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In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication

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The transition from mitosis to meiosis is a defining juncture in the life cycle of sexually reproducing organisms. In yeast, the decision to enter meiosis is made before the single round of DNA replication that precedes the two meiotic divisions[1]. We present genetic evidence of an analogous decision point in the germ line of a multicellular organism. The mouse *Stra8* gene is expressed in germ cells of embryonic ovaries, where meiosis is initiated, but not in those of embryonic testes, where meiosis does not begin until after birth[2]. Here we report that in female embryos lacking *Stra8* gene function, the early, mitotic development of germ cells is normal, but these cells then fail to undergo premeiotic DNA replication, meiotic chromosome condensation, cohesion, synapsis and recombination. Combined with previous findings, these genetic data suggest that active differentiation of ovarian germ cells commences at a regulatory point upstream of premeiotic DNA replication.

Mechanisms by which diploid cells transition from mitosis to meiosis have been investigated most thoroughly in the budding yeast *Saccharomyces cerevisiae* and in the fission yeast *Schizosaccharomyces pombe*[1]. In these two species, the molecular pathways governing meiotic initiation are dissimilar, and there is no evidence that either initiation pathway is conserved in multicellular organisms. Nonetheless, genetic studies of meiotic initiation in *S. cerevisiae* and *S. pombe* uncover an underlying commonality: in both species, the decision to enter meiosis is made before the round of DNA replication that precedes the meiotic divisions. An analogous decision point upstream of premeiotic DNA replication might, in principle, exist in multicellular organisms, but genetic evidence of this has been lacking.

The embryonic ovary of the mouse presents an opportunity to explore genetic control of meiotic initiation *in vivo*[3–6]. In mice, the timing of meiotic initiation differs markedly between the sexes: ovarian germ cells enter meiosis during embryogenesis, whereas testicular germ cells begin meiosis only after birth. Nonetheless, germ cells of embryonic ovaries remain uncommitted to meiosis until about 24–36 h before meiotic prophase is observed there[4]. Within this defined temporal window, the decision to initiate the meiotic program is made in germ cells of the ovary but not in those of the testis.

The mouse *Stra8* gene is expressed exclusively in premeiotic germ cells in both sexes. In females, *Stra8* is expressed in the embryonic ovary in an anterior-to-posterior wave that precedes a similar wave of

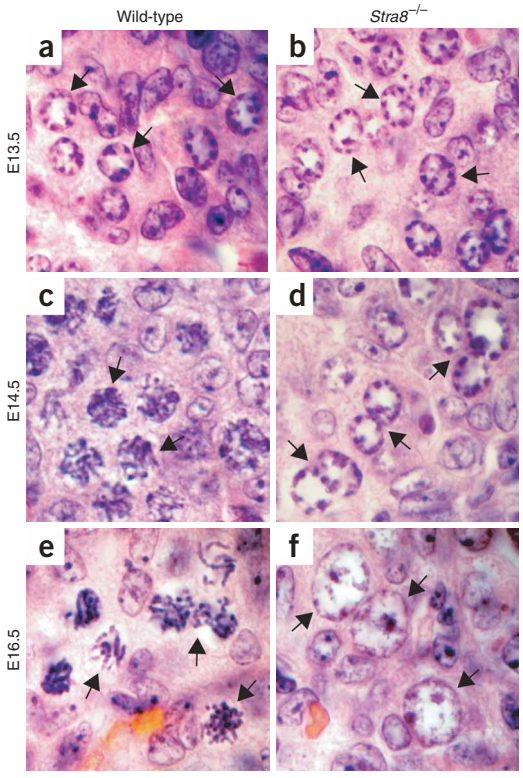

Figure 1 Photomicrographs of hematoxylin and eosin–stained ovarian sections from wild-type and *Stra8*-deficient littermate embryos. (**a**,**b**) E13.5. (**c**,**d**) E14.5. (**e**,**f**) E16.5. Arrows indicate representative germ cells.

