

Cell-cycle and Age-Related Modulations of Mouse Chromosome Stiffness

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eLife Assessment

This **valuable** paper describes the stiffness of meiotic chromosomes in both oocytes and spermatocytes. The authors identify differences in stiffness between meiosis I and II chromosomes, as well as an age-dependent increase in stiffness in meiosis I (and meiosis II) chromosomes, results that are highly significant for the field of chromosome biology. The report is, however, mostly descriptive and the mechanisms underlying age-dependent changes in chromosome stiffness remain unclear. The evidence suggesting that changes in stiffness are independent of cohesin, which is known to deteriorate with age, is still **incomplete**.

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Abstract

Chromosome structure is complex, and many aspects of its organization remain poorly understood. Measuring chromosome stiffness offers valuable insight into their structural properties. In this study, we analyzed the stiffness of chromosomes from metaphase I (MI) and metaphase II (MII) oocytes. Our results revealed a ten-fold increase in stiffness (Young's modulus) of MI chromosomes compared to somatic chromosomes. Furthermore, the stiffness of MII chromosomes was lower than that of MI chromosomes. We examined the role of meiosis-specific cohesin complexes in regulating chromosome stiffness. Surprisingly, chromosomes from three meiosis-specific cohesin mutants exhibited stiffness comparable to that of wild-type chromosomes, indicating that these cohesins are not the primary determinants of chromosome stiffness. Additionally, our findings revealed an age-related increase in chromosome stiffness in MI oocytes. Since aging is associated with elevated levels of DNA damage, we investigated the impact of etoposide-induced DNA damage on oocyte chromosome stiffness and found that it led to a reduction in MI chromosome stiffness. Overall, our study underscores the dynamic and cyclical nature of chromosome stiffness, modulated by both the cell cycle and age-related factors.

Introduction

DNA serves as the carrier of genomic information, interacting with a wide variety of proteins to form chromatin during interphase. Before cell division, chromatin is compacted into thick, noodle-like chromosome structures, a process which plays a critical role in ensuring the equitable distribution of genetic material [1]. This process is essential for maintaining genome integrity, as any errors in chromosome behavior during meiosis or mitosis can lead to conditions such as cancer, infertility, miscarriage, or congenital diseases [2,3]. Despite many decades of research, chromosome structure and organization are not yet understood.

Chromosome compaction is not a random process, but a tightly regulated sequence of events. In mammals, inches-long segments of DNA are compacted into a chromosome less than 10 μm long via a multi-level process [4]. This process begins with the formation of nucleosomes, wherein DNA wraps around histone octamers, following the “beads on a string” model [5]. Linker histone H1 binds to the entry and exit points of DNA on the nucleosome and interacts with the linker DNA region between nucleosomes.

However, the interaction between histones and DNA alone cannot explain the full extent of chromosome compaction, suggesting that other factors must be involved in this process [6–8]. There are different models for chromosome organization, with the most popular being the “Scaffold/Radial-Loop” model [9–12] and the “Chromatin Network” model [13]. The Scaffold/Radial-Loop model proposes a continuous central protein core with chromatin loops stacked around it, based on electron microscopy observations. The Chromatin Network model suggests that structure proteins link chromatin segments without forming a continuous central core. MNase, an enzyme that degrades DNA/chromatin, ablated chromosome stiffness, in discord with a pure Scaffold/Radial-Loop model and instead indicating a key role for chromatin cross-bridges [14]. Experiments with DNases, which affect chromosome stiffness depending on their cutting frequencies, lend support to the Chromatin Network model (or equivalently, a chromosome radial looping model without a continuous proteinaceous scaffold).

Chromosome compaction and decompaction occur in different phases of the cell cycle [15], and the shape and size of chromosomes vary accordingly [16]. It is reasonable to hypothesize that chromosome stiffness also differs between stages, and between mitotic and meiotic chromosomes, which have similar shapes but experience different molecular events [17,18]. Chromosome stiffness measurements have shown that prophase I spermatocyte chromosomes are approximately ten times stiffer than those in mitosis, indicating differences in chromosome organization between meiosis I and mitosis [19].

During meiosis I, a unique railroad-track-like protein structure, known as the synaptonemal complex (SC), forms between the two homologous chromosomes [20,21]. Although the SC was thought to be a potential factor increasing stiffness of meiotic chromosomes, it has been observed that SYCP1, a key SC component, does not contribute to chromosome stiffness [19]. SYCP1 laterally connects the two “rails” of the SC, axial elements (AEs), but may not impact the longitudinal stiffness. It is more likely AEs provide mechanical strength to the meiotic chromosomes longitudinally. Cohesin proteins, which connect sister chromatids in both mitosis and meiosis, are fundamental components of AEs [22,23]. In meiosis, meiosis-specific cohesin proteins, such as SMC1 β , RAD21L, REC8, and STAG3, may play a role in chromosome structure and stiffness [24–26].

Aging can also induce significant changes in chromosomes, potentially leading to apoptosis, senescence, or cancer, all of which affect the lifespan and well-being of both animals and humans [27,28]. Moreover, chromosome-associated protein levels change with age, with some

increasing while others decreasing [29 [↗](#), 30 [↗](#)]. For instance, cohesin proteins along chromosome axes decreases with age, potentially contributing to increased rates of aneuploidy and unsuccessful gamete production [31 [↗](#), 32 [↗](#)]. Given the elevated aneuploidy rates in oocytes from elder individuals, it is essential to explore how aging impacts chromosome mechanics and to gain a better understanding of the molecular mechanisms driving these changes.

Results

Chromosome stiffness measurement for metaphase

I (MI) and metaphase II (MII) mouse oocytes

To measure oocyte chromosome stiffness, we isolated chromosomes from oocytes collected from 3- to 4-week-old mice. These oocytes were cultured for 6 hours to reach MI or 14 h to reach MII. The zona pellucida was removed by treating the oocytes with Tyrode's solution for approximately 3 minutes (**Figure 1A** [↗](#)). The oocyte membranes were then lysed via microspraying Triton X-100, allowing the oocyte contents to flow out spontaneously. Using this technique, we successfully isolated the spindle from the oocytes (**Figure 1B** [↗](#) Left) and subsequently separated the chromosomes from the spindle (**Figure 1B** [↗](#) Middle).

Next, we used two pipettes with small openings to grasp the two ends of the chromosome (**Figure 1B** [↗](#) Right). This setup allowed us to measure chromosome stiffness by moving one pipette and observing and calibrating the bending of the other (see Materials and Methods for further details). We stretched and relaxed the chromosomes to monitor its length change under an applied force, ultimately determining their Young's modulus (a measure of material stiffness which is independent of geometry, i.e., chromosome thickness or number of chromatids). These experiments were carried out with extensions of less than twice the chromosome's native length, ensuring a reversible mechanical response - i.e., the force versus extension curve was similar during both stretching and retraction (Supplement Fig. 1).

Chromosome stiffness in MI oocytes is about ten times higher than that in mitotic cells

Chromosome stiffness has been studied for a variety of mitotic cells, revealing similarities and differences across different cell types [33 [↗](#)–35 [↗](#)]. However, a comprehensive analysis of chromosome stiffness through either mitotic or meiotic cell cycles has not been done. For comparison with the meiotic case, we measured the chromosome stiffness of Mouse Embryonic Fibroblasts (MEFs) at late pro-metaphase (just slightly before their attachment to the mitotic spindle) and found that the average Young's modulus was 340 ± 80 Pa (**Figure 2B** [↗](#)). The value is consistent with our previously published data, where the modulus for MEFs was measured to be 370 ± 70 Pa [19 [↗](#)].

Next, we isolated chromosomes from mouse MI oocytes and measured their stiffness (**Figure 2A** [↗](#)), obtaining a Young's modulus of 3790 ± 700 Pa, roughly tenfold higher than that of MEF chromosomes (**Figure 2B** [↗](#)). This finding was comparable to previous results demonstrating that spermatocyte prophase I chromosomes are approximately 10 times stiffer than MEF chromosomes [19 [↗](#)]. These results suggest that the high chromosome stiffness observed in meiotic cells is a feature of gametes, common to both sexes. To further explore this, we investigated chromosome stiffness in MII oocytes and explored potential factors that might contribute to the high stiffness observed for gamete chromosomes.

