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Aurora Kinase B Modulates Chromosome Alignment in Mouse Oocytes

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SUMMARY

The elevated incidence of aneuploidy in human oocytes warrants study of the molecular mechanisms regulating proper chromosome segregation. The Aurora kinases are a well-conserved family of serine/threonine kinases that are involved in proper chromosome segregation during mitosis and meiosis. Here we report the expression and localization of all three Aurora kinase homologs, AURKA, AURKB, and AURKC, during meiotic maturation of mouse oocytes. AURKA, the most abundantly expressed homolog, localizes to the spindle poles during meiosis I (MI) and meiosis II (MII), whereas AURKB is concentrated at kinetochores, specifically at metaphase of MI (Met I). The germ cell-specific homolog, AURKC, is found along the entire length of chromosomes during both meiotic divisions. Maturing oocytes in the presence of the small molecule pan-Aurora kinase inhibitor, ZM447439 results in defects in meiotic progression and chromosome alignment at both Met I and Met II. Over-expression of AURKB, but not AURKA or AURKC, rescues the chromosome alignment defect suggesting that AURKB is the primary Aurora kinase responsible for regulating chromosome dynamics during meiosis in mouse oocytes.

INTRODUCTION

In humans, 1–4% of sperm from healthy human males are aneuploid (Brandriff et al., 1994), whereas approximately 20% of human oocytes are aneuploid (Pacchierotti et al. 2007). If an aneuploid gamete fertilizes or is fertilized by a gamete of the opposite sex, the resulting aneuploid preimplantation embryo may fail to develop (Pellestor et al., 1994; Iwarsson et al., 1999; Sandalinas et al., 2001) or implant (Munne et al., 2003). If implantation occurs, the embryo may undergo spontaneous abortion (Fritz et al., 2001), and if development goes to term, congenital disorders may be observed (Hassold and Chiu, 1985). This difference in aneuploidy incidence most likely involves the difference in timing of meiosis between the two sexes. Males undergo spermatogenesis continuously beginning at puberty with a stem cell population generating the supply of male germ cells that continuously give rise to

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daughter cells that undergo meiosis. In contrast, oocytes in females enter the first meiotic prophase during fetal life and the female is born with the full complement of oocytes that are contained in primordial follicles and become arrested in the dictyate stage of meiosis I (MI). In humans, the onset of puberty initiates both growth of primordial oocytes and resumption of meiosis in response to a gonadotropin surge. The ovulated oocytes arrest at metaphase II (Met II), and only complete the second meiotic division upon fertilization.

Accurate chromosome segregation depends upon proper chromosome condensation, bipolar spindle formation, chromosome alignment, and cytokinesis. Aneuploidy can arise from errors in any of these cellular events. In oocytes, MI spindle formation and chromosome alignment abnormalities are linked to aneuploidy and increase with maternal age (Hunt and Hassold, 2008). In mice, the MI spindle forms de novo from a network of cytoplasmic microtubules (Schuh and Ellenberg, 2007) and microtubules nucleate to make connections with chromosome through a proteinaceous structure called the kinetochore that is associated with centromeric regions of DNA. In somatic cells, improper attachments of microtubules to kinetochores are common and are corrected by Aurora kinase B (Lampson et al., 2004). Disruption of Aurora kinase B function leads to chromosome segregation defects that include nondisjunction and lagging chromosomes (Kallio et al., 2002; Murata-Hori and Wang, 2002; Ditchfield et al., 2003; Hauf et al., 2003).

The Aurora kinases are a conserved family of serine/threonine kinases that function in mitosis and meiosis. Mammals contain three homologs—Aurora kinase A (AURKA), Aurora kinase B (AURKB), and Aurora kinase C (AURKC), whose expression and activity levels are up-regulated in a vast array of human cancers (Sen et al., 1997; Bischoff et al., 1998; Vader and Lens, 2008). In mitotic NIH3T3 cells, AURKA localizes to centrosomes, the organelle that nucleates and organizes microtubules to form a spindle, and spindles where it regulates centrosome separation, bipolar spindle assembly, and chromosome segregation (Gopalan et al., 1997). In human cell lines AURKB is a chromosomal passenger protein that localizes to kinetochores (Hauf et al., 2003) and in mouse and rat cell lines AURKB is found in the spindle midzone (Shindo et al., 1998; Terada et al., 1998). In human cell lines, AURKB similarly functions in chromosome condensation, alignment, and segregation, as well as cytokinesis (Adams et al., 2001). Little is known about AURKC and although AURKC was originally identified as a testis-specific homolog in mice (Gopalan et al., 1997, 1999; Yanai et al., 1997; Tseng et al., 1998), it is also over-expressed in a number of human cancer cell lines, including HeLa cells, where it localizes to centrosomes with AURKA (Kimura et al., 1999). In human tissue culture cell lines, however, AURKC colocalizes with AURKB at centromeres and expression of AURKC can rescue the multinucleation phenotype observed in cells depleted for AURKB suggesting that AURKC function can overlap with that of AURKB (Sasai et al., 2004). Interestingly, AURKB and AURKC have nonoverlapping functions in mouse spermatogenesis. Testis sections from mice expressing catalytically inactive AURKB contain spermatocytes with increased apoptosis and meiotic arrest whereas mice lacking AURKC form mature sperm with abnormal heads and chromatin condensation defects (Kimmins et al., 2007).

