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Report

Evidence that Weakened Centromere Cohesion Is a Leading Cause of Age-Related Aneuploidy in Oocytes

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Summary

Aneuploidy arising early in development is the leading genetic cause of birth defects and developmental disabilities in humans. Most errors in chromosome number originate from the egg, and maternal age is well established as the key risk factor. Although the importance of this problem for reproductive health is widely recognized, the underlying molecular basis for age-related aneuploidy in female meiosis is unknown. Here we show that weakened chromosome cohesion is a leading cause of aneuploidy in oocytes in a natural aging mouse model. We find that sister kinetochores are farther apart at both metaphase I and II, indicating reduced centromere cohesion. Moreover, levels of the meiotic cohesin protein REC8 are severely reduced on chromosomes in oocytes from old mice. To test whether cohesion defects lead to the observed aneuploidies, we monitored chromosome segregation dynamics at anaphase I in live oocytes and counted chromosomes in the resulting metaphase II eggs. About 90% of age-related aneuploidies are best explained by weakened centromere cohesion. Together, these results demonstrate that the maternal age-associated increase in aneuploidy is often due to a failure to effectively replace cohesin proteins that are lost from chromosomes during aging.

Results and Discussion

Down syndrome was shown to be associated with advanced maternal age over 70 years ago [1]. It is now well established that the probability of a trisomic pregnancy (i.e., an embryo with an extra copy of a chromosome) increases dramatically with age, from only 2% for a woman in her twenties to about 35% by age 40 [2]. Most trisomies and all autosomal monosomies are inviable, and the few that are viable result in severe developmental disabilities. Aneuploidies in the embryo usually originate from the egg instead of sperm, and, more specifically, from chromosome segregation errors in meiosis I (MI) of oocyte maturation [3]. The frequency of these errors increases with maternal age, which is likely related to the long meiotic arrest that occurs during female meiosis in mammals. Primary oocytes enter meiosis during fetal development and remain arrested until ovulation in the adult, which in humans can be as long as 50 years. Although the phenomenon of age-related aneuploidy is well known, the molecular basis for the meiotic chromosome segregation errors is unclear.

Leading hypotheses to explain maternal age-related aneuploidy include defects in recombination, failure of the spindle assembly checkpoint, and premature loss of chromosome cohesion [3, 4]. Experimental evidence has depended on introducing perturbations that lead to an age-dependent increase in aneuploidy. Examples include the mating of two closely related mouse species to reduce meiotic recombination, mutation of the spindle checkpoint protein BUB1, and knockout of the meiosis-specific cohesin *Smc1*β [5–7]. These studies have shown that various defects can be introduced to produce an age-dependent increase in aneuploidy, but what actually occurs during the natural aging process remains unknown.

We previously showed that 60- to 70-week-old mice, corresponding to women ages 38–45 based on a linear extrapolation estimate, have a higher incidence of aneuploid eggs compared to 6- to 8-week-old mice [8]. Unlike many experimental systems, the aging mouse oocyte is one in which nature has already made the perturbation, and careful observation will be required to determine how age leads to chromosome segregation errors. Rather than test the contributions of various candidates to chromosome segregation in oocytes, our approach was to determine the defect that already exists in a natural aging mouse model.

We initially took an unbiased approach to this problem by identifying transcripts that are differentially expressed in oocytes from old and young mice, which suggested the spindle assembly checkpoint, kinetochore function, and spindle assembly as processes that might become defective with age [8]. We subsequently found, however, that a spindle checkpoint defect is unlikely to be the primary cause of age-related aneuploidy [9]. We also have not found clear defects in spindle assembly or kinetochore-microtubule attachment that would explain the increased aneuploidy (see Figure S1A available online). During these studies, however, we noticed that sister kinetochores are farther apart in metaphase II (MII) eggs from old mice (16–19 months of age) compared to MII eggs from young mice (6–14 weeks of age) (referred to as old or young eggs). This observation suggested that centromere cohesion might be weakened in old oocytes. Because cohesion is established during premeiotic S phase, which occurs during fetal development, and must remain functional until meiotic resumption in adult life (e.g., up to ~50 years later in humans or 15 months later in mice), defective cohesion is a good candidate for a process that might fail with increasing maternal age [4]. Though this hypothesis is appealing, it remains largely untested in a natural aging model. If cohesion defects do contribute to age-associated aneuploidy, there are clear predictions that can be tested by direct observations in old oocytes.