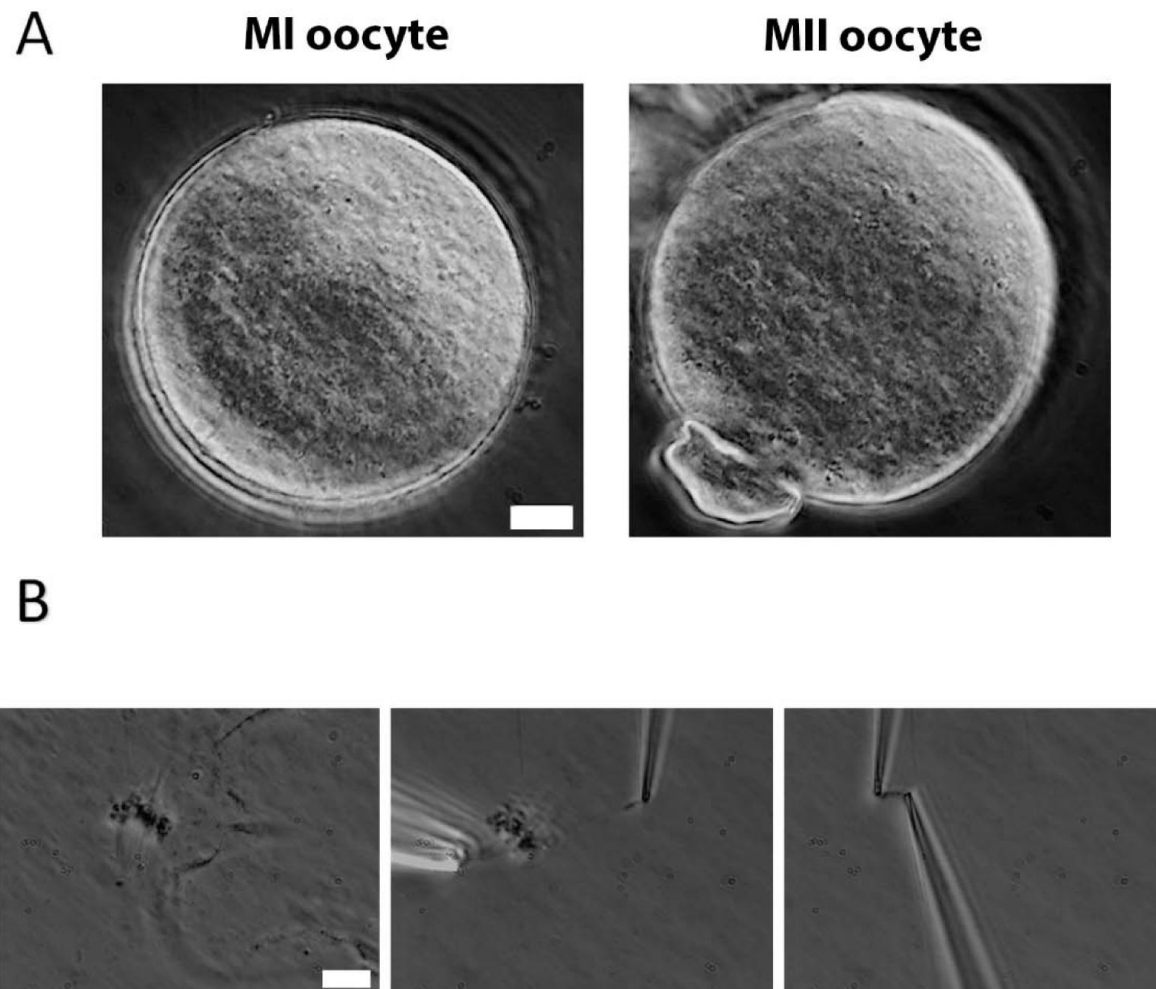
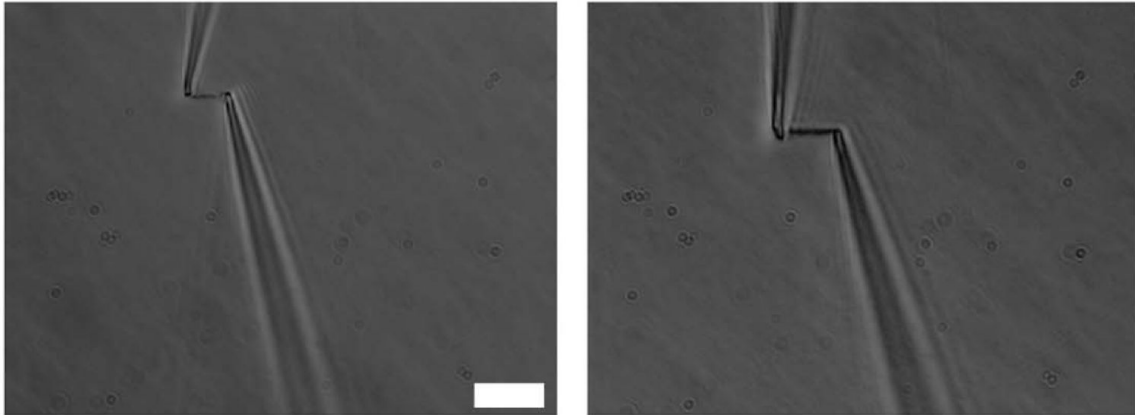


Figure 1.

Chromosome isolation from oocytes.

(A) Oocytes after zona pellucida removal. Left panel: MI oocyte. Right panel: MII oocyte with visible polar body. Scale bar = 10 μ m. (B) Spindle isolation process. Left panel: Spindle flowing out from the oocyte after oocyte lysis. Middle panel: a chromosome being isolated from the spindle-chromosome complex. Right panel: chromosome captured between two pipettes. Scale bar = 10 μ m.

A



B

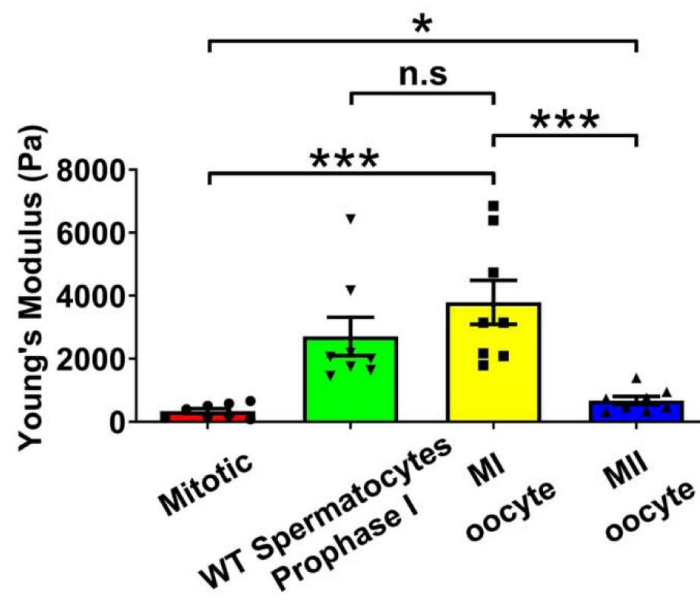


Figure 2.

Chromosome stiffness measurement.

(A) Example images of chromosome isolation. Left: MII oocyte chromosome. Right: MI oocyte chromosome. Scale bar = 10 μ m. (B) Chromosome stiffness comparison across different cell types: mitotic cells (n=8), WT spermatocytes at prophase I (n=8), MI oocytes (n=8) and MII oocytes (n=8). Young's Modulus of MI oocyte chromosomes (3790 ± 700 Pa) is much higher than that of mitotic cells (370 ± 70 Pa, $P=0.0002$) and MII oocytes (670 ± 130 Pa, $P=0.0006$). Data are presented as mean $\pm$ SEM. All statistical analyses were performed via t-test.

The stiffness of chromosomes in MI mouse oocytes is significantly higher than that of MII oocytes

To study the effect of meiotic cell cycle stage on chromosome stiffness, we measured the chromosome stiffness for the MII oocytes, as we did for the MI chromosomes. We found that chromosome stiffness in MII oocytes was significantly lower than that in MI oocytes: the Young's Modulus of MII oocytes was 670 ± 130 Pa, while that of MI oocytes was 3790 ± 700 Pa ($P < 0.001$; **Figure 2B**). Surprisingly, despite this reduction, the stiffness of MII oocyte chromosomes was still significantly higher than that of mitotic cells (**Figure 2B**). This finding challenges the conventional view that meiosis II is closely analogous to mitosis, since we observe that chromosome mechanics in meiosis II quantitatively differs from that observed in mitotic cells [36]. Our results affirm that chromosome stiffness varies dynamically across different cell cycle stages.

To verify the consistency of chromosome measurements, we compared our data with previously published results [19], in terms of the “doubling force” (the force required to double the length of a chromosome, which is expected to be dependent on chromosome thickness). MEF chromosomes in the published study exhibited a doubling force of 190 ± 40 pN, while WT prophase I spermatocytes had a doubling force of 2130 ± 440 pN [19]. Our measurements closely agreed with these values, showing a doubling force of 210 ± 40 pN for mitotic MEFs and 1690 ± 450 pN for WT prophase I spermatocytes (Supplement Fig. 2), indicating quantitative reproducibility of our results. Here, we found that the doubling forces of chromosomes from MI and MII oocytes are 3770 ± 940 pN and 510 ± 50 pN, respectively. We conclude that chromosomes from MI oocytes are much stiffer than those from both mitotic cells and MII oocytes (Supplement Fig. 2), in terms of either Young's modulus or doubling force.

Meiosis-specific cohesins do not contribute to chromosome stiffness

We previously demonstrated that the central elements of the synaptonemal complex do not contribute to longitudinal chromosome stiffness, so we shifted our focus to the role of meiosis-specific cohesins during meiosis I [19]. Cohesin can load onto chromosomes before synaptonemal complexes form [37]. During mammalian mitosis, cohesin proteins bind along the chromosome axis during S phase, staying at the centromeres until anaphase, when they are cleaved by separase. Most chromosome-arm cohesin proteins are removed early before metaphase-anaphase transition by a separase-independent pathway [38]. During meiosis I, cohesin proteins are removed from chromosome arms at anaphase I by separase, and only a small amount remains at the centromere until anaphase II [39]. While cohesin proteins disappear from chromosome arms by metaphase in both mitosis and MII, they are retained along chromosome arms during MI.

Given the higher cohesin levels along chromosome arms during MI, we hypothesized that this might contribute to the greater stiffness observed in MI oocyte chromosomes compared to those from MII. To test this hypothesis, we examined the effects of mutations of meiosis-specific cohesins (REC8, STAG3, RAD21L) on chromosome stiffness, utilizing *Rec8*^{-/-}, *Stag3*^{-/-}, and *Rad21l*^{-/-} mutant mice [40]. These cohesins are essential for sister chromatid cohesion, and their absence disrupts gametogenesis, resulting in arrest at prophase I [41–43]. Consequently, our experiments were restricted to prophase I chromosomes. Furthermore, we conducted these experiments in males, as spermatocytes are continuously produced and surgically accessible. In contrast, female oocyte prophase I occurs *in utero*, making isolation and genotyping challenging. Despite these constraints, we were able to perform comparative analyses between wild-type and mutant spermatocytes.

We isolated chromosomes from *Rec8*^{-/-} prophase I spermatocytes, which displayed large cell size, a round shape, and thick chromosomal threads, indicative of advanced chromosome compaction after stalling at a zygotene-like prophase I stage (Supplement Fig. 3). These features, particularly the combination of large cell size and chromosome compaction, allowed us to reliably identify *Rec8*^{-/-} prophase I chromosomes. Using micromanipulation, we measured chromosome stiffness by stretching the chromosomes (Supplement Fig. 3) [13]. Surprisingly, there was no significant difference in chromosome stiffness between wild-type (WT) control and *Rec8*^{-/-} mutant (2710 ± 610 Pa in WT spermatocytes versus 2580 ± 620 Pa in *Rec8*^{-/-} spermatocytes, $P=0.8884$) (Figure 3B).

Similarly, for both *Stag3*^{-/-} (2710 ± 610 Pa in WT spermatocytes versus 2240 ± 210 Pa in *Stag3* mutant spermatocytes, $P = 0.4533$) and *Rad21*^{-/-} (2710 ± 610 Pa in WT spermatocytes versus 2050 ± 370 Pa in *Rad21*^{-/-} spermatocytes, $P = 0.3514$) mutants, no significant differences in chromosome stiffness relative to wild-type was observed (Figure 3C and Supplement Fig. 4). Therefore, we concluded that meiosis-specific cohesins do not play an important role in determining chromosome stiffness.

