Initial spindle positioning at the oocyte center protects against incorrect kinetochore-microtubule attachment and aneuploidy in mice

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ABSTRACT

Spindle formation and positioning are two critical events that must be regulated tightly to avoid erroneous chromosome segregation. Following nuclear envelope breakdown (NEBD), the spindle is predominantly assembled centrally before migrating towards the cortex to allow the first asymmetric division. The biological significance of primary central positioning of the spindle has not yet been studied. Because the spindle forms where NEBD occurs, nucleus position greatly influences the site of spindle assembly. We find that nucleus positioning is a dynamic process that depends on maternal age. Moreover, we show that preferential initial spindle positioning at the oocyte center is an insurance mechanism to avoid premature exposure to cortical Cdc42 signaling that hinders proper kinetochore-microtubule attachments, thereby protecting against the development of aneuploid gametes. These findings will help in understanding why female gametes are notoriously associated with high rates of aneuploidy, the leading genetic cause of miscarriage and infertility in women.

INTRODUCTION

Infertility is a globally prevalent issue affecting approximately 122 million women worldwide ¹.

Errors in chromosome segregation during meiosis result in aneuploidy, the leading genetic cause of infertility, spontaneous abortion, and congenital birth defects such as down syndrome^{2,3}.

Aneuploidy occurs in females of all ages, but women of advanced age experience elevated risk of aneuploidy ⁴. Given that chromosome segregation errors occur most frequently in female gametes (as opposed to male gametes), particularly within the first meiotic division (Meiosis I, MI)^{5,6}, it is crucial to understand the molecular mechanisms regulating female MI.

Oogenesis is a complex process that begins during fetal development in female mammalian species, shortly after conception. During late pregnancy, oocytes enter MI and duplicate their chromosomes ⁷. Shortly after birth, all germ cells undergo a prolonged arrest that is maintained until puberty. Hormonal cues prior to ovulation stimulate a subset of oocytes to resume meiosis ⁷. In fully-grown prophase I-arrested oocytes, the nucleus (germinal vesicle, GV) localizes mainly at the center ⁸. *In vitro*, peripherally localized nuclei migrate towards the center of the oocyte, regulated by a meshwork of cytoplasmic actin. This meshwork of cytoplasmic actin creates a gradient of velocity (higher near the cortex, lower near the center) induced by Myosin Vb activity and relocates the nucleus centrally through gentle movement governed by active diffusion, rather than exerting a force on the nucleus ⁹⁻¹¹. However, the biological significance of central nucleus positioning is largely unknown.

Upon meiotic resumption, prophase I-arrested oocytes undergo nuclear envelope breakdown (NEBD). Throughout the NEBD process, the microtubule organizing centers (MTOCs) undergo

a three-step fragmentation before clustering and sorting into two dominant spindle poles that are integral in forming a bipolar spindle at the center of metaphase I (Met I) oocytes¹²⁻¹⁵. The spindle dictates the plane of cell division¹⁶, and, therefore, must migrate to the oocyte cortex to ensure the highly asymmetric meiotic division^{16,17}. Extruding a small polar body through a highly asymmetrical division is critical as it allows the egg to retain a majority of the cytoplasmic store of maternal RNAs and proteins synthesized during the growth phase which are essential for fertilization and early embryonic development^{18,19}. However, the necessity of central spindle assembly prior to cortical spindle migration is unknown.

Here we show that nucleus positioning is a dynamic process that is affected by maternal age. A majority of oocytes collected from sexually mature mice had a centrally positioned nucleus, whereas a majority of oocytes from prepubertal mice had a peripherally positioned nucleus. We find that peripheral nucleus positioning is associated with chromosome misalignment, abnormal kinetochore-microtubule (K-MT) attachments and aneuploidy. Importantly, we find that central spindle positioning post-NEBD is critical to avoid deleterious cortical Cdc42 signals that hinder the fidelity of forming kinetochore-microtubule (K-MT) attachments, thereby protecting against aneuploidy in mammalian eggs.

RESULTS

Nucleus Positioning – A dynamic process

Because the spindle forms where NEBD occurs whether at the center (Supplementary Fig. 1A) or at the cortex (Supplementary Fig. 1B), we sorted full-grown prophase I-arrested oocytes according to the positioning of the nucleus into three groups: central, intermediate, and

peripheral; herein after, these groups are referred to as central GV oocytes, intermediate GV oocytes, or peripheral GV oocytes. Peripheral GV oocytes had a distance of 5 μ m or less from the nucleus to the nearest cortex, intermediate GV oocytes remained at a distance of 5 to 15 μ m from the nucleus to the nearest cortex, and central GV oocytes maintained more than 15 μ m distance from the nucleus to the nearest cortex (Fig. 1A). First, we observed the proportions of these groups of oocytes *in vitro* after collection. Oocytes with a centrally located nucleus were the most prevalent, contributing nearly 50.96% of all oocytes collected. Oocytes with an intermediately positioned nucleus accounted for nearly 24.22% of all collected oocytes, while peripheral oocytes represented only 24.81% of collected oocytes (Fig. 1B).

