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Reduced Chromosome Cohesion Measured by Interkinetochore Distance Is Associated with Aneuploidy Even in Oocytes from Young Mice[1]

Julie A. Merriman,[3,6] Simon I.R. Lane,[3,6] Janet E. Holt,[3] Phoebe C. Jennings,[3] Irene García-Higuera,[5] Sergio Moreno,[5] Eileen A. McLaughlin,[4] and Keith T. Jones[2,3,6]

[3]*School of Biomedical Sciences & Pharmacy, University of Newcastle, Callaghan, New South Wales, Australia*
[4]*School of Environmental & Life Sciences, University of Newcastle, Callaghan, New South Wales, Australia*
[5]*Instituto de Biología Molecular y Celular del Cáncer, CSIC/Salamanca University, Campus Miguel de Unamuno, Salamanca, Spain*
[6]*Centre for Biological Sciences, Faculty of Natural and Environmental Sciences, University of Southampton, Southampton, United Kingdom*

ABSTRACT

It is becoming clear that reduced chromosome cohesion is an important factor in the rise of maternal age-related aneuploidy. This reduction in cohesion has been observed both in human and mouse oocytes, and it can be measured directly by an increase with respect to maternal age in interkinetochore (iKT) distance between a sister chromatid pair. We have observed variations in iKT distance even in oocytes from young mice and wondered if such differences may predispose those oocytes displaying the greatest iKT distances to be becoming aneuploid. Therefore, we used two methods, one pharmacological (Aurora kinase inhibitor) and one genetic (*Fzr1* knockout), to raise aneuploidy rates in oocytes from young mice (age, 1–3 mo) and to examine if those oocytes that were aneuploid had greater iKT distances. We observed that for both Aurora kinase inhibition and *Fzr1* knockout, iKT distances were significantly greater in those oocytes that became aneuploid compared to those that remained euploid. Based on these results, we propose that individual oocytes undergo loss in chromosomal cohesion at different rates and that the greater this loss, the greater the risk for becoming aneuploid.

aneuploidy, cell cycle, meiosis, meiotic maturation, meiotic spindle

INTRODUCTION

Aneuploidy from meiotic segregation errors in human eggs is very common, and advanced female age is an established risk factor. Various influences have been suggested to contribute to this effect of maternal age; however, a number of recent studies suggest that the gradual loss in the cohesive ties holding chromosomes together during prophase arrest may be important [1–3].

Homologous chromosomes (bivalents) are prevented from undergoing premature separation by virtue of having undergone homologous recombination and having sister chromatids distal to this crossover event locked together by cohesin. Components of the cohesin complex, however, are loaded onto chromosomes in oogonia during the premeiotic S phase, and the resulting oocytes appear to have little-to-no capacity to reload them later in life [4, 5]. With age, therefore, and dependent on the extent of cohesin loss, the two sister chromatids of the bivalent may fall apart, generating two univalents or four single chromatids. In addition, a reduction in cohesion may allow the less tightly associated kinetochores of a bivalent to form erroneous attachments to spindle microtubules. Either the formation of univalents/single chromatids in meiosis I or erroneous microtubule attachment would predispose the oocyte to becoming aneuploid.

Support for a cohesin-deterioration model of aneuploidy has come from studies that correlated advanced maternal age in mice with a loss in the cohesin subunit Rec8 [6, 7]. In both studies, chromosome cohesion was also assessed by measurement of the distance between sister kinetochores, hereafter referred to as interkinetochore (iKT) distance. This was measured either on bivalent spreads of maturing oocytes [6] or on metaphase II-arrested mature eggs following an in situ spreading technique [7]. For either type of iKT measurement, the same trend was observed, with distances being greater in older mice. Importantly the same relationship between increased iKT distance and age was confirmed in oocytes from women between 16 and 50 years of age [8], so the phenomenon of age-associated chromosome cohesion loss likely is conserved in mammals.