Chromosomes from older MI oocytes have higher stiffness than those from younger MI oocytes

We next examined the effects of aging on chromosome mechanics. Initially, we hypothesized that chromosomes from aged oocytes would be less stiff based on previous findings that aging is associated with decreased levels of cohesin, particularly REC8 [44,45]. To test this hypothesis, we isolated chromosomes from MI oocytes of 48-week-old mice (nearing the end of fertility, roughly equivalent to 40-year-old humans) and compared them to chromosomes from 3-to 4-week-old mice (Figure 4A). Contrary to our hypothesis, our measurement revealed that chromosomes from older mice were much stiffer than those from younger mice (8150 ± 1590 Pa in older MI oocytes versus 3790 ± 700 Pa in younger MI oocytes, $P = 0.0150$) (Figure 4B). This result further supports the conclusion that cohesins are not the main contributors to chromosome stiffness.

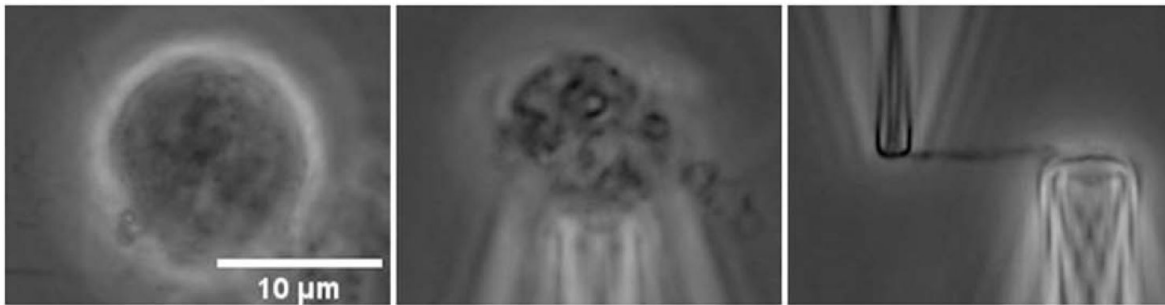
Our findings are consistent with previous studies that observed increased chromosome stiffness in aged MII oocytes when compared to their counterparts from younger oocytes [33]. The doubling force for chromosomes in 3-to 4-week-old MII oocytes was measured at 510 ± 50 pN (see Supplement Fig. 2), while for 6-to 8-week-old MII oocytes, it was significantly higher at 830 ± 100 pN [33]. These findings underscore a trend of increased chromosome stiffness with advancing age, common to both MI and MII oocytes.

At the MII stage, most cohesin complexes have already dissociated from chromosome arms, and only a small amount remains, connecting sister chromosomes at their centromeres until anaphase II. Therefore, the observed age-related increase in chromosome stiffness is unlikely to be driven by cohesin levels. This suggests that other age-related factors, possibly linked to chromosome structural changes, contribute to the increased stiffness in older oocytes. Future investigations are needed to identify these age-related factors and their impact on chromosome mechanics.

DNA damage reduces chromosome stiffness in oocytes

Oocytes from older individuals are known to exhibit higher levels of DNA damage compared to those from younger individuals [46,47]. In response to DNA damage on chromosomes, several DNA repair mechanisms are activated, which recruits various DNA repair proteins to the damage sites [48]. We hypothesized that this recruitment could affect chromosome stiffness. To test this hypothesis, we used etoposide, a chemotherapy drug used to treat a variety of cancers, including testicular and ovarian cancer [49,50]. We treated the oocytes with etoposide to introduce DNA damage and investigated its impact on chromosome stiffness [46].

A



B

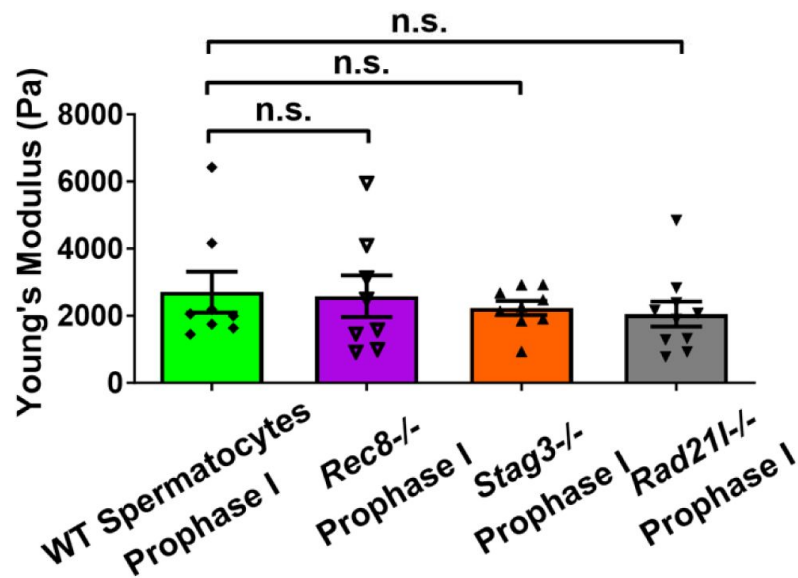
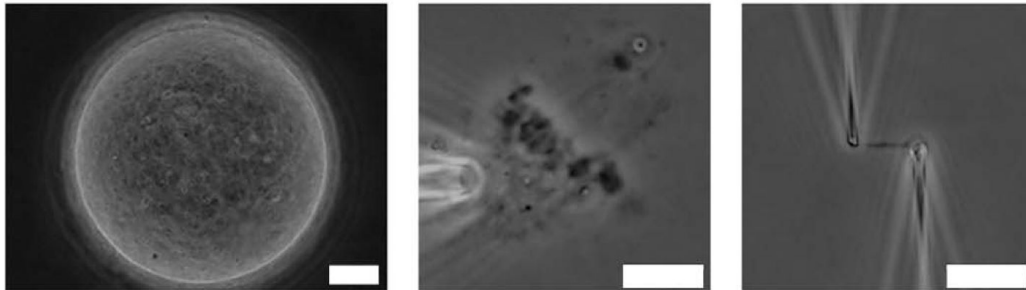


Figure 3.

Chromosome stiffness measurement in meiosis-specific cohesin mutants.

(A) Images of chromosome isolation from *Stag3*^{-/-} spermatocytes. Scale bar = 10 µm. (B) Chromosome stiffness comparison across various cell types: WT spermatocytes at prophase I (n=8), *Rec8*^{-/-} spermatocytes at prophase I (n=8), *Stag3*^{-/-} spermatocytes at prophase I (n=9) and *Rad21I*^{-/-} spermatocytes at prophase I (n=10). Young's Modulus of WT spermatocyte chromosomes (2710 ± 610 Pa) is not significantly different from that of *Rec8*^{-/-} spermatocyte chromosomes (2580 ± 620 Pa, P= 0.8884), *Stag3*^{-/-} spermatocyte chromosomes (2240 ± 210 Pa, P= 0.4533) and *Rad21I*^{-/-} spermatocyte chromosomes (2050 ± 370 Pa, P= 0.3514). Data are presented as mean ± SEM. All statistical analyses were conducted using t-test.

A



B

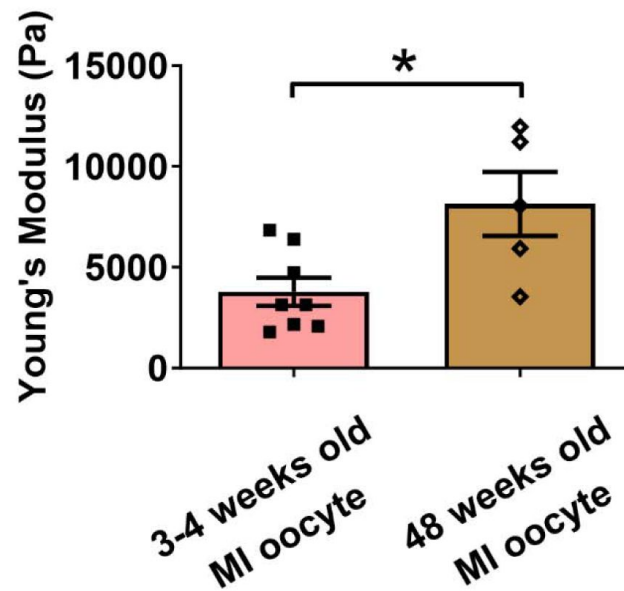


Figure 4.

Chromosomes in aged oocytes are stiffer than those in younger oocytes.

(A) Images showing the isolation of chromosomes from an aged MI oocyte. Left panel: aged MI oocyte images. Middle panel: spindle isolated from aged MI oocyte. Right panel: MI chromosome isolated from the spindle-chromosome complex. Scale bars = 10 μ m. (B) Chromosome stiffness comparison between MI oocytes from 3–4-week-old mice (n=8) and 48-week-old mice (n=5). Young's Modulus of 3–4-week-old MI oocyte chromosomes (3790 $\pm$ 700 Pa) is significantly lower than that of 48-week-old MI oocyte chromosomes (8150 $\pm$ 1590 Pa, P=0.0150). Data are presented as mean $\pm$ SEM and statistical analysis was performed using t-test.

We cultured oocytes from the GV (germinal vesicle) stage for 6 hours to MI stage in the presence of a high concentration of etoposide (50 $\mu\text{g/ml}$). Following treatment, the spindle was isolated from the oocytes. Notably, the etoposide-treated chromosomes were unevenly distributed, forming large clusters in the spindle, unlike the well-aligned chromosomes in the control group (**Figure 5A** [↗](#)). DAPI staining further confirmed that the etoposide-treated oocytes exhibited disrupted chromosome compaction and alignment compared to the control group (**Figure 5B** [↗](#)).

Upon measuring the stiffness of the chromosomes, we found that chromosomes from MI oocytes treated with 50 $\mu\text{g/ml}$ etoposide were significantly less stiff than those from untreated control oocytes (1710 ± 430 Pa versus 3780 ± 700 Pa, $P = 0.0245$) (**Figure 5C** [↗](#)). At lower etoposide concentrations, the stiffness of chromosomes from untreated control oocytes was not significantly different from that of oocytes treated with 5 $\mu\text{g/ml}$ etoposide (3780 ± 700 Pa versus 3930 ± 400 Pa, $P = 0.8624$). However, chromosome stiffness in untreated oocytes was significantly higher than that in oocytes treated with 25 $\mu\text{g/ml}$ etoposide (3780 ± 700 Pa versus 1640 ± 340 Pa, $P = 0.015$) (**Figure 5C** [↗](#)).

Overall, these findings suggest that DNA damage reduces chromosome stiffness in oocytes instead of increasing it, which aligns with previous studies showing that DNA damage can soften chromosomes [\[51\]](#). Thus, the increased chromosome stiffness observed in aged oocytes is not due to DNA damage.