Because the Aurora kinases are over-expressed in many cancers, several pharmacological inhibitors have been developed (Gautschi et al., 2008). However, the high percentage of amino acid conservation in the catalytic domains of the three mammalian Aurora kinases prevents many of these inhibitors from specifically targeting one kinase. ZM447439 (4-(4-(N-benzoylamino)anilino)-6-methoxy-7-(3-(1-morpholino)propoxy)quinazoline) inhibits recombinant AURKA and AURKB in in vitro kinase assays with IC₅₀ values of 110 and 130 nM, respectively (Ditchfield et al., 2003). Both human cancer cell lines and spermatocytes treated with ZM447439 exhibit chromosome alignment, segregation, and cytokinesis defects (Ditchfield et al., 2003; Wang et al., 2006b). Mouse oocytes treated with

ZM447439 fail to progress to Met II and contain improperly condensed and misaligned chromosomes possibly due to the hypo-phosphorylation of histone H3 on S10 and S28 (Wang et al., 2006a; Swain et al., 2008).

To understand the molecular mechanism(s) that lead to the high incidence of aneuploidy in human oocytes, we studied the requirement of the Aurora kinases during meiotic maturation in mouse oocytes where the rates of aneuploidy range from 8% to 12% (Zuccotti et al., 1998; Pan et al., 2008). We report for the first time the localization of all three AURKs in mouse oocytes. AURKA co-localizes with Microtubule Organizing Centers (MTOCs), which are acentriolar and with polar microtubules at both Met I and Met II, whereas AURKB concentrates at kinetochore regions of chromosomes, specifically at Met I and not at Met II. During the MI–MII transition, both AURKA and AURKB re-localize to the spindle midzone. AURKC, the germ cell-specific homolog, localizes along the entire length of chromosomes, including the centromere region at Met I and Met II. Consistent with previous reports, inhibition of the Aurora kinases with ZM447439 retards meiotic progression and causes chromosome misalignment at Met I and Met II. Importantly, over-expression of AURKB in ZM447439-treated oocytes, but not AURKA or AURKC, partially restores chromosome alignment at Met I suggesting that the observed chromosome alignment defects can be specifically attributed to AURKB.

RESULTS

Aurka-c mRNAs Are Present in Mouse Oocytes and Eggs

To determine the relative abundance of *Aurka*, *Aurkb*, and *Aurkc* transcripts we isolated mRNA from fully grown oocytes and Met II-arrested eggs from sexually mature mice. Normalizing against Protein Kinase A (*Prkaca*) mRNA, a critical regulator of meiotic resumption in oocytes (Bornslaeger et al., 1986) and assuming that the different Taqman probes prime with similar efficiency, we found that *Aurka* mRNA is more abundant at both stages compared to *Aurkb* and *Aurkc* mRNAs (Fig. 1). *Aurka* mRNA is 9- and 7-fold more abundant than *Aurkb* mRNA at the GV and Met II stages, respectively, whereas it is 18- and 20-fold more abundant than *Aurkc* mRNA at the GV and Met II stages, respectively. In contrast to many maternal mRNAs whose degradation is triggered by initiation of oocyte maturation (Schultz, 1993; Su et al., 2007), all three *Aurk* mRNAs appear relatively stable. These data indicate that all three AURKs are expressed in the oocyte and their relative abundances are consistent with a previously published report which also found that *Aurka* is the most abundantly expressed isoform (Swain et al., 2008). In contrast to Swain et al., however, we found that *Aurkc* is not expressed at equal levels as *Aurkb*. The difference of these results may reflect differences the assay (i.e., Sybr Green vs. Taqman).

AURKA Localizes to Meiotic MTOCs and Spindle Poles

To assess the spatial-temporal localization of AURKA during oocyte maturation, we isolated GV-intact oocytes, matured them in vitro and performed immunocytochemistry at the indicated meiotic stages (Fig. 2). AURKA staining was restricted to sharp, punctuate spots surrounding the nucleus in GV-stage oocytes (Fig. 2A). Many of these spots co-localized with γ -tubulin (arrows), consistent with a previous report demonstrating that AURKA co-localizes with MTOCs (Saskova et al., 2008). AURKA remained in punctate spots surrounding the region of spindle formation during germinal vesicle breakdown (GVBD) and all of the observed AURKA spots co-localized with γ -tubulin. At metaphase I (Met I) AURKA associated with the spindle poles. At anaphase I (Ana I) AURKA was dispersed throughout the cytoplasm (data not shown) and was then observed at the spindle midbody during telophase I (Telo I) when the first polar body is formed. By Met II, AURKA was once again localized to the spindle poles (Fig. 2A).

