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Report

Oocyte Cohesin Expression Restricted to Predictyate Stages Provides Full Fertility and Prevents Aneuploidy

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Summary

To ensure correct meiotic chromosome segregation, sister chromatid cohesion (SCC) needs to be maintained from its establishment in prophase I oocytes before birth until continuation of meiosis into metaphase II upon oocyte maturation in the adult. Aging human oocytes suffer a steep increase in chromosome missegregation and aneuploidy, which may be caused by loss of SCC through slow deterioration of cohesin [1–3]. This hypothesis assumes that cohesin expression in embryonic oocytes is sufficient to provide adequate long-term SCC. With increasing age, mouse oocytes deficient in the meiosis-specific cohesin SMC1β massively lose SCC and chiasmata [3, 4]. To test the deterioration hypothesis, we specifically and highly efficiently inactivated the mouse *Smc1*β gene at the primordial follicle stage shortly after birth, when oocytes had just entered meiosis I dictyate arrest. In the adult, however, irrespective of oocyte age, chiasma positions and SCC are normal. Frequency and size of litters prove full fertility even in aged females. Thus, SMC1β cohesin needs only be expressed during prophase I prior to the primordial follicle stage to ensure SCC up to advanced age of mice.

Results and Discussion

Aneuploidy constitutes a major health problem because it leads to embryonic death or severe syndromes such as Down's syndrome. Aneuploidy predominantly arises through errors in female meiosis, and loss of sister chromatid cohesion is thought to play a major role because it may lead to chromosome missegregation. In humans, the frequency of aneuploidy greatly increases with advancing age of the mother. The incidence of trisomy in clinically recognized pregnancies increases from less than 4% at the age of 25 to more than 30% at age 40 [1, 2].

To avoid chromosome missegregation during cell division, two copies of each chromosome, sister chromatids, need to be properly positioned at the division plane and be attached to the spindle microtubules. To achieve correctly oriented attachment, the sister chromatids have to stay connected to each other by sister chromatid cohesion (SCC). In meiotic prophase I, recombination between nonsister chromatids creates obligate physical links between homologous chromosomes. These sites of exchange can be visualized cytologically as chiasmata [5].

In somatic cells, SCC depends on the essential cohesin complex that consists of four proteins, which, in mammals, are named SMC1α, SMC3, RAD21, and SA1 (or SA2). RAD21 closes the cohesin ring, preformed by the V-shaped SMC dimer. The precise function of SA1/SA2 remains to be elucidated (for recent reviews, see [6, 7]).

Meiotic cells express homologs of the SMC1α, RAD21, and SA proteins, called SMC1β, REC8, and STAG3, that associate with SMC3 to form additional cohesin complexes. The meiotic kleisin REC8 replaces RAD21, STAG3 replaces SA1/SA2, and SMC1β replaces SMC1α. Early in meiosis, however, the canonical cohesin featuring SMC1α is still present [8]. The meiosis-specific cohesins, including the SMC1β-based cohesin complex, are present in mouse spermatocytes and oocytes throughout meiosis [9–11]. At the metaphase-anaphase transition of the first meiotic division, dissolution of SSC in chromosome arms allows separation of homologs, but sister chromatids remain linked together by centromeric cohesion. SCC at centromeres persists until anaphase II.

In mammals, oocytes start undergoing meiosis in the fetal ovary and then enter meiosis I arrest, a stage of quiescence called the dictyate, when they form the storage pool awaiting the hormonal signal for continuation of meiosis (reviewed in [12]). In the mouse, the first cohorts of oocytes leave dictyate arrest within a few weeks after birth and resume meiosis I by entering into metaphase I. Oocytes become again arrested at metaphase of the second meiotic division prior to ovulation. Anaphase II, and thus dissolution of the remaining SCC, happens upon fertilization. Thus, the time interval from establishment to final dissolution of SCC in female meiosis is remarkably long and, in humans, may last for more than 40 years. Degradation of cohesin over time, accompanied by a failure to reload new cohesin complexes, may cause premature separation of sister chromatids, leading to an age-dependent increase in aneuploidy.