Discussion

In this study, we aspired to identify how meiotic cell cycle stage and aging influence meiotic chromosome stiffness. Prior studies found a large difference between mitotic and spermatocyte meiotic chromosome stiffness [\[19\]](#). Spermatocyte meiotic prophase chromosomes were found to be ten times stiffer than somatic mitotic chromosomes, which may be due to chromosome compaction or folding mechanisms specific to meiosis. During prophase I of meiosis, the synaptonemal complex (SC) zips up the two homologous chromosomes with components that are meiosis-specific [\[52\]](#). Previously, we found that SYCP1, an essential component of the synaptonemal complex that connects the lateral and central elements, does not contribute to the high prophase I chromosome stiffness in spermatocytes [\[19\]](#), consistent with a “liquid crystal” organizational scheme of the SC [\[53\]](#).

Here, we found that high chromosome stiffness is also a feature of MI oocytes, which have a Young’s modulus approximately ten times larger than that of somatic mitotic chromosomes, five times larger than that of MII chromosomes, and comparable to that of prophase I chromosomes (**Fig. 2** [↗](#)). Notably, the Young’s modulus takes chromosome thickness/numbers into consideration, providing a volume- and geometry-independent measure of elasticity. A similar trend is observed when examining the force needed to double the length of chromosomes: stiffness increases dramatically from somatic metaphase to meiotic prophase I, persists through MI, and decreases at MII (Supplement Fig. 2). This robust pattern of chromosome stiffening during prophase I and MI aligns well with the chromosome compaction/relaxation and mechanics progression model proposed by Kleckner *et al.* [\[54\]](#). To further validate this model, it would be desirable to measure the elasticity of somatic prophase chromosomes. However, technical challenges have thus far precluded successful measurements in this context.

The SC central element dissociates from chromosomes before MI, in accord with our earlier results that SYCP1 does not contribute to high meiotic chromosome stiffness [\[19\]](#). We also measured chromosome stiffness in MII oocytes and found that it is significantly lower than in MI oocytes. This result aligns quantitatively with previous work [\[33\]](#), which reported a doubling force of MII chromosomes approximately twice that of somatic mitotic chromosomes. Given the high and

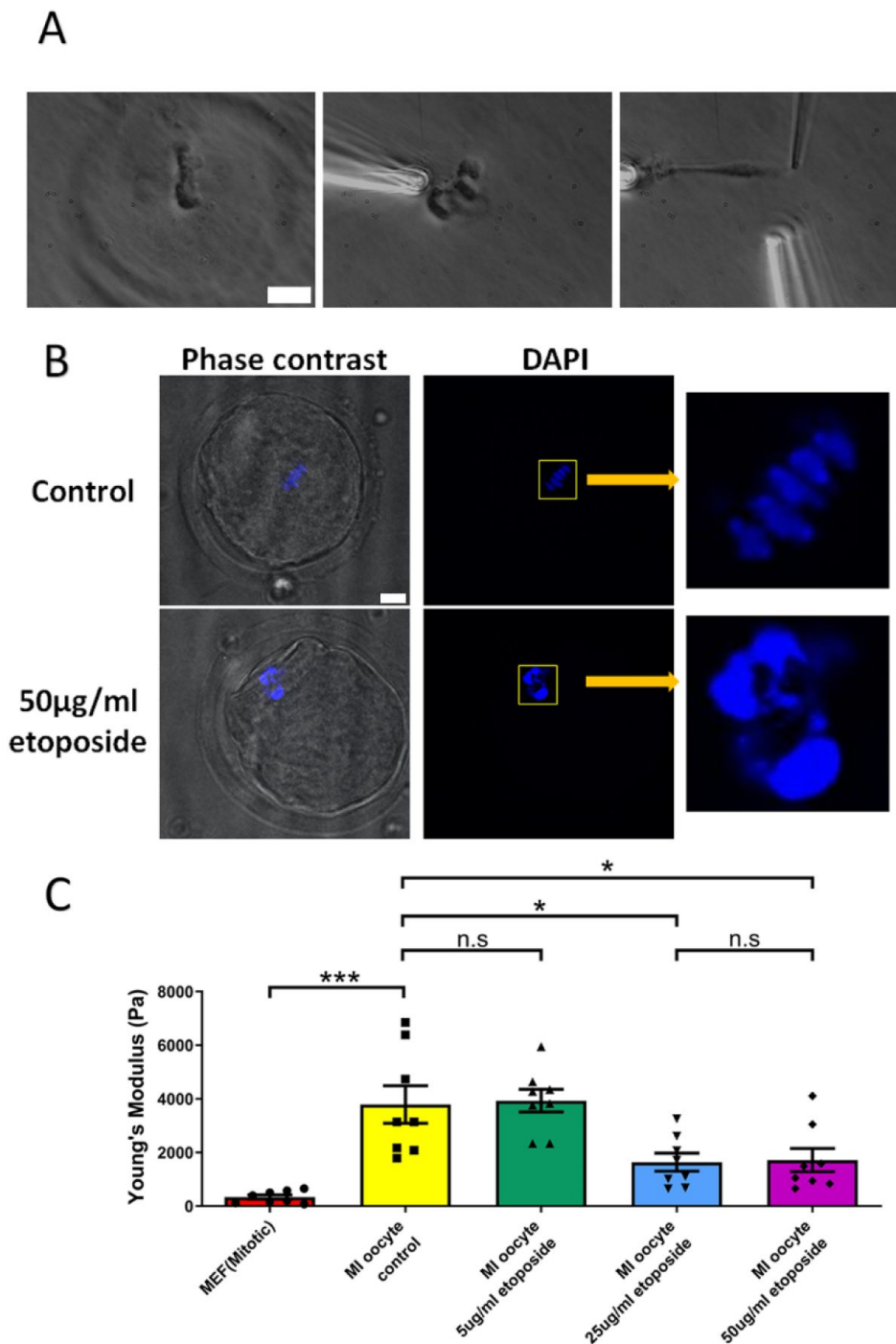


Figure 5.

Etoposide treatment reduces chromosome stiffness.

(A) Images of chromosome isolation from MI oocyte treated with 50 µg/ml etoposide. Left panel: a spindle after cell lysis. Middle panel: a spindle captured with pipettes. Right panel: chromosome isolation. Scale bar = 10 µm. (B) DAPI staining of control and 50 µg/ml etoposide-treated MI oocytes. Scale bar = 10 µm. (C) Chromosome stiffness comparison between mitotic cells (n=8), control MI oocytes (n=8), 5 µg/ml etoposide-treated MI oocytes (n=8), 25 µg/ml etoposide-treated MI oocytes (n=8) and 50 µg/ml etoposide-treated MI oocyte (n=8). Young's Modulus of control MI oocyte chromosomes (3790 ± 700 Pa) did not differ significantly from that of 5 µg/ml etoposide-treated MI oocyte chromosomes (3930 ± 400 Pa, $P = 0.8624$). However, it was significantly higher than that of 25 µg/ml etoposide-treated MI oocyte chromosomes (1640 ± 340 Pa, $P = 0.015$) and 50 µg/ml etoposide-treated MI oocyte chromosomes (1710 ± 430 Pa, $P=0.0245$). Data are presented as mean ± SEM, with statistical analysis conducted using t-test.

comparable stiffness of prophase I and MI chromosomes, we focused on factors specific to meiosis I. Notably, axial elements of the SC can still load onto chromosomes in *Sycp1*^{-/-} spermatocytes, which exhibit WT prophase I chromosome elasticity [19].

Cohesin proteins are components of the meiotic prophase chromosome axis, and load onto chromosomes during DNA replication. Some meiosis-specific cohesins, such as REC8, persist on chromosome arms until anaphase I. We hypothesized that cohesins rather than the central element might contribute to meiotic chromosome stiffness, at least through MI. However, comparing results from experiments on wild-type and cohesin-deficient (*Rec8*^{-/-}, *Rad21*^{-/-}, and *Stag3*^{-/-}) spermatocytes, we found no difference in chromosome stiffness. We conclude that the meiosis-specific cohesins do not account for high stiffness in meiotic chromosomes. While we do not yet understand the molecular origin of the high stiffness of meiotic chromosomes, we hypothesize that factors present during prophase I and persisting through MI, drive high meiotic chromosome stiffness.

A limitation of the current experiments is that we were restricted to studying meiotic cohesin mutants at prophase I in spermatocytes. While it would be valuable to extend these comparisons to prophase I oocytes, this is currently not feasible. We also note that the prophase I chromosome mechanics of WT CD-1 spermatocytes were compared to cohesin mutants on a C57BL6/J background. Ideally, control experiments would be conducted using C57BL6/J WT or heterozygous spermatocytes. However, the absence of any significant differences in prophase I chromosome mechanics across the four genetic cases studied (Fig. 3) strongly suggests that cohesin mutations do not strongly affect chromosome mechanics at prophase I.

Another limitation is that our mechanics experiments are extracellular, following removal of chromosomes from MI and MII oocytes, prophase I spermatocytes and mitotic cultured somatic cells. The extracellular environment (PBS, or cell culture buffer for somatic cells) is different from the environment inside the cell, and one might hypothesize that this changes chromosome mechanics, *e.g.*, via loss of chromosome-folding proteins after their isolation. In addition to past experiments indicating that mitotic chromosomes are stable for long periods after their isolation [55], we carried out control experiments on mouse oocyte chromosomes where we incubated them for 1 hour in PBS, or exposed them to a flow of Triton X-100 solution for 10 minutes; there was no change in chromosome stiffness in either case (Methods and Supplementary Fig. 5). Across all our experiments we found quantitative agreement across trials under corresponding conditions, arguing against there being large uncontrolled changes in chromosome structure resulting from our isolation method.

Previous research on mitotic chromosome stiffness revealed that chromosomes have a chromatin network organization [14]. Several factors, including condensin, have been found to affect chromosome stiffness [34]. Condensin exists in two distinct complexes, condensin I and condensin II, and both are active during meiosis. Published studies indicate that condensin II is more sharply defined and more closely associated with the chromosome axis from anaphase I to metaphase II [56]. Additionally, condensin II appears to play a more significant role in mitotic chromosome mechanics compared to condensin I [34]. Thus, condensin II likely contributes more significantly to meiotic chromosome stiffness than condensin I. It would be interesting to determine to what extent condensin defects affect meiotic chromosome structure.

Age also plays a role in altering chromosome stiffness. We found that chromosomes from aged MI oocytes had higher stiffness compared to those from younger oocytes, in accord with previous observations for MII chromosomes [33]. This supports our conclusion that cohesins are not the primary factor making meiotic chromosomes stiffer, as cohesin-protein levels reduce with age. The molecular mechanisms underlying the age-related stiffening of oocyte meiotic chromosomes need further investigation.