Sister Kinetochores Are Farther Apart in Old Oocytes

To measure distances between sister kinetochores (interkinetochore distance), we matured oocytes in vitro to MII, treated them with a kinesin-5 inhibitor to create monopolar spindles, and then fixed and stained them for DNA and kinetochores. Because the chromosomes are dispersed on the monopolar spindle rather than tightly clustered at metaphase, this assay allows us to clearly visualize individual kinetochore pairs and accurately count chromosomes based on the number of kinetochores [9]. In addition, pulling forces on kinetochores are

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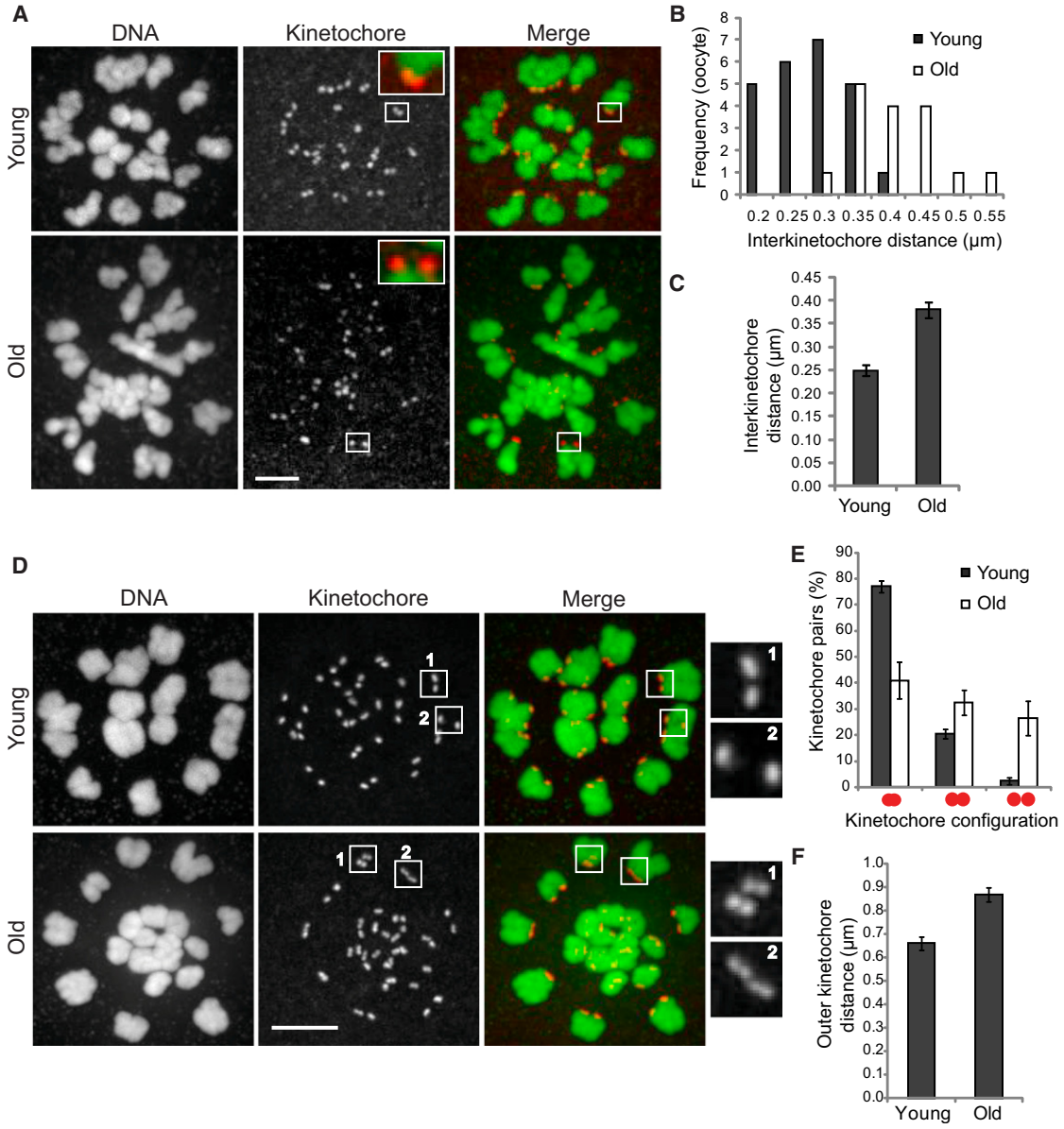

Figure 1. Sister Kinetochores Are Farther Apart in Old Oocytes Both at MII and MI

(A–C) Oocytes from young (6–14 weeks of age) or old (16–19 months of age) mice were matured in vitro, treated with monastrol to disperse the chromosomes at MII, and then fixed and stained for DNA (Sytox, green) and kinetochores (CREST, red). Representative images (A) show increased distance between sister kinetochores, with insets magnified to show sister kinetochore pairs. Interkinetochore distances were measured for all sister kinetochore pairs in an oocyte and averaged over each oocyte. The populations of young (n = 24) and old (n = 17) oocytes are represented by histograms (B) and by the mean (±standard error of the mean) for each group (C).