To analyze a meiotic cohesin deficiency model, we previously created a transgenic mouse with an allele of the *Smc1*β gene, *Smc1*β^{-}, in which exon 10 was replaced with the *neo* gene, causing loss of SMC1β [4]. Male and female *Smc1*β$^{-/-}$ mice are completely sterile. In prophase I, *Smc1*β$^{-/-}$ oocytes and spermatocytes show drastically shortened axial elements and synaptonemal complexes, incomplete synapsis, reduced numbers of MLH1 foci diagnostic for sites of recombination, and aberrant telomere structures [4, 13]. Spermatocytes die in pachytene, whereas oocytes can survive until metaphase II but suffer a complete loss of sister chromatid cohesion at this stage [3, 4]. Thus, SMC1β is essential for continuous meiotic sister chromatid cohesion, and endogenous SMC1α cannot rescue a deficiency in SMC1β. Importantly, with increasing age of female *Smc1*β$^{-/-}$ mice, a dramatic loss of chiasmata holding the two pairs of sister chromatids together is observed. As *Smc1*β$^{-/-}$ oocytes get older, loss of sister chromatid cohesion in metaphase I oocytes dramatically increases in parallel [3]. This strongly

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correlates with an increasingly distal localization of chiasmata on metaphase chromosomes, suggesting that without SMC1β-mediated SCC, chiasmata cannot be prevented from moving and slipping off the chromosomes. Indeed, the shorter mouse chromosomes show the highest frequency of loss of chiasmata, consistent with this hypothesis. Therefore, the SMC1β-deficient mouse was considered a model that, in certain respects, reflects the age-dependent increase in aneuploidies seen in humans [14, 15]. However, why should human oocytes—proficient in SMC1β cohesin—suffer loss of cohesion?

One may speculate that in human oocytes, slow degradation of cohesin starts to cause significant increase in aneuploidy after several decades. Thus, a key question is whether cohesin that is loaded onto chromosomes at the initial stages of meiotic prophase I during embryogenesis is sufficient to maintain sister chromatid cohesion through years of the dictyate arrest.

In the *Smc1*$\beta^{-/-}$ mouse, this question cannot be answered because the protein is absent from the onset of meiosis, and thus establishment of SMC1β-dependent SCC is initially impaired. We therefore addressed this question by generation and analysis of a mouse strain carrying a floxed *Smc1*β allele, *Smc1*β^{fl}, which, after Cre-mediated recombination, becomes *Smc1*β^{ex} and lacks exon 10, similar to the loss-of-function allele *Smc1*β^{-} [4] (see Figure S1A available online). Cre recombinase is provided by a transgenic mouse strain expressing Cre under control of the *Gdf9* promoter [16]. In this strain, *GDF-9-iCre*, Cre is expressed and functional in oocytes at the primordial follicle stage, starting between day 1 and day 3 after birth [16]. *Smc1*β inactivation very soon after birth allows assessment of the putative requirement for *Smc1*β expression during the dictyate arrest, which lasts for many months thereafter.

Smc1$\beta^{fl/fl}$ and *Smc1*$\beta^{fl/-}$ males and females were healthy and fertile. To obtain *Smc1*$\beta^{fl/-}$ *or Smc1*$\beta^{fl/fl}$ females expressing Cre in their oocytes, we crossed them with *Smc1*$\beta^{+/-}$ *GDF-9-iCre* males. *Smc1*$\beta^{fl/-}$ *GDF-9-iCre* females are all fertile, which initially raised the question of whether inactivation of *Smc1*β^{fl} by Cre-mediated excision was efficient and timely. Efficiency of Cre-mediated excision in the germline was evaluated by genotyping the progeny of *Smc1*$\beta^{fl/-}$ *GDF-9-iCre* or *Smc1*$\beta^{fl/fl}$ *GDF-9-iCre* females crossed with wild-type (Figures S1B and S1C). Among the 218 progeny of five *Smc1*$\beta^{fl/-}$ *GDF-9-iCre* female mice genotyped by polymerase chain reaction (PCR), 110 inherited the *loxP*-containing allele and, in 108 of them, exon 10 was excised. Of the progeny of six *Smc1*$\beta^{fl/fl}$ *GDF-9-iCre* females whose oocytes contained two floxed alleles to be converted, all 183 genotyped mice inherited the excised allele. Overall, the conversion of *Smc1*β^{fl} to *Smc1*β^{ex} by Cre-mediated recombination was 99% efficient.