To confirm our immunocytochemistry data, we microinjected an mRNA encoding *Aurka-eGfp* into GV-intact oocytes. The localization of AURKA-eGFP was consistent with the results seen using immunocytochemistry because the fluorescent signal was detected on the poles of the Met I spindle (Fig. 2B). These data also indicated that a stronger AURKA signal was always observed at one pole compared to the other. Thus, AURKA is asymmetrically localized on the MI spindle, as are several other proteins (e.g., pericentrin, γ -tubulin, and phospho-MARCKS) (Carabatsos et al., 2000; Meng et al., 2004; Michaut et al., 2005); the functional consequence of this asymmetry is not clear. In somatic cells, AURKA co-localizes with centrosomes and spindle poles during prophase and metaphase where it plays a role in centrosome maturation and bipolar spindle assembly. AURKA also associates with the spindle during telophase (Bischoff et al., 1998). AURKA localization in oocytes appears identical to that of somatic cells suggesting that AURKA may play a similar role in spindle formation and cytokinesis during meiotic maturation.

AURKB-eGFP Is Concentrated at Kinetochores

We attempted to determine the localization of AURKB using immunocytochemistry but were unable to detect a specific signal despite using several different antibodies and fixation conditions (data not shown). As an alternative, we generated *Aurkb-eGfp* mRNA that was microinjected into GV-intact oocytes, which were then matured in vitro. Upon meiotic resumption and through Met I, AURKB-eGFP localized with chromosomes (Fig. 3A). At higher resolution, AURKB-eGFP was enriched at centromeres/kinetochores as indicated (Fig. 3B) and co-localized with a portion of signal from staining with CREST, an anti-serum that recognizes several components of the kinetochore complex (Fig. 3C). This partial co-localization can be explained by the fact that CREST anti-sera recognizes proteins in both the kinetochore and centromere (inner kinetochore); in somatic cells AURKB is found in the outer kinetochore. At Ana I, however, AURKB-eGFP relocated to the spindle midzone, and was found at the midbody at Telo I. In Met II eggs, AURKB-eGFP was dispersed throughout the cytoplasm (Fig. 3A). In somatic cells, AURKB is a chromosomal passenger protein that co-localizes to kinetochores through metaphase where it regulates microtubule-kinetochore attachment and bi-orientation of chromosomes (Bischoff et al., 1998). The similar localization of AURKB to the centromere/kinetochore in Met I oocytes and its absence from the kinetochores at MII suggests that AURKB regulates meiotic chromosome dynamics and that this hypothesized role may be specific to MI.

AURKC Localizes With Chromosomes

AURKC, as detected by immunocytochemistry, was dispersed in the cytoplasm of GV-intact oocytes (data not shown) and was found on chromosomes at Met I and Met II (Fig. 4A). Moreover, AURKC co-localized with centromeres marked by CREST anti-serum at Met I (Fig. 4B) and Met II (data not shown) suggesting that AURKC is important in chromosome segregation during both meiotic divisions. The AURKC-eGFP fusion protein localization on chromosomes confirmed our immunocytochemistry data (Fig. 4C).

Inhibition of the Aurora Kinases Retards Meiotic Progression and Causes Chromosome Misalignment

To investigate the function of the Aurora kinases during oocyte maturation, we matured GV-intact oocytes in the presence of increasing concentrations of ZM447439, a small molecule inhibitor that has a similar affinity for AURKA and AURKB (Ditchfield et al., 2003). The affinity of ZM447439 for AURKC has not been reported in the literature. Because AURKC is highly identical in amino acid sequence to AURKB, ZM447439 likely has a similar affinity for AURKC. At lower concentrations (0.1 and 1 μ M), the percentages of oocytes that reached Met I and Met II after 16 hr of treatment were indistinguishable from control DMSO-treated oocytes (Fig. 5A). At higher concentrations (2, 5, and 10 μ M), however, a

significantly larger percentage of oocytes remained at Met I (ranging from 73% to 92%) whereas a significantly smaller percentage of oocytes progressed to Met II when compared to controls. Furthermore, we assessed the effect of the inhibitor (2, 5, and 10 μ M) on chromosome alignment at either Met I or Met II and noted that a significantly greater percentage of oocytes exhibited misaligned chromosomes (ranging from 72% to 86%; Fig. 5B). Although the percentage of oocytes with misaligned chromosomes significantly increased between the 1 and 2 μ M concentrations, there was no significant difference in the percentage of oocytes with misaligned chromosomes following treatment with 2, 5, or 10 μ M ZM447439. We also did not observe any striking differences in the severity of chromosome misalignment amongst oocytes treated with the higher concentrations of ZM447439 (Fig. 5C).