(D–F) Oocytes were treated with monastrol at MI, then fixed and stained as above. Insets (D) show increased separation of sister kinetochores; each box includes all four kinetochores of a bivalent. Sister kinetochore configurations in MI were classified as indistinguishable, overlapping, or distinct (E, left to right). Outer kinetochore distances were measured from the outer edges of sister kinetochore pairs for young and old oocytes (n = 200 kinetochore pairs from 10 oocytes in each group; F). Images (A) and (D) are maximal intensity projections of confocal Z series, and insets are single optical sections; scale bars represent 5 μm. See also Figure S1.

minimized in a monopolar spindle, so the distance between kinetochores reflects differences in cohesion holding the centromeres together. The interkinetochore distance was increased in old eggs (0.38 ± 0.02 μm, mean ± standard error of the mean, n = 17 eggs, 340 kinetochore pairs) compared to young eggs (0.25 ± 0.01 μm, n = 24, 480 kinetochore pairs; $p < 0.05$) (Figures 1A–1C). Furthermore, increased interkinetochore distances were not limited to aneuploid eggs but occurred consistently in old eggs (Figure 1B), suggesting that this difference reflects a general age-associated phenomenon. We also found that aneuploidies in old eggs are often due to loss or gain of a single chromatid (4 out of 7 aneuploidies, n = 23 eggs), indicating complete loss of centromere cohesion. In another mouse strain, CB6F1, interkinetochore distance also increased with age (0.33 ± 0.02 μm in old eggs versus 0.27 ± 0.02 μm in young eggs; $p < 0.05$; Figures S1B and S1C), and 11% of old eggs (n = 72) contained a pair of completely separated chromatids, compared to 3% of young

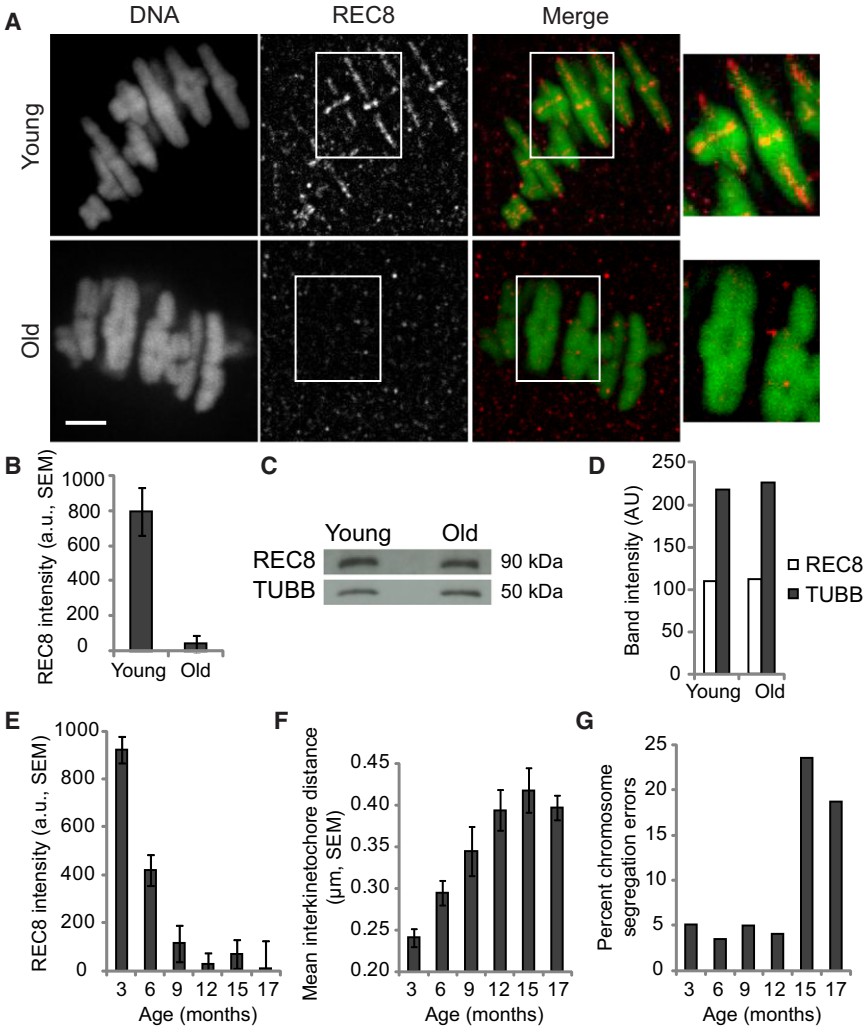

Figure 2. REC8 Protein Levels on Chromosomes Are Severely Reduced in Old Oocytes, whereas Total REC8 Is Constant

(A and B) Chromosome-associated REC8 was detected by immunocytochemistry in young and old oocytes at MI. Representative images (A) show DNA (Sytox, green) and REC8 (red); scale bar represents 5 μm. Similar results were obtained with multiple young (n = 11) and old (n = 9) oocytes, and the REC8 fluorescence intensity was quantified (B).

