygous *cyclin A2<sup>fl/fl</sup>* mice (C57BL/6J) were obtained from the laboratory of P. Sicinski and crossed with Zp3-Cre transgenic mice (C57BL/6J; provided by E. McLaughlin, University of Newcastle, New South Wales, Australia) that specifically express Cre in growing oocytes to generate *cyclin A2<sup>−/−</sup>* oocytes. Control animals were littermates possessing two loxP-flanked alleles without Cre (*cyclin A2<sup>+/+</sup>), as shown in Fig. S1a. Genotyping of cyclin A2 knockout mice was performed as described previously (Kalaszczyńska et al., 2009) with primers p1 (5′-CGCAGCAAAAGCTCAAGACTCGAC-3′) and p2 (5′-CTTACATCTAATGGAATGCCTTG-3′; Fig. S1b).

#### Oocyte culture and manipulation

Oocytes were collected from 4–6-wk-old female mice. For in vitro maturation, GV oocytes were collected in M2 medium (Sigma-Aldrich) containing 200 µM IBMX. Culture was performed in drops of M16 medium (Sigma-Aldrich) under mineral oil (Sigma-Aldrich) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. For cold treatment, oocytes were incubated in 4°C M2 medium for 10 min (MI stage) or 8 min (MII stage). For collection of MII-stage oocytes, mice were superovulated by sequential intraperitoneal injections of 8 IU pregnant mare’s serum gonadotropin (PMSG; Intervet) and 8 IU human chorionic gonadotropin (hCG; Intervet) at an interval of 48 h before culling the mice and collecting the eggs from oviducts. Parthenogenesis was performed using 7% ethanol for 5 min at 37°C at 16–18 h after hCG or after 18 h of in vitro culture from the GV stage. MII-stage oocytes from *cyclin A2<sup>fl/fl</sup>* and *cyclin A2<sup>−/−</sup>* mice were collected and exposed to ethanol to induce parthenogenetic activation. For collection of one-cell zygotes, mice were placed with a male immediately after hCG, and fertilized eggs were released from the oviduct after 24 h.

#### In vitro transcription and microinjections

Cyclin A2–GFP mRNA (a gift from J. Pines, The Institute of Cancer Research, London, England, UK) was transcribed using the T7 mMessage mMachine kit (Ambion) according to the manufacturer’s protocol and resuspended in RNase-free water. mRNA (1.2 mg/ml) was delivered to GV-stage–arrested oocytes using a picopump. After injection, oocytes were washed out of IBMX and transferred in M16 medium. For Western blot, the injected oocytes were incubated in 4°C M2 medium for 10 min (MI stage) or 8 min (MII stage). For collection of MII-stage oocytes, 8 min (MII stage). For collection of MII-stage oocytes, mice were superovulated by sequential intraperitoneal injections of 8 IU pregnant mare’s serum gonadotropin (PMSG; Intervet) and 8 IU human chorionic gonadotropin (hCG; Intervet) at an interval of 48 h before culling the mice and collecting the eggs from oviducts. Parthenogenesis was performed using 7% ethanol for 5 min at 37°C at 16–18 h after hCG or after 18 h of in vitro culture from the GV stage. MII-stage oocytes from *cyclin A2<sup>fl/fl</sup>* and *cyclin A2<sup>−/−</sup>* mice were collected and exposed to ethanol to induce parthenogenetic activation. For collection of one-cell zygotes, mice were placed with a male immediately after hCG, and fertilized eggs were released from the oviduct after 24 h.

#### Western blot

Oocytes were collected in SDS sample buffer and heated for 10 min at 70°C. Proteins were fractionated at 200 V for 30 min using a 10% NuPAGE Bis-Tris precast gel (Invitrogen) and MOPS running buffer.
Data analysis was performed using a t test or a χ² test with Microsoft Excel and GraphPad Prism Software. Analysis was performed for at least three replicate experiments and is represented as means and SEMs unless otherwise stated. Differences at P < 0.05 were considered significant. Level of significance is denoted by *, P < 0.05; **, P < 0.01; *** P < 0.001. Columns with different letters are significantly different.

Online supplemental material

Fig. S1 shows the generation of mice with disruption of cyclin A2 in oocytes and the deletion of cyclin A2 in oocytes. Fig. S2 shows that the exogenous expression of cyclin A2 rescues the egg activation phenotype. Fig. S3 shows MII spindle formation in cyclin A2−/− oocytes using live-cell imaging. Fig. S4 shows no increase in aneuploidy in cyclin A2−/− oocytes. Videos show anaphase I to MII transition of cyclin A2−/− (Video 1) and cyclin A2+/− (Videos 2 and 3) oocytes.

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Proteins were blotted onto polyvinylidene fluoride membranes for 1.5 h at 30 V, and then the membrane was blocked in TBST containing 5% skimmed milk for 2 h, followed by incubation overnight at 4°C with mouse monoclonal anti-cyclin A antibody (1:500; e4710; Sigma-Aldrich). Mouse anti-securin (1:500; ab3305; Abcam) and mouse anti–β-actin (1:1,000; ab3220; Abcam) antibodies were also used for Western blot. For primary antibody detection, we used a goat HRP–conjugated anti–mouse secondary antibody (1:1,000; 1 h at 37°C; P0447; Dako). Finally, the membrane was processed using standard ECL techniques (GE Healthcare) and processed using the ChemiDoc MP imaging system (Bio-Rad). Densitometric analyses of band intensities were performed with ImageJ software.

