ABSTRACT

SMC complexes include three major classes: cohesin, condensin and SMC5/6. However, the localization pattern and genetic requirements for the SMC5/6 complex during mammalian oogenesis have not previously been examined. In mouse oocytes, the SMC5/6 complex is enriched at the pericentromeric heterochromatin, and also localizes along chromosome arms during meiosis. The infertility phenotypes of females with a Zp3-Cre-driven conditional knockout (cKO) of Smc5 demonstrated that maternally expressed Smc5 protein is essential for early embryogenesis. Interestingly, protein levels of SMC5/6 complex components in oocytes decline as wild-type females age. When SMC5/6 complexes were completely absent in oocytes during meiotic resumption, homologous chromosomes failed to segregate accurately during meiosis I. Despite what appears to be an inability to resolve concatenation between chromosomes during meiosis, localization of topoisomerase IIβ to bivalents was not affected; however, localization of condensin along the chromosome axes was perturbed. Taken together, these data demonstrate that the SMC5/6 complex is essential for the formation of segregation-competent bivalents during meiosis I, and findings suggest that age-dependent depletion of the SMC5/6 complex in oocytes could contribute to increased incidence of oocyte aneuploidy and spontaneous abortion in aging females.

KEY WORDS: SMC5/6, Cohesin, Condensin, Meiosis, Chromosome segregation, Aneuploidy

INTRODUCTION

Meiosis is a specialized cell division required for the formation of haploid gametes. Following pre-meiotic DNA replication, homologous chromosomes pair and recombine. DNA recombination occurs within the context of a proteinaceous scaffold known as the synaptonemal complex (SC), which ensures close juxtaposition of homologs (Handel and Schimenti, 2010). After desynapsis, homologous chromosomes remain linked via chiasmata, which are a visible manifestation of crossover recombination. Chiasmata are biologically essential as they ensure that homologous chromosomes bi-orient and thus segregate from each other during the first meiotic division (meiosis I). Subsequently, sister chromatids segregate during meiosis II, resulting in the formation of haploid gametes.

Regulation of meiosis is sexually dimorphic in mammals. Research using the mouse as a model has helped to delineate the dimorphic features that are also observed in humans. In most male mammals, meiosis is initiated postnatally, with continual production of spermatocytes undergoing meiosis throughout life. In female mice, meiosis is initiated during fetal development but arrests in a prolonged diplotene, or dictyate, stage of prophase I. Cohorts of dictyate stage oocytes begin growth shortly after birth and meiosis does not resume in vivo until after the preovulatory surge of luteinizing hormone (LH) in post-pubescent mice. However, fully grown oocytes undergo spontaneous, LH-independent, resumption of meiosis after isolation and culture under supportive conditions (Pincus and Enzmann, 1935). Meiosis, whether occurring in vivo or in vitro, becomes arrested again after progression to metaphase II and is completed only after fertilization or parthenogenetic activation.

Cohorts of oocytes resume meiosis throughout the reproductive lifespan and therefore can reflect aging effects. As women age, their oocytes become more susceptible to chromosome mis segregation, which can lead to infertility and developmental abnormalities (Hassold and Hunt, 2001). Therefore, it is important to determine the molecular pathways that are prone to error in oocytes, especially the proteins required for monitoring and facilitating chromosome segregation (MacLennan et al., 2015).

The structural maintenance of chromosomes (SMC) complexes are important regulators of chromosome dynamics and structure during mitosis and meiosis. Each member of the SMC family, which includes cohesin, condensin and SMC5/6, comprises a V-shaped SMC protein heterodimer. The SMC proteins each have a hinge domain that is flanked by long coiled-coil domains, which allows the proteins to fold back on themselves. The C and N globular heads interact with each other, forming an ATP-binding and ATP hydrolysis site. The ATPase domains are bridged together by non-SMC elements (Nasmyth and Haering, 2005).

Cohesin is a SMC1/3 heterodimer that is linked by an α-kleisin and a stromal antigen protein. During mitosis, cohesin is required to maintain sister chromatid cohesion before the metaphase-to-anaphase transition (Remeseiro and Losada, 2013). However, to ensure that sister chromatids segregate together during meiosis I, centromeric cohesin is maintained until meiosis II (Petronczki et al., 2003). In addition, cohesin complexes are required for accurate recombination and synopsis between homologous chromosomes (Rankin, 2015). Meiosis-specific cohesin components, including SMC1β, two α-kleisins (REC8 and RAD21L) and a stromal antigen protein (STAG3), are important for these additional requirements of cohesins during meiosis (Bannister et al., 2004; Fukuda et al., 2014; Herrán et al., 2011; Hopkins et al., 2014; Llano et al., 2014; Revenkova et al., 2004; Winters et al., 2014; Xu et al., 2005). Mutation of meiosis-specific cohesin components in female mice results in an increased frequency of oocyte aneuploidy and premature ovarian failure (Herrán et al., 2011; Hodges et al., 2005; Murdoch et al., 2013).
The two condensin complexes (I and II) are composed of the SMC2 and SMC4 heterodimers, but their kleisin subunit and pair of HEAT repeat elements are unique (Hirano, 2015). Condensins localize to the longitudinal axes of bivalents following meiotic resumption in mouse oocytes, and both complexes are required for chromosome compaction before meiosis I (Houlard et al., 2015; Lee et al., 2011). However, only condensin II is essential for disentanglement of chromosomes prior to their segregation.