To create the *Smc1*β^{-} allele, we replaced exon 10 with the *neo* gene, and consequently the SMC1β protein disappeared [4]. As a result of splicing, the *neo* sequence is removed from the transcript and exons 9 and 11 become joined, as demonstrated by reverse transcriptase PCR (RT-PCR) and sequencing of the PCR product (Figure S2A). Exon 10 codes for a part of the SMC1 hinge domain that is indispensable for SCC [6]. Thus, the deletion of exon 10 renders any residual *Smc1*β^{-} transcript nonfunctional. We found that expression of the *Smc1*β^{ex} allele also leads to appearance of an mRNA containing the exon 9–exon 11 junction (Figure S2B), which provides an opportunity for testing for the presence of *Smc1*β^{ex} by RT-PCR.

To prove that Cre recombinase-mediated conversion of the *Smc1*β^{fl} allele to the *Smc1*β^{ex} allele occurs in oocytes, we performed RT-PCR analysis of RNA isolated from single germinal vesicle-stage oocytes collected from *Smc1*$\beta^{fl/-}$ or *Smc1*$\beta^{fl/fl}$ *GDF-9-iCre* mice or their Cre-negative littermates (Figure 1A). The mRNA transcribed from the *Smc1*β^{fl} allele was present in Cre-negative oocytes but was not detected in any of the Cre-expressing cells tested. We conclude that in Cre-expressing oocytes, the *Smc1*β exon 10 deletion and degradation of the wild-type *Smc1*β transcript occurs before germinal vesicle breakdown.

We also investigated expression of *Smc1*β at various time points after birth to determine the kinetics of excision of the floxed allele (Figure 1B). RT-PCR was performed on total RNA isolated from ovary, in which *Smc1*β is expressed only in oocytes. Provided that the *GDF-9-iCre* transgene is present, mRNA transcribed specifically from the *Smc1*β^{ex} allele can already be detected at day 2 after birth. A weak signal from the wild-type transcript was still observed at day 4 but was not found at day 6 or thereafter. At day 6, more than 90% of oocytes are at the primordial follicle stage, and only a tiny fraction developed to primary and secondary follicles [17]. Thus, as expected, Cre-mediated deletion of the *Smc1*β exon 10 and degradation of wild-type *Smc1*β mRNA happen early after birth at the primordial follicle stage.

In *Smc1*$\beta^{-/-}$ oocytes, all cohesion is lost in metaphase II, and the mice are sterile at all ages [4]. Thus, there is no other cohesin-like activity that could even partially complement SMC1β deficiency. If reloading of SMC1β would be necessary for maintenance of SCC in oocytes throughout adulthood and for progression through meiosis I and II, the *Smc1*$\beta^{fl/-}$ *GDF-9-iCre* or *Smc1*$\beta^{fl/fl}$ *GDF-9-iCre* female mice would be compromised in their fertility or would be sterile. However, we observed full fertility of these mice (Table 1).

*Smc1*β inactivation in oocytes of *Smc1*$\beta^{fl/-}$ *GDF-9-iCre* or *Smc1*$\beta^{fl/fl}$ *GDF-9-iCre* mice did not affect their fertility with respect to litter size or number of offspring at any age tested. Two-way analysis of variance (ANOVA) showed that effects of age or genotype were statistically insignificant. Some *Smc1*$\beta^{fl/-}$ *GDF-9-iCre* or *Smc1*$\beta^{fl/fl}$ *GDF-9-iCre* females were kept breeding for up to 15 months, and no difference to wild-type mice was observed. Thus, the conversion of the *Smc1*β^{fl} allele to the *Smc1*β^{ex} allele in dictyate-arrested oocytes right after birth does not impair oocyte maturation.