We recently reported that iKT distance was observed to increase in mice between the ages of 1 and 15 mo [9]. Furthermore, for the majority of ages examined, greater iKT distance correlated with oocytes that had become aneuploid at the completion of meiosis I. From this previous study, it seems clear that a wide range of iKT distances can be measured in oocytes even from young mice, suggesting that the rate of chromosome cohesion loss with age is not necessarily constant among oocytes. In the present study, we wanted to test the hypothesis that agents capable of raising aneuploidy rates in oocytes from young mice may select out those with higher iKT distances. We used 1-mo-old mice, for which the aneuploidy rate is the lowest of all age ranges we have tested. ZM447439, a pan-inhibitor of Aurora kinases [10], and FZR1 knockout [11, 12] were two approaches used to increase aneuploidy rates. In both instances, we examined if those oocytes that became aneuploid had larger iKT distances.

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[2]Correspondence: E-mail: k.t.jones@soton.ac.uk or keith.jones@newcastle.edu.au

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MATERIALS AND METHODS

Ethics Statement

All animal procedures were performed with approval from the animal ethics committee of the University of Newcastle.

Animals

Mice were either B6CBF1 mice (C57BL6/6JLac × CBA/CaLac; University of Newcastle Animal Services Unit) or oocyte-specific *FZR1* knockout mice that were produced as previously described [13].

Oocyte Collection and Culture

Germinal vesicle (GV)-stage oocytes were used for in vitro maturation and collected 44–52 h after hormonally priming mice by intraperitoneal injection of 10 IU of equine chorionic gonadotropin (Intervet) as described previously [14]. For all other studies, metaphase II mature oocytes were collected from primed animals 12–14 h after a further intraperitoneal injection of 5 IU of human chorionic gonadotropin (Intervet) [14, 15]. Oocytes were cultured in M2 medium with 4% bovine serum albumin (BSA) for bench handling or in Minimum Essential Medium-α (Life Technologies), with 20% fetal calf serum, 40 U/ml of penicillin, and 40 μg/ml of streptomycin with 5% CO_2 in a humidified incubator at 37°C for vitro maturation [11, 13]. Milrinone (10 μM) was used to maintain GV arrest. ZM447439 (10 mM; Tocris) in dimethyl sulfoxide was used at a dilution of 0.1% [10].

Kinetochore Counting

Oocytes were treated for 2 h in 200 μM monastrol before fixation and permeabilization as described previously [9, 10]. Immunofluorescence was performed using antibodies to tubulin (1:400; Molecular Probes) and CREST (1:400; Cortex Biochem) in PHEM (60 mM PIPES, 25 mM HEPES, 25 mM EGTA, 4 mM $MgSO_4$) supplemented with 1% BSA and 0.2% Tween 20 overnight at 4°C and an Alexa 555-conjugated secondary antibody (Molecular Probes) [16]. Oocytes were briefly stained with Hoechst dye (20 μg/ml) before mounting on glass slides with Citifluor (Citifluor Ltd.). Confocal microscopy was performed using an Olympus FV1000 fitted with a 60× objective, and images were analyzed using FV10-ASW 2.0 Viewer software (Olympus) and Metamorph (Universal Imaging). Kinetochore counts were made by comparing CREST and Hoechst staining in each *z*-plane through a stack encompassing the entire spindle [9]. The iKT measurements were only made when sister kinetochores were in the same *z*-plane. Three-dimensional rendering was performed to confirm the structures being analyzed. All oocytes were counted blind, and counts were confirmed by an independent second count.

Statistical Analysis

All statistical analysis was performed using GraphPad Prism 5.0 software. Normality was analyzed using the D'Agostino-Pearson omnibus test. The iKT distance analysis between two groups was performed using a two-tailed Mann-Whitney *U*-test. ANOVA, with Tukey post test, was used for repeated measures between three or more groups. Dichotomous data, such as percentage aneuploidy, were analyzed using a Fisher exact test.

RESULTS

Association of Raised iKT Distance with Aneuploidy in Oocytes Cultured with an Aurora Kinase Inhibitor

Oocytes were collected from the antral follicles of hormonally primed mice (age, 4–6 wk) and in vitro cultured with or without the pan-Aurora kinase inhibitor ZM447439 [17], which was added 4 h after nuclear envelope breakdown to allow progression through meiosis I [10]. Those oocytes that had extruded polar bodies were further processed for in situ chromosome counting by adding the kinesin inhibitor monastrol, which disperses the sister chromatids sufficiently to allow accurate analysis and dissipates any tension across the pair. We and others have used this in situ chromosome counting assay both because oocytes are not susceptible to chromosomal loss during preparation [7, 9, 10, 18] and because it allows accurate measurement of iKT distance without the influence of microtubule stretch across the kinetochores. Kinetochores of the sister chromatids were labeled using CREST antibodies and chromatin visualized using Hoechst dye. Individual oocytes were then analyzed for kinetochore number by confocal microscopy.