(C and D) Total REC8 levels in young and old oocytes at MI (n = 80 oocytes in each lane) were determined by western blot, with TUBB as a loading control. A representative blot is shown; similar results were obtained in two independent experiments, and band intensities were quantified (D). Shown are results from one experiment.

(E) Levels of chromosome-associated REC8 were measured as in (A) with oocytes from mice at different ages (in months): 3 (n = 21), 6 (n = 21), 9 (n = 19), 12 (n = 21), 15 (n = 7), 17 (n = 3).

(F and G) MII eggs from mice at various ages were analyzed to determine interkinetochore distances (F) as in Figures 1A–1C, and the percentage of eggs with chromosome segregation errors, including aneuploidies and separated sister chromatids, was determined (G). Mice were used at the following different ages (in months): 3 (n = 39), 6 (n > 28), 9 (n = 20), 12 (n > 24), 15 (n > 14), 17 (n = 16). See also Figure S2. For (B), (E), and (F), the data are expressed as mean ± standard error of the mean.

eggs (n = 31). Interkinetochore distance was also increased in untreated old MII eggs with normal bipolar spindles (1.76 ± 0.05 μm, n = 4 eggs, 71 kinetochore pairs) compared to young eggs (1.52 ± 0.04 μm, n = 6 eggs, 103 kinetochore pairs; $p < 0.05$; Figures S1D–S1F). Together, these observations indicate that centromere cohesion is weakened overall in old eggs based on increased interkinetochore distance and, occasionally lost completely, as indicated by aneuploidies of ± a single chromatid.

Cohesion between sister chromatids distal to crossover sites holds homologous chromosomes together after recombination in prophase I and remains intact until anaphase I onset. Centromere cohesion, on the other hand, must keep sister chromatids together until anaphase II [10, 11]. Loss of cohesion would clearly lead to premature chromatid separation, but cohesion also has another important function in MI. In fission yeast, cohesion at the core centromere physically links sister kinetochores in MI to facilitate mono-orientation [12, 13], in which both sister kinetochores attach to spindle microtubules from the same pole. Loss of centromere cohesion can lead to a failure to hold sister kinetochores together and thus promote biorientation at MI [12], resulting in segregation errors at anaphase I and aneuploidy at MII. Similarly, in plants, cohesion is required for mono-orientation of sister kinetochores, and increased separation of sister kinetochores in MI promotes biorientation, leading to lagging chromosomes and chromosome segregation errors in anaphase I [14, 15].

We tested whether sister kinetochores are held together less tightly in old oocytes at MI. Based on kinetochore staining, nearly all sister kinetochore pairs were indistinguishable or overlapping in young oocytes, whereas sister kinetochores in old oocytes were often distinct (Figures 1D and 1E). Distances between sister kinetochores, which were measured from the outer edges of the pairs because sister kinetochores are often overlapping, were increased in old oocytes (0.87 ± 0.03 μm) compared to young oocytes (0.66 ± 0.02 μm; n = 10 oocytes, 200 kinetochore pairs in each group; $p < 0.05$; Figure 1F). These results indicate that weakened centromere cohesion at MI fails to hold sister kinetochores together in old oocytes, which could increase the frequency of incorrect biorientation in MI.

Chromosome-Associated Cohesin REC8, but Not Total REC8, Is Reduced in Old Oocytes

To test whether reduced cohesin protein levels explain the weakened centromere cohesion phenotype in old oocytes, we analyzed levels of REC8, a meiosis-specific cohesin component. We found that chromosome-associated REC8 is severely reduced in old oocytes (Figures 2A and 2B), consistent with a previous report [16]. Cohesion on chromosome arms was also reduced, as indicated by an increase in distal chiasmata in old oocytes (Figure S2), consistent with previous studies that reported a shift toward distal chiasmata both in