To determine whether nucleus positioning proportions *in vitro* are similar to those *in vivo*, ovaries were collected at four ages (2 weeks, 3 weeks, 5 weeks, and 8 weeks) in order to display nucleus positioning throughout reproductive development. All oocytes and their corresponding nucleus positioning were examined from histological sections of ovaries stained with Hematoxylin and Eosin (Fig. 1C,D). Similar to our *in vitro* results, ovaries from mice aged 8 weeks showed a majority of oocytes with a centrally positioned nucleus (63.52%). Interestingly, younger mice (2 & 3 weeks of age) showed a majority of peripherally located nucleus (50.46% and 55.63%, respectively), while mice aged 5 weeks showed very similar proportions of oocytes with both a centrally and peripherally located nucleus (42.86% and 43.25%, respectively, Fig. 1D,E), suggesting that, before sexual maturation, oocytes have an eccentrically positioned nucleus (likely incompetent) prior to central relocation after sexual maturation.

Oocytes with a peripherally located nucleus associate with higher rates of chromosome misalignment and aneuploidy

Upon meiotic resumption, NEBD is shortly followed by the formation of a bipolar spindle and the alignment of chromosomes at the metaphase plate. The occurrence of peripheral nucleus positioning is correlated with a decreased maturation rate⁸. To assess the meiotic progression, oocytes were collected from mice aged 6-8 weeks and matured for 16 h (Met II). The majority (~92%) of centrally located GV oocytes were able to undergo NEBD. In contrast, peripherally located GV oocytes showed a significant decrease (~61%) in the percentage of NEBD (Figure 2A). However, using time-lapse microscopy, we found no significant difference in the timing of NEBD between centrally located GV oocytes and peripherally located GV oocytes (Figure 2B). Similarly, a majority (~81%) of central GV oocytes were able to extrude the first polar body (PBE), while peripheral GV oocytes were less successful in extruding the first polar body (~59%, Fig. 2C). Once again, there was no significant difference in the timing of PBE when both centrally located GV oocytes and peripherally located GV oocytes were compared (Fig. 2D). To assess the chromosome alignment and spindle morphology among groups, oocytes were matured *in vitro* for either 7 h (Met I) or 16 h (Met II), fixed, and immunostained with α -tubulin to label the spindle and stained with DAPI to label DNA. Chromosome alignment was assessed according to the parameters previously described²⁰. We found a significant increase in the percentage of chromosome misalignment at both Met I or Met II in oocytes with a more peripherally positioned nucleus when compared to oocytes with a centrally located nucleus. Intermediate GV oocytes also showed significantly higher rates of chromosome misalignment when compared to central GV oocytes at Met I, but not at Met II (Fig. 2E,F,H,I). Spindle morphology was assessed using the criterion that a normal meiotic spindle is barrel shaped with two clearly defined spindle poles. Spindle morphology did not vary significantly among groups either at Met I or Met II (Fig. 2E,G,H,J). Because chromosome misalignment is highly

associated with aneuploidy, we then assessed aneuploidy in Met II eggs using *in situ* chromosome counting^{21,22}. Strikingly, segregation errors occurred in oocytes with a peripherally located nucleus (~50.15%) approximately 5 times more frequently than in oocytes with a centrally located nucleus (~10.37%, Fig. 2K,L).

Nuclear envelope breakdown occurs during central relocation of peripherally located nucleus

To understand the behavior of the nucleus and its positioning during MI, we employed time-lapse microscopy to image live oocytes. When peripheral GV oocytes were arrested at prophase I (using milrinone, a phosphodiesterase inhibitor) to prevent NEBD, only ~15% of the oocytes remained peripheral within 3 h of incubation, while the majority (~85%) relocated to the center of the oocyte (Supplementary Fig. 2 and Movie 1), confirming that the nucleus favors central movement, as previously reported^{9,23,24}. When the oocytes were allowed to mature in milrinone-free medium, nuclei initially located at the periphery of the cell underwent $10 \pm 1.256 \mu\text{m}$ on average of central movement before experiencing NEBD. Intermediately located nucleus and centrally located nucleus underwent central movement and traveled a distance of $6 \pm 0.6024 \mu\text{m}$ and $4 \pm 0.6783 \mu\text{m}$, respectively, before NEBD (Supplementary Fig. 3A). However, chromosome alignment in oocytes with a peripherally located nucleus remained relatively closer to the oocyte cortex in comparison to chromosome alignment in oocytes with a central nucleus (Supplementary Fig. 3B,C). It is of note that the timing of chromosome alignment did not vary significantly among groups (Supplementary Fig. 3B,D). Why oocytes favor central nucleus positioning is not fully understood.