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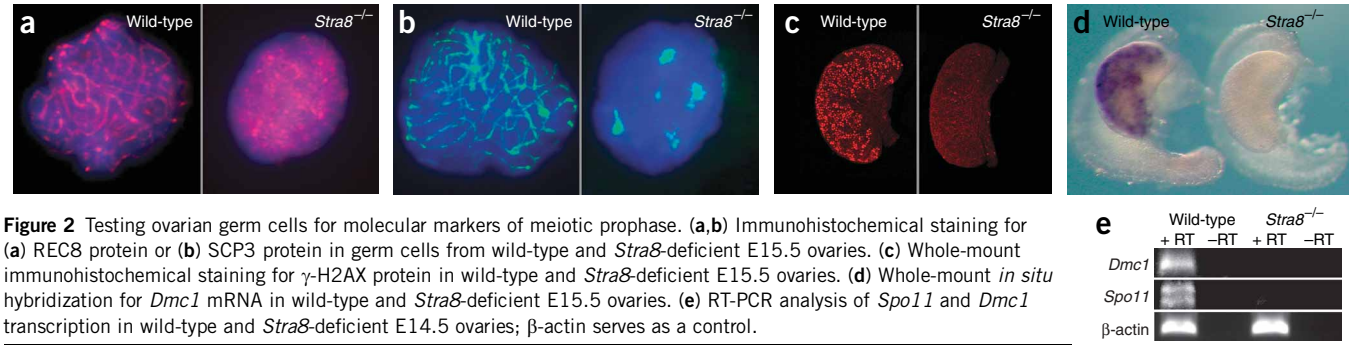

Figure 2 Testing ovarian germ cells for molecular markers of meiotic prophase. (**a**,**b**) Immunohistochemical staining for (**a**) REC8 protein or (**b**) SCP3 protein in germ cells from wild-type and *Stra8*-deficient E15.5 ovaries. (**c**) Whole-mount immunohistochemical staining for γ-H2AX protein in wild-type and *Stra8*-deficient E15.5 ovaries. (**d**) Whole-mount *in situ* hybridization for *Dmc1* mRNA in wild-type and *Stra8*-deficient E15.5 ovaries. (**e**) RT-PCR analysis of *Spo11* and *Dmc1* transcription in wild-type and *Stra8*-deficient E14.5 ovaries; β-actin serves as a control.

expression of early meiotic genes[2,7,8]. In males, *Stra8* is expressed in pubertal and adult testes, in premeiotic spermatogenic cells[9]. Thus, *Stra8* might have a role early in meiosis or perhaps in the transition from mitosis to meiosis, in one or both sexes.

To explore the role of *Stra8* in germ cell development, we used gene targeting to derive mice that lack *Stra8* function (**Supplementary Fig. 1** online). We found that both female and male homozygotes for the targeted *Stra8* allele were invariably infertile; heterozygotes were fertile. Inspection of the reproductive organs of *Stra8*-deficient adults showed a marked reduction in the size of ovaries and testes (**Supplementary Fig. 2** online). Apart from the gonads, we detected no phenotypic abnormalities in *Stra8*-deficient animals. Although the ovaries of heterozygous females showed a normal distribution of maturing ovarian follicles at 8 weeks of age, the ovaries of *Stra8*-deficient females contained no oocytes or follicles. Although heterozygous males showed normal spermatogenesis at 8 weeks of age, with a full range of premeiotic, meiotic and postmeiotic cells, the testicular germ cells of *Stra8*-deficient animals were severely reduced in number, and nearly all appeared to be premeiotic.

Having found no oocytes in 8-week-old *Stra8*-deficient females, we compared histologically the gonadal germ cells of wild-type and *Stra8*-deficient female embryos (**Fig. 1**). We did not observe any differences through embryonic day 13.5 (E13.5). In wild-type ovaries, germ cells proliferate mitotically until about E13.5, by which time they acquire a distinctive morphology, with patches of condensed chromatin at the periphery of the nucleus[3,10] (**Fig. 1a**). *Stra8*-deficient germ cells also show this premeiotic morphology at E13.5 (**Fig. 1b**). Notably, testicular germ cells show a similar morphology at about this time, which, as some studies have suggested, may be a pivotal period of germ cell sensitivity to extrinsic meiosis-inducing or inhibiting factors that differ between female and male gonads[3,11]. We conclude that *Stra8* function is not required for germ cells to develop the premeiotic morphology observed in wild-type females at E13.5.