Hundreds of DNA double-stranded breaks are spontaneously introduced by the SPO11 protein at the onset of meiosis and aging also induces DNA damage [57,58]. How can meiotic chromosomes still become stiffer even in the presence of hundreds of DNA breaks *in vivo*? The answer may lie in the DNA repair proteins that are recruited to or near the DNA damage sites to repair the DNA damage and maintain genome integrity. Therefore, we hypothesized that DNA repair proteins contribute to meiotic chromosome stiffness. To test this hypothesis, we used a chemotherapy drug, etoposide, to induce DNA damage in MI oocytes. Etoposide can increase the levels of TOP2–DNA covalent complexes, which generate DNA damage [59]. We found that etoposide treatment caused chromosome stiffness to decrease. This result is consistent with our previous study that DNA breaks can reduce chromosome stiffness *in vitro* [14], reaffirming that meiotic chromosomes integrity relies on DNA continuity rather than the linkage of chromosome axis components [13].

Since DNA repair proteins do not increase chromosome stiffness, other factors must be responsible for the high level of meiotic chromosome stiffness. During senescence, the amount of nuclear protein increases by around two-fold, even though the cohesin level is decreased [45,60]. Thus, it is possible that some nuclear proteins increase chromosome stiffness with age. However, further investigation is needed to determine which nuclear proteins contribute to chromosome stiffness. Additionally, histone methylation might influence chromosome stiffness because the level of histone methylation is proportional to chromosome stiffness [13]. Given that histone methylation levels are altered during aging, it is also plausible that some histone methyltransferases and demethylases regulate chromosome stiffness [61,62]. Moreover, histone methylation, especially H3K4, H3K9 and H3K36, plays a very important role in synapsis and recombination and has been found on meiotic chromatin from leptotene stage onward [63–65]. Further experiments could test the hypothesis that histone methylation regulates meiotic chromosome stiffness across different cell types.

No matter what factors are involved in stiffening meiotic chromosomes, they must alter chromosome organization to regulate chromosome stiffness. This indicates that chromosome stiffness is an important parameter for understanding chromosome structure. Defective chromosome organization is often related to various diseases, such as cancer, infertility, and senescence [66–68], and can be expected to cause changes in chromosome mechanics. By using micromanipulation experiments to study chromosome stiffness, we can better understand the mechanisms underlying these chromosome defects, potentially leading to new treatments and therapies.

A well-documented characteristic of aged oocytes is their higher rate of aneuploidy compared to that of younger oocytes [69,70]. The majority of aneuploidies can be traced to meiosis I, especially anaphase I, because it is an error-prone process [71]. It would be intriguing to investigate whether the increased chromosome stiffness contributes to this issue. A common cause of aneuploidy is lagging chromosomes during anaphase I [72]. Chromosome stiffness may be related to the occurrence of lagging chromosomes, as chromosome separation relies on the pulling forces exerted by microtubules [73,74]. During anaphase I, homologous chromosomes, rather than sister chromatids, segregate. Thus, the chromosome region between the centromere and crossover must resist the tension applied by the spindle [73]. Chromosomes must be stiff enough to prevent breakage under the pulling force. If the chromosomes are excessively stiff, the tightly connected homologous chromosomes may not properly separate, potentially causing aneuploidy. This suggests that chromosome stiffness change may be related to the high rate of aneuploidy observed in aged oocytes. Modulating factors altering chromosome stiffness may help reduce the incidence of aneuploidy. Moreover, further investigation into the relationship between chromosome stiffness and lagging chromosomes could provide insight into the mechanisms underlying aneuploidy in oocytes.

Materials and Methods

Animals

Wild-type CD-1 mice (Charles River Laboratories, Wilmington, MA) were used for all chromosome measurements, except for *Rec8*, *Stag3*, and *Rad21l* mutants, which were maintained on a C57BL/6 background. All mice were housed in the Pancoe CCM rooms of Northwestern University under a 12 h dark/light cycle at $22 \pm 1^\circ\text{C}$, with unrestricted access to food and water. All animal handling and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Northwestern University.

Mouse oocyte *in vitro* culture

Oocytes were harvested from 3-to 4-week-old mice (48-week-old mice for aging studies) and culture *in vitro*. Both ovaries were immediately dissected and washed with M2 medium. The ovaries were then placed in prewarmed M2 medium containing $100 \mu\text{M}$ IBMX at 37°C . Sterilized needles were used to release cumulus-oocyte complexes (COCs). Next, COCs were pipetted in and out several times with a mouth pipette under dissection microscope to remove the cumulus cells surrounding the oocyte, yielding denuded oocytes at the germinal vesicle (GV) stage. These oocytes were then placed in prewarmed M2 medium containing $100 \mu\text{M}$ IBMX.

Oocytes with irregular shapes and abnormal sizes were discarded, while healthy oocytes were selected and washed three times in M16 medium. The oocytes were then transferred to small drops of M16 medium covered with mineral oil in a petri dish and incubated at 37°C in a 5% CO_2 incubator. Depending on the experimental plan, oocytes were cultured with or without additional chemicals for 6 hours to reach the MI stage or 14 hours to reach the MII stage. After culture, the oocytes were rinsed three times with M2 medium and transferred to Tyrode's solution to remove the zona pellucida. Finally, the oocytes were transferred to PBS (phosphate buffered saline) solution for chromosome stiffness measurements.

Mitotic cell culture

Mouse embryonic fibroblasts (MEFs) were used for mitotic chromosome measurements. The MEF cells were cultured in DMEM (Corning) supplemented with 10% fetal bovine serum (FBS) (HyClone) and 1% penicillin-streptomycin (100 X, Corning). Cultures were maintained at 37°C in a 5% CO_2 incubator and passaged every 3-5 days, with a maximum of 20 passages.

For chromosome measurements, cells were transferred to prepared culture wells, which were made by fixing rubber rings on coverslips using wax. Each well was filled with 2 mL of culture media. The cells were cultured in these wells for 1-3 days to allow attachment to the coverslips. Chromosome measurements were conducted directly within these culture wells.

Spermatocyte preparation

Testes were dissected from adult mice [19]. After removing the tunica albuginea, small clusters of seminiferous tubules were isolated and rinsed in PBS solution. These tubules were then finely chopped with a surgical blade to release spermatocytes, which were transferred into a culture well containing 2 mL of PBS. The spermatocytes were allowed to settle at the bottom of the well and subsequently used for chromosome measurements.

Chromosome isolation

Hold pipettes, force pipettes, and stiff pipettes were prepared for manipulating chromosomes. They were made using a micropipette puller (Sutter P-97) and cut to appropriate sizes [19]. Chromosomes were isolated and measured under an inverted microscope (IX-70; Olympus) with a 60x 1.42 NA oil immersion objective and a 1.5x magnification pullout. All experiments were conducted at room temperature within 3 hours to minimize the effects of water evaporation from the culture well.

MEF cells and spermatocytes were visually identified under phase-contrast microscopy. These cells were lysed with 0.05% Triton X-100 in PBS, applied using a spray pipette to remove cell membranes. After lysis, meiotic or mitotic chromosome bundles were released and captured by a pipette.

To isolate individual chromosomes, a force pipette was used to attach and extract a single chromosome from meiotic or mitotic chromosome bundles. Then, a stiff pipette was used to grab the other end of the chromosome, while the remaining chromosome bundles were carefully moved away.

For oocytes, a notable difference was that the spindle, along with its chromosomes, could be isolated as a unit. In this case, a force pipette was inserted into the spindle to capture chromosomes and drag them out from the spindle. After isolation, the chromosomes were ready for measurements.

Chromosome stiffness measurement and calculation

Once the chromosome was held between the floppy force and moving stiff pipettes, it was stretched by moving the stiff pipettes perpendicularly. The process was recorded by LabVIEW software [19]. Before stretching, an image of the chromosome was captured to calculate the deflection of the force pipette. The stiff pipette was moved around 6.0 μm and then returned to its original position at a constant rate of 0.20 $\mu\text{m}/\text{second}$, with 0.04 μm steps controlled by the LabVIEW program. This measurement process was repeated six times.

The position of the stiff and force pipettes were recorded during the experiment. From the captured image, the original length and radius (r) of the chromosome were measured using ImageJ. The cross-section area (A) was calculated using the formula $A = \pi r^2/2$. The force constant of the force pipette was calibrated using a reference pipette with a premeasured spring constant [19]. Young's modulus (E) was calculated according to the formula $E = (F/A)/(\Delta L/L_0)$.

E was the Young's modulus, F was the force applied to stretch the chromosome, A was the cross-section area of the chromosome, ΔL was changed in chromosome length during stretching, and L_0 was the original length of the chromosome.

All measurements of meiotic chromosome stiffness were conducted in PBS solution. To investigate the effects of PBS exposure on chromosome stiffness, the Young's modulus of spermatocyte chromosomes was measured and compared before and after 1 hour of incubation in PBS (Supplement Fig. 5 Left panel). Similarly, to assess the impact of Triton X-100, the Young's modulus of spermatocyte chromosomes was measured and compared before and after microspraying with 0.05% Triton X-100 for 10 minutes (Supplement Fig. 5 Right panel). We observed no significant change of chromosome stiffness in either of these cases, leading us to the conclusion that chromosome stiffness is not strongly affected by exposure to PBS or to Triton X-100.

Chromosome staining

Isolated oocytes were fixed in 4% (W/V) paraformaldehyde in PBS solution for 30 min at room temperature (RT). The oocytes were then washed three times with a washing buffer (0.1% tween-20 and 0.01% Triton X-100 in PBS). Next, the oocytes were permeabilized in PBS containing 0.5% Triton X-100 for 20 min at RT. After that, the oocytes were transferred into 3% BSA (bovine serum albumin) for blocking for one hour at RT. After blocking, the oocytes were washed three times and counterstained with 1 µg/ml of 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) for 10 min at RT. Finally, the oocytes were washed twice with the washing buffer, mounted on glass slides in 80% glycerol, and examined using a Nikon A1R confocal microscope. Images were processed with NIS-Elements software.

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Additional information

Author contributions

H. Qiao and J. F. Marko conceived the project and designed the experiments; N. Liu performed all the experiments and analyzed the data; P. Jordan provided experimental direction and cohesin mutant mice for this study. W. Qiang provided technical assistance. P. Jordan and W. Qiang helped with manuscript editing; and N. Liu, H. Qiao, and J.F. Marko wrote the manuscript. All authors reviewed and approved the final version.

Additional files

Supplemental Figures 

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Reviewer #1 (Public review):

Summary:

By using the biophysical chromosome stretching, the authors measured the stiffness of chromosomes of mouse oocytes in meiosis I (MI) and meiosis II (MII). This study was the follow-up of previous studies in spermatocytes (and oocytes) by the authors (Biggs et al. *Commun. Biol.* 2020; Hornick et al. *J. Assist. Rep. and Genet.* 2015). They showed that MI chromosomes are much stiffer (~10 fold) than mitotic chromosomes of mouse embryonic fibroblast (MEF) cells. MII chromosomes are also stiffer than the mitotic chromosomes. The authors also found that oocyte aging increases the stiffness of the chromosomes. Surprisingly, the stiffness of meiotic chromosomes is independent of meiotic chromosome components, Rec8, Stag3, and Rad21L, and aging increases the stiffness.