Incorrect kinetochore-microtubule attachments are increased in oocytes with a peripherally located nucleus

While aneuploidy can occur as a result of many different errors, one important cause of aneuploidy is a weakened or non-functional spindle assembly checkpoint (SAC). Typically, the SAC is inactivated when MTs are attached correctly to all kinetochores, but a weakened or non-functional SAC may allow the progression of the cell to anaphase without proper K-MT attachments, a phenomenon that is highly associated with aneuploidy. To assess the SAC function in oocytes with a central, intermediate, or peripheral nucleus, oocytes were treated with nocodazole, a MT depolymerizing agent that is known to maintain the SAC active and thereby prevent PBE by inducing Met I arrest^{22,25}. Expectedly, positive control oocytes treated by ZM447439, an Aurora Kinase B/C inhibitor that is known to disrupt the SAC function at a high dose (10 μ M), extruded a PB even in the presence of nocodazole²⁶. In contrast to ZM447439 treated oocytes, all other oocytes (central, intermediate, and peripheral) failed to extrude a PB in the presence of nocodazole, suggesting that the SAC is intact, functional, and therefore not contributing to aneuploidy in peripheral GV oocytes (Supplementary Fig. 4).

Improper K-MT attachment is another major contributor of aneuploidy. Because cortically facing centromeres have relatively high rates of unstable K-MT attachments when compared to those away from the oocyte cortex²⁷ and peripheral nucleus positioning correlates with peripheral spindle assembly (near the cortex), we hypothesized that K-MT attachments are perturbed in peripheral GV oocytes. In meiotic oocytes, even if the SAC is satisfied by present K-MT attachment, it is not necessary that all attachments are correct and homologous chromosomes are bioriented^{20,28}. To assess K-MT attachments, Met I oocytes (~7 h) were exposed to a brief cold shock (6 minutes) in order to depolymerize labile unattached MTs and maintain stable MT fibers

that are attached to kinetochores. The oocytes were immunostained with α -tubulin (to label MTs), CREST (to label kinetochores), stained with DAPI (to label DNA), and imaged using confocal microscopy. K-MT attachments were scored as normal, unattached, or abnormally attached (merotelic or syntelic) (Fig. 3A). Nucleus positioning had no effect on the percentage of unattached kinetochores among all groups (Fig. 3A,B). Importantly, oocytes with peripherally located nucleus displayed ~ four-fold increase in the percentage of incorrect K-MT attachments, compared to oocytes with a centrally located nucleus (Fig. 3A,C). Moreover, the percentages of peripheral ($37.93 \pm 9.17\%$) and intermediate ($39.13 \pm 10.41\%$) oocytes having two or more abnormally attached kinetochores were significantly higher than those in the central group ($7.14 \pm 4.02\%$) (Fig. 3D). These results suggest that improper K-MT attachments are the likely cause, at least in part, of increased aneuploidy in oocytes with a peripherally located nucleus. These results led us to hypothesize that initial spindle formation and its positioning at the oocyte center serves as mechanism to ensure avoidance of cortical influence on the establishment of K-MT attachments, prior to timely spindle migration to the cortex.

Incidence of aneuploidy directly associated with the proximity of early spindle positioning to the cortex

To confirm that the cortical influence during spindle formation is the cause, at least partially, of the high rate of aneuploidy in peripheral GV oocytes, we tracked the movement of the nucleus using live time-lapse microscopy. Oocytes with a peripherally located nucleus demonstrated one of three types of movement: oocytes that were able to achieve complete relocation of the nucleus to the center prior to NEBD were termed ‘corrected’, oocytes that demonstrated central movement prior to NEBD but did not achieve full relocation were termed ‘migratory’, and

oocytes that failed to make a relocation attempt and remained at the cortex through NEBD were termed ‘peripheral’ (Fig. 4A,B and supplementary movies 2-4). Strikingly, oocytes that were able to successfully relocate their nucleus to the center showed relatively lower rates of aneuploidy (comparable to that of oocytes beginning with a central nucleus) when compared to oocytes that failed nucleus relocation and underwent NEBD at the periphery of the oocyte (Fig. 4C).

It is technically feasible to shift the position of the spindle within the oocyte without noticeable adverse effects^{29,30}. Therefore, to further confirm the relationship between aneuploidy and the proximity of early spindle positioning to the cortex, we relocated the spindle prematurely to the cortex through mild centrifugation, a technique proven successful in murine oocytes (Fig. 4D)³¹. Central GV oocytes were matured *in vitro* for 4 h, until prometaphase (the time prior to establishing correct K-MT attachments³²). These prometaphase oocytes were centrifuged, examined live under the microscope and resorted into two groups according to their spindle positioning: those maintaining central positioning (control central) and those with induced peripheral positioning (induced peripheral) prior to *in vitro* maturation for an additional 13 h (Met II). Notably, oocytes with induced peripheral positioning showed significantly higher rates of aneuploidy (58.33%) when compared to oocytes maintaining central spindle positioning after centrifugation (16.67%, Fig. 4E); thus suggesting that peripheral GV oocytes are not inherently erroneous, but instead are subjected to increased levels of cortical influence that is detrimental to the fidelity of chromosome segregation.