We observed a wide variety of phenotypes associated with Aurora kinase inhibition ranging from a single to multiple unaligned chromosomes and multi-polar to apolar meiotic spindles (Fig. 5C). The majority of ZM447439-treated oocytes exhibited the “severe misalignment” phenotype (~75%) whereas the remaining ~25% either had no spindle and collapsed DNA or a “mild misalignment” phenotype. Thus, these data indicate that at least one of the Aurora kinases is required for proper chromosome alignment and meiotic progression in mouse oocytes.

To determine if the abnormal phenotypes observed when AURKs were inhibited could be reversed, we matured oocytes in vitro in the presence of the inhibitor for 8 hr, a time in which most oocytes reach Met I, washed out the drug and then continued maturation for an additional 10 hr. We found that following transfer of oocytes to inhibitor-free medium, significantly fewer oocytes contained misaligned chromosomes (Fig. 5D). Removal of the drug did not, in general, affect the percentage of oocytes that progressed to Met II with the exception of treatment with 5 μ M of ZM447439 (Fig. 5E). Thus, although the misalignment phenotype could be corrected upon removal of the inhibitor, the oocytes still exhibited meiotic progression defects.

Inhibition of the Aurora Kinases Perturbs Chromosome Alignment at Both Met I and Met II

To further investigate the effect of ZM447439 on chromosome alignment, specifically at Met I, we matured GV-intact oocytes in the presence of the inhibitor for 8 hr, a time by which most oocytes have reached Met I. We found that the same concentrations of the drug that affected chromosome alignment after 16 hr of treatment, namely, 2, 5, and 10 μ M, also caused chromosome misalignment at Met I (Fig. 6A). To assess specifically the effect of ZM447439 on chromosome alignment at Met II, we matured oocytes for 10 hr in the absence of the ZM447439 to allow completion of MI, and then matured them to Met II (8 hr) in the presence of the drug. Interestingly, only the 5 and 10 μ M concentrations of the inhibitor caused significant chromosome alignment defects (Fig. 6B). Because a higher concentration of the drug was required to cause chromosome misalignment at Met II than at Met I, the Aurora kinases may play a greater role in properly aligning chromosomes on the first meiotic spindle than the second. This result also suggests that there is something inherently different (i.e., which isoform acts at MI compared to MII or mechanistic and/or substrate differences) about how Aurora kinases regulate chromosome alignment at Met I as compared to chromosome alignment at Met II.

Over-Expression of AURKB Partially Rescues the Alignment Defect Caused by ZM447439 at Met I

ZM447439 has similar affinities for the three Aurora kinases. Therefore, to determine if one Aurora kinase homolog was the major target responsible for chromosome misalignment, each kinase was over-expressed in ZM447439-treated oocytes, and following maturation

were scored to ascertain if the defects in chromosome alignment were mitigated. Accordingly, we microinjected GV-intact oocytes with mRNA encoding GFP-tagged versions of each kinase, matured GV-intact oocytes in the presence of the inhibitor for 8 hr, and then assessed chromosome alignment at Met I. Over-expression of AURKA and AURKC did not improve the percentage of oocytes with misaligned chromosomes compared to *Gfp*-injected controls (Fig. 7). In contrast, significantly fewer oocytes contained misaligned chromosomes when AURKB was over-expressed (Fig. 7). In somatic cells treated with ZM447439 the observed phenotype was due to an effect on AURKB activity but not AURKA (Ditchfield et al., 2003; Girdler et al., 2006). Consistent with this conclusion, our data suggest that AURKB is responsible for the Met I chromosome alignment defect seen with ZM447439 treatment and that AURKB has a more significant role in aligning chromosomes on the first meiotic spindle than either AURKA or AURKC.

DISCUSSION

We report here for the first time that all three AURK homologs localize to distinct structures in the oocyte during meiotic maturation. Consistent with Yao et al. (2004) we found AURKA on the spindles at Met I and Met II. We did not however find AURKA in the nucleus of GV-intact oocytes. Instead AURKA co-localizes to spots characteristic of MTOCs in GV-intact oocytes and following GVBD (Fig. 2A), and with γ -tubulin at spindle poles during Met I and Met II. In addition, AURKA was found at the midbody during Telo I. Because our immunocytochemistry data of endogenous AURKA was also confirmed and identical to that found using a GFP-tagged AURKA, these discrepancies may reflect differences in fixation techniques and/or sources of AURKA antibodies.