ZM447439 significantly raised aneuploidy rates in oocytes from 2% to 15% (n = 2/89 vs. 6/39; $P = 0.001$, Fisher exact test) (Fig. 1A). This aneuploidy, as previously reported, manifests as nondisjunction [10]. Its ability to do this may be due to inhibition of Aurora kinase B, which is normally involved in the destabilization of erroneous microtubule-kinetochore interactions during prometaphase [19, 20]. Oocytes also contain the gamete-specific Aurora kinase C that may also contribute to this error correction pathway and would be predicted to be similarly inhibited by this drug [21]. Persistence of such errors at anaphase onset would consequently lead to chromosome missegregation.

Previously, we observed that iKT distance in metaphase II oocytes collected from superovulated mice increases with female age [9]. Therefore, in the present study, we wanted to measure iKT distances in euploid young oocytes and compare them to oocytes of the same age treated with ZM447439. For this analysis, we randomly selected 20 euploid oocytes from those that had (n = 20/39) or had not (n = 20/89) been treated with ZM447439 during in vitro maturation as well as all of the six aneuploid oocytes that had resulted from Aurora kinase inhibition. The iKT measurements were not possible on all sister kinetochore pairs because either the pair was not contained in the same focal plane or the intensity was not sufficiently strong for accurate measurement. Therefore, in the 46 total oocytes analyzed, we were able to measure iKT distance in 75% (n = 692/920) of all sister kinetochore pairs.

Visual inspection of the frequency distribution plot of iKT distances for each of the three groups (euploid, vehicle-control; euploid, ZM447439-treated; and aneuploid, ZM447439-treated) showed that iKT distances did not follow a normal distribution but, instead, were heavily right-skewed (Fig. 1B). Applying the D'Agostino-Pearson omnibus normality test gave high K2 values due to this skewness, and all frequency distributions thus were not Gaussian ($P < 0.0001$). Therefore, iKT distance data are amenable only to nonparametric analysis.

No significant difference was found in the mean iKT measurements made between the euploid, vehicle-control oocytes and those treated with ZM447439 that ended up being euploid ($P > 0.05$, Kruskal-Wallis test, Dunn multiple comparison). Therefore, these measurements would not support the idea that the drug treatment per se has any capacity to raise iKT distance. However, in oocytes that became aneuploid following addition of ZM447439, iKT distances were significantly larger when compared either to those oocytes that remained euploid or those that were incubated in vehicle alone ($P < 0.05$, Kruskal-Wallis test, Dunn multiple comparison) (Fig. 1C).

Raised iKT Distance Associated with Aneuploidy in FZR1-Knockout Oocytes

FZR1 (aka CDH1) is a coactivator of the anaphase-promoting complex (APC) and in a number of cells, including mouse oocytes, is involved in preventing missegregation of chromosomes [12, 22, 23]. We have previously generated an oocyte-specific knockout of *Fzr1* (*Fzr1*$^{-/-}$) by mating mice with a floxed allele *Fzr1*$^{fl/fl}$ to those expressing Cre-recombinase driven by the *zona pellucida 3* (ZP3) promoter [11, 13] and established that in these oocytes, progression through meiosis I is accelerated due to premature spindle