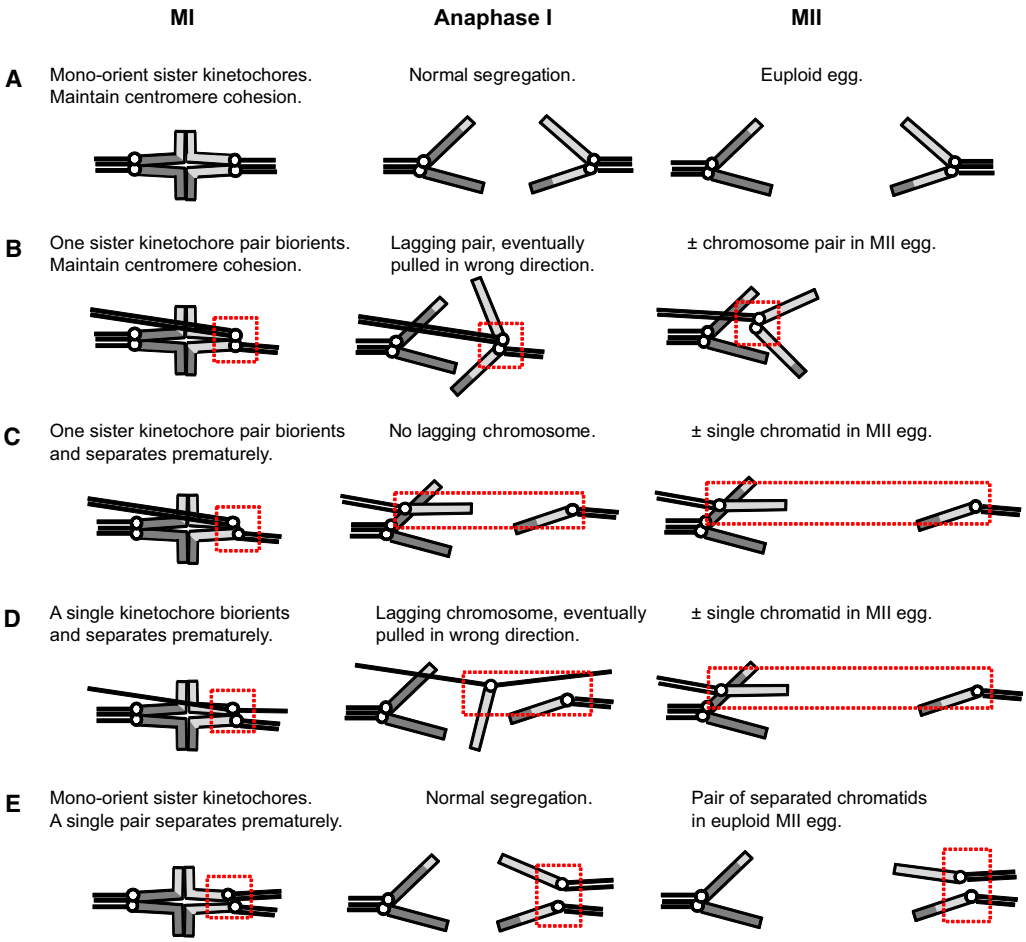

Figure 3. Proposed Outcomes of Weakened Centromere Cohesion in MI

(A–E) Schematics show the predicted outcomes of normal (A) or weakened (B–E) centromere cohesion in MI. Red boxes follow the progression of a sister kinetochore pair. Cohesion status and sister kinetochore orientation on the MI spindle determine chromosome segregation dynamics in anaphase I and the chromosome content of the MII egg. For prediction (E), sister kinetochore separation is shown at MI but could alternatively arise at a later time. Note that mouse chromosomes are telocentric.

old oocytes and in oocytes from SMC1β-deficient mice [7, 17]. Despite the reduced arm cohesion, we rarely observed unpaired univalents (2 of 57 old MI oocytes) and never saw single chromatids in intact old oocytes at MI. These results indicate that, although REC8 levels are below what we can detect reliably by immunofluorescence, there is still sufficient REC8 to prevent premature chromosome separation in MI. It is possible that other factors may compensate for the loss of REC8; however, most evidence points to REC8-dependent cohesion as crucial [12, 13, 18]. Remarkably, total REC8 protein levels are similar between young and old oocytes (Figures 2C and 2D), which demonstrates that cohesins are lost from chromosomes during aging and are not effectively replaced.

To further compare loss of REC8 cohesin protein and functional cohesion, we analyzed oocytes from mice at different ages. We are able to quantify a gradual decrease in chromosome-associated REC8 out to 9 months of age (Figure 2E), and the decrease likely continues beyond 9 months but is below the level that we can accurately quantify. Consistent with this interpretation, interkinetochore distances gradually increase from 3 to 15 months (Figure 2F). However, chromosome segregation errors remain low until 12 months and then increase dramatically (Figure 2G). These results suggest a threshold level of REC8 necessary to prevent errors. Based on what we are able to quantify, this threshold is ~10% or less of the starting amount (Figure 2E). Only after chromosome-associated REC8 reaches this threshold do chromosome segregation errors increase.