In ovaries of wild-type embryos, germ cells enter meiotic prophase in a spatiotemporal wave that begins at E13.5–E14.5 (refs. 2,7,8,10,12,13). By E14.5, many germ cells in wild-type ovaries show the thread-like chromosome condensation that defines leptotene, the initial stage of meiotic prophase (**Fig. 1c**). By E16.5, most germ cells in wild-type ovaries show the more advanced nuclear morphologies that characterize the zygotene and pachytene stages of meiotic prophase (**Fig. 1e**). By contrast, the germ cells of *Stra8*-deficient ovaries do not advance beyond the premeiotic morphology observed in wild-type ovaries at E13.5 (**Fig. 1d**,**f**). Instead, *Stra8*-deficient germ cells retain this morphology until at least E16.5, by which point most of their nuclei are enlarged (**Fig. 1f**). (Oocytes with a similar morphology are sometimes observed in age-matched wild-type ovaries, but at much lower frequency.) By birth, *Stra8*-deficient ovaries are severely depleted of germ cells (data not shown). Taken as a whole, these histological findings suggest that, in female embryos, *Stra8*-deficient germ cells develop normally to the brink of meiosis but do not undergo the chromosome condensation indicative of early meiotic prophase.

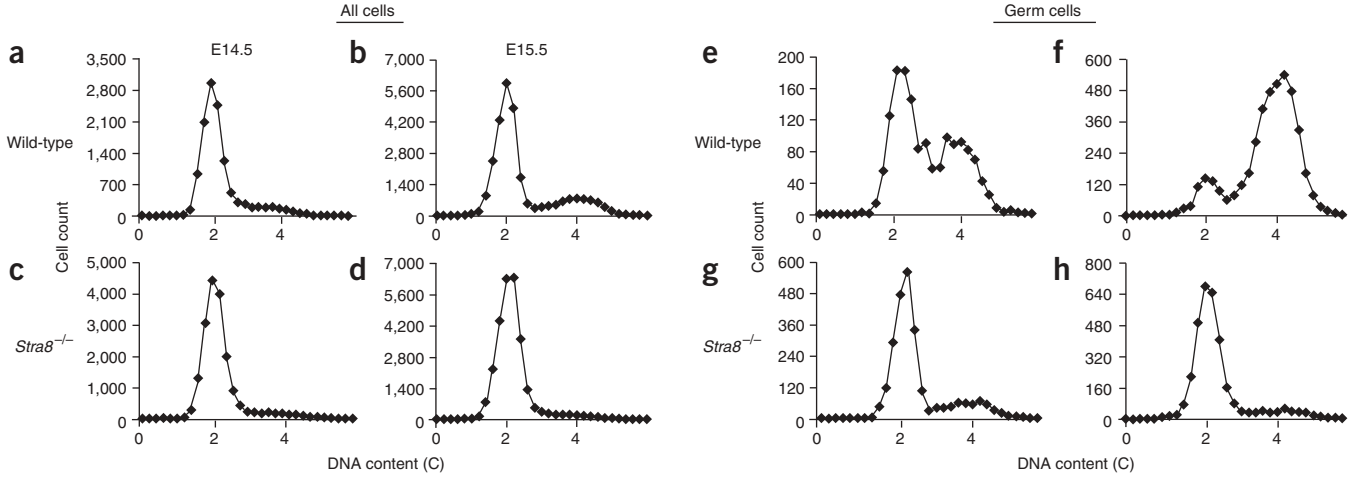

Figure 3 Histograms of cellular DNA content in embryonic ovaries. (**a**–**d**) All cells from (**a**) wild-type E14.5, (**b**) wild-type E15.5, (**c**) *Stra8*-deficient E14.5 or (**d**) *Stra8*-deficient E15.5 ovaries. (**e**–**h**) Germ cells from (**e**) wild-type E14.5, (**f**) wild-type E15.5, (**g**) *Stra8*-deficient E14.5 or (**h**) *Stra8*-deficient E15.5 ovaries. Total numbers of cells plotted: **a**, 12,255; **b**, 27,626; **c**, 18,160; **d**, 27,711; **e**, 1,532; **f**, 4,329; **g**, 2,493; **h**, 3,224.