Immunofluorescence and imaging

Oocytes or eggs were fixed in a solution of 4% paraformaldehyde and 2% Triton X-100 in PBS for 40 min at 25°C. Blocking was performed in PBS with 10% BSA and 2% Tween for 60 min at 25°C. The following antibodies were used for immunolabeling: CREST (1:50; 90C-CS1058; Fitzgerald) and FITC–conjugated mouse anti–α-tubulin (1:200; Alexa Fluor 488; Invitrogen). Goat anti–human IgG antibody (1:100; Alexa Fluor 555; Invitrogen) was used to detect CREST. For analysis of cold-stable MTs, MI-stage oocytes were exposed to ice-cold M2 media for 10 min immediately before fixation, and MI-stage oocytes were exposed to ice-cold M2 media for 8 min immediately before fixation. DNA was labeled using 10-min incubation in Hoechst 33342 (10 µg/ml; Sigma-Aldrich). Serial Z sections of fixed oocytes/eggs were acquired at room temperature in a glass-bottomed dish using a laser-scanning confocal microscope imaging system (SP8; Leica). Imaging of fixed samples was performed using a 63× oil immersion objective (1.4 NA) at 25°C. For live-cell imaging, eggs were microinjected with or without EB1-GFP mRNA and incubated in drops of M2 medium containing Hoechst 33342 (5 µg/ml) under mineral oil. Live-cell imaging was performed using a Leica SP8 confocal microscope with a 40× water immersion objective (1.1 NA) at 37°C. Image acquisition was performed using Las X software (Leica). Imaris software was used to orient 3D confocal stacks for measurement of spindle length and width.

Photoactivated fluorescence imaging and analysis

PAGFP-α-tubulin mRNA (a gift from G. FitzHarris [University of Montreal, Montreal, Quebec, Canada] and S. Nakagawa [Centre Hospitalier de l’Université de Montréal, Montreal, Canada]) was transcribed using the T7 mMessage mMachine kit according to the manufacturer’s protocol and resuspended in RNase-free water. X-rhodamine–labeled tubulin protein (Cytoskeleton, Inc.) was dissolved in general tubulin buffer (Cytoskeleton, Inc.) containing 1 mM GTP sodium salt (Cytoskeleton, Inc.). The final concentration for microinjection was 70 µg/ml. PAGFP-α-tubulin mRNA and X-rhodamine–labeled tubulin were co-injected as described previously. Photoactivation was performed by using the FRAP function on a Leica SP8 confocal microscope with a 40× water immersion objective (1.1 NA) at 37°C. Throughout the experiment, oocytes were in M2 medium containing Hoechst 33342 (5 µg/ml) under mineral oil. Fluorescence decay curves and data analysis were performed as described previously (Kabeche and Compton, 2013). Specifically, for each egg, the decay in PAGFP fluorescence intensity was plotted against time and fitted to a double exponential curve $f(t) = A \times \exp(-k_1t) + B \times \exp(-k_2t)$ using MATLAB’s cftool, where $t$ represents time; $A$ is the less stable non-kMT population; and $B$ is the kMT population with decay rates of $k_1$ and $k_2$, respectively. The half-life for each process was calculated as $\ln(2)/k$. Data for which $A > 95%$ (two oocytes) or where $Re^2 < 0.99$ (one oocyte) were not included in the analysis.

Chromosome spreads

Oocytes were exposed to Acid Tyrodes solution (Sigma-Aldrich) for 1 min at 25°C to remove the zona pellucida. After a 2-min recovery in M2 medium, the oocytes were transferred onto a clean microscope slide that had been previously dipped in a solution of 1% paraformaldehyde in distilled H2O, pH 9.2, containing 0.15% Triton X-100 and 3 mM diethiothreitol (Hodges and Hunt, 2002). The slides were allowed to dry slowly in a humid chamber overnight, and the fixed oocytes were blocked with 10% BSA and 2% Tween for 1 h at 25°C. The oocytes were then incubated with anti–Bub3 antibody (1:50; H-100; Santa Cruz Biotechnology) overnight at 4°C before being incubated with the goat anti–rabbit IgG antibody (1:100, Alexa Fluor 488) for 2 h at 25°C. DNA on the slides was stained with Hoechst 33342 (10 µg/ml) for 10 min, and slides were mounted for observation using a laser-scanning confocal microscope imaging system as described previously.

Polysome array

Data were obtained from GEO under accession no. GSE35106 (Chen et al., 2011).

Statistical analysis

Data analysis was performed using a $t$ test or a $\chi^2$ test with Microsoft Excel and GraphPad Prism Software. Analysis was performed for at least three replicate experiments and is represented as means and SEMs unless otherwise stated. Differences at $P < 0.05$ were considered significant. Level of significance is denoted by *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Columns with different letters are significantly different.

Online supplemental material

Fig. S1 shows the generation of mice with disruption of cyclin A2 in oocytes and the deletion of cyclin A2 in oocytes. Fig. S2 shows that the exogenous expression of cyclin A2 rescues the egg activation phenotype. Fig. S3 shows MII spindle formation in cyclin A2+/− and cyclin A2−/− oocytes using live-cell imaging. Fig. S4 shows no increase in aneuploidy in cyclin A2−/− oocytes. Videos show anaphase I to MII transition of cyclin A2−/− (Video 1) and cyclin A2+/− (Videos 2 and 3) oocytes.

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Author contributions: Most of the experiments were performed by Q.-H. Zhang, W.S. Yuen, D. Adhikari, G. FitzHarris, I Nabti, and P. Marangos provided support and discussions throughout; J. Flegg analyzed the photoactivation data; M. Conti performed the experiments in Fig. 3 c; and P. Sicinski provided the floxed cyclin A2 mice and contributed to the interpretation of data. J. Carroll coordinated the project and wrote the paper with Q.-H. Zhang.

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