Strengths

This provides a new insight into the biophysical property of meiotic chromosomes, that is chromosome stiffness. The stiffness of chromosomes in meiosis prophase I is ~10-fold higher than that of mitotic chromosomes, which is independent of meiotic cohesin. The increased stiffness during oocyte aging is a novel finding.

Weaknesses:

A major weakness of this paper is that it does not provide any molecular mechanism underlying the difference between MI and MII chromosomes (and/or prophase I and mitotic chromosomes).

Comments on revisions:

The main text lacks the first page with the authors' names and their affiliations (and corresponding authors etc).

<https://doi.org/10.7554/eLife.97403.2.sa2>

Reviewer #2 (Public review):

Initial Review:

This paper reports investigations of chromosome stiffness in oocytes and spermatocytes> the paper shows that prophase I spermatocytes and MI/MII oocytes yield high Young Modulus values in the assay the authors applied. Deficiency in each one of three meiosis-specific cohesins they claim did not affect this result and increased stiffness was seen in aged oocytes but not in oocytes treated with the DNA-damaging agent etoposide.

The paper reports some interesting observations which are in line with a report by the same authors of 2020 where increased stiffness of spermatocyte chromosomes was already shown. In that sense, if the current manuscript is an extension of that previous paper and thus novelty is somewhat limited. The paper is also largely descriptive as it does neither propose mechanism nor report factors that determine the chromosomal stiffness.

There are several points that need to be considered.

Limitations of the study and the conclusions are not discussed in "Discussion"; that's a significant gap. Even more so as the authors rely on just one experimental system for all their data - no independent verification - and that in vitro system may be prone to artefacts.

It is somewhat unfortunate that they jump between oocytes and spermatocytes to address the cohesin question. Prophase I (pachytene) spermatocytes chromosomes are not directly comparable to MI or MII oocyte chromosomes. In fact, the authors report Young Modulus values of 3700 for MI oocytes and only 2700 for spermatocyte prophase chromosomes, illustrating this difference. Why not using oocyte-specific cohesin deficiencies?

It remains unclear whether the treatment of oocytes with the detergent TritonX-100 affects the spindle and thus the chromosomes isolated directly from the Triton-lysed oocytes. In fact, it is rather likely that the detergent affects chromatin-associated proteins and thus structural features of the chromosomes.

Why did the authors use mouse strains of different genetic background, CD-1 and C57BL/6? That makes comparison difficult. Breeding of heterozygous cohesin mutants will yield the ideal controls, i.e. littermates.

How did the authors capture chromosome axes from STAG3-deficient spermatocytes which feature very little if any axes? How representative are those chromosomes that could be captured?

Line 135: that statement is not substantiated; better to show retraction data and full reversibility.

Line 144: the authors claim that the Young Modulus of MII oocytes is "slightly" higher than that of mitotic cells (MEFs). Well, "slightly" means it is rather similar and therefore the commonly used statement that MII is similar to mitosis is OK - contrary to the authors claim.

There are a lot of awkward sentences in this text. Some sentences lack words, are not sufficiently precise in wording and/or logic, and there are numerous typos. Some examples can be found in lines 89 (grammar), 94, 95 ("looked"), 98, 101 ("difference" - between what?), and some are commonplaces or superficial (lines 92/93, 120...,). Occasionally the present and past tense are mixed (e.g. in M&M). Thus the manuscript is quite badly written.

Comments on revisions:

In their revised paper, Liu et al have addressed a number of my concerns and thus the paper is clearly improved in several details, e.g. in showing a control for a potential effect of the detergent (new supplies. fig. 5). Other points were not sufficiently addressed though.

I remain sceptical about using mice of a substantially different genetic background (CD1) as controls in the analysis of the cohesin mutants (C57BL/6). The argument that C57BL/6 yield smaller litter size is, frankly, ridiculous. Hundreds of labs worldwide extensively and successfully work with C57BL/6. Further, the paper Liu et al. cite to argue that there are no (or minor) differences in chromosome structure (Biggs et al., 2020, which is from the same lab) of the two mouse strains deals with spermatocyte chromosomes only. Nothing there on oocyte chromosomes. And there is no direct comparison within the same experimental setting since in Biggs et al only C57BL/6 is used (sic!). Thus, this is not a convincing argument. It would also be reassuring to see an independent reference directly comparing different genetic backgrounds (authors may have a look at older papers of Pat Hunt/Terry Hassold where they may find some data). In my experience, differences in genetic background do play a very clear role in meiosis, e.g. in the timing of juvenile spermatogenesis, in the onset of puberty, in the kinetics of oocyte maturation, in the success of PBE, and in biophysical properties as seen in the stability of oocytes during experimental handling. In fact, the authors themselves indicate differences in reproduction by stating the low litter size of C57BL/6. Thus, I strongly advise carrying out at least a few key experiments using C57BL/6 control mice (which can very easily and cheaply be obtained from vendors; the authors have used C57BL/6 wt before - see their 2020 paper).

The answer to my question #5 is not really satisfactory. I asked specifically how the authors isolated the very small chromosomes from Stag3^{-/-} spermatocytes, where the axes are almost non-existing. The authors refer to suppl. fig. 3, but that shows isolation from Rec8^{-/-} spermatocytes, which still have nicely visible, well-formed, shortened axes. Suppl. fig. 4 shows this for Rad21^{-/-}. Why not show this for the Stag3^{-/-}, which in this respect is the most critical and difficult, and specifically answer my question?

The overall criticism of the lack of conceptual novelty of the basic message of the paper and of very little if any insights into the mechanisms and factors determining the changes in chromosome stiffness remains.

<https://doi.org/10.7554/eLife.97403.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

Summary:

By using the biophysical chromosome stretching, the authors measured the stiffness of chromosomes of mouse oocytes in meiosis I (MI) and meiosis II (MII). This study was the follow-up of previous studies in spermatocytes (and oocytes) by the authors (Biggs et al. Commun. Biol. 2020; Hornick et al. J. Assist. Rep. and Genet. 2015). They showed that MI chromosomes are much stiffer (~10 fold) than mitotic chromosomes of mouse embryonic fibroblast (MEF) cells. MII chromosomes are also stiffer than the mitotic chromosomes. The authors also found that oocyte aging increases the stiffness of the chromosomes. Surprisingly, the stiffness of meiotic chromosomes is independent of meiotic chromosome components, Rec8, Stag3, and Rad21L. with aging.

Strengths:

This provides a new insight into the biophysical property of meiotic chromosomes, that is chromosome stiffness. The stiffness of chromosomes in meiosis prophase I is ~10-fold higher than that of mitotic chromosomes, which is independent of meiotic cohesin. The increased stiffness during oocyte aging is a novel finding.

Weaknesses:

A major weakness of this paper is that it does not provide any molecular mechanism underlying the difference between MI and MII chromosomes (and/or prophase I and mitotic chromosomes).

We acknowledge that our study does not provide a comprehensive explanation for the stage-related alterations in chromosome stiffness; however, we believe that the observation of these changes is itself of broad interest. Initially, we hypothesized that DNA damage or depletion of meiosis-specific cohesin might contribute to the observed increase in chromosome stiffness. However, our experimental finding did not support these hypotheses, indicating that neither DNA damage nor cohesion depletion is responsible for the stiffness increase. The molecular basis underlying the stage-related stiffness increase remains elusive and requires exploration in future studies. In the Discussion, we propose that factors such as condensin, nuclear proteins, and histone methylation may play a role in regulating meiotic chromosome stiffness. The involvement of these factors in stage-related chromosome stiffening requires future investigation.

Reviewer #2 (Public Review):

This paper reports investigations of chromosome stiffness in oocytes and spermatocytes. The paper shows that prophase I spermatocytes and MI/MII oocytes yield high Young Modulus values in the assay the authors applied. Deficiency in each one of three meiosis-specific cohesins they claim did not affect this result and increased stiffness was seen in aged oocytes but not in oocytes treated with the DNA-damaging agent etoposide.

The paper reports some interesting observations which are in line with a report by the same authors of 2020 where increased stiffness of spermatocyte chromosomes was already shown. In that sense, the current manuscript is an extension of that previous paper, and thus novelty is somewhat limited. The paper is also largely descriptive as it

does neither propose a mechanism nor report factors that determine the chromosomal stiffness.

There are several points that need to be considered.

(1) Limitations of the study and the conclusions are not discussed in the "Discussion" section and that is a significant gap. Even more so as the authors rely on just one experimental system for all their data - there is no independent verification - and that in vitro system may be prone to artefacts.

Our experimental system has been used to study different types of chromosome stiffness as well as nuclear stiffness. We have compared our results with previously published data and found the data is consistent across different experiments. To address the reviewer's concern, we describe the limitations of our in vitro experimental approach in the Discussion section.

(2) It is somewhat unfortunate that they jump between oocytes and spermatocytes to address the cohesin question. Prophase I (pachytene) spermatocytes chromosomes are not directly comparable to MI or MII oocyte chromosomes. In fact, the authors report Young Modulus values of 3700 for MI oocytes and only 2700 for spermatocyte prophase chromosomes, illustrating this difference. Why not use oocyte-specific cohesin deficiencies?

In this study, our goal was to investigate the mechanism underlying the increased chromosome stiffness observed during prophase I. Ideally, we would have compared wild-type and cohesin-deleted mouse oocytes at the metaphase I (MI) stage. However, experimental constraints made this approach unfeasible: spermatocytes and oocytes from *Rec8*^{-/-} and *Stag3*^{-/-} mutant mice cannot reach MI stage, and *Rad21*^{-/-} mutant mice are sterile in males and subfertile in females, because cohesin proteins are crucial for germline cell development.

Additionally, collecting prophase I chromosomes from oocytes is exceptionally challenging and requires fetal mice as prophase I oocyte sources because female oocytes progress to the diplotene stage during fetal development. The process is further complicated by the difficulty of genotyping fetal mice, making the study of female prophase I impracticable. By contrast, spermatocytes are continuously generated in males throughout life, with meiotic stages readily identifiable, making them more accessible for analysis.

Our findings consistently showed increased chromosome stiffness in both prophase I spermatocytes and MI oocytes, suggesting that the phenomenon is not sex-specific. This observation implies that similar effects on chromosome stiffness may occur across meiotic stages, from prophase I to MI.