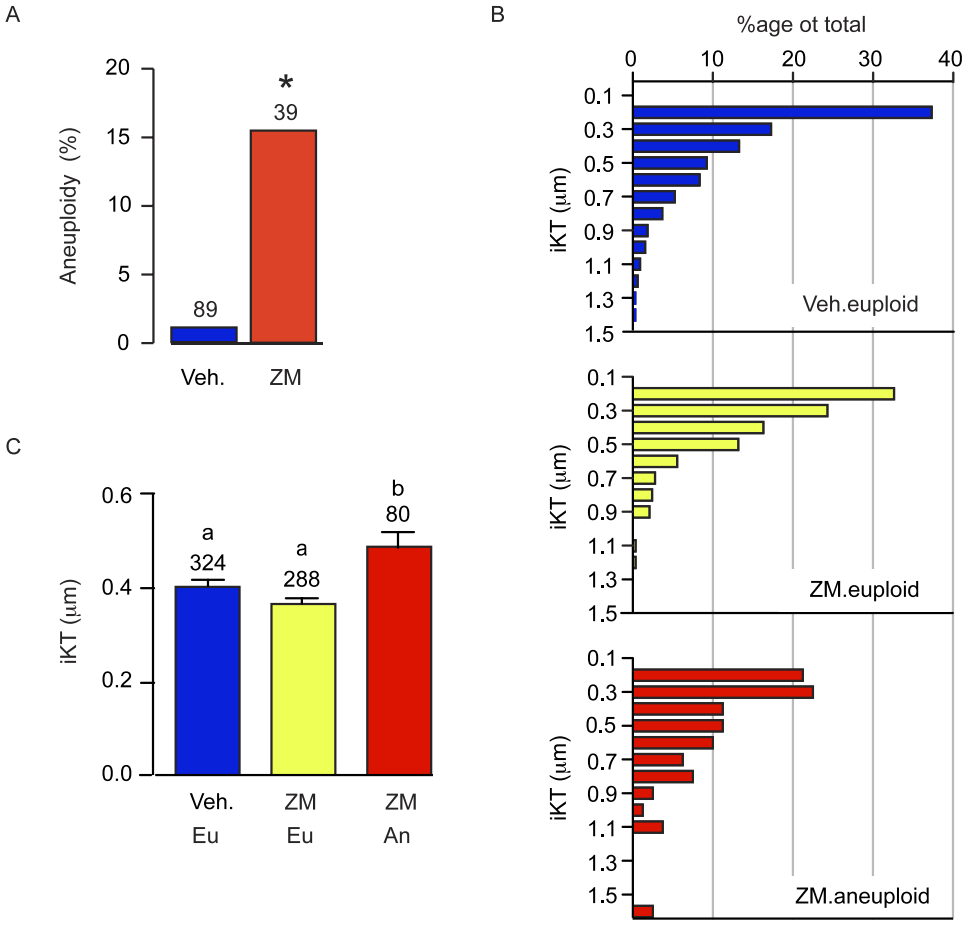

FIG. 1. Association of aneuploidy with iKT distance in oocytes cultured with ZM447439. ZM447439 (10 μM) or dimethyl sulfoxide (vehicle control) were added 4 h after GV breakdown. Numbers of oocytes (**A**) or sister kinetochores (**C**) analyzed are indicated above the bars. **A**) Aneuploidy rates in metaphase II oocytes following in vitro maturation with ZM447439. Asterisk indicates significant difference ($P = 0.001$, Fisher exact test). **B** and **C**) The iKT distances in a random pool of metaphase II oocytes taken from **A** (euploid, untreated; aneuploid and euploid treated with ZM447439) presented as a histogram (**B**) and a bar graph (**C**; mean ± SEM). Different letters denote significant difference ($P < 0.05$, Kruskal-Wallis test, Dunn multiple comparison).

assembly. In mouse oocytes, APC activity is switched on by establishment of microtubule-kinetochore interaction [16, 24–26], which often is initially incorrect (i.e., bivalents are not bioriented) and needs to be corrected during the course of prometaphase I [16, 27]. The accelerated spindle assembly observed in *Fzr1*$^{-/-}$ oocytes probably accentuates erroneous attachments, which are able to switch on the APC but, due to their number, fail to get corrected before the onset of anaphase [11].

Metaphase II oocytes superovulated from *Fzr1*$^{-/-}$ mice had nearly 3-fold rates of aneuploidy (11%, n = 8/72) compared to *Fzr1*$^{fl/fl}$ controls (4%, n = 3/73). This aneuploidy is nondisjunction, as previously reported [11, 12]. Therefore, we measured iKT distances in all 11 aneuploid oocytes (3 *Fzr1*$^{fl/fl}$ and 8 *Fzr1*$^{-/-}$) and in 20 randomly selected euploid oocytes from the *Fzr1*$^{fl/fl}$ and *Fzr1*$^{-/-}$ mice. In total, we were able to measure 83% of the sister kinetochore pairs (n = 853/1029).