SMC5/6 heterodimers are linked by NSMCE4, a kleisin subunit (Verver et al., 2015). Two additional subunits, NSMCE1 and NSMCE3, interact with one another and with NSMCE4 (Palecek et al., 2006; Pebernard et al., 2008). NSMCE1 contains a RING-finger domain, common to E3 ubiquitin ligases, and NSMCE3 contains a MAGE (melanoma-associated antigen gene) domain. NSMCE3 enhances the E3 ubiquitin ligase activity of NSMCE1 (Doyle et al., 2010). NSMCE2, which contains an SP-RING domain, binds to the coiled-coil region of SMC5 and can function as an E3 SUMO ligase (Andrews et al., 2005; Potts and Yu, 2007; Zhao and Blobel, 2005).

Studies assessing the SMC5/6 complex in mammalian germ cells have been limited to analyses of its localization pattern during mammalian spermatogenesis (Gómez et al., 2013; Verver et al., 2014, 2013). Because the regulation of meiosis is sexually dimorphic, there may be temporal and functional differences in the roles of SMC5/6 in females versus males. This study demonstrates that the SMC5/6 complex is enriched at the pericentromeric regions and is also detected along chromosome arms during female meiosis. To determine the function of the SMC5/6 complex following meiotic resumption in mouse oocytes, an oocyte-specific conditional knockout (cKO) mouse was created, deleting a floxed Smc5 allele using the Zp3-Cre transgene, which is expressed in growing oocytes before meiotic resumption (Lan et al., 2004; Lewandoski et al., 1997). Analysis of the female Smc5 cKO mutants led to two major findings: (1) maternal expression of SMC5 before meiotic resumption is essential for embryogenesis; and (2) absence of SMC5/6 during meiotic resumption results in oocyte aneuploidy due to an inability to resolve chromosomes during meiosis I. Furthermore, protein levels of SMC5/6 components in oocytes decline as wild-type females age, implicating the SMC5/6 complex as a potential contributor to oocyte aneuploidy and infertility in aging females.

RESULTS

SMC5/6 is enriched at oocyte pericentromeric heterochromatin during meiosis

Chromatin spreads were prepared to assess the localization of the SMC5/6 complex during female meiosis via immunofluorescence microscopy with antibodies raised against SMC5, SMC6 and NSMCE1 (Fig. 1; Fig. S1). Meiotic prophase sub-stages were determined by assessing chromosome axis morphology (synaptonemal complex protein, SYCP3) and centromere pairing (anti-centromere autoantibody, CEN; also known as ACA and CREST). During leptotena, SMC6 localized throughout the spread chromatin (Fig. 1A). By early zygonema, SMC6 was enriched at pericentromeric heterochromatin. At pachynema, SMC6 remained enriched at pericentromeric heterochromatin, and was also evident at lower intensity along the arms of chromosomes. These localization patterns were partially resistant to DNase treatment (Fig. S2). Additionally, SMC6 was observed as foci along chromosome axes and chromosome ends (Fig. 1B). SMC6 foci were not always evident on pachytene stage chromatin spreads, and did not overlap with MLH1 foci (Fig. S3), suggesting that they may be transient and stage specific. At early diplonema, SMC6 remained enriched at the pericentromeric heterochromatin; however, this enrichment was decreased by late diplonema. Analysis of SMC5, NSMCE1 and an additional antibody raised against SMC6 resulted in similar localization patterns (Fig. S1). Differences in localization patterns are likely to be due to epitope accessibility, as is the case with mouse prophase spermatocytes (Gómez et al., 2013), SMC6 localization to the pericentromeric heterochromatin in oocytes overlaps with that observed for TOP2A (Fig. 1C).

Following meiotic resumption, SMC6 was enriched at the pericentromeric heterochromatin during meiosis I and remained present at metaphase II (MII), when oocytes arrest (Fig. 1D). Chromosome spread preparations of metaphase I (MI) oocytes demonstrated that there was also some SMC6 protein along chromosome arms (Fig. 1E).

Contrasting data have been reported on whether mutation of cohesin component, REC8, affects SMC5/6 axis loading during meiosis in budding yeast (Copsey et al., 2013; Lilienthal et al., 2013). Localization of SMC6 was assessed using a Rec8 mouse mutant (Bannister et al., 2004). The enrichment of SMC6 to the pericentromeric heterochromatin and localization to chromosome arms was not affected in Rec8 mutants (Fig. 1F), demonstrating that REC8 was not required for SMC6 localization. This finding is supported by observations made using mouse spermatocytes, where mutation of Smc1β did not affect SMC5/6 localization (Gómez et al., 2013).

Oocyte-specific conditional mutation of Smc5 results in infertility

Mice that harbored a conditional knockout (cKO) allele of Smc5 were used to assess the requirement of the SMC5/6 complex for the meiotic divisions and formation of blastocysts (Fig. 2A,B, see Materials and Methods). Exon 4 of Smc5 was flanked by loxP Cre recombinase target sequences and this allele was termed Smc5 flox (Fig. 2A). Breeding heterozygous SMC5 flox mice to mice expressing the Cre recombinase transgene generated a KO allele termed Smc5 del. The heterozygous Smc5 del mice exhibited no gross morphological abnormalities during development and adult life. No offspring homozygous for the Smc5 del allele were produced, indicating that homozygosity for the deletion allele is lethal. Therefore, to determine whether Smc5 is essential for oogenic meiotic divisions, a hemizygous Cre recombinase transgene under the control of the promoter for the zona-pelucida protein 3 gene (Zp3-Cre tg/0) was used. This transgene is expressed exclusively in growing oocytes before resumption of the first meiotic division (Lan et al., 2004; Lewandoski et al., 1997). Breeding Smc5 +/- flox, Zp3-Cre tg/0 (control) females to wild-type males showed that mutation of the Smc5 flox allele mediated by Zp3-Cre was 100% efficient (Table 1). The Smc5 flox/del, Zp3-Cre tg/0 (Smc5 cKO) females failed to produce litters (n=5), despite having normal ovarian morphology and equivalent oocyte numbers (Fig. S4A,B).