When *Smc1*$\beta^{ex/+}$ mice were crossed with *Smc1*$\beta^{+/-}$, their male and female *Smc1*$\beta^{ex/-}$ descendants were sterile, as assessed in extensive breeding trials. Similarly, breeding of *Smc1*$\beta^{ex/-}$ mice (direct descendants of *Smc1*$\beta^{fl/-}$ *GDF-9-iCre* or *Smc1*$\beta^{fl/fl}$ *GDF-9-iCre* breeding with *Smc1*$\beta^{+/-}$) with wild-type mice did not produce any litters in multiple crosses over >5 months. Histological examination of the *Smc1*$\beta^{ex/-}$ testes confirmed that spermatogenesis was arrested at the same stage as in the *Smc1*$\beta^{-/-}$ mutant (Figure S3). This demonstrates that the *Smc1*β^{ex} allele does not complement the *Smc1*β^{-} allele and thus is nonfunctional, and it again illustrates the efficiency of Cre-mediated *Smc1*β deletion. We conclude that expression of SMC1β cohesin is essential only in embryonic, predictyate oocytes and is dispensable at later stages of oocyte development.

Although the *Smc1*$\beta^{fl/-}$ *GDF-9-iCre* and *Smc1*$\beta^{fl/fl}$ *GDF-9-iCre* female mice retained full fertility despite loss of *Smc1*β expression very soon after birth, their oocytes may still be affected by a mild impairment of meiotic SCC, which may become apparent only with increasing age. Therefore, we

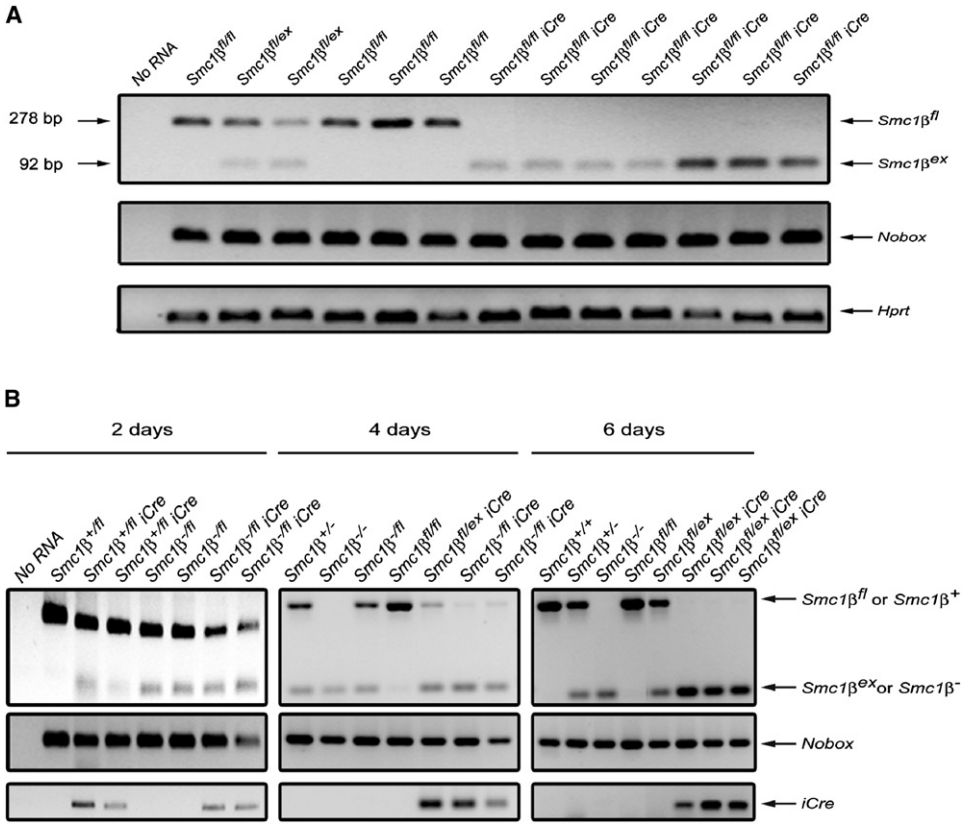

Figure 1. Absence of Full-Length *Smc1*β Transcript in Oocytes from the *Smc1*β$^{fl/fl}$ *GDF-9-iCre* Mice