Inhibition of the Cdc42 cortical gradient permits normal formation of K-MT attachment in oocytes with peripheral spindle formation

Recent study showed that Cdc42 signals coming from the oocyte cortex can influence K-MT attachments through tyrosination of MTs close to the cortex²⁷. In mitotic cells, tyrosinated MTs are associated with abnormal K-MT attachments³³. From these findings, we hypothesized that premature exposure to increased cortical Cdc42 signals in oocytes with a peripherally positioned spindle during prometaphase could hinder the oocyte's ability to establish and/or stabilize correct K-MT attachments. To this end, oocytes were matured *in vitro* for 5 h. Based on spindle position using live imaging, oocytes were sorted into two groups: central and peripheral oocytes. At this point, the treatment groups were supplemented with a Cdc42 inhibitor (ML141), whereas control oocytes were supplemented with dimethyl sulfoxide (DMSO) for an additional 2 h (Fig. 4F). Met I oocytes were exposed to a 6 minutes cold shock followed by the assessment of K-MT attachments using immunocytochemistry and confocal microscopy. K-MT attachments were scored as normal, unattached, or abnormally attached (merotelic or syntelic). The number of unattached kinetochores did not differ significantly among groups (Fig. 4G). Expectedly, oocytes with peripherally positioned spindles (during prometaphase I) exhibited a significant increase in the percentage of abnormal K-MT attachments, compared to those with a centrally positioned spindle. Importantly, inhibition of cortical Cdc42 signals using ML141 rescued, at least in part, the percentage of abnormal K-MT attachments in non-treated oocytes with a peripherally positioned spindle (Fig. 4H). These results suggest that avoidance of the cortical cdc42 signals during prometaphase I is critical in protecting the oocyte's ability to establish correct K-MT attachments and further undergo faithful chromosome segregation (Fig. 5).

DISCUSSION

Most errors contributing to aneuploid embryos occur in female gametes, specifically within the first meiotic division ^{5,6}; therefore, it is crucial to understand the tightly regulated mechanisms that ensure faithful chromosome segregation. Our findings suggest that central nucleus positioning serves as a preemptive insurance mechanism to ensure that the spindle forms at the center of the oocyte and avoids deleterious cortical Cdc42 signals. These cortical signals are capable of affecting the fidelity of proper K-MT attachment formation. Indeed, when the spindle is prematurely exposed to cortical signals, due to peripheral nucleus positioning or premature induction of cortical positioning of the spindle, the oocyte experiences significantly increased levels of abnormal K-MT attachment and aneuploidy.

Abnormal K-MT attachments during Met I is a major cause of aneuploidy in mammalian oocytes ^{22,34,35}. Our results showed that peripheral GV oocytes that displayed a correction mechanism by relocating their nuclei to the center (before spindle assembly) displayed a significant decrease in the percentage of abnormal K-MT attachments when compared to uncorrected oocytes, suggesting that the cortical signals may have an influence on the ability of the oocyte to establish correct K-MT attachments. In mitotic cells, tyrosinated MTs form early K-MT attachments, but correctly established attachments become detyrosinated and stabilize as spindle tension increases. Incorrect attachments remain tyrosinated (unstable) and are thus more likely to depolymerize ³³. In addition, recent evidence suggests that cortical Cdc42 signals have the capability to influence K-MT attachments and centromeric selection in meiotic oocytes through spindle asymmetry and MT tyrosination. Consistently, we found that inhibition of Cdc42 largely rescued increased abnormal K-MT attachments in peripherally assembled and positioned spindle. Taken together, our results suggest that primary spindle formation and positioning at the center

of the oocyte may serve as an essential protection mechanism against cortical signals capable of influencing improper K-MT attachments.

Nucleus positioning in meiotic oocytes is often studied only *in vitro* or at a single time point *in vivo* and is thus not well understood. There has been a long-standing debate regarding the position of the nucleus in mammalian oocytes. For example, a previous study found that the majority of prophase I-arrested oocytes have a peripherally located nucleus, and this cortical positioning of the nucleus is likely influenced by gap junctions and increased cumulus cell contact ³⁶. On the other hand, other studies demonstrated that the majority of prophase I-arrested oocytes have a centrally positioned nucleus ^{8,37,38}. Interestingly, in the first study ³⁶, the oocytes were collected from mice aged 21 days, whereas in the other studies ^{8,37,38}, the oocytes were collected from mice aged more than 6-8-week old. Our results— that the majority of oocytes collected from 2-3-week old mice exhibited a peripherally located nucleus, whereas the majority of oocytes from 6-8-week old mice exhibited a centrally located nucleus— clearly explain these previous findings and advance our understanding of an important phenomenon in mammalian oocytes.