The mean iKT distance between sister kinetochores was significantly greater in aneuploid oocytes than in those that remained euploid (mean ± SEM, 0.578 ± 0.026 vs 0.403 ± 0.008 μm; $P < 0.0001$, Mann-Whitney *U*-test) (Fig. 2A). For this analysis, the genotypes were pooled, so comparison was made of all euploid against all aneuploid oocytes, not distinguishing between *Fzr1*$^{fl/fl}$ and *Fzr1*$^{-/-}$ mice. Next, further analysis was done by genotype, with box-and-whisker plots presented for individual oocytes grouped by genotype and by whether aneuploid or euploid (Fig. 2B). Further analysis of the mean iKT distance between sister kinetochores demonstrated that these measurements were significantly greater in both the *Fzr1*$^{fl/fl}$ and *Fzr1*$^{-/-}$ aneuploid oocytes compared with the euploid oocytes ($P < 0.05$, Kruskal-Wallis test, Dunn multiple comparison) (Fig. 2C).

DISCUSSION

The present study tested the idea that techniques used to raise aneuploidy rates in oocytes from young mice would tend to predispose those oocytes with reduced chromosome cohesion (i.e., raised iKT distance) to become aneuploid. Using two methods to increase aneuploidy rates, ZM447439 addition and *Fzr1* knockout, we observed that this hypothesis appeared to be upheld, with those oocytes that were aneuploid having the higher iKT distances.

Mechanisms for ZM447439 and FZR1 Loss-Induced Aneuploidy

Biorientation of the bivalent at onset of anaphase will achieve faithful segregation of each sister chromatid pair to opposite poles. However, when bivalents first attach to microtubules on the meiotic spindle during meiosis I, these connections are often erroneous, and on average, each bivalent