**Smc5 cKO oocytes are incapable of mature blastocyst formation following IVF**

*In vitro* oocyte maturation (IVM) and fertilization (IVF) was used to determine whether blastocysts could be obtained from Smc5 cKO oocytes. Fully grown germinal vesicle (GV) oocytes were isolated from the large antral follicles of Smc5 cKO (Smc5 flox/del, Zp3-Cre tg/0) and control (Smc5 +/- flox, Zp3-Cre tg/0) female ovaries aged between 4 and 12 weeks, and cultured in media that supported meiotic resumption in *vitro* (IVM). There was no observable delay...
in GV breakdown (GVBD), indicative of meiotic resumption (Fig. S4C), and likewise no reduction in frequency of oocytes that underwent polar body extrusion (PBE) and metaphase II (MII) arrest (Fig. 3A,B). However, following IVF using sperm from a wild-type mouse, fertilized oocytes from Smc5 cKO females failed to form mature blastocysts, with many embryos arresting at the 4- to 16-cell stages (Fig. 3A-C). Intriguingly, there was a difference in IVF results between oocytes from mice that were 4 weeks of age (considered as the ‘juvenile’ cohort), and mice that were between 12 and 16 weeks of age (considered the ‘adult’ cohort). In the ‘juvenile’ cohort, fertilized oocytes progressed to the 2-cell stage at levels comparable with their littermate controls (Fig. 3A). By contrast, the cohort of ‘adult’ fertilized oocytes displayed a significant decrease in 2-cell stage embryos following IVF (Fig. 3B). In addition, although there was a significant decrease in embryos progressing beyond the 2-cell stage compared with the littermate control, the ‘juvenile’ cohort of embryos collectively progressed further than the ‘adult’ cohort (Fig. 3A,B). Embryos from the ‘juvenile’ cohort were assessed via light and immunofluorescence microscopy. Cells and nuclei from the control embryos displayed similar shape and size, and the nuclei harbored an SMC6 signal (Fig. 3C,D; Fig. S5). By contrast, embryos from the Smc5 cKO embryos contained low or undetectable levels of SMC6 protein, and nuclei were irregular in size, which is consistent with defects during mitosis and imbalanced chromosome segregation during cell division.

Only the ‘adult’ Smc5 cKO oocytes display aneuploidy at metaphase II
To determine whether the observed failure to form blastocysts was due to defects in chromosome segregation during meiosis, the number and morphology of chromosomes in oocytes arrested at MII were assessed. Owing to the age-related differences observed in the IVF studies, MII oocytes from ‘juvenile’ and ‘adult’ mice were assessed separately. MII chromosome spread preparations of the ‘juvenile’ Smc5 cKO embryos did not exhibit significant increases in aneuploidy or chromosome abnormalities (Fig. 4A, Table 2). By contrast, chromosome spread preparations from the ‘adult’ Smc5 cKO females displayed abnormal chromosome number and morphology, and separated sister chromatids were observed (Fig. 4A, Table 2). Chromosome number and morphology were also assessed within the confines of the cell by treating the oocytes...
with monastrol. Monastrol binds to and disrupts the function of the kinesin protein KIF11, resulting in monopolar spindles making it easier to distinguish each sister chromatid pair (Stein and Schindler, 2011). Centromere number was counted using an anti-centromere autoantibody (CEN). In addition, the presence of the SMC5/6 complex was determined using an SMC6 antibody. Complementary to the chromosome spread preparations (Fig. 4A, Table 2), the monastrol-treated MII oocytes from the ‘juvenile’ Smc5 cKO cohort did not exhibit significant differences compared with the control oocytes with respect to centromere counts or chromatin morphology (Fig. 4B-D). Furthermore, most (83%) of the oocytes from the ‘juvenile’ Smc5 cKO harbored SMC6 protein signal. By contrast, the majority (61%) of monastrol treated oocytes from the ‘adult’ Smc5 cKO cohort lacked SMC6 signal, and presented significant differences with regards to centromere counts compared with littermate controls (Fig. 4B,C; Fig. S6A). Additionally, it was not possible to obtain centromere counts from more than 50% of the monastrol-treated Smc5 cKO oocytes from the ‘adult’ mice, because the chromatin was grossly abnormal, demonstrating stretched morphology, and indistinguishable sister chromatid pairs (Fig. 4B-D; Fig. S6A). Furthermore, 5% of Smc5 cKO MII oocytes displayed abnormal morphology that was indicative of oocyte degeneration (Fig. S6B,C).