Weakened Centromere Cohesion Explains Most Aneuploidies in Old Eggs

We next considered the possible repercussions of weakened cohesion to determine whether it could explain the observed aneuploidies in MII eggs. Because chromosomes rarely separate prematurely in MI in old oocytes, even with reduced REC8, we focused on defects associated with reduced centromere cohesion and biorientation of sister kinetochores. For example, if centromere cohesion is weakened but remains functional and sister kinetochores biorient at MI, the chromosome pair would be pulled in opposite directions by spindle microtubules, leading to a lagging chromosome pair at anaphase I (Figure 3B). In this case, the entire chromosome pair could be pulled toward the wrong pole, resulting in gain or loss of a chromosome pair in the MII egg. On the other hand, if the bioriented sisters separate prematurely, then there would be

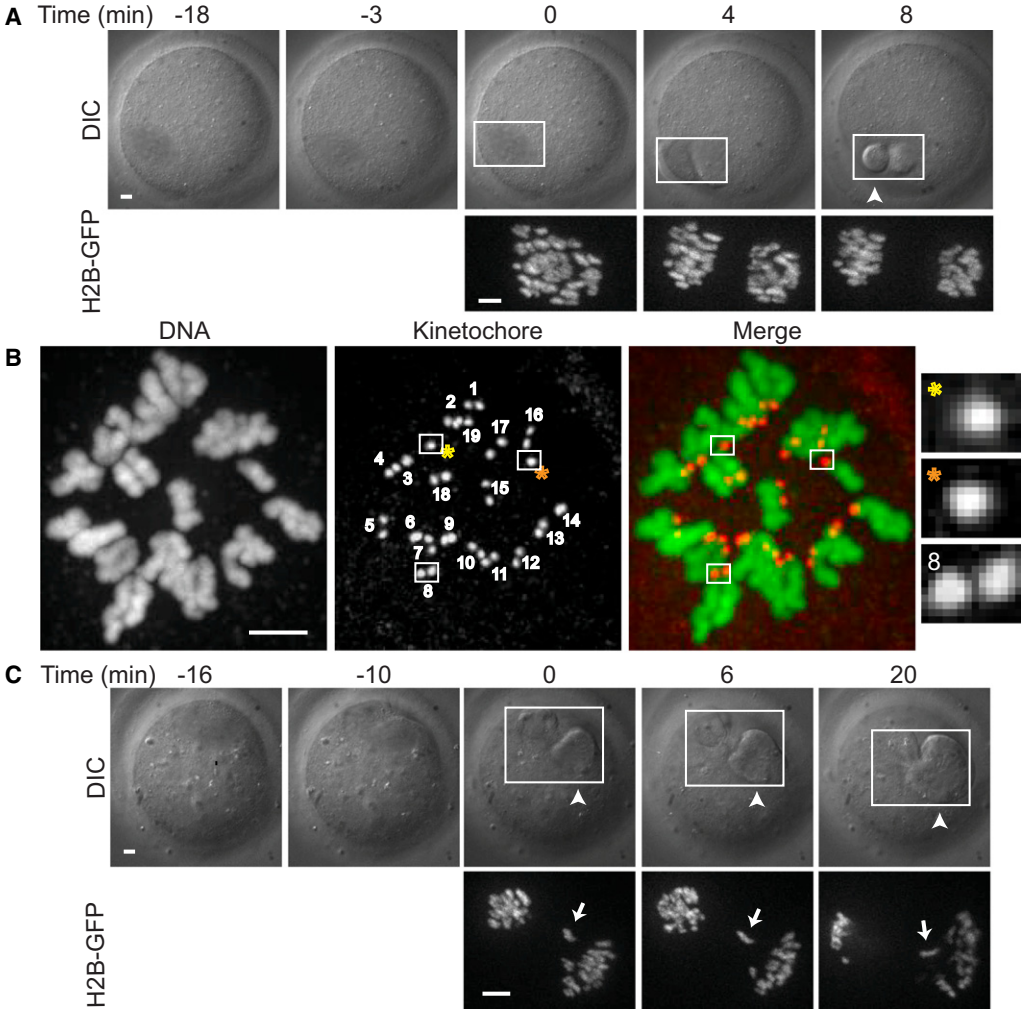

Figure 4. Analysis of Anaphase I Chromosome Segregation Dynamics and MII Chromosome Numbers in Old Oocytes

(A) An old oocyte was microinjected with *H2b-gfp* cRNA and then matured in vitro and imaged live, initially by differential interference contrast (DIC) to determine the time of anaphase onset (t = 0) and subsequently by fluorescence to detect lagging chromosomes in anaphase I. GFP images are magnified from white boxes in the DIC images. No lagging chromosomes are detected in this example.