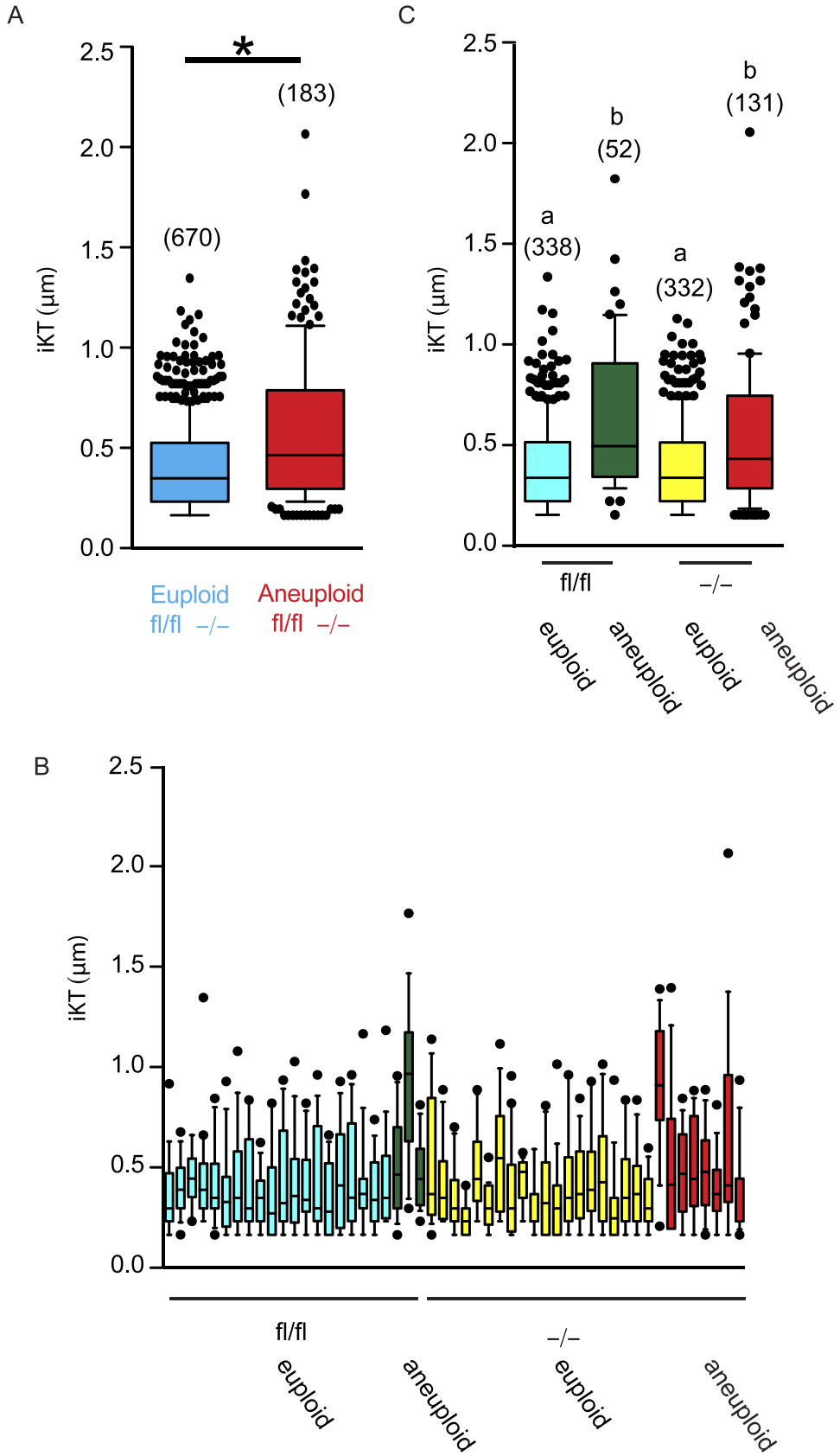

FIG. 2. Increased iKT distance in aneuploid oocytes from *Fzr1*$^{-/-}$ knockout mice. **A**) The iKT distance measurements in oocytes superovulated from *Fzr1*$^{fl/fl}$ and *Fzr1*$^{-/-}$ mice. All the euploid oocytes were pooled and compared to all the aneuploid oocytes, irrespective of genotype. Asterisk indicates significant difference ($P < 0.0001$, Mann-Whitney *U*-test). **B** and **C**) The iKT distance measurements in individual euploid and aneuploid oocytes (**B**) from *Fzr1*$^{fl/fl}$ and *Fzr1*$^{-/-}$ mice or pooled groups (**C**). Different letters denote significant difference ($P < 0.05$, Kruskal-Wallis test, Dunn multiple comparison).