(3) It remains unclear whether the treatment of oocytes with the detergent TritonX-100 affects the spindle and thus the chromosomes isolated directly from the Triton-lysed oocytes. In fact, it is rather likely that the detergent affects chromatin-associated proteins and thus structural features of the chromosomes.

Regarding the use of Triton X-100, it is important to emphasize that the concentration used (0.05%) is very low and unlikely to significantly affect chromosome stiffness. To support this assertion, we have provided additional evidence in the revised manuscript demonstrating that this low concentration of Triton X-100 has a negligible effect on chromosome stiffness (Supplement Fig. 5, Right panel).

(4) Why did the authors use mouse strains of different genetic backgrounds, CD-1, and C57BL/6? That makes comparison difficult. Breeding of heterozygous cohesin mutants

will yield the ideal controls, i.e. littermates.

The genetic mutant mice, all in a C57BL/6 background, were generously provided by Dr. Philip Jordan and delivered to our lab. As our lab does not currently maintain C57BL/6 colony and given that this strain typically produces small litter sizes - which would have complicated the remainder of the study - we chose CD-1 mice as the control group and used C57BL/6 mice specifically for the cohesin study. To address potential concerns regarding genetic background differences, we compared our results with previously published data from C57BL/6 mice and found no significant differences (2710 ± 610 Pa versus 3670 ± 840 Pa, $P=0.4809$) (Biggs et al., 2020). Furthermore, prophase I spermatocytes from CD-1 mice showed no significant difference compared to any of the three cohesin-deleted C57BL/6 mutant mice, suggesting that chromosome stiffness is not significantly influenced by genetic background.

(5) How did the authors capture chromosome axes from STAG3-deficient spermatocytes which feature very few if any axes? How representative are those chromosomes that could be captured?

We isolated chromosomes from prophase I mutant spermatocytes, which were identified by their large size, round shape, and thick chromosomal threads - characteristics indicative of advanced condensation and a zygotene-like stage during prophase I (Supplemental Fig. 3). The methodology for isolating these chromosomes has been described in details in our previous publication (Biggs et al., 2020), which is referenced in the current manuscript.

Reviewer #3 (Public Review):

Summary:

Understanding the mechanical properties of chromosomes remains an important issue in cell biology. Measuring chromosome stiffness can provide valuable insights into chromosome organization and function. Using a sophisticated micromanipulation system, Liu et al. analyzed chromosome stiffness in MI and MII oocytes. The authors found that chromosomes in MI oocytes were ten-fold stiffer than mitotic ones. The stiffness of chromosomes in MI mouse oocytes was significantly higher than that in MII oocytes. Furthermore, the knockout of the meiosis-specific cohesin component (Rec8, Stag3, Rad21) did not affect meiotic chromosome stiffness. Interestingly, the authors showed that chromosomes from old MI oocytes had higher stiffness than those from young MI oocytes. The authors claimed this effect was not due to the accumulated DNA damage during the aging process because induced DNA damage reduced chromosome stiffness in oocytes.

Strengths:

The technique used (isolating the chromosomes in meiosis and measuring their stiffness) is the authors' specialty. The results are intriguing and informative to the chromatin/chromosome and other related fields.

Weaknesses:

(1) How intact the measured chromosomes were is unclear.

Currently, a well-calibrated chromosome mechanics experiment requires the extracellular isolation of chromosomes. In experiments conducted parallel to those in our previous study (Biggs et al., 2020), we obtained quantitatively consistent results, including measurements of the Young modulus for prophase I spermatocyte chromosomes. Our isolation approach is significantly gentler than bulk methods that rely on hypotonic buffer-driven cell lysis and centrifugation. If substantial chromosomal damage had occurred during isolation, we would

expect greater variation between experiments, as different amounts or types of damage could influence the results.

| (2) *Some control data needs to be included.*

We used wild-type prophase I spermatocytes and metaphase I (MI) oocytes as controls. To validate our findings, we compared some of our results with those reported in a previous study and observed consistent outcomes (Biggs et al., 2020).

| (3) *The paper was not well-written, particularly the Introduction section.*

We have revised the paper and improved the overall quality of the manuscript.

| (4) *How intact were the measured chromosomes? Although the structural preservation of the chromosomes is essential for this kind of measurement, the meiotic chromosomes were isolated in PBS with Triton X-100 and measured at room temperature. It is known that chromosomes are very sensitive to cation concentrations and macromolecular crowding in the environment (PMID: 29358072, 22540018, 37986866). It would be better to discuss this point.*

As suggested, we investigated the impact of PBS and Triton X-100 on chromosome stiffness. Our findings indicate that neither PBS nor Triton X-100 caused significant changes in chromosome stiffness (Supplemental Fig. 5).

| **Recommendations For The Authors:**

Major points of Reviewers that the Editor indicated should be addressed

| (1) *Reviewer's point 3, the effect of the high concentration of etoposide: It would be advisable to use lower concentrations of etoposide to observe the effect of DNA damage on chromosome stiffness more accurately.*

The effect of etoposide on oocyte is dose-dependent (Collins et al., 2015). Oocytes are generally not highly sensitive to DNA damage, and even at relatively high concentrations, not all may exhibit a response. To ensure that sufficient DNA damage in the oocytes we isolated, we used relatively high concentration of etoposide for the experiment. This concentration (50 $\mu\text{g/ml}$) falls within the typical range reported in the literature (Marangos and Carroll, 2012)(Cai et al., 2023)(Lee et al., 2023). As the reviewer suggested, we tested two additional lower concentrations of etoposide (5 $\mu\text{g/ml}$ and 25 $\mu\text{g/ml}$) (see Fig. 5 C). We did not observe any significant differences in chromosome stiffness in 5 $\mu\text{g/ml}$ etoposide-treated oocytes compared to the control. However, higher concentrations of etoposide (25 $\mu\text{g/ml}$) significantly reduced oocyte chromosome stiffness compared to the control.

Revision to manuscript:

“Results at lower etoposide concentrations revealed that chromosome stiffness in untreated control oocytes was not significantly different from that in oocytes treated with 5 $\mu\text{g/ml}$ etoposide (3780 ± 700 Pa versus 3930 ± 400 Pa, $P = 0.8624$). However, chromosome stiffness in untreated oocytes was significantly higher than that in oocytes treated with 25 $\mu\text{g/ml}$ etoposide (3780 ± 700 Pa versus 1640 ± 340 Pa, $P = 0.015$) (Figure 5C).”

| (2) *Reviewer's point 3, the effect of Triton X-100: This is related to the concern of the #3 reviewer. It is critical to check whether the detergent does not affect the stiffness indirectly or not.*

To demonstrate that the low concentration of Triton X-100 does not influence chromosome stiffness, we conducted additional experiments. First, we isolated chromosomes and measured their stiffness. Then, we treated the chromosomes with 0.05% Triton X-100 via micro-spraying and remeasured the stiffness. The results showed no significant difference (see Supplement Fig. 5 right panel).

Revision to manuscript:

“In addition to past experiments indicating that mitotic chromosomes are stable for long periods after their isolation (Pope et al., 2006), we carried out control experiments on mouse oocyte chromosomes where we incubated them for 1 hour in PBS, or exposed them to a flow of Triton X-100 solution for 10 minutes; there was no change in chromosome stiffness in either case (Methods and Supplementary Fig. 5).”

(3) Reviewer's point 1, the effect of the buffer composition: Please describe how the composition affects the stiffness of the chromosomes.

PBS is an economical and effective buffer solution that closely mimics the osmotic conditions of the cytoplasm, which is crucial for maintaining chromosomal structural integrity. Appropriate ion concentrations are crucial for preserving chromosome integrity, as imbalances—either too high or too low—can alter chromosome morphology (Poirier and Marko, 2002). When chromosomes are stored in PBS, their stiffness remains relatively stable, even with prolonged exposure, ensuring minimal changes to their physical properties. To confirm this, we isolated chromosomes and measured their stiffness. After one-hour incubation in PBS, we remeasured stiffness and observed no significant differences, which demonstrated that chromosomes remain stable in PBS (see Supplement Fig. 5 left panel).

Revision to manuscript:

“In this study, we developed a new way to isolate meiotic chromosomes and measure their stiffness. However, one concern is that the measurements were conducted in PBS solution, which is different from the intracellular environment. To address this, we monitored chromosome stiffness overtime in PBS solution and found that it remained stable over a period of one hour (Supplement Fig. 5 Left panel).”

Reviewer #1 (Recommendations For The Authors):

Major points:

(1) Previously, the role of condensin complexes in chromosome stiffness is shown (Sun et al. Chromosome Research, 2018). Thus, at least the authors described the condensin staining on MI and MII chromosomes.

We have added sentences in the discussion to elaborate on the role of condensin.

Revision to manuscript:

“Several factors, including condensin, have been found to affect chromosome stiffness (Sun et al., 2018). Condensin exists in two distinct complexes, condensin I and condensin II, and both are active during meiosis. Published studies indicate that condensin II is more sharply defined and more closely associated with the chromosome axis from anaphase I to metaphase II (Lee et al., 2011). Additionally, condensin II appears to play a more significant role in mitotic chromosome mechanics compared to condensin I (Sun et al., 2018). Thus, condensin II likely contributes more significantly to meiotic chromosome stiffness than condensin I.”

(2) Although the authors nicely showed the difference in the stiffness between MI and MII chromosomes (Figure 2), as known, MI chromosomes are bivalent (with four chromatids) while MII chromosomes are univalent (with two chromatids). The physical property of the chromosomes would be affected by the number of chromatids. It would be essential for the authors to measure the physical properties of a univalent of MI chromosomes from mice defective in meiotic recombination such as Spo11 and/or Mlh3 KO mice.

The reviewer correctly pointed out that the number of chromatids in chromosomes differs between metaphase I (MI) and metaphase II (MII) stages. We have addressed this difference by calculating Young's modulus (E), a mechanical property that describes the elasticity of a material, independent of its geometry. Young's modulus describes the intrinsic properties of the material itself, rather than the specific characteristics of the object being tested. It is calculated as $E = (F/A) / (\Delta L/L_0)$, where F was the force given to stretch the chromosome, A was the cross-section area, ΔL was the length change of the chromosome, and L_0 was the original length of the chromosome. While an increase in chromosome or chromatid numbers, results in a larger cross-sectional area, leading to a higher doubling force (F). This variation in chromosome number or cross-sectional area does not impact the calculation of chromosome stiffness/Young's modulus (E). While study of the mutants suggested by the referee would certainly be interesting, it would be likely that the absence of these key recombination factors would impact chromosome stiffness in a more complex way than just changing their thickness; this type of study is beyond the scope of the present manuscript and is an exciting direction for future studies.