(B) The MII egg resulting from the oocyte shown in (A) was fixed and stained for DNA (Sytox, green) and kinetochores (CREST, red) to count chromosomes. The egg is euploid with 19 paired sister kinetochores (numbered 1–19) and two unpaired kinetochores (yellow and orange asterisks). Insets show the two unpaired kinetochores and an intact sister kinetochore pair.

(C) Another old oocyte was imaged live in MI as in (A). In this example, a lagging chromosome (arrow) eventually segregates to the polar body. The final kinetochore count in the MII egg is 39, indicating loss of a single chromatid. Arrowheads mark the polar bodies; GFP images are maximal intensity projections of confocal Z series; scale bars represent 5 μm.

no lagging chromosomes at anaphase I, and the egg would gain or lose a single chromatid (Figure 3C). If a single kinetochore biorients and separates prematurely, the single bioriented chromatid would lag at anaphase I and result in aneuploidy of a single chromatid in the MII egg (Figure 3D). Finally, if sister kinetochores mono-orient correctly at MI but a single pair separates prematurely, then segregation would be normal at anaphase I, and the MII egg would be euploid with a pair of separated chromatids (Figure 3E). In these eggs, segregation errors are likely to occur during anaphase II, leading to aneuploidy after fertilization.

To test the proposed outcomes of weakened cohesion, we designed an experiment to directly observe both chromosome segregation dynamics at anaphase I and chromosome numbers at MII. Young and old oocytes were microinjected with *H2b-gfp* cRNA to label chromosomes, matured in vitro to MI, and then imaged at anaphase I to monitor chromosome segregation. Each egg was then treated with monastrol, fixed, and processed for immunocytochemistry to determine an MII chromosome count for the same oocyte that was previously imaged live. By observing both anaphase I and MII outcomes described in Figure 3, we can deduce the underlying problem at MI.

The most common abnormal outcome (7 of 36 old oocytes) was normal segregation at anaphase I, but with a pair of separated chromatids in a euploid MII egg (Figures 4A and 4B). This outcome is consistent with correct mono-orientation but loss of centromere cohesion at a single kinetochore pair at MI (Figure 3E). Lagging chromosomes were observed in 7 old oocytes, often leading to aneuploidy of a single chromatid (4 of 36 old oocytes). In the example shown in Figure 4C, a lagging chromosome at anaphase I is pulled toward the polar

Table 1. Results of Anaphase I Live Imaging and Chromosome Counting Experiments in Young and Old Oocytes

No Lagging Chromosomes	Young	Old	Explanation
Euploid	25	19	Normal segregation (Figure 3A).
Euploid, separated chromatids	0	7	Sister kinetochores mono-orient correctly, but one pair separates prematurely (Figure 3E).
±single chromatid	1	1	One sister kinetochore pair biorients and separates prematurely (Figure 3C).
±chromosome pair	0	2	All four kinetochores of a bivalent attach to one pole, not predicted by cohesion defect.
Lagging Chromosomes			
Euploid, separated chromatids	0	1[a]	A single kinetochore biorients and separates prematurely from its sister (Figure 3D).
±single chromatid	1	4[b]	
±chromosome pair	0	2	One sister kinetochore pair biorients because of weakened centromere cohesion (Figure 3B).
Total	27	36	

Each oocyte is categorized as "No lagging chromosomes" or "Lagging chromosomes" depending on the presence of lagging chromosomes in anaphase I. Chromosome counts at MII designate whether an egg is euploid (with or without separated chromatids) or aneuploid (±single chromatid or chromosome pair). Explanations for the results refer back to possible outcomes from Figure 3.

[a] This outcome is a variation of the prediction in Figure 3D. This oocyte contained multiple lagging chromosomes at anaphase I.

[b] In one of these oocytes, the extra chromatid lacked a kinetochore. The same oocyte also contained multiple lagging chromosomes in anaphase I and a pair of separated chromatids at MII.

body, resulting in an MII egg missing a single chromatid (19 kinetochore pairs and 1 single chromatid). This outcome is consistent with loss of centromere cohesion and biorientation of a single chromatid at MI (Figure 3D).