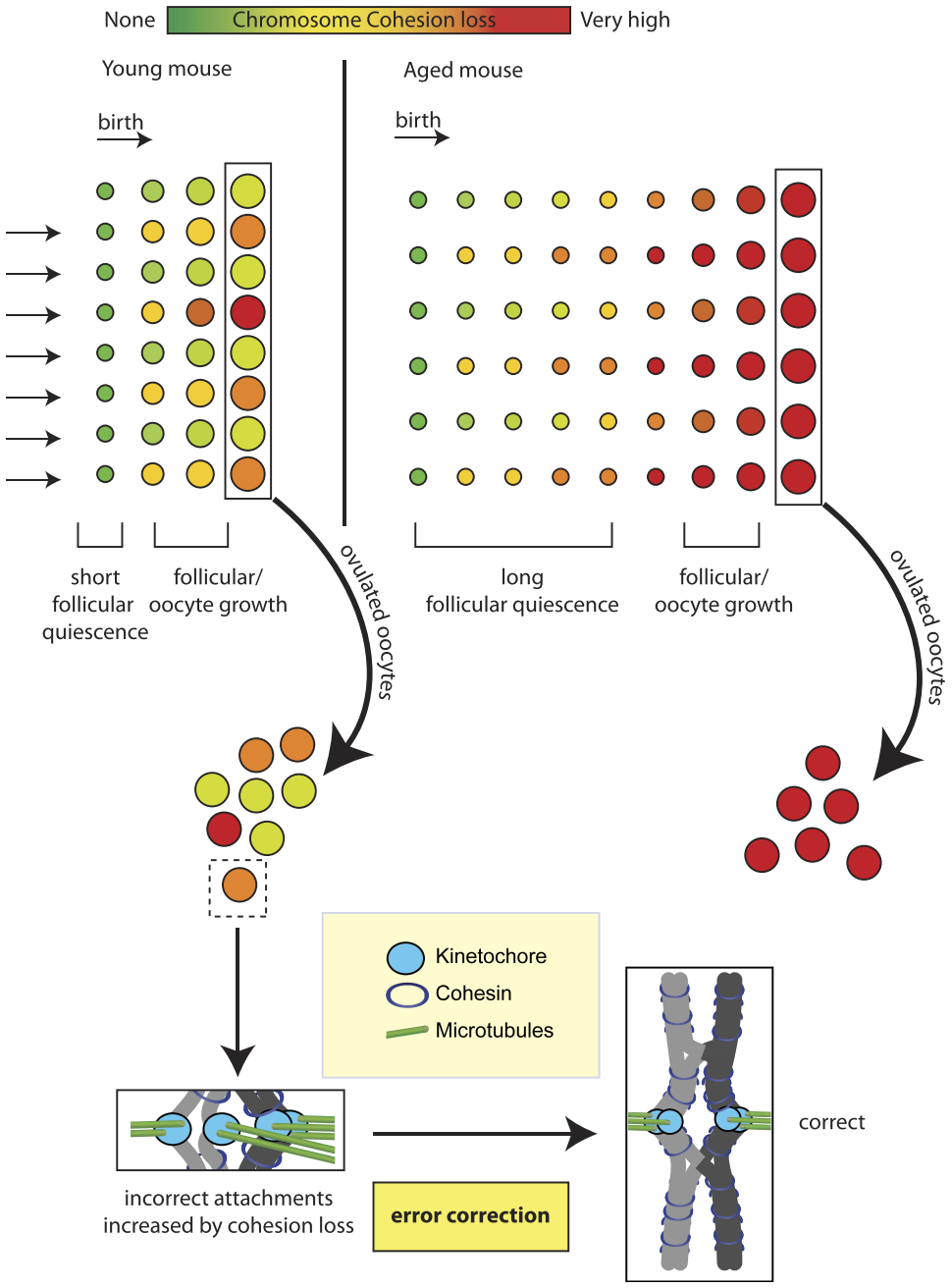

FIG. 3. Model for chromosome cohesion loss predisposing oocytes from young mice to becoming aneuploid. The extent of cohesion loss in individual oocytes is depicted at two ages (young and aged). Rates of loss vary between oocytes, but the long follicular quiescence observed for aged mice results in extensive loss in all oocytes. Elevated iKT distances due to some cohesion loss could predispose the oocyte to greater levels of incorrect attachment to microtubules. Normally, these would be corrected, but if the error-correction process is compromised or overwhelmed, these errors would remain and the bivalent undergo missegregation at meiosis I (see *Discussion* for further details).

undergoes three rounds of error correction [27]. Critically, although these initial attachments would not lead to faithful chromosome division, they are nonetheless sufficient to lead to spindle assembly checkpoint satisfaction [24, 25]; consequently, they activate the APC [16]. This latter event therefore starts the countdown to anaphase onset. In mitosis, the error-correcting mechanism is mediated by Aurora kinase B, a member of the chromosomal passenger complex that sits at the inner kinetochore and destabilizes any microtubule attachment to the outer kinetochore that has not resulted in biorientation, a process sensed through tension [19, 20, 28]. The equivalent error-sensing process in oocytes remains to be fully investigated, but it likely involves both Aurora kinase B and the germ cell-specific Aurora kinase C [21, 29, 30]. ZM447439, an Aurora kinase inhibitor added in the present study to oocytes during their maturation, would induce higher rates of aneuploidy by inhibiting this error-correcting process.

In mitotic cells, FZR1 is involved in maintaining genome integrity [22, 23]. We recently reported that FZR1 loss causes the spindle in meiosis I to be assembled more quickly by an unidentified pathway [11]. We do not know as yet if FZR1 loss interferes with the microtubule-kinetochore error-correcting process normally associated with bivalents during meiosis I. However, it remains a feasible hypothesis that the accelerated

spindle assembly accentuates the rate of kinetochore-microtubule attachment errors to such an extent that these are not all repaired by the onset of anaphase.