(3) In Figure 5, the authors measure the stiffness of etoposide-treated MI chromosomes. The concentration of the drug was 50 ug/ml, which is very high. The authors should analyze the different concentrations of the drug to check the chromosome stiffness. Moreover, etoposide is an inhibitor of Topoisomerase II. The effect of the drug might be caused by the defective Top2 activity, rather than Top2-adducts, thus DNA damage. It is very important to check the other Top2 inhibitors or DNA-damaging agents to generalize the effect of DNA damage on chromosome stiffness. Moreover, DNA damage induces the DNA damage response. It is important to check the effect of DDR inhibitors on the damage-induced change of stiffness.

The reviewer is correct in noting that etoposide can induce DNA damage and inhibit Top2 activity. To address this concern, our previous DNase experiment provided further clarity and supports our results of this study (Biggs et al., 2020). This experiment was conducted *in vitro*, where DNase treatment caused DNA damage on chromosomes without affecting Top2 activity or triggering DNA damage response. The results demonstrated that DNase treatment led to reduced chromosome stiffness, which aligns with the findings presented in our manuscript.

(4) In the same line as the #3 point, the authors also need to check the effect of etoposide on the stiffness of mitotic chromosomes from MEF.

Experiments on MEF mitotic chromosomes were designed to serve as a reference for the meiotic chromosome studies. The etoposide experiments on meiotic chromosomes specifically aimed to investigate how DNA damage affects meiotic chromosome structure. While it would be interesting to explore the effects of etoposide-induced DNA damage on mitotic chromosomes, it represents a distinct research question that falls outside the scope of the current study.

Minor points:

(1) Line 141-142: Previous studies by the author analyzed the stiffness of mitotic chromosomes from pro-metaphase. Which stage of cell cycles did the authors analyze here?

To ensure consistency in our experiments, we also measured the stiffness of mitotic chromosomes at the prometaphase stage. The precise stage used is very near to metaphase, at the very end of the prometaphase stage. We have modified the manuscript to clarify this point.

Revision to manuscript:

“For comparison with the meiotic case, we measured the chromosome stiffness of Mouse Embryonic Fibroblasts (MEFs) at late pro-metaphase (just slightly before their attachment to the mitotic spindle) and found that the average Young’s modulus was 340 ± 80 Pa (Figure 2B). The value is consistent with our previously published data, where the modulus for MEFs was measured to be 370 ± 70 Pa (Biggs et al., 2020).”

(2) Line 157: Here, the doubling force of MI (and MII) oocytes should be described in addition to those of spermatocytes.

The purpose of this paragraph is to demonstrate the reproductivity and consistency of our experiments. In this section, we compared our data with previously published findings. Published data do not include chromosome stiffness measurement from MI mouse oocytes. Our experiment is the first to assess this. Therefore, we did not include MI mouse oocytes in that comparison. To clarify this, we have added sentences to highlight the comparison of doubling force.

Revision to manuscript:

“Here, we found that the doubling forces of chromosomes from MI and MII oocytes are 3770 ± 940 pN and 510 ± 50 pN, respectively. We conclude that chromosomes from MI oocytes are much stiffer than those from both mitotic cells and MII oocytes (Supplement Fig. 2), in terms of either Young’s modulus or doubling force.”

(3) Line 202: What stage of prophase I do the authors mean by the spermatocyte stage here? Diakinesis, Metaphase I or prometaphase I? I am not sure how the authors can determine a specific stage of prophase I by only looking at the thickness of the chromosomes. Please show the thickness distribution of WT and $Rec8^{-/-}$ chromosomes.

We have reworded the sentence and clarified that the spermatocyte stage is prophase I stage. Since $Rec8^{-/-}$ spermatocytes cannot progress beyond the pachytene stage of prophase I, the isolated chromosomes must be in prophase I rather than diakinesis, metaphase I, prometaphase I, or any later stages (Xu et al., 2005). Based on the cell size and degree of chromosome condensation (Biggs et al., 2020), it is most likely that the measured chromosomes are at the zygotene-like stage. However, as we cannot definitively determine the exact substage of prophase I, thus, we have referred to them simply as prophase I.

Revision to manuscript:

“We isolated chromosomes from $Rec8^{-/-}$ prophase I spermatocytes, which displayed large and round cell size and thick chromosomal threads, indicative of advanced chromosome compaction after stalling at a zygotene-like prophase I stage (Supplement Fig. 3). The combination of large cell size and degree of chromosome compaction allowed us to reliably identify $Rec8^{-/-}$ prophase I chromosomes. Using micromanipulation, we measured chromosome stiffness by stretching the chromosomes (Supplement Fig. 3) (Biggs et al., 2019).”

Reviewer #2 (Recommendations For The Authors):

(1) Line 135: that statement is not substantiated; better to show retraction data and full reversibility.

We added a figure showing oocyte chromosome stretching, which showed that the oocyte chromosome is elastic, and that the stretching process is reversible (Supplement Fig.1).

(2) Line 144: the authors claim that the Young Modulus of MII oocytes is "slightly" higher than that of mitotic cells (MEFs). Well, "slightly" means it is rather similar, and therefore the commonly used statement that MII is similar to mitosis is OK - contrary to the authors' claim.

We have removed the word "slightly" in the manuscript. The difference is statistically significant.

Revision to manuscript:

"Surprisingly, despite this reduction, the stiffness of MII oocyte chromosomes was still significantly higher than that for mitotic cells (Figure 2B)."

(3) There are a lot of awkward sentences in this text. Some sentences lack words, are not sufficiently precise in wording and/or logic, and there are numerous typos. Some examples can be found in lines 89 (grammar), 94, 95 ("looked"), 98, 101 ("difference" - between what?), and some are commonplaces or superficial (lines 92/93, 120...,). Occasionally the present and past tense are mixed (e.g. in M&M). Thus the manuscript is quite poorly written.

Thanks for the comments of the reviewer. We have revised all the sentences highlighted by the reviewer and polished the entire manuscript.

Reviewer #3 (Recommendations For The Authors):

(1) Line 48. "We then investigated the contribution of meiosis-specific cohesin complexes to chromosome stiffness in MI and MII oocytes." There is no data on oocytes with meiosis-specific cohesin KO. This part should be corrected.

We have corrected this error.

Revision to manuscript:

"We examined the role of meiosis-specific cohesin complexes in regulating chromosome stiffness."

(2) Lines 155-157. The result of MI mouse oocyte chromosomes should also be mentioned here (Supplementary Figure 1).

Please see our response to Reviewer 1 – Minor Point 2.

(3) Line 163. "The stiffness of chromosomes in MI mouse oocytes is significantly higher compared to MII oocytes." Is this because two homologs are paired in MI chromosomes (but not in MII chromosomes)? The authors may want to discuss the possible mechanism.

Please see our response to Reviewer 1 – Major Point 2.

(4) Line 188: "We hypothesized that MI oocytes... would have higher chromosome stiffness than MII oocytes." Why did the authors measure chromosomes from spermatocytes but not MI oocytes?

Both spermatocytes and oocytes from *Rec8^{-/-}*, *Stag3^{-/-}*, and *Rad21^{-/-}* mutant mice cannot reach MI stage because cohesin proteins are crucial for germline-cell development. We chose to use spermatocytes in our study because collecting fetal meiotic oocytes is extremely difficult, and genotyping fetal mice adds another layer of complexity to the experiments. In females, all oocytes complete prophase I and progress to the dictyotene stage during the fetal stage. Obtaining individual oocytes at this stage is challenging. In contrast, spermatocytes are continuously generated at all stages in males.

(5) To support the authors' conclusion, verifying the KO of REC8, STAG3, and RAD21L by immunostaining or other methods is essential.

These mice are provided by one of the authors, Dr. Philip Jordan, who has published several papers using these knockout mice (Hopkins et al., 2014)(Ward et al., 2016). The immunostaining of these models has already been well-characterized in those previous studies. In addition to performing double genotyping, we also use the size of the collected testes as an additional verification of the mutant genotype. These knockout mice have significantly smaller testes compared to their wild-type counterparts, providing a clear physical indicator of the mutation.

(6) Some of the cited papers and descriptions in the Introduction are not appropriate and confusing. This part should be improved:

Line 79. Recent studies have revealed that the 30-nm fiber is not considered the basic structure of chromatin (e.g., review, PMID: 30908980; original papers, PMID: 19064912, 22343941, 28751582). This point should be included.

We have corrected the references as needed. Additionally, thank you for the updated information regarding the 30-nm fiber. We have removed all the descriptions about the 30-nm fiber to ensure the information is accurate and up to date.

(7) Line 83. Reviews on mitotic chromosomes, rather than Ref. 9, should be cited here. For instance, PMID: 33836947, 31230958.

We have corrected it and added references according to the review's suggestion.

(8) Line 85. Refs. 10 and 11 are not on the "Scaffold/Radial-Loop" model. For instance, PMID: 922894, 277351, 12689587. The other popular model is the hierarchical helical folding model (PMID: 98280, 15353545).

We have corrected it and added appropriate references according to the review's suggestion. Regarding the hierarchical helical folding model, our experiments do not provide data that either support or refute this model. Thus, we have opted not to include any discussion of this model in our manuscript.

(9) Figure legends. There is no description of the statistical test.

We have added the description of the statistical test at the end of the figure legends for clarity.

(10) Line 156. The authors should mention which stages in spermatocyte prophase I (pachytene?) were used for their measurement.

We cannot precisely determine the substage of prophase I in the spermatocytes although it is most likely in the pachytene stage.

(11) Line 241. "DNA damage reduces chromosome stiffness in oocytes." It would be better to show how much damage was induced in aged and etoposide-treated chromosomes, for example, by gamma-H2AX immunostaining. In addition, there are some papers that show DNA damage makes chromatin/chromosomes softer (e.g., PMID: 33330932). The authors need to cite these papers.

The effects of etoposide and age on meiotic oocytes has been published (Collins et al., 2015) (Marangos et al., 2015)(Winship et al., 2018).

We are grateful for the citation information provided by the reviewer and have added it to our manuscript.

Revision to manuscript:

"Overall, these findings suggest that DNA damage reduces chromosome stiffness in oocytes instead of increasing it, which aligns with other studies showing that DNA damage can make chromosomes softer (Dos Santos et al., 2021). These results suggest that the increased chromosome stiffness observed in aged oocytes is not due to DNA damage."

(12) Line 328. Senescence?

This error is corrected in the revised manuscript.

Revision to manuscript:

"Defective chromosome organization is often related to various diseases, such as cancer, infertility, and senescence (Thompson and Compton, 2011; Harton and Tempest, 2012; He et al., 2018)."

References:

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