**Oocyte SMC5/6 protein levels decrease in aging females**

Excision of the floxed 4th exon of Smc5 driven by the Zp3-Cre transgene was shown to be 100% efficient based on mating tests, PCR analysis and the IVF data (Fig. 2B,C, Fig. 3A). However, data from monastrol-treated MII oocytes demonstrated that the SMC6 protein was still present in most oocytes of the ‘juvenile’ Smc5 cKO cohort (Fig. 4B). These data suggest that SMC5/6 protein levels present before Cre-mediated deletion of Smc5 are sufficient to support proficient meiosis, but not embryogenesis. Furthermore, the majority of oocytes from the ‘adult’ cohort do not harbor residual SMC6 protein, and fail to form chromosomally normal MII oocytes (Fig. 4, Table 2). As fertility and genome integrity are negatively correlated with age, it can be postulated that SMC5/6 levels within GV oocytes of wild-type mice may decrease with age. To test this hypothesis, oocyte protein extracts from three groups of C57BL/6J wild-type mice aged 4, 12 and 24 weeks were assessed for SMC5, SMC6, NSMCE1 and NSMCE2 protein levels (Fig. 5A,B). From this analysis, it was determined that protein levels for all four SMC5/6 components decreased significantly in oocytes isolated from older mice.

**Smc5 is a maternal-effect gene**

As there were residual levels of SMC6 detected in the oocytes isolated from ‘juvenile’ Smc5 cKO mice, it was hypothesized that SMC5/6 levels were adequate to facilitate chromosome segregation during meiosis, but was insufficient for sustaining proper mitotic segregation during the early embryogenesis. To further assess the relationship between Smc5 mutation and the capacity to form mature blastocysts, wild-type, heterozygous Smc5 del male and female mice were used for IVF to test effects of paternal versus maternal inheritance of the mutant allele. The oocytes used in these assays were from 4-week-old mice, and therefore equivalent to the designated ‘juvenile’ age group. In addition, Smc5 cKO male mice (Smc5 flox/del, Hspa2-Cre tg/0), which are fertile and produce sperm that almost exclusively carry the Smc5 del allele, were used for IVF. Based on mating tests with C57BL/6J wild-type females, 98% of progeny from the Smc5 flox/del, Hspa2-Cre males carry the Smc5 del allele (Table 3). When sperm from the heterozygous Smc5 del and Smc5 flox/del, Hspa2-Cre males were combined with wild-type oocytes the levels of mature blastocysts obtained were equivalent to the wild-type IVF (Table 4), showing that presence of the paternally inherited Smc5 del allele does not affect early embryogenesis. When female heterozygous Smc5 del oocytes were fertilized with wild-type sperm, levels of mature blastocysts were equivalent to the wild-type IVF results, suggesting that the expression of Smc5 during oocyte growth is essential for supporting early stages of development.
embryogenesis. When the heterozygous Smc5 del oocytes were fertilized with sperm from the Smc5 flox/del, Hspa2-Cre males, the level of blastocysts obtained reduced by approximately half, which supports the fact that early stages of embryonic development are affected in embryos homozygous for mutation of Smc5. Homozygous mutation of other components of the SMC5/6 complex, Smc6 and Nsmce2, have also been shown to cause embryonic lethality (Jacome et al., 2015; Ju et al., 2013). Taken together with the IVF and MII data obtained for the ‘juvenile’ Smc5 cKO females (Figs 3 and 4, Table 2; Fig. S5), these results suggest that Smc5 expression during oocyte growth, before meiotic resumption, is crucial for embryogenesis and, therefore, Smc5 is a maternal-effect gene.

Oocyte-specific cKO of Smc5 causes chromosome stretching during meiosis I

Because abnormal chromosome morphology was observed in oocytes from the ‘adult’ Smc5 cKO group at metaphase II arrest, it is possible that chromosome morphology and segregation earlier, during meiosis I, was perturbed. The localization of SMC5/6 components in the Smc5 cKO and control oocytes during meiosis I was assessed. SMC5/6 components SMC5, SMC6 and NSMCE1 were enriched at the pericentromeric heterochromatin during the metaphase-to-anaphase I transition in control oocytes, but were absent in the Smc5 cKO oocytes (Fig. 6A). Oocytes were assessed during the metaphase-to-anaphase I transition (Fig. 6D). In the majority (95%, n=144) of the control oocytes, proficient segregation of homologous chromosomes was observed. In sharp contrast, the majority (62%, n=220) of Smc5 cKO experimental oocytes displayed chromosome stretching and lagging chromosomes. The severe chromatin stretching observed between homologous chromosomes (Fig. 6C) suggests that deletion of Smc5 prevented decatenation of homologous chromosomes.

Given the meiotic abnormalities described above, the spindle assembly checkpoint (SAC) was assessed in the Smc5 cKO oocytes. SAC satisfaction during the metaphase to anaphase I transition was indirectly determined by assessing the SAC protein MAD2, which normally localizes to kinetochores during prometaphase, and remains there until ubiquitous bipolar microtubule-kinetochore attachment satisfies the SAC (Lara-Gonzalez et al., 2012). MAD2 staining was present at the kinetochores at prometaphase in both control and Smc5 cKO oocytes (Fig. 6F). MAD2 signal at the kinetochore was absent in both control and Smc5 cKO oocytes undergoing the metaphase to
anaphase I transition (Fig. 6G). These observations suggest that mutation of Smc5 does not affect the temporal pattern of MAD2 localization, and therefore may not affect SAC function, consistent with the lack of MI oocyte arrest. Additionally, a cell cycle kinase, PLK1, localized to kinetochores in control and Smc5 cKO oocytes (Fig. S7).