We also report for the first time localization of a GFP-tagged AURKB as well as endogenous AURKC and a GFP-tagged AURKC. Similar to its localization in mitotic cells, AURKB localizes to chromosomes and is enriched at kinetochores specifically at Met I, suggesting it plays a role in homologous chromosome alignment (Fig. 3). Interestingly, AURKB is not found on chromosomes or kinetochores at Met II, the more mitotic-like division where sister chromatids segregate. It was, however, found in the spindle midzone at Ana I, and like AURKA, at the midbody during Telo I, suggesting that both AURKA and AURKB take part in the asymmetric cytokinesis that occurs during first polar body formation. AURKC, which was originally identified as a testis-specific homolog in mouse (Gopalan et al., 1997, 1999; Yanai et al., 1997; Tseng et al., 1998), is found on chromosomes including centromeres at both Met I and Met II (Fig. 4). This chromosomal localization is similar to that seen in cancer cell lines that aberrantly express AURKC (Kimura et al., 1999). It has been suggested that AURKB and AURKC functions overlap in mitosis as expression of AURKC rescues AURKB-depleted cells (Sasai et al., 2004). However, the enrichment of AURKB at kinetochores and the enrichment of AURKC on chromosomes at Met I suggest that they regulate different aspects of homologous chromosome alignment and segregation during the first meiotic division. This hypothesis is also consistent with our data indicating that over-expression of AURKB, but not AURKC, rescues the Met I chromosome alignment defect in ZM447439-treated oocytes (Fig. 7). Further, the absence of AURKB from kinetochores at Met II supports a unique role for AURKC in sister chromatid alignment and segregation during the second meiotic division. Generation of mice lacking either AURKB specifically in the oocyte or AURKC would help to resolve the unique meiotic functions of each of these AURKs.

We found that treatment of mouse oocytes with ZM447439, a pan Aurora kinase inhibitor, retards meiotic progression and perturbs chromosome alignment in a concentration-dependent manner, confirming the results of a previous study (Swain et al., 2008). Our data expand upon that study by finding that Aurora kinase activity is required for chromosome

alignment at both Met I and Met II (Fig. 6). Moreover, removing ZM447439 from the culture medium after 10 hr restores chromosome alignment at Met I, but prevents the oocytes from reaching Met II (Fig. 5D,E). Most importantly, we find that over-expression of AURKB-GFP, but not AURKA-GFP or AURKC-GFP, rescues the chromosome alignment defect at Met I (Fig. 7), a result that is consistent with the finding that the phenotype seen in ZM447439 treated mitotic cells is due to AURKB, and not AURKA (Ditchfield et al., 2003; Girdler et al., 2006). Expression levels of the GFP-tagged AURKs were similar (data not shown) and therefore differences in expression are unlikely to account for the ability of AURKB, but not AURKA or AURKC, to rescue the phenotype. Finally, we find that a higher concentration of ZM447439 is required to perturb chromosome alignment at Met II, where AURKB is absent from kinetochores. This suggests that higher doses of ZM447439 inhibit AURKC at Met I and Met II and that because of its localization on the chromosomes, AURKC may be responsible for chromosome alignment at Met II.

Phosphorylation of histone H3 is associated with chromosome condensation (Hendzel et al., 1997). In mitotic cells AURKB phosphorylates histone H3 (Crosio et al., 2002) and mouse oocytes treated with ZM447439 show hypo-phosphorylation of histone H3 on S10 and S28 (Swain et al., 2008). In contrast, Jelinkova and Kubelka (2006) found that although ZM447439 treatment eliminated phosphorylation of AURKB and histone H3 on S10, the drug did not affect chromosome condensation in porcine oocytes. However, chromosome alignment could not be assessed due to what appears to be a species-specific arrest at the GV stage. If chromosome condensation in mouse oocytes is not affected by ZM447439, the chromosome alignment defect must be due to an AURKB function other than phosphorylation of histone H3. In mitosis, AURKB is a chromosomal passenger protein that, along with INCENP, survivin and borealin regulates kinetochore-microtubule attachment to chromosomes and is essential for proper chromosome tension, and thus, chromosome segregation (Honda et al., 2003; Gassmann et al., 2004; Sampath et al., 2004). Disruption of AURKB's function causes chromosome alignment defects that are an early sign of aneuploidy because cells are unable to correct improper microtubule-kinetochore attachments (Kallio et al., 2002; Murata-Hori and Wang, 2002; Ditchfield et al., 2003; Hauf et al., 2003). The enrichment of AURKB at kinetochores at Met I and its partial rescue of the chromosome misalignment phenotype caused by ZM447439 suggests that AURKB is responsible for regulating chromosome alignment at Met I. Future studies on the role of AURKB at Met I kinetochores will be important for elucidating the molecular mechanisms that contribute to the high degree of aneuploidy due to nondisjunction during the first meiotic division in oocytes.