Although the nucleus shifts from a peripheral position in prepubertal oocytes towards a central position in sexually mature mouse oocytes, this is likely not the only shift during the reproductive life. Indeed, oocytes from aged mice have a majority of peripherally positioned nuclei ⁸. Why and how nucleus positioning is a dynamic process during oocyte development remains unknown. It is unlikely that age-dependent nucleus positioning is due to the difference in the response to gonadotropins because FSH β knockout mice showed similar proportions of

central vs. peripheral GV oocytes to those of WT mice (Kincade, J., Almamun, Marshall, K., M., Rivera, R. and Balboula, A., unpublished data). During oocyte meiosis, prophase I-arrested oocytes have a cytoplasmic mesh of F-actin-coated vesicles that experience increased movement near the cortex and decreased movement near the center. Accordingly, the gradient of F-actin creates a gentle force that encourages the nucleus to position at the center of the cell through active diffusion^{9,10}. Analyzing the cytoskeletal changes within the oocyte during its development and growth may reveal more insight into the underlying mechanisms behind nucleus positioning throughout oogenesis.

Current *in vitro* fertilization (IVF) technologies maintain much room for improvement, as only 12.7% of naturally ovulated IVF cycles in humans produce a pregnancy and only 8.8% of those pregnancies result in live births³⁹. Although technologies and protocols have advanced since the first IVF birth in 1978, the success rate in producing a live birth in women under the age of 35 through IVF remains less than 50%⁴⁰. To date, there are no definitive morphological markers of predictive oocyte quality and successful IVF that are particularly non-invasive⁴¹. Gametes from human females are notoriously associated with high rates of aneuploidy^{5,42}. In addition, human oocytes with a peripherally located nucleus are associated with poor maturation rates compared to those with a centrally located nucleus³⁷. Therefore, understanding the association between peripherally located nucleus and the incidence of aneuploidy may allow for the selection of oocytes predisposed to correct segregation of chromosomes. Additionally, arresting the cell and enabling the nucleus to relocate to central positioning may be a promising approach to increase the overall efficacy of IVF practices, allowing for increases in blastocyst rate, pregnancy rate, and live birth rate in all species.

306

307 MATERIAL AND METHODS

308 *Ethics*

309 All laboratory animals were managed, and experiments conducted, in compliance with the
310 University of Missouri (Animal Care Quality Assurance Reference Number, 9695).

311

312 *Mouse strains*

313 Unless otherwise specified, female CF-1 WT mice were used. *Cep192-eGfp* reporter mice were
314 generated by integrating a construct harboring the EGFP reporter gene into CF-1 mouse genome
315 via CRISPR/Cas9-mediated homology-directed repair. The EGFP reporter was fused at the C-
316 terminus of the endogenous mouse Cep192. Homozygous breeding pairs of *Cep192-eGfp*
317 reporter mice were used to maintain the colony.

318

319 *Oocyte collection and in vitro maturation (IVM)*

320 Full-grown GV oocytes were collected from CF-1 female mice (not primed by pregnant mare
321 serum gonadotropin) aged 6-8 weeks. Oocyte collection and culture were carried out as
322 previously described^{22,43}. Cumulus oocyte complexes (COCs) were collected and cultured in
323 bicarbonate-free minimal essential medium (MEM) supplemented with 3mg/ml
324 polyvinylpyrrolidone (PVP) and 25 mM Hepes (pH 7.3) under mineral oil (MilliporeSigma, St.
325 Louis, MO, USA # M4659, # P2307, # H3784, # M8410). Oocytes were manually denuded and
326 sorted into 3 groups: Central GV, Intermediate GV, Peripheral GV oocytes. Careful rolling of the
327 oocyte ensured correct classification. Oocytes were then transferred to Chatot, Ziomek, and

Bavister (CZB) medium ⁴⁴ supplemented with 1 μ M glutamine (MilliporeSigma, # G8549) under mineral oil and matured at 37°C with humidified 5% CO₂ in an incubator for either 7, 14, or 16 h. Nocodazole (Sigma #M1404), monastrol (Sigma #M8515), ML141 (Sigma #SML0407) and ZM447439 (Tocris #2458) were dissolved in DMSO and added to CZB culture medium at a final concentration of 5 μ M, 100 μ M, 500 nM and 10 μ M, respectively. SiR-tubulin and SiR-DNA were added to the maturation medium (at a final concentration of 100 nM) during live imaging to label MTs and DNA, respectively.

Immunocytochemistry

After maturation, oocytes were fixed in a freshly prepared solution of 3.7% paraformaldehyde (MilliporeSigma, # P6148) dissolved in phosphate buffer saline (PBS). Oocytes were incubated in permeabilization solution (0.1% Triton X-100 in PBS) for 20 minutes and blocking solution (0.3% BSA and 0.01% Tween-20 in PBS) for 20 minutes before staining. Oocytes were incubated at room temperature for one h with the primary antibody before undergoing 3 consecutive washes in blocking solution for 9 minutes each. Oocytes were then incubated at room temperature for one h in the secondary antibody solution. Following another 3 consecutive washes in blocking solution at 9 minutes each, oocytes were mounted on glass slides using Vectashield containing 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI; Vector Laboratories, Burlingame, CA, USA) in order to stain the DNA. Fluorescence was observed using a 40X oil objective using Leica TCS SP8 confocal microscope or Leica DMI8 fluorescence microscope and oocytes were imaged using 3 μ m Z-intervals. Oocytes were analyzed using NIH ImageJ software (National Institute of Health, Bethesda, MD USA).