Our histological findings suggested that *Stra8* might be required for female germ cells to enter meiotic prophase. If so, then the chromosomes of *Stra8*-deficient female germ cells should not be decorated with the cohesion or synaptonemal complexes characteristic of meiotic prophase. To test this, we immunostained chromosomes from ovarian germ cells of wild-type and *Stra8*-deficient E15.5 littermates with antibodies to either REC8, a meiotic cohesin[14,15], or SCP3, a synaptonemal complex protein[16]. As expected, REC8 and SCP3 proteins were found along the lengths of chromosomes in most wild-type oocytes at this time (**Fig. 2a**,**b**). In all *Stra8*-deficient germ cells examined, REC8 and SCP3 were localized not along the lengths of chromosomes but in the patterns recently reported in premeiotic germ cells of the embryonic ovary (**Fig. 2a**,**b**)[17] (**Supplementary Note** online). We conclude that *Stra8* is required in female germ cells for meiotic cohesion and synaptonemal complex formation.

If *Stra8* is required for female meiotic prophase, then *Stra8*-deficient cells should not engage in meiotic recombination. Accordingly, we asked whether *Stra8*-deficient female germ cells form DNA double-strand breaks (DSBs), a hallmark of meiotic recombination. When DNA DSBs are formed, cells respond by phosphorylating H2AX, a histone H2A isoform, to yield γ-H2AX[18]. As expected, immunostaining for γ-H2AX demonstrated the presence of DNA DSBs throughout the ovaries of wild-type embryos at E15.5 (**Fig. 2c**). By contrast, the ovaries of *Stra8*-deficient embryos were negative for γ-H2AX, indicating that DNA DSBs had not formed (**Fig. 2c**).

In additional tests of engagement in meiotic recombination, we asked whether *Stra8*-deficient female germ cells express *Spo11* and *Dmc1*, which are genes required to form and to repair meiotic DSBs, respectively[19–22]. As previously demonstrated by whole-mount *in situ* hybridization, *Stra8* is expressed shortly before *Dmc1* in wild-type embryonic ovaries[2]. By this same assay, we found that *Stra8*-deficient germ cells do not express *Dmc1* (**Fig. 2d**), a result confirmed by RT-PCR analysis (**Fig. 2e**). Likewise, *Spo11* was not expressed in *Stra8*-deficient female germ cells (**Fig. 2e**). Taken together, the absence of γ-H2AX staining and the absence of *Spo11* and *Dmc1* expression provide strong evidence that *Stra8*-deficient female germ cells do not undertake meiotic recombination.

Immediately before meiotic prophase, germ cells replicate their DNA and thus increase their DNA content from 2C to 4C, which then remains constant throughout the extended prophase of the first meiotic division. Given the weight of evidence that *Stra8* is required for female germ cells to enter meiotic prophase, the question arises whether *Stra8* is also required for premeiotic DNA replication. Although no markers specific to premeiotic DNA replication are known in mammals, this replication has been shown to occur during the 15 h before meiotic prophase[23]. By image cytometry, we compared the DNA content of cells from wild-type and *Stra8*-deficient ovaries at E14.5 and E15.5, when wild-type 4C germ cells should accumulate in meiotic prophase. (The numbers of germ cells in embryonic ovaries are insufficient for DNA content analysis via flow cytometry.) We first analyzed DNA content in cells of the entire ovary, where germ cells are greatly outnumbered by somatic cells. In wild-type ovaries, the proportion of 4C cells increases from E14.5 (**Fig. 3a**) to E15.5 (**Fig. 3b**), reflecting the increasing number of germ cells that have completed premeiotic DNA replication. By contrast, *Stra8*-deficient ovaries contain few if any 4C cells, and their numbers do not increase (**Fig. 3c**,**d**), implying that *Stra8*-deficient germ cells have not undergone premeiotic DNA replication.