MATERIALS AND METHODS

Oocyte Collection and Culture

Six-week-old female CF-1 mice (Harlan, Indiananapolis, IN) were injected intraperitoneally with 5 IU of eCG (Sigma-Aldrich, St. Louis, MO). Meiotically competent, germinal vesicle-intact oocytes were collected as previously described (Schultz et al., 1983) into MEM/PVP (bicarbonate-free minimal essential medium supplemented with 100 µg/ml pyruvate, 10 µg/ml gentamicin, 3 mg/ml polyvinylpyrrolidone (PVP), and 25 mM HEPES at pH 7.3) containing 2.5 µM milrinone to inhibit meiotic resumption (Tsafiriri et al., 1996). Cumulus cells were removed by pipetting and oocytes were transferred into milrinone-free CZB (Chatot, Ziomek, and Bavister medium) (Chatot et al., 1989) for meiotic maturation at 37°C and 5% CO₂. All animal experiments were approved by the Institutional Animal Use and Care Committee and were consistent with NIH guidelines.

Quantitative RT-PCR

Total RNA was extracted from GV-intact oocytes and MII eggs using the Absolutely RNA Microprep Kit (Stratagene, Santa Clara, CA) with the addition of 2 ng of *Egfp* RNA to the lysis buffer (Romanova et al., 2006). Reverse transcription was performed using random hexamers and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) as previously described (Anger et al., 2004). The C_t values were determined by real-time PCR using an ABI Prism 7000 (Applied Biosystems) and the following custom Taqman assays (Applied Biosystems): AurkA F 5'GTCTCACTGTGGGAACTTACCA 3', R 5'GCCTGGTGACCCAATAAGTTATACA 3', probe FAM CTG-TGTCGTAGCCT; TCA; AurkB F 5'GGTGCTCACGACCACTGT 3', R 5'CTCGAAGGCCCCAGATTCC; 3', probe FAM CCAGGACTGGGTGTTACA; AurkC F 5' AAGCGGATCTGGAAAC; CT 3', R 5'GACACACACACTGGTAATCCACTAG 3', probe FAM ACAGCGGCACT; CAAG. Assay on demand, Mm00660092 m1, was used to detect *Prkaca*. Relative expression was calculated using the comparative C_t method where the samples were normalized to *Egfp* levels and the *Prkaca* level in a GV-intact oocyte was set to 1. Three independent samples were collected and C_t values were determined in duplicate from 4 oocyte equivalents.

Immunocytochemistry

Following meiotic maturation to the indicated stage, oocytes and eggs shown in Figures 2, 4, and 5 were washed in phosphate buffered solution (PBS) and fixed for 10 min in cold methanol (Electron Microscopy Services, Hatfield, PA). The oocytes and eggs were incubated in blocking buffer consisting of 10% normal goat serum (Vector Labs, Burlingame, CA), 4% BSA, and 0.1% Triton X-100 (Sigma-Aldrich) either overnight at 4°C or for 1 hr at room temperature (RT), respectively. The cells were then incubated with a 1:500 dilution of rabbit anti-AURKA antibody (Bethyl, Montgomery, TX) or a 1:200 dilution of rabbit anti-AURKC antibody (Bethyl) in blocking buffer for 1 hr at RT followed by a 1:200 dilution of monoclonal anti- β -tubulin clone TUB2.1 antibody (Sigma-Aldrich), 1:100 dilution of monoclonal anti- γ -tubulin (Sigma-Aldrich), or a 1:30 dilution of CREST anti-serum (Immunovision, Springfield, AR) in blocking buffer for 1 hr at RT. These incubations were followed by a 1:250 dilution of goat anti-rabbit antibody conjugated to AlexaFluor568 (Invitrogen) and a 1:200 dilution of goat anti-mouse antibody conjugated to FITC (Southern Biotech, Birmingham, AL) or a 1:100 dilution of goat anti-human antibody conjugated to Cy5 (Jackson, West Grove, PA) in blocking buffer for 1 hr at RT. Finally, the oocytes and eggs were stained with a 1:1,000 dilution of 1 mg/ml DAPI in PBS for 20 min at RT before mounting in Vectashield (Vector Labs).

Following microinjection and meiotic maturation to the indicated stage, oocytes and eggs from Figures 3 and 7 were fixed in 3.7% paraformaldehyde in PBS for 1 hr at RT prior to mounting in Vectashield containing 3 μ g/ml propidium iodide (PI).

Cloning and In Vitro Transcription

Egfp was subcloned from pEGFP-N1 (Clontech, Mountain View, CA) into pDP19 (Ambion, Foster City, CA). *Aurka*, *Aurkb*, and *Aurkc* were PCR amplified from a C57BL/6 12.5d embryo cDNA library, TA-cloned into pGEM-TEasy (Promega, Madison, WI), and finally subcloned in frame into pDP19:*Egfp* to create *Aurka*, *Aurkb*, and *Aurkc* fusion constructs with carboxy-terminal eGFP tags. The pDP19: *Aurk*—*Egfp* plasmids were linearized then in vitro transcribed using the mMessage mMachine T7 Ultra Kit (Ambion). *Aurk*—*Egfp* RNA was checked on a denaturing agarose gel for size and polyA tailing.