In total, we analyzed 27 young and 36 old oocytes and observed 7% and 25% aneuploidy in young and old eggs, respectively (Table 1), consistent with our previous findings [8, 9]. Out of 36 old MII eggs, 17 were either aneuploid or contained a pair of separated chromatids. Of these 17 problematic eggs, 13 contained either a single chromatid or a pair of separated chromatids, indicating that centromere cohesion was lost more frequently in old oocytes compared to young oocytes (Fisher's exact test, $p = 0.01$). It is possible that erroneous attachments may generate enough force from the opposite poles to rip apart sister kinetochores at anaphase I, independently of a centromere cohesion defect. In this case, a lagging chromosome pair would be pulled apart at anaphase I, which was never observed in our live imaging experiments. Two of the 17 problematic eggs had lagging chromosomes at anaphase I and aneuploidy of a chromosome pair at MII, which indicates that even though centromere cohesion was maintained, sister kinetochores bioriented at MI (Figure 3B). Overall, we observed an increased frequency of anaphase I lagging chromosomes in old oocytes (7 of 36, compared to 1 of 27 in young oocytes), consistent with our prediction that partially separated sister kinetochores are more likely to attach incorrectly at MI. In the last two problematic eggs, we observed normal segregation at anaphase I and aneuploidy of a chromosome pair in the MII egg. This outcome indicates that all four kinetochores of a bivalent oriented to one pole, which is not predicted by weakened cohesion. There may be multiple pathways contributing to age-related aneuploidy, but most (~90%) aneuploidies seen in old eggs are consistent with weakened cohesion as the underlying cause, through either improper kinetochore biorientation in MI or premature chromatid separation.

Conclusions

We provide multiple lines of evidence demonstrating that centromere cohesion is weakened in old oocytes: increased separation of sister kinetochores at both MI and MII, severe loss of REC8 from chromosomes, and unpaired chromatids at MII. Moreover, weakened cohesion can explain most of the observed aneuploidies because of a failure to effectively hold sister centromeres together in MI. The frequency of unpaired univalents in old oocytes at MI is much lower than that of single chromatids in MII, which suggests that, even though cohesin levels are reduced globally, centromere cohesion is more vulnerable than arm cohesion. Because there are more cohesin binding sites along chromosome arms than at centromeres, a small fraction of the initial cohesins may be sufficient to keep the bivalents intact.

We find that chromosomal REC8 levels decrease with age while interkinetochore distances increase, indicating a gradual reduction in cohesins and cohesion (Figures 2E and 2F). In contrast, aneuploidy rises sharply after 12 months (Figure 2G), which is similar to the dramatic increase in humans that occurs around 35 years of age [2]. These results suggest that cohesins are initially loaded in large excess of what is sufficient to maintain functional cohesion, and errors arise only after cohesin levels fall below a critical threshold. The REC8 and interkinetochore distance measurements are averaged over all chromosomes, whereas most aneuploidies involve a single chromatid or chromosome pair. Some chromosomes may be more susceptible to weakened cohesion, which would be missed in our analyses that average over all chromosomes. Chromosome-specific effects have been documented in human aneuploidies [2] but have not been systematically examined in mouse models.

Our findings are consistent with previous studies that have examined genetic perturbations of cohesion. Oocytes from SMC1β-deficient mice are unable to maintain meiotic cohesion, and the magnitude of the effect increases dramatically with age [7, 19]. Similarly, reduced cohesion in *Drosophila* oocytes leads to an age-dependent increase in meiotic nondisjunction [20]. Although these experimental systems are not models of natural aging, they do indicate that cohesion declines with age.

Most human trisomies are classified as MI errors based on analyses of pericentromeric markers [2]. Taking trisomy 21 as an example, if the two copies of chromosome 21 inherited from the same parent are heterozygous at the centromere, there must have been a segregation error in MI. In addition, analyses of unfertilized human MII eggs and polar bodies show that the most common problems associated with advanced maternal age are separated sister chromatids and loss or gain of single chromatids [21–24], indicating premature loss of centromere cohesion. The MI errors depicted in Figures 3C and 3D are consistent with the observations of both heterozygosity and single chromatids at MII, suggesting that these may be the most common errors leading to human trisomies. Other factors also likely contribute to human aneuploidy, because altered recombination and chromosome-specific effects have been documented [3, 25], though some of

these effects might make chromosomes more susceptible to reduced cohesion. Defects in spindle formation and chromosome alignment have also been reported in human oocytes [26, 27]. Although there may be multiple causes of human aneuploidy, the prevalence of unpaired chromatids in MII indicates that centromere cohesion defects must make a major contribution, consistent with our findings. The molecular explanation for the loss of cohesion is not clear, in large part because almost nothing is known about how cohesion is normally maintained during the long prophase arrest in mammalian oocytes. Outstanding questions, such as what the stability is of cohesin complexes on chromosomes during the MI arrest and whether new cohesins load and mature during the arrest, are now under investigation.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at doi:10.1016/j.cub.2010.06.069.

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