To corroborate and refine this model, we used a germ cell–specific antibody (α-mouse Vasa homolog (MVH))[24] to label and distinguish the germ cell population in our image-cytometric analyses of DNA content in wild-type and *Stra8*-deficient ovaries. We observed that a substantial fraction of germ cells in wild-type ovaries are 4C at E14.5 (**Fig. 3e**), and the great majority are 4C at E15.5 (**Fig. 3f**), again reflecting the increasing number of germ cells that have completed premeiotic DNA replication by E15.5. In *Stra8*-deficient ovaries, by contrast, only a very small fraction of germ cells are 4C at E14.5 (**Fig. 3g**), and even this small fraction has disappeared by E15.5 (**Fig. 3h**). We suggest that, in *Stra8*-deficient (and wild-type) ovaries, a small population of (4C) germ cells has not completed the last mitotic division by E14.5 (**Fig. 3g**) but has done so by E15.5 and thus returns to a 2C state (**Fig. 3h**). Taken together, our findings indicate that in the female germline, *Stra8* is not required for completion of the final mitotic division, but it is required for premeiotic DNA replication and the subsequent events of meiotic prophase.

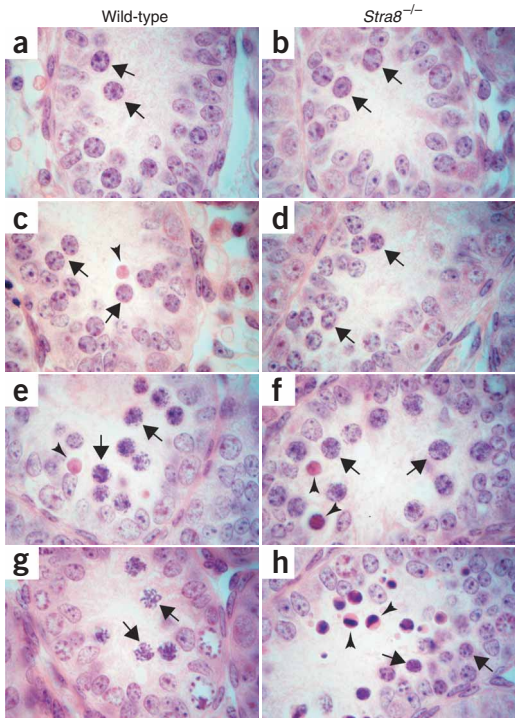

Figure 4 Photomicrographs of hematoxylin and eosin–stained sections from wild-type and *Stra8*-deficient testes at 7 or 8 d after birth. In each section, arrows indicate the most advanced spermatogenic cell type; arrowheads indicate apoptotic cells. (**a**,**b**) B-type spermatogonia in wild-type and *Stra8*-deficient testes. (**c**,**d**) Preleptotene spermatocytes in wild-type and *Stra8*-deficient testes. (**e**) Leptotene spermatocytes in wild-type testes. (**f**) Preleptotene spermatocytes in *Stra8*-deficient testes, with no meiotic chromosome condensation. (**g**) Zygotene spermatocytes in wild-type testes. (**h**) Preleptotene spermatocytes and apoptotic spermatocytes in *Stra8*-deficient testes.

Although many genes in addition to *Stra8* are known to be required for mammalian meiosis, these genes function during or after meiotic prophase and are not required for premeiotic DNA replication. For example, germ cells lacking either *Rec8*, *Scp3*, *Spo11* or *Dmc1* readily progress into meiotic prophase in both females and males[19–22,25–27].

Is *Stra8* required for male meiosis? In wild-type male mice, spermatogenesis is initiated shortly after birth (**Fig. 4**). Spermatogonia (**Fig. 4a**) undertake a series of mitotic divisions, eventually giving rise to a population of spermatocytes, which undergo meiosis. In wild-type mice of the strain that we used, spermatocytes first appear 7 or 8 d after birth (**Fig. 4c**) and quickly progress into meiotic prophase. In

Stra8-deficient males, spermatogonial divisions seem to proceed normally (**Fig. 4b**), with premeiotic spermatocytes appearing as expected 7 or 8 d after birth (**Fig. 4d**). These *Stra8*-deficient spermatocytes (**Fig. 4d**) show premeiotic chromatin condensation as in wild-type testes (**Fig. 4c**), but here the development of *Stra8*-deficient spermatocytes seems to stall. Whereas wild-type cells readily progress through leptotene (**Fig. 4e**) and zygotene (**Fig. 4g**) stages of meiotic prophase, most *Stra8*-deficient spermatocytes undergo apoptosis before reaching these stages (**Fig. 4f**,**h**). Admixture with mitotically active spermatogonia precluded our determining whether *Stra8*-deficient spermatocytes replicate their DNA before undergoing apoptosis. Nonetheless, we conclude that male germ cells, like female germ cells, require *Stra8* for normal progression into meiotic prophase.