Absence of the Smc5/6 complex causes aberrant localization of condensin

Premature depletion of REC8 before the meiosis I division in oocytes is associated with chromosome missegregation (Chiang et al., 2010; Tachibana-Konwalski et al., 2010). Therefore, localization of REC8 was assessed using metaphase I chromosome spreads. REC8 was present along the axes of the bivalents in control and Smc5 cKO oocytes from ‘adult’ mice, with no apparent difference between them (Fig. 7A). These results suggest that mutation of Smc5 before meiotic resumption does not significantly affect localization of REC8-containing cohesins.

Table 2. Chromosome count data from chromosome spreads

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>&lt; 20 (%)</th>
<th>20 (%)</th>
<th>&gt; 20 (%)</th>
<th>Single chromatid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (4 weeks old)</td>
<td>83</td>
<td>15 (18.1)</td>
<td>68 (81.9)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Smc5 cKO (4 weeks old)</td>
<td>90</td>
<td>17 (18.9)</td>
<td>71 (78.9)</td>
<td>1 (1.1)</td>
<td>1 (1.1)</td>
</tr>
<tr>
<td>Control (≥ 12 weeks old)</td>
<td>76</td>
<td>14 (18.4)</td>
<td>62 (82.5)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Smc5 cKO (≥ 12 weeks old)</td>
<td>80</td>
<td>30 (37.5)</td>
<td>40 (50.0)</td>
<td>6 (7.5)</td>
<td>4 (5.0)</td>
</tr>
</tbody>
</table>

SMC5/6 colocalizes with TOP2A in mouse oocytes (Fig. 1C), and similar to the Smc5 cKO oocytes from the ‘adult’ cohort, inhibition of TOP2A function results in severe defects in chromosome condensation and homologous chromosome separation (Li et al., 2013). Therefore, the effect of Smc5 cKO on the localization of TOP2A during meiosis I was determined using ‘adult’ mice. TOP2A was enriched at the pericentromeric regions in control oocytes, and was also detected along chromosome arms (Fig. 7B). No detectable change in TOP2A localization was observed in the Smc5 cKO oocytes (Fig. 7B).

Condensins are required to ensure chromosome segregation during meiosis I in mouse oocytes (Houlard et al., 2015). Similar to the results presented here for Smc5 cKO oocytes, conditional mutation of a condensin II component, Ncaph2, resulted in chromosome stretching during meiosis I due to an inability to disentangle chromosomes. To determine whether condensin localization is affected in the absence of the SMCS/6 complex, localization of the condensin I and II subunit SMC4 was assessed using ‘adult’ cohorts of mice. In control metaphase I chromosome spread preparations, SMC4 was present along the longitudinal axes of each bivalent (Fig. 7C). By contrast, in chromosome spread preparations from Smc5 cKO metaphase I oocytes, there was a significant reduction in SMC4 signal along chromosome arms (Fig. 7C,D). In addition, the SMC4 signal on chromosome arms was discontinuous and the normal linear pattern along chromosomes was difficult to distinguish. However, there was no apparent reduction in condensin signal that colocalized with the kinetochore/
centromeric regions in Smc5 cKO metaphase I chromosome spread preparations compared with control (Fig. 7E).

**DISCUSSION**

This study of a genetic model for oocyte depletion of SMC5 has demonstrated that the SMC5/6 complex is essential for ensuring accurate chromosome segregation following meiotic resumption and during early embryogenesis. Furthermore, the data suggest that SMC5/6 complex protein levels diminish as mice age, and Smc5 is a maternal-effect gene.

**SMC5/6 localization pattern implicates multiple functions during meiosis**

SMC5/6 is enriched at the pericentromeric heterochromatin regions throughout meiosis in mouse oocytes, which is consistent with what was found in mouse spermatocytes (Gomez et al., 2013; Verver et al., 2013). The pericentromeric heterochromatin region consists of densely packed repetitive sequences and is at high risk of aberrant recombination events when double-strand breaks within these regions are repaired via homologous recombination (HR) (Goodarzi and Jeggo, 2012). SMC5/6 prevents HR within repetitive sequences such as rDNA in yeast, and heterochromatin in Drosophila mitotic cells (Torres-Rosell et al., 2007; Chiolo et al., 2011). Taken together, studies using mouse spermatocytes and oocytes suggest that SMC5/6 performs a similar function at the pericentromeric heterochromatin during meiosis (Gomez et al., 2013; Verver et al., 2013).

Although lower in signal intensity, SMC5/6 also localized throughout the chromatid during meiosis. This is consistent with what has been reported for mouse spermatocytes (Gomez et al., 2013; Verver et al., 2013). SMC5/6 was also visible along chromosome axes at pachynema in oocytes, which was also detected in mouse spermatocytes (Gomez et al., 2013; Verver et al., 2013). In mammals, every chromosome pair obtains many recombination sites but generally yields only one to two crossover sites (Kauppi et al., 2004). Designations of which recombination sites become crossovers involve antagonistic roles between ubiquitin E3 ligase HEI10 and SUMO E3 ligase RNF212 (Reynolds et al., 2013; Qiao et al., 2014; Rao et al., 2017; Ahuja et al., 2017). It is possible that SMC5/6 complex protein levels diminish as mice age and during early embryogenesis. Furthermore, the data suggest that SMC5/6 complex protein levels diminish as mice age, and SMC5/6 is a maternal-effect gene.