Cohesion Loss and Predisposition to Aneuploidy

The above discussion explains how Aurora kinase inhibition and *Fzr1* loss would lead to a rise in aneuploidy rate. In fact, such associations with aneuploidy are not unique to oocytes [23, 31]. It is important to stress that we do not propose the two procedures used here actually raise iKT distance in a population of oocytes. Instead, we suggest that these procedures preferentially predispose oocytes containing bivalents with weaker chromosome cohesion to become aneuploid. How would this be achieved? It seems most likely, though still needing direct validation, that the weakened cohesion encourages the two kinetochores normally associated with a sister chromatid pair to establish independent microtubule attachment. Within the microtubule milieu that constitutes the spindle, it is not technically feasible for us to image directly those microtubules attaching to kinetochores. Therefore, proof of such erroneous connection is lacking. However, at least with respect to *Fzr1* loss, many nonaligned chromosomes can be imaged during meiosis I for which their position would be consistent with sister chromatid biorientation [11].

Rate of Cohesion Loss in Individual Oocytes May Vary and Occur Early in Life

The mice used in the present study were very young (age, 1–1.5 mo). As such, it is initially surprising that individual oocytes show such marked variation in levels of chromosome cohesion as measured through iKT distance (see Fig. 2B). It suggests that loss in chromosome cohesion may be more dramatic in some oocytes than in others or, alternatively, that the initial level of cohesion can vary between oocytes. At present, we cannot distinguish between these two alternatives. However, evidence suggests that cohesion does not follow a linear loss with advancing mouse age but, instead, undergoes an extensive loss in the first few months of life. Such a conclusion is drawn from knockout mice of the meiosis-specific cohesin SMC1B (structural maintenance of chromosomes 1B), in which 98% of bivalents are normal in 1-mo-old knockout mice but two-thirds of bivalents have lost their integrity and become either univalents or single chromatids by 3 months of age [32]. This suggests a rapid decline of chromosome cohesion occurs in oocytes during the first few months in life, which in the absence of SMC1B; can be visualized as a dissolution of the bivalent.

Clearly, wild-type mice, having normal levels of SMC1B, do not undergo such a dramatic loss in bivalent integrity at a young age. Such young oocytes likely are protected from aneuploidy because components of the cohesin complex are associated with chromatin in a large excess of that ultimately needed to maintain bivalent integrity [7]. However, the SMC1B knockout mice nonetheless suggest that cohesion loss may not be linear with age but, instead, occur more rapidly over the early months of life in mice.

Based on the observations presented here for young mice and those presented previously for aged mice [9], we propose the following model (Fig. 3). Oocytes from newborn mice all have levels of cohesion that are adequate to maintain bivalent integrity and allow passage through meiosis without chromosome segregation defects. As mice age, the rate of cohesion loss varies from animal to animal and from oocyte to oocyte. The factors that could control this rate may be associated with the overall health of the animal and/or of individual primordial follicles during their quiescence. Regardless of individual rates of loss, all oocytes experience cohesion deterioration as animals age. Ovulated oocytes from aged mice tend to contain chromatids with greater iKT distance, as observed previously [9], which predisposes to incorrect microtubule attachment that, if not corrected, leads to a segregation error at anaphase onset. We propose that with age, loss is extensive and the iKT distance is raised sufficiently that the error-correcting process, which has no capacity to stall oocytes in meiosis I, is overwhelmed. Therefore, some incorrect attachment remains at anaphase, resulting in segregation errors. In contrast, ovulated oocytes from young mice tend not to become aneuploid, even though they possess some cohesion loss, because the error-correcting process is sufficiently developed that the attachment errors so generated are adequately corrected by the onset of anaphase. However, any perturbation of meiosis I that may lead to enhance the rate of incorrect attachments or compromise error correction—in the present study, Aurora kinase inhibition and FZR1 loss—would raise aneuploidy rates and tend to predispose those oocytes with the greater extent of cohesion loss. Further work is needed to determine the extent to which iKT distance can vary in individual oocytes from mice of different ages and, ultimately, uncover what factors contribute to such variation.

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