(A) Total RNA was isolated from single oocytes, and reverse transcriptase-polymerase chain reaction (RT-PCR) products generated with primers specific to *Smc1*β exons 9 and 11 were analyzed on an agarose gel (top). Mouse genotypes are shown above the lanes. In oocyte samples from the *Smc1*β$^{fl/fl}$ mice, PCR generated the fragment of wild-type size (278 bp). In oocyte samples from the *Smc1*β$^{fl/fl}$ *GDF-9-iCre* mice, only the truncated fragment (92 bp), which originated from the *Smc1*β^{ex} transcript, was amplified. Primers specific for an oocyte-specific gene *Nobox* (middle) and a housekeeping gene *Hprt* (bottom) were used as sample quality controls.

(B) Full-length *Smc1*β transcript disappears from oocytes between days 4 and 6 postpartum as a result of Cre-mediated excision of exon 10 from the *Smc1*β^{fl} allele. Total RNA was isolated from ovaries of different ages and analyzed by RT-PCR with primers specific to *Smc1*β exons 9 and 11 (top), an oocyte-specific gene *Nobox* as positive control (middle), and *iCre* (bottom). Mouse genotypes are shown above the lanes. In *Cre*-positive ovaries, faint signals from the *Smc1*β^{fl} allele still persist at day 4 postpartum but completely vanish at day 6, whereas the intensity of the *Smc1*β^{ex} fragments increases.

analyzed the position of chiasmata in oocytes from *Smc1*β$^{fl/-}$ *GDF-9-iCre* and *Smc1*β$^{fl/fl}$ *GDF-9-iCre* mice, with a focus on older animals (6–8 months) to assess a potential age-dependent effect. At that age, the *Smc1*β$^{-/-}$ females show massive oocyte loss, and about 80% of those oocytes that still exist show loss of sister chromatid cohesion and of chiasmata [3]. However, oocytes obtained from the *Smc1*β$^{fl/-}$ *GDF-9-iCre* and *Smc1*β$^{fl/fl}$ *GDF-9-iCre* females did not show any increased loss of chiasmata, i.e., appearance of univalents, and the positions of chiasmata were not skewed toward the more distal locations, as seen previously very prominently in the *Smc1*β$^{-/-}$ mice [3] (Table 2; Figure 2). We conclude that expression of the *Smc1*β gene during and after dictyate arrest is not required for maintenance of sister chromatid cohesion and chiasma position in aging mice.

Our results do not exclude the possibility that SMC1β—were it expressed—could, in principle, be reloaded during the long period of arrest of oocytes, but there is no evidence for such a mechanism. In *Smc1*β$^{-/-}$ oocytes, cohesin is partially intact during meiotic prophase I, but SCC is entirely lost upon oocyte

Table 1. Loss of *Smc1*β Expression in Oocytes during the Dictyate Stage Does Not Affect Fertility

Genotype	Age (Months)	No. of Females	No. of Pups	No. of Litters	Litter Size, Mean (SD)	Offspring Number, Mean (SD)
Floxed +iCre	2–3	5	113	11	10.3 (3.0)	22.6 (6.3)
	3–5	2	50	6	8.3 (2.3)	25.0 (2.8)
	5–7.5	4	75	11	6.8 (2.7)	18.8 (6.7)
Control	2–3	7	156	20	7.8 (2.9)	22.3 (9.7)
	3–5	6	121	17	7.1 (3.1)	20.2 (6.9)
	5–7.5	4	72	9	8.0 (2.7)	18.0 (4.5)

Females with the *Smc1*β gene inactivated in the oocytes by Cre (floxed + *iCre*) or females with intact *Smc1*β (control) at the specified age were mated with wild-type or *Smc1*β$^{+/-}$ males, and the number of pups born during the 3 month mating trial was counted. The effects of age or genotype on the litter size or offspring number were not significant, as determined by analysis of variance ($0.3 < p < 0.9$); SD denotes standard deviation.