**SMC6 localization pattern implicates multiple functions during meiosis**

SMC6 was detected along the chromosome arms in female germ cells during pachynema, suggesting a role during meiotic recombination, which has previously been reported using budding yeast and Caenorhabditis elegans (Bickel et al., 2010; Hong et al., 2016; Checchi et al., 2014; Copsey et al., 2013; Lilienthal et al., 2013; Xaver et al., 2013). In mammals, every chromosome pair obtains many recombination sites but generally yields only one to two crossover sites (Kauppi et al., 2004). Designations of which recombination sites become crossovers involve antagonistic roles between ubiquitin E3 ligase HEI10 and SUMO E3 ligase RNF212 (Reynolds et al., 2013; Qiao et al., 2014; Rao et al., 2017; Ahuja et al., 2017). It is possible that the SMC6 complex is a substrate of HEI10 and RNF212. Therefore, these SMC6 foci could indicate that SMC6/3 plays a role in regulating recombination during mammalian meiosis.

**Differences between the Smc5 cKO oocytes isolated from ‘juvenile’ and ‘adult’ mice**

SMC6 protein was detected in the majority of oocytes in the ‘juvenile’ Smc5 cKO cohort. However, SMC6 was not detected in the majority of ‘adult’ Smc5 cKO oocytes. As a consequence of this difference, oocytes from ‘juvenile’ Smc5 cKO mice progress to MII without aberrant chromosome configurations (Fig. 4, Table 2), whereas oocytes from ‘adult’ Smc5 cKO mice fail to accurately segregate chromosomes during meiosis I (Fig. 6). Despite evidence for proficient meiosis from analysis of MII ploidy and chromosome morphology in oocytes from the ‘juvenile’ Smc5 cKO cohort, these oocytes failed to form mature blastocysts when fertilized with sperm bearing a wild-type Smc5 gene. This failure to form mature blastocysts is attributed to aberrant chromosome segregation during mitosis (Fig. 3; Fig. S5). This phenotype is reminiscent of the mitotic catastrophe observed in Smc5 cKO mouse embryonic stem cells (Pryzhkova and Jordan, 2016).

Table 3. Mating test and genotyping data for Smc5 flox/del (control) and Smc5 flox/del, Hspa2-Cre cKO males mated to C57BL6/J females

<table>
<thead>
<tr>
<th>Strain</th>
<th>Smc5 +/flox</th>
<th>Smc5 +/del</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>♂ Smc5 flox/del × ♀ wild type</td>
<td>49% (51 pups)</td>
<td>51% (53 pups)</td>
<td>104 pups (6.9/litter)</td>
</tr>
<tr>
<td>♀ Smc5 flox/del, Hspa2-Cre × ♂ wild type</td>
<td>2% (1 pup)</td>
<td>98% (64 pups)</td>
<td>65 pups (7.2/litter)</td>
</tr>
</tbody>
</table>

n=5 males tested, three litters.

Table 4. Mature blastocyst counts following IVF of MII oocytes

<table>
<thead>
<tr>
<th>Cross</th>
<th>Percentage of blastocysts</th>
<th>Fraction</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>♂ Wild type × ♀ wild type</td>
<td>52</td>
<td>34/66</td>
<td>3</td>
</tr>
<tr>
<td>♂ Smc5 flox/del × ♀ wild type</td>
<td>48</td>
<td>19/38</td>
<td>2</td>
</tr>
<tr>
<td>♂ Wild type × ♀ Smc5 flox/del</td>
<td>55</td>
<td>41/75</td>
<td>4</td>
</tr>
<tr>
<td>♂ Smc5 flox/del, Hspa2-Cre × ♀ wild type</td>
<td>50</td>
<td>39/77</td>
<td>3</td>
</tr>
<tr>
<td>♂ Smc5 flox/del, Hspa2-Cre × ♀ Smc5 flox/del</td>
<td>23</td>
<td>10/43</td>
<td>2</td>
</tr>
<tr>
<td>♂ Wild type × ♀ Smc5 flox/del, Zp3-Cre</td>
<td>&lt;0.02</td>
<td>0/79</td>
<td>4</td>
</tr>
</tbody>
</table>
The phenotypes observed and differences between ‘juvenile’ and ‘adult’ Smc5 cKO mice implies the following hypotheses. First, SMC5/6 protein levels before oocyte growth are important for proficient chromosome segregation during meiotic resumption (Figs 4 and 6, Table 2). Second, SMC5/6 protein levels present in oocytes diminish as mice age (Fig. 5, Tables 3 and 4). Third, there is a critical level of SMC5 protein that is required for proficient chromosome segregation during oocyte meiosis (Fig. 4, Table 2). Fourth, expression of Smc5 during the oocyte growth phase is crucial during early embryogenesis (Figs 3 and 4, Table 2).