The following primary antibodies were used in immunofluorescence: conjugated α -tubulin-AlexaFluor 488 (Life Technologies # 322 588; 1:75), CREST autoimmune serum (Antibodies Incorporated # 15-234; 1:25).

In situ chromosome counting

Oocytes matured *in vitro* for 14 h in CZB medium supplemented with L-glutamine under mineral oil were transferred to CZB containing 100 μ m monastrol (MilliporeSigma, # M8515) and incubated an extra 2 h; monastrol is an Eg5-kinesin inhibitor which induces monopolar spindle formation and thus results in a rosette of chromosome distribution^{21,22}. After treatment with monastrol, oocytes at Met II stage were fixed in a freshly prepared solution of 2.5% paraformaldehyde (MilliporeSigma, # P6148) and stained with CREST autoimmune serum (Antibodies Incorporated # 15-234; 1:25) to label the kinetochores. DAPI was used to label the DNA. Fluorescence was observed using a 40X oil objective using a Leica DMI8 microscope and oocytes were imaged using 0.5 μ m Z-intervals in order to observe all kinetochores. Oocytes were analyzed individually and were scored either as euploid (containing 40 kinetochores) or as aneuploid (containing $\pm$ 40 kinetochores).

Assessment of kinetochore-microtubule attachment

Cells were matured *in vitro* for 7 h in CZB medium supplemented with L-glutamine under mineral oil. The oocytes were placed on ice for 6 minutes in a 96 well dish containing chilled MEM. Exposing the oocytes to cold shock depolymerizes unattached MTs; however, when MTs have established end-on attachment to a kinetochore, it becomes cold stable⁴⁵. Oocytes were fixed using a freshly prepared 2.5% paraformaldehyde solution and immunostained with anti-

human CREST (to label kinetochores) and α -tubulin (to label MTs) antibodies. DAPI was used to label DNA. Cells were imaged using a Leica TCS SP8 confocal microscope and a x63 oil objective, taking images at 0.5 μ m Z-intervals. Kinetochores were analyzed for unattached, normal, or abnormal attachments (syntelic - both kinetochores attached to one side of the spindle or merotelic - a kinetochore maintaining attachment to both sides of the spindle). Oocytes were analyzed using NIH ImageJ Software.

Time-lapse microscopy

Full-grown oocytes were imaged using a 40X oil objective on Leica TCS SP8 confocal microscope or Leica DMI8 microscope within a microenvironmental chamber maintaining an atmosphere of 5% CO₂ and temperature of 37°C in humidified air. Imaging acquisition began before NEBD. Images were captured at 5 μ m Z-intervals. Image sequences were analyzed using NIH ImageJ Software.

Ovary collection and histological evaluation

Ovaries from CF-1 mice of varying ages (2 weeks, 3 weeks, 5 weeks, and 8 weeks) were collected, submerged, and stored in buffered zinc formalin overnight. Tissues were then moved to 70% ethanol for storage until undergoing a 10-h protocol using a Leica ASP300S enclosed tissue processor. After processing, tissues were embedded in paraffin wax and cooled fully using the Leica EG1150 C/H pair. Embedded tissues were sectioned at 8 μ m thickness using a Leica Histocore AutoCut microtome. Representative samples were taken for each age by taking 3 consecutive sections periodically (every 4, 6, 8, or 10 sections respectively). Tissues were stained with Hematoxylin and Eosin (Abcam, # 245880) before being mounted with permount

resin (Thermo Fisher Scientific, Inc. # SP15). Oocytes and their corresponding nucleus positioning were examined using 10X or 40X objective on a Leica DMI8 microscope.

Statistical analysis

Tests used to evaluate the statistical significance of the findings reported include One-way ANOVA and Student t-test using GraphPad Prism. The Tukey post hoc test was utilized to determine statistical significance between groups. P values of < 0.05 were considered significant. All data are displayed as means $\pm$ SEM.

Declaration of interest

All authors declare that there are no conflicts of interest that may prejudice or bias the research reported.

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Author Contributions

AZB conceived the project. AZB and JK designed and analyzed experiments. JK performed experiments. AZB and JK wrote the manuscript.

Figure Legends

Figure 1: Nucleus positioning is a dynamic process throughout the development

(A) Full-grown GV oocytes were collected, imaged and classified into one of three groups: central GV, intermediate GV, or peripheral GV according to the distance measured from the nucleus to the nearest cortex. The scale bar represents 50 μm . Quantification of the average distance from the nucleus to the nearest cortex within groups (right graph). One-way ANOVA and Tukey's post hoc test were performed to analyze the data. (B) Representative images of oocytes with a central, intermediate, and peripherally located nucleus. The scale bars represent 100 μm . Quantification of the contribution of each group to all oocytes collected (right graph). One-way ANOVA and Tukey's post hoc test were performed to analyze the data. (C) Histological analysis of nucleus positioning *in vivo* at different ages. Ovaries from 2, 3, 5, and 8 weeks old mice were collected, fixed, and stained with Hematoxylin and Eosin. Sections were observed through 10X and 40X objective magnification and classified into one of the three positioning groups: central, intermediate, peripheral. The scale bar represents 100 μm . (D) Representative images of nucleus positioning in 3 and 8 weeks old mice. The white box emphasizes the location of the magnified image within the ovary. The scale bar represents 100 μm . (E) Quantification of the proportion of oocytes sorted according to their nucleus positioning (central, intermediate, peripheral) in 2, 3, 5, and 8 week old mice. Two-way ANOVA and Sidak's multiple comparison test were performed to determine if the groups differed

significantly. Data are displayed as mean $\pm$ SEM. Values with asterisks vary significantly, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. The total number of analyzed oocytes (from 3 independent replicates) is specified above each graph.