Table 2. Analysis of Chiasma Positions and Univalent Chromosomes in Metaphase I Spread Oocytes

Mouse Genotype	Chiasma Position			Univalents/ Oocyte
	Proximal (%)	Interstitial (%)	Distal (%)	
*Smc1*β$^{fl/-}$ *GDF9-iCre*	15.1	56.2	28.6	0.3
*Smc1*β$^{fl/-}$	11.8	61.1	27.1	0.4
	χ^2 = 5.637, p = 0.060			t test, p = 0.37

Chiasma position was determined in 940 bivalents (47 oocytes) from five *Smc1*β$^{fl/-}$ *GDF9-iCre* mice and 740 bivalents (37 oocytes) from five *Smc1*β$^{fl/-}$ mice. Univalents were counted in 63 oocytes from eight *Smc1*β$^{fl/-}$ *GDF9-iCre* mice and 56 oocytes from eight *Smc1*β$^{fl/-}$ mice. The animals were 7–8 months old. The results did not differ significantly between the two genotypes.

maturation [4]. The SMC1β-independent cohesion, likely accomplished by the SMC1α complex still present early in meiosis, is not sufficient to maintain cohesion and cannot rescue loss of chiasmata and SCC in *Smc1*β$^{-/-}$ or *Smc1*β$^{ex/-}$ oocytes. Real-time PCR analysis of the *Smc1*α transcript level did not show any significant increase in single oocytes from *Smc1*β$^{fl/fl}$ *GDF-9-iCre* mice compared to Cre-negative oocytes collected from 3- or 6-month-old mice. Neither genotype nor age affected *Smc1*α expression (data not shown; two-way ANOVA, p > 0.2).

These data show that SMC1β cohesin—shown earlier to be essential for sister chromatid cohesion and chiasma maintenance—needs only be expressed at the initial stage of meiosis. Recent observations in age-accelerated mouse strains fit this model, for there is a decline in chromosome-associated SMC1β with increasing age in these mice, which suffer from enhanced rates of meiotic aneuploidies [10].

Together, the data presented here show that in oocytes, the key requirements for SMC1β cohesin are met prior to the primordial follicle stage. Thus, the decline in faithful chromosome segregation observed with increasing age in humans is very likely not due to a failure to continuously express and reload SMC1β, unless mice and humans differ fundamentally in this respect. The most straightforward interpretation based on data presented here is that slow degradation of SMC1β-mediated cohesion, established early in embryonic meiosis, significantly contributes to the age-dependent increase in aneuploidy.

Experimental Procedures

Generation of Mice for Conditional Inactivation of *Smc1*β

To generate an allele of the *Smc1*β gene suitable for conditional targeting, we modified the targeting vector used by us previously [4] by inserting the *Smc1*β exon 10 flanked by *loxP* sites and the *FRT*-flanked *tk-neo* selection cassette between the long and short arms of homology (Figure S1A). The *tk* negative selection marker was replaced by the diphteria toxin A fragment gene controlled by the *Pgk1* promoter and polyadenylation signal. The sequence of the targeting vector is available upon request. Mice carrying the *Smc1*β allele with the floxed exon 10 and the insertion of the *FRT*-flanked *tk-neo* (*Smc1*β$^{fl::neo}$) were generated as described previously [4]. To remove the *tk-neo* selection marker, we crossed the *Smc1*β$^{fl::neo/+}$ mice with the *ACTB::Flpe* mice [18] (Figure S1A). In the progeny, the *FRT*-flanked *neo* was deleted by the FLP recombinase, and the resulting *Smc1*β^{fl} allele was transmitted through the germline. *Smc1*β$^{+/-}$ mice [4] were crossed with *GDF-9-iCre* transgenic mice [16], and the resulting *Smc1*β$^{+/-}$; *GDF-9-iCre* males were mated with *Smc1*β$^{fl/+}$ females to obtain *Smc1*β$^{fl/-}$; *GDF-9-iCre* females. Mice were genotyped by PCR analysis of tail DNA using Taq polymerase (Invitrogen) and primers shown in Figures S1B and S1C. Primers were designed by Primer3 software [19]. Sequences of the primers are listed in Table S1. *iCre* PCR primers and conditions were as described [16]. *Smc1*β PCR conditions were as follows: denaturation at 94°C for 3 min, 12 cycles of denaturation at 94°C for 30 s, annealing at 60°C–0.5°C/cycle for 30 s, elongation at 72°C for 2 min, followed by 25 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 2 min, and final elongation at 72°C for 2 min.