**SMC5/6 protein levels are diminished in aging oocytes**

Frequency of meiotic segregation errors increases as women age, especially after the age of ~35, resulting in dramatically increased incidence of miscarriage and birth defects (Hassold and Hunt, 2001). During the long prophase arrest that precedes meiosis I in female mammals, cohesin levels decline gradually and in aged oocytes this reduction in cohesin causes destabilization of chiasmata and separation of sister centromeres, which can result in chromosome missegregation during meiosis I (Lister et al., 2010; Tachibana-Konwalski et al., 2010; Tsutsumi et al., 2014). This current study determined that SMCS/6 protein levels decrease in oocytes isolated from aging mice.
from older mice, and by correlation of phenotypes, this could also contribute to age-related aneuploidy and infertility (Fig. 8). Using an inducible transgene of \textit{Rec8}, it was recently shown that cohesin is established in fetal oocytes during DNA replication, and there is no detectable turnover of cohesin in arrested oocytes, or during meiotic resumption (Burkhardt et al., 2016). Development of inducible, tagged version of an SMC5/6 component could be used to determine whether the SMC5/6 complex is replenished during meiotic resumption, or it remains stably associated with the chromatin for months following meiotic arrest.

Heterozygous mutants of cohesin components lead to age-related increases in oocyte aneuploidy (Murdoch et al., 2013). Therefore, it is possible that a heterozygous mutation of a SMC5/6 component could lead to age-related errors during oogenesis too. Supporting this notion, it has been shown that heterozygous mutation of \textit{Nscme2} results in increased incidences of micronuclei and polynucleation in MEFs (Jacome et al., 2015).

\textbf{Smc5 is a maternal-effect gene}

Early stages of embryogenesis are almost entirely dependent on the oocyte for subcellular organelles and proteins before the robust activation of the embryonic genome at cleavage-stage development (Fig. 8A). These maternal proteins are encoded by maternal-effect genes (Li et al., 2010). Approximately, 45-50 maternal-effect genes have been identified in mammals, and many of these are involved in chromatin structure, modification and genome integrity (Zhang and Smith, 2015). Reduced levels of maternal-effect genes have been associated with the reduced oocyte developmental competence that is characteristic of ovarian aging (Guglielmino et al., 2011; Hamatani et al., 2004; Pan et al., 2008; Zhang and Smith, 2015). The IVF experiments presented in this study show that embryogenesis is aberrant only when \textit{Smc5} is mutated during the oocyte growth phase, and provision of a functional \textit{Smc5} gene from sperm is insufficient to facilitate embryogenesis. These data suggest that \textit{Smc5} is a maternal-effect gene in mouse. Recently, it was reported that \textit{smc5} and \textit{smc6} of \textit{Drosophila melanogaster} are also maternal-effect genes (Tran et al., 2016), suggesting that this feature is conserved in many sexually reproducing organisms.

\textbf{SMC5/6 may be required to assist condensin functions and TOP2A-dependent decatenation}

Inhibition of TOP2A function in mouse oocytes and RNAi-mediated depletion in fly oocytes during meiosis I cause similar chromosome segregation defects observed in the \textit{Smc5} conditional
knockout mouse oocytes (Hughes and Hawley, 2014; Li et al., 2013). Components of the SMC5/6 complex colocalize with TOP2A during prophase and following meiotic resumption in mouse oocytes. This is supported by previous observations made using mouse spermatocytes (Gómez et al., 2013). RNAi knockdown of SMC5 and SMC6 in human RPE-1 cells alters chromosomal localization properties of TOP2A (Gallego-Paez et al., 2013). Therefore, it was hypothesized that mutation of Smc5 would affect TOP2A localization in mouse oocytes. However, no defects in TOP2A localization were observed, which corresponds to what has been reported for Smc5 cKO in mouse embryonic stem cells (Pryzhkova and Jordan, 2016). Studies of yeast SMC5/6 have shown that the complex is linked with TopoII-dependent catenation/decatenation functions (Jeppsson et al., 2014; Kanno et al., 2015; Kegel et al., 2011). Furthermore, meiotic depletion of Top2 in budding yeast affects Smc5 localization (Copsey et al., 2013). Although TOP2A localization is unaffected by mutation of Smc5 in mouse oocytes, the functionality of TOP2A may still be affected.

Analysis of metaphase I chromosome spreads revealed that SMC5/6 is required for normal localization of condensin along chromosome arms. The phenotypes observed here for the Smc5 cKO mutant are reminiscent of the Ncaph2 condensin II cKO mutant (Houlard et al., 2015), as both display abnormal chromosome morphology, similar stretching of chromosomes and chromosome segregation defects during meiosis I. There is mounting evidence for a functional link between SMC5/6 and condensin. RNAi depletion of SMC5 and SMC6 in human RPE-1 cells resulted in defective axial localization of condensin (Gallego-Paez et al., 2013). Abnormal condensin localization was also observed using Smc5 cKO mouse embryonic stem cells (Pryzhkova and Jordan, 2016). Furthermore, mutation of smc-5 in C. elegans leads to abnormal distribution of condensin along bivalents during meiosis I (Hong et al., 2016). However, previous studies were not able to determine whether the defects in condensin localization were specific to the prophase to metaphase transition. Using the Zp3-Cre transgene to mutate Smc5 suggests that there is a functional relationship between condensin and SMC5/6 that is specific to meiotic resumption.

It has been shown that condensin and TOP2A activities are coordinated to ensure efficient chromosome condensation, sister chromatid decatenation and subsequent segregation in budding yeast (Charbin et al., 2014; Leonard et al., 2015). Based on the collective observations made using human and mouse systems, it is proposed that the aberrant localization of condensin observed in Smc5 mutant oocytes results in the loss of coordination between condensin and TOP2A, leading to an inhibition of chromosome resolution during meiosis (Fig. 8B).