Microinjection

Oocytes were microinjected in bicarbonate-free Whitten's medium supplemented with 10 mM Hepes (pH 7.3), 0.01% poly-vinylalcohol (Whitten, 1971), and 2.5 μ M milrinone with approximately 7 pl of 1 μ g/ μ l mRNA as previously described (Kurasawa et al., 1989). Oocytes were returned to CZB containing 100 μ M of L-glutamate, 3 mg/ml PVP and 2.5 μ M milrinone for 14 hr and then transferred to milrinone-free CZB for meiotic maturation at 37°C and 5% CO₂.

Microscopy and Image Acquisition

Oocytes and eggs shown in Figures 2, 3, and 5 were viewed on a Zeiss Axiovert 200 M inverted microscope and images were acquired with a Zeiss LSM 510 Meta confocal laser system. Oocytes and eggs from Figure 4 were viewed on a Leica TCS SP laser-scanning confocal microscope (Leica Microsystems, Wetzlar, Germany). Most images were viewed under a 40 $\times$ oil immersion objective (NA 1.25). Images that focus on the chromosomes and kinetochores were viewed under a 63 $\times$ oil immersion objective (NA 1.32). Images were processed using Photoshop software (Adobe Systems, Inc., San Jose, CA).

ZM447439 Treatment

ZM447439 (Tocris, Ellisville, MO) was dissolved in dimethyl sulfoxide (DMSO) at 10 mM and stored in aliquots at -20°C. Appropriate concentrations were prepared in DMSO so that the final concentrations indicated were achieved with a 1:100 dilution in CZB culture medium. A humidified chamber was used for oocyte culture during treatment.

Scoring and Statistical Analyses

Chromosome alignment was scored blind to treatment and percentages from three separate experiments were used for the analyses. Two-way ANOVA or Student *t*-test was used to evaluate the difference between groups using Prism software (Graph Pad Software, Inc., San Diego, CA) with specific test and significance as indicated in the figure legends.

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Abbreviations

Ana I	anaphase I
GV	germinal vesicle
GVBD	germinal vesicle breakdown
MI	meiosis I
MII	meiosis II
Met I	metaphase I
Met II	metaphase II
Telo, I	telophase

ZM447439 4-(4-(N-benzoylamino)anilino)-6-methoxy-7-(3-(1-morpholino)propoxy)quinazoline

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Figure 1.

Relative mRNA levels of *Aurka*, *Aurkb*, and *Aurkc* in GV-intact oocytes and MII-arrested eggs. RNA was isolated from GV-intact oocytes and MII-arrested eggs from sexually mature mice. Following reverse transcription, mRNA levels of *Aurka*, *Aurkb*, and *Aurkc* were determined using quantitative RT-PCR and were normalized against *Prkaca*. Data are shown as mean $\pm$ SEM from three independent experiments.

Figure 2.

Immunocytochemical detection of AURKA during meiotic maturation. **A:** GV-intact oocytes were collected from sexually mature mice and matured in vitro for 0 hr (GV), 3 hr (GVBD), 7 hr (Met I), 10 hr (Telo I), or 16 hr (Met II) prior to fixation in cold methanol and staining with anti-AURKA and anti- γ -tubulin (TUBG1) antibodies. DNA was visualized with DAPI. Merged images show AURKA in red, γ -tubulin in green, and DNA in blue. The arrows point to spots where AURKA and γ -tubulin co-localized. The asterisk indicates the midbody. **B:** GV-intact oocytes were microinjected with *Aurka-eGfp* mRNA, held for 1 hr in maturation medium containing milrinone, and matured in vitro for 7 hr (Met I) prior to fixation in 3.7% paraformaldehyde. Experiments were repeated at least three times with approximately 20 oocytes per stage.

Figure 3.

AURKB-eGFP expression during meiotic maturation. **A:** GV-intact oocytes were microinjected with *Aurkb-eGfp* mRNA, held for 1 hr in maturation medium containing milrinone, and matured in vitro for 0 hr (GV), 3 hr (GVBD), 7 hr (Met I), 9 hr (Ana I), 10 hr (Telo I), or 16 hr (Met II) prior to fixation in cold methanol. DNA was visualized with DAPI. Merged images show AURKB-eGFP in green and DNA in blue. In the GVBD panel, a region containing one chromosome was zoomed in to highlight the association of AURKB and DNA. **B,C:** GV-intact oocytes were microinjected with *Aurkb-eGfp* mRNA, held for 1 hr in maturation medium containing milrinone, and matured in vitro for 7 hr (MI) prior to fixation in 3.7% paraformaldehyde. **C:** Merged image shows AURKB-eGFP in green and CREST in red. The experiments were repeated at least three times with approximately 20 oocytes analyzed at each stage.