Figure 2: Oocytes with a peripherally located nucleus have higher rates of chromosome misalignment and aneuploidy.

Full-grown prophase I-arrested oocytes were collected and sorted into central, intermediate and peripheral groups based on nucleus positioning and imaged live using time-lapse microscopy during *in vitro* maturation. (A) Quantification of the percentage of oocytes that underwent NEBD. (B) Quantification of the average time of NEBD. (C) Quantification of the percentage of first polar body extrusion (1st PBE). (D) Quantification of the average time of PBE calculated from oocytes successfully extruded the PB. (E-J) Full-grown prophase I oocytes were collected and sorted into central, intermediate and peripheral groups based on nucleus positioning and *in vitro* matured for 7 h (Met I) or 16 h (Met II). Oocytes were fixed and immunostained with α -tubulin to label the microtubules (the spindle). DNA was labeled by DAPI. (E) Representative confocal images of Met I oocytes. (F) Quantification of the percentage of chromosome misalignment in Met I oocytes. (G) Quantification of the percentage of abnormal spindle morphology in Met I oocytes. (H) Representative confocal images of Met II oocytes. (I) Quantification of the percentage of chromosome misalignment in Met II oocytes. (J) Quantification of the percentage of abnormal spindle morphology in Met II oocytes. (K) Full-grown prophase I oocytes were collected and sorted into central, intermediate and peripheral groups based on nucleus positioning and *in vitro* matured for 14 h (Met II). Oocytes were treated with monastrol for 2 h, fixed and immunostained with CREST antibody to label kinetochores.

DNA was labeled by DAPI. Oocytes were analyzed individually and scored either as euploid (containing 40 kinetochores) or as aneuploid (containing $\pm$ 40 kinetochores). Shown are representative images. (L) Quantification of the percentage of aneuploid eggs. The scale bar represents 10 μ m. One-way ANOVA and Tukey's post hoc tests were performed to determine statistical significance among groups. Data are displayed as mean $\pm$ SEM. Values with asterisks vary significantly, * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$. The total number of analyzed oocytes (from at least 3 independent replicates) is specified above each graph.

Figure 3: Oocytes with peripherally located nucleus experience higher levels of incorrect kinetochore-microtubule attachments

(A) Representative images of kinetochore-microtubule (K-MT) attachments in oocytes with a central, intermediate, or peripherally located nucleus. Panels on the right (from top to bottom) display normal K-MT attachment, unattached kinetochores and abnormal K-MT attachment. (B) Quantification of the percentage of unattached kinetochores and (C) abnormal K-MT attachments. (D) Quantification of the oocytes with 2 or more incorrect K-MT attachments. One-way ANOVA and Tukey's post hoc test were performed to analyze the data. Data are displayed as mean $\pm$ SEM. Values with asterisks vary significantly, * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. The total number of analyzed oocytes (from 4 independent replicates) is specified above each graph.

Figure 4: Incidence of incorrect K-MT attachments and aneuploidy associate with spindle proximity to the cortex

(A) Schematic depicting the determination of corrected, migratory, and peripheral oocytes. (B) Representative images of the nucleus movement prior to NEBD. Images were taken every 30 minutes (Z-interval of 5 μ m). The black circle denotes the primary nucleus positioning, while the green circle denotes the final nucleus position prior to NEBD. (C) Quantification of the incidence of aneuploidy in metaphase II oocytes from the oocytes categorized in B. One-way ANOVA and Tukey's post hoc test were performed to determine statistical significance. (D) Schematic depicting the induction of peripheral spindle positioning by mild centrifugation in oocytes collected with a centrally positioned nucleus. (E) Quantification of the incidence of aneuploidy in oocytes with induced peripherally positioned nucleus and centrally positioned nucleus after centrifugation. Student t-test was performed to determine statistical significance. Data are displayed as mean $\pm$ SEM. Values with asterisks vary significantly, * $P < 0.05$. (F) Schematic depicting the treatment of collected oocytes with ML141, a Cdc42 inhibitor, in order to inhibit the cortical influence upon kinetochore-microtubule (K-MT) attachment formation. (G,H) Quantification of the average incidence of unattached kinetochores and abnormal K-MT attachment, respectively. One-way ANOVA and Tukey's post hoc test were performed to determine statistical significance. Data are displayed as mean $\pm$ SEM. Values with asterisks vary significantly, * $P < 0.05$, ** $P < 0.01$. The total number of analyzed oocytes (from 3 independent replicates) is specified above each graph.