RT-PCR on Single Oocyte Samples or Ovary RNA

Total RNA was isolated from oocytes using RNeasy Micro Kit (QIAGEN). Single oocytes from 7-month-old animals were frozen in 80 μl of lysis buffer RLT. For real-time PCR, oocytes from 3- or 6-month-old animals were frozen in pools of 10 in 80 μl of buffer RLT. Upon defrosting, poly-A RNA carrier was added to 0.25 ng/μl. To lyse cells, we pipetted the mixture up and down 10 times. RNA was eluted with 14 μl of elution buffer. Total RNA was isolated from ovaries or liver using RNeasy Mini kit (QIAGEN). Two ovaries were homogenized in 350 μl of lysis buffer RLT. RNA was eluted in 40 ul of water. Reverse transcription was performed in final volume of 20 μl using Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen), in accordance with the manufacturer's instruction, either with 4–9 μl of single-oocyte RNA and random hexamer primers or with 500 ng of total ovary RNA and oligo dT_{15} primer. For single-oocyte cDNA, PCR amplification was carried out using Ex Taq (Takara Bio USA) with 5 μl of single-oocyte cDNA in a 20 μl reaction, according to the manufacturer's instruction. PCR conditions were as follows: cDNA was denatured at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s. Final elongation step was at 72°C for 1 min. For ovary cDNA samples, PCR was performed using DreamTaq Green DNA Polymerase (Fermentas) with 1 μl of a 1:10 cDNA dilution in a 25 μl reaction, according to the manufacturer's instruction. The PCR program for *Smc1*β and *iCre* was 95°C for 2 min, 35 cycles of 94°C for 1 min, 55°C for 30 s, 72°C for 45 s, and final elongation at 72°C for 5 min. For *Nobox*, 25 cycles of the same program were used. Sequences of the

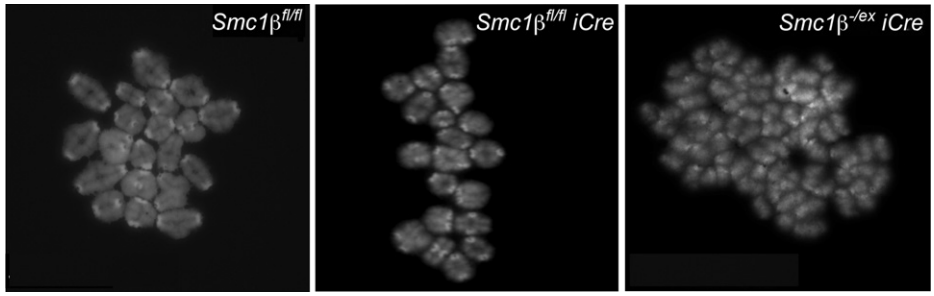

Figure 2. Cessation of *Smc1*β Expression in Dictyate-Stage Oocytes Does Not Impair Sister Chromatid Cohesion