Figure 4.

Immunocytochemical detection of AURKC during meiotic maturation. **A:** GV-intact oocytes were collected from sexually mature mice and matured in vitro for 7 hr (Met I), or 16 hr (Met II) prior to fixation in cold methanol and staining with anti-AURKC and anti- β -tubulin antibodies. DNA was visualized with DAPI. Merged images show AURKC in red, β -tubulin in green, and DNA in blue. **B:** Staining with anti-AURKC antibody and CREST serum to mark centromeres. DNA was visualized with DAPI. Merged images show AURKC in red, CREST in green, and DNA in blue. **C:** GV-intact oocytes were microinjected with *Aurkc-eGfp* mRNA, held for 1 hr in maturation medium containing milrinone, and matured in vitro for 7 hr (Met I) prior to fixation in 3.7% paraformaldehyde. Experiments were repeated at least three times with approximately 20 oocytes per stage.

Figure 5.

Effect of ZM447439 on meiotic progression and chromosome alignment. **A:** GV-intact oocytes were treated with concentrations of ZM447439 ranging from 0 to 10 μ M for 1 hr in maturation medium containing milrinone, and matured in vitro in the same concentration of ZM447439 for 16 hr prior to fixation in cold methanol. The spindle was detected with an anti- β -tubulin antibody and DNA was visualized with DAPI. Confocal microscopy was used to determine the stage of meiosis. Data are shown as mean $\pm$ SEM from three independent experiments and were analyzed using two-way ANOVA. *** P < 0.001. **B:** As in (A) but confocal microscopy was used to determine chromosome alignment. Data are shown as mean $\pm$ SEM from three independent experiments and were analyzed using two-way ANOVA. ** P < 0.01, *** P < 0.001. **C:** Representative images for scoring chromosome misalignment and spindle abnormalities. Merged images show β -tubulin in green and DNA in blue. **D:** GV-intact oocytes were treated with concentrations of ZM447439 ranging from 2 to 10 μ M for 1 hr in maturation medium containing milrinone, and matured in vitro in the same concentration of ZM447439 for 8 hr. ZM447439 was washed out of the media (W) in some groups of oocytes and all groups were allowed to continue maturation in vitro for 10 hr prior to fixation in cold methanol. The spindle was detected with an anti- β -tubulin antibody and DNA was visualized with DAPI. Confocal microscopy was used to determine chromosome misalignment. Data are shown as mean $\pm$ SEM from three independent experiments and were analyzed using two-way ANOVA. ** P < 0.01, *** P < 0.001. **E:** As in (D) but confocal microscopy was used to determine the stage of meiosis. Data are shown as mean $\pm$ SEM from three independent experiments and were analyzed using two-way ANOVA. * P < 0.05.

Figure 6.

Effect of ZM447439 on chromosome alignment at Met I and Met II. **A:** GV-intact oocytes were treated with concentrations of ZM447439 ranging from 0 to 10 μ M for 1 hr in maturation medium containing milrinone, and matured in vitro in the same concentration of ZM447439 for 8 hr prior to fixation in cold methanol. The spindle was detected with an anti- β -tubulin antibody and DNA was visualized with DAPI. Confocal microscopy was used to determine chromosome alignment at Met I. Data are shown as mean $\pm$ SEM from three independent experiments and were analyzed using two-way ANOVA. * $P < 0.05$. **B:** GV-intact oocytes were held for 1 hr in maturation medium containing milrinone and matured in vitro for 10 hr, a time at which most oocytes have passed Met I. Concentrations of ZM447439 ranging from 0 to 10 μ M were added to the media and oocytes were allowed to continue maturation in vitro for 8 hr prior to fixation in cold methanol. The spindle was detected with an anti- β -tubulin antibody and DNA was visualized with DAPI. Confocal microscopy was used to determine chromosome alignment at Met II. Data are shown as mean $\pm$ SEM from three independent experiments and were analyzed using two-way ANOVA. *** $P < 0.001$.

Figure 7.

AURKB rescue of chromosome alignment defect caused by ZM447439. GV-intact oocytes were microinjected with *Gfp*, *Aurka-eGfp*, *Aurkb-eGfp*, or *Aurkc-eGfp* mRNA and held for 14 hr in medium containing milrinone. These oocytes were treated with 1.5 μ M ZM447439 for 1 hr in maturation medium containing milrinone and matured in vitro in the same concentration of ZM447439 for 8 hr prior to fixation in 3.7% paraformaldehyde. DNA was visualized with propidium iodide. Confocal microscopy was used to determine chromosome alignment. Data are shown as mean $\pm$ SEM from three independent experiments and were analyzed using Student's *t*-test. **P* < 0.05.