Conclusions

The data demonstrate that SMC5/6 levels diminish in oocytes as mice age, leading to increased incidence of chromosome missegregation during meiosis (Fig. 8A). Furthermore, Smc5 is a maternal-effect gene and its expression during oocyte maturation is crucial for early stages of embryogenesis (Fig. 8A). The SMC5/6 complex ensures that chromosomes are accurately resolved and segregated during female meiosis (Fig. 8A). The SMC5/6 complex ensures that chromosomes are accurately resolved and segregated during female meiosis (Fig. 8A). Although TOP2A localization is unaffected by mutation of Smc5 in mouse oocytes, the functionality of TOP2A may still be affected. The SMC5/6 complex ensures that chromosomes are accurately resolved and segregated during female meiosis (Fig. 8A). The SMC5/6 complex ensures that chromosomes are accurately resolved and segregated during female meiosis (Fig. 8A). Like cohesin and condensin, the

![Fig. 8. Smc5 is a maternal-effect gene, and SMC5/6 is required for the formation of bivalent chromosomes capable of segregation during meiosis I in mouse oocytes.](image-url)
SMC5/6 complex is crucial to chromosome integrity in oocytes following their long arrested state. Protein levels of SMC5/6 components in oocytes are diminished in aging mice, suggesting that SMC5/6 levels are correlated with age-related oocyte and embryo chromosomal abnormalities. These data present the possibility that genetic and expression variations of SMC5/6 components are linked with fertility differences between individuals and defects may cause premature ovarian failure.

**MATERIALS AND METHODS**

**Ethics statement**
All mice were bred at The Jackson Laboratory (JAX) and Johns Hopkins University (JHU) in accordance with the National Institutes of Health and US Department of Agriculture criteria. Protocols for their care and use were approved by the Institutional Animal Care and Use Committees (IACUC) of JAX and JHU.

**Mice**
Mice harboring Smc5 with a floxed exon 4 (designated Smc5^floxed^ and deleted exon 4 (designated Smc5^del^) have been previously described (Pryzhkova and Jordan, 2016). Heterozygous Smc5^del^ mice were bred to mice harboring the Zp3-Cre transgene ([C57BL/6-Tg(Zp3-cre)93Knm/J], which resulted in progeny heterozygous for the Smc5^del^ allele and hemizygous for the Zp3-Cre transgene (Smc5^del^, Zp3-Cre tg/0). Male Smc5^del^, Zp3-Cre tg/0 mice were bred to homozygous Smc5^floxed^ female mice to derive Smc5^cKO^ (Smc5^floxed^, Zp3-Cre tg/0) and control (Smc5^floxed^, Zp3-Cre tg/0) genotypes. The Smc5^floxed^ genotype was used as an additional control. The same mating strategy was employed to create the male Smc5^floxed^, Hspa2-Cre tg/0 cKO mice, using mice harboring the Hspa2-Cre transgene ([C57BL/6-Tg(Hspa2-cre)1Eddy/J]).

**PCR genotyping**
Primers used are described in Fig. 2 and Table S1. PCR conditions: 90°C for 2 min; 30 cycles of 90°C for 20 s; 58°C for annealing; and 72°C for 1 min.

**Oocyte harvesting, culture and IVF**
Female mice were injected intraperitoneally with 5 IU of equine chorionic (eCG; Sigma) to stimulate ovarian follicle development. GV-staged oocytes were harvested from ovaries 44 to 48 h later. Oocytes were cultured in MEMα medium supplemented with 5% fetal bovine serum (FBS; Gibco) and 3 mg/ml bovine serum albumin (BSA; Sigma-Aldrich). To harvest oocytes at metaphase II (MII) stage, mice were injected intraperitoneally with 5 IU of eCG (Sigma) and then with human chorionic gonadotropin (hCG; Sigma) 44-48 h later. After 15-16 h, MII oocytes were harvested from ovaries with 5 IU of eCG (Sigma) and then with human chorionic gonadotropin (hCG; Sigma) 44-48 h later. Oocytes were cultured in MEMα medium supplemented with 5% FBS, 3 mg/ml BSA and 20 µl of sperm. Oocytes were washed in MEMα medium prior to fixation. All cultures were washed with a fusion medium containing 150 oocytes were run on 4-15% gradient SDS PAGE gels (Bio-Rad) and transferred to PVDF membranes. Primary antibodies and dilutions used are presented in Table S2. At a 1:10,000 dilution, goat anti-mouse and goat anti-rabbit horseradish peroxidase-conjugated antibodies (Invitrogen) were used as secondary antibodies. Antibody signal was detected via treatment with Bio-Rad ECL western blotting substrate and captured using Syngene XR5 system. Protein levels were assessed using ImageJ (NIH).

**Western blot analyses**
Protein lysate from eCG-primed oocytes were isolated from C57BL/6J mice using methods previously described (Maranogs, 2016). Protein extracts containing 150 oocytes were run on 4-15% gradient SDS PAGE gels (Bio-Rad) and transferred to PVDF membranes. Primary antibodies and dilutions used are presented in Table S2. At a 1:10,000 dilution, goat anti-mouse and goat anti-rabbit horseradish peroxidase-conjugated antibodies (Invitrogen) were used as secondary antibodies. Antibody signal was detected via treatment with Bio-Rad ECL western blotting substrate and captured using Syngene XR5 system. Protein levels were assessed using ImageJ (NIH).

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**Supplementary information**
Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.145607.supplemental

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