Metaphase I chromosome preparations from adult females were stained with Hoechst 33342. In oocytes from *Smc1*β$^{fl/fl}$ *iCre* females after birth, *Smc1*β^{fl} alleles were converted to nonfunctional *Smc1*β^{ex} alleles. Oocytes from *Smc1*β$^{-/ex}$ *iCre* females lacked SMC1β throughout all stages of meiosis. In both *Smc1*β$^{fl/fl}$ (left) and *Smc1*β$^{fl/fl}$ *iCre* (middle) oocytes from 8-month-old females, no unpaired bivalents were found. In contrast, in *Smc1*β$^{-/ex}$ oocytes from a 5-month-old female (right), most of the chromosomes were present as univalents or single chromatids, as observed previously in SMC1β-deficient females [3].

primers are listed in Table S1. Real-time PCR amplification was done using LightCycler 480 II Instrument (Roche Applied Science) with PerfeCTa SYBR Green FastMix (Quanta Biosciences). Amplification was carried out with 5 μl of oocyte cDNA in a 20 μl reaction, according to the manufacturer's instructions. The cDNA was denatured at 95°C for 2 min. PCR conditions were as follows: denaturation at 95°C for 10 s, annealing at 56°C for 15 s, and elongation at 72°C for 20 s. Fluorescence was acquired at the end of the elongation step. To confirm specificity, we carried out melting curve analysis after 45 cycles and analyzed PCR products by agarose gel electrophoresis. Quantification was done using LightCycler Software version LCS480 1.5.0.39. We generated standard curves using serial dilutions of total ovary cDNA. We used the *Hprt* transcript as a reference to allow comparisons of *Smc1α* levels between individual oocyte samples. Relative quantification results were expressed as the *Smc1α:Hprt* ratio. Real-time PCR was performed in triplicates for each sample. Fourteen samples from 3-month-old and 8 samples from 6-month-old *Smc1β*$^{fl/fl}$; *GDF-9-iCre* or *Smc1β*$^{fl/-}$; *GDF-9-iCre* mice, and the same number of samples from Cre-negative *Smc1β*$^{fl/fl}$ or *Smc1β*$^{fl/-}$ animals, were analyzed.

Oocyte Isolation, Culture, and Chromosome Spreads

Oocytes from adult mice in dioestrus stage were isolated and matured in M2 medium at 37°C for 8 hr. Oocytes with germinal vesicle breakdown were incubated for 5 min in 0.75% sodium citrate before they were fixed in methanol:acetic acid (3:1) and dropped onto slides. After drying at room temperature, chromosomes were stained with Hoechst 33342 (1 μg/ml in phosphate-buffered saline; Sigma) and mounted with Vectashield (Vector Laboratories).

Mating Trials

Depending on the age at the start of breeding, females were divided into three groups: (1) from 2 to 3 months old, (2) older than 3 months but younger than 5 months, and (3) older than 5 months but younger than 8 months. Each female was housed with one male for 3 months, and the number of pups in each litter was recorded. The matings that tested oocytes upon the *Smc1β* gene inactivation by Cre consisted of *Smc1β*$^{fl/fl}$ *GDF-9-iCre* or *Smc1β*$^{fl/-}$ *GDF-9-iCre* females mated with *Smc1β*$^{fl/fl}$, *Smc1β*$^{+/-}$, or *Smc1β*$^{+/+}$ males. The control crosses were reciprocal. In addition, some *Smc1β*$^{fl/fl}$ *GDF-9-iCre* or *Smc1β*$^{fl/-}$ *GDF-9-iCre* females were left for up to 15 months with such males; we occasionally put in younger males of the above-specified genotypes to provide the best chance for mating.

Histology

For hematoxylin and eosin staining, testes were fixed in Bouin's solution (Sigma-Aldrich Chemie) at 4°C overnight and washed several times in 70% EtOH. Tissues were then embedded in paraffin and cut in sections of 8 μm. Staining was performed according to a standard protocol.

Statistical Analysis

Two-way ANOVA was done using GraphPad Prism 5 software.

Supplemental Information

Supplemental Information includes three figures and one table and can be found with this article online at doi:10.1016/j.cub.2010.08.024.

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