Figure 5: A summary schematic depicting the deleterious influence of cortical Cdc42 upon proper K-MT attachments in oocytes with a peripherally positioned spindle that is at least partially contributory to the development of aneuploid gametes. The lower section of the

schematic demonstrates the ability of the oocyte to establish correct K-MT attachments in oocytes with a centrally positioned spindle and thus avoid chromosome mis-segregation.

Supplementary Figure 1: Full-grown prophase I-arrested oocytes were collected from *Cep192-eGfp* reporter mice, sorted into central (A) and peripheral (B) groups based on the positioning of their nucleus and *in vitro* matured in CZB medium containing SiR-tubulin (to label microtubules, magenta). Spindle poles were labeled by CEP192-EGFP (MT organizing centers, green). Oocytes were imaged live using time-lapse confocal microscopy. Shown are representative fluorescence and bright-field (lower panels) image sequence depicting the location of NEBD and consequent spindle formation. Images were taken every 1 h (Z-projection of 13 sections every 5 μ m). The scale bar represents 50 μ m.

Supplementary Figure 2: (A) Representative bright-field images of time-lapse microscopy of the movement of a peripherally positioned nucleus during prophase I arrest in oocytes incubated with milrinone. Images were taken every 10 minutes using a Z-interval of 5 μ m. The black circle identifies the initial positioning of the nucleus, while the green circle denotes the final positioning of the nucleus immediately prior to NEBD. (B) Quantification of the percentage of peripheral and intermediate GV oocytes achieving full relocation of the nucleus to a central position within 3 h of incubation with milrinone.

Supplementary Figure 3: Full-grown prophase I-arrested oocytes were collected and sorted into central, intermediate, and peripheral groups based on the positioning of their nucleus and imaged live using time-lapse microscopy during *in vitro* maturation. (A) Quantification of the change in

the distance of the nucleus to the nearest cortex from the initial position of the nucleus at the prophase I-arrested stage to the final destination of the nucleus immediately prior to NEBD. (B) Representative fluorescence and bright-field (lower panels) image sequence depicting chromosome alignment (labeled by SiR-DNA, red) in oocytes with a central (upper) and peripheral (lower) nucleus. Images were taken every 45 minutes using a Z-interval of 7 μm . Scale bars represent 50 μm . (C) Quantification of the distance of chromosomes at the time of alignment to the nearest cortex. (D) Quantification of the time of chromosome alignment. One-way ANOVA and Tukey's post hoc test were performed to determine statistical significance. Data are displayed as mean $\pm$ SEM. Values with asterisks vary significantly, ** $P < 0.01$, *** $P < 0.001$. The total number of analyzed oocytes (from 3 independent replicates) is specified above each graph.

Supplementary Figure 4: (A) Full-grown prophase I-arrested oocytes were collected, sorted (into central, intermediate, and peripheral groups based on the positioning of their nucleus) and *in vitro* matured in the presence of nocodazole. Randomly selected oocytes were *in vitro* matured in the presence of nocodazole and ZM447439 (positive control group). The percentage of first polar body extrusion (PBE) was assessed at 16 h (Met II). One-way ANOVA and Tukey's post hoc test were performed to determine statistical significance. Data are displayed as mean $\pm$ SEM. Values with asterisks vary significantly, **** $P < 0.0001$. The total number of analyzed oocytes (from 4 independent replicates) is specified above the graph.

Supplementary Movie 1: Time-lapse microscopy of germinal vesicle (GV) oocytes cultured in milrinone-containing medium to maintain prophase I arrest. DIC images were captured every 10 minutes at Z-intervals of 5 μm . The scale bar represents 50 μm .

Supplementary Movie 2: Time-lapse microscopy of peripheral germinal vesicle (GV) oocyte showing corrected nucleus relocation during *in vitro* maturation (Same oocyte used in the top panels of Fig. 4B). DIC images were captured every 10 minutes at Z-intervals of 5 μm . The scale bar represents 50 μm .

Supplementary Movie 3: Time-lapse microscopy of peripheral germinal vesicle (GV) oocyte showing migratory nucleus relocation during *in vitro* maturation (Same oocyte used in the middle panels of Fig. 4B). DIC images were captured every 10 minutes at Z-intervals of 5 μm . The scale bar represents 50 μm .

Supplementary Movie 4: Time-lapse microscopy of peripheral germinal vesicle (GV) oocyte that failed to relocate its nucleus during *in vitro* maturation (Same oocyte used in the lower panels of Fig. 4B). DIC images were captured every 10 minutes at Z-intervals of 5 μm . The scale bar represents 50 μm .

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Fig. 2

Fig. 3**A** **B** **C** **D**

Fig. 4

Fig. 5

A

Cep192/SiR-tubulin/Bright-field

B

Cep192/SiR-tubulin/Bright-field

A

B

A

B

C

D

Supplementary Figure 3