Our analyses of *Stra8*-deficient female germ cells shed light on two fundamental but poorly understood processes in mammalian reproduction: meiotic initiation and the beginnings of ovarian differentiation. Classical descriptions of mammalian meiosis begin with prophase of the first division. We suggest that, in embryonic mouse ovaries, the decision to engage in meiosis is made considerably earlier. We have shown that *Stra8* function is required for premeiotic DNA replication, as well as for the ensuing meiotic prophase, in embryonic ovaries. Recent studies have demonstrated that *Stra8* expression in embryonic ovaries is the result of induction by retinoic acid, which is present there at higher levels than in embryonic testes[5,6]. We conclude that, in ovarian germ cells, the decision to initiate meiosis, made in response to retinoic acid, is made before or early in the round of DNA replication that precedes the meiotic divisions. The fact that *Stra8* is not required for mitotic DNA replication in either germ or somatic lineages underscores the specificity with which premeiotic DNA replication is regulated in mammals.

These insights have ramifications for mammalian sex determination and the divergent paths by which ovary and testis arise from a common precursor. Genetic understanding of ovary determination lags behind that of testis determination[28], and early ovarian development is sometimes portrayed as a relatively uneventful process. To the contrary, our present results imply that premeiotic DNA replication—a regulated act of terminal differentiation dependent on *Stra8*—distinguishes germ cells of the early embryonic ovary from those of its sexually undifferentiated precursor as well as from those of the early embryonic testis. Premeiotic DNA replication, in gonadal germ cells, may be among the earliest manifestations of active feminization during mammalian embryogenesis.

We note that the regulatory logic, if not the molecular specifics, of the mitosis-to-meiosis transition are shared between yeasts and mice. In mouse ovaries, as in budding and fission yeasts, the decision to engage the meiotic program is taken upstream of premeiotic DNA replication[1]. In mouse ovaries, as in yeasts, germline cells initiate the meiotic program in response to molecular cues offered by their environment: retinoic acid in mammals and nutrient depletion in yeast. To be sure, the molecules involved in the mitosis-to-meiosis transition are not widely conserved. The *Stra8* gene and the protein it encodes, for example, seem to be restricted to vertebrates (**Supplementary Fig. 3** online), and retinoic acid is limited to chordates[29]. Nonetheless, the common logic of meiotic initiation—from single-celled yeasts to a sexually dimorphic animal with a physically and embryologically segregated germline—attests to the unity of sexual reproduction among eukaryotes.

METHODS

Targeted disruption of the *Stra8* locus. A *Stra8 IRES-LacZ/PGK-Neo* targeting construct was generated using PCR products amplified with Advantage HF2 polymerase (Clontech). All PCR products were sequenced to ensure the absence of point mutations. The v6.5 embryonic stem (ES) cell line was electroporated with 40 μg of linearized targeting construct DNA, and selection was performed with 300 μg/ml G418 (GIBCO-BRL). ES cell colonies were picked and screened by long-distance PCR specific for homologous targeting at the 5′ arm. Homologous targeting at the 3′ arm was confirmed by DNA blot analysis. Correctly targeted ES cell clones were injected into Balb/c or C57Bl/6 blastocysts and transferred to pseudopregnant Swiss Webster females. Germline transmission was obtained with three independent clones. Primers Stra8_p1, Stra8_p2 and Stra8_p3 (**Supplementary Table 1** online) were used for PCR genotyping. The wild-type allele gives rise to a PCR product of 280 bp. The mutant allele gives rise to a PCR product of 180 bp.

Histology. Dissected tissues were fixed overnight in Bouin's solution, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Images were taken with a Nikon Optiphot 2 microscope, a Kodak DC290 Zoom digital camera and Adobe Photoshop 7.

Cell spreads for meiotic chromosome analysis. Gonads removed from embryos between E14.5 and E15.5 and dissected free of mesonephroi were incubated in 50 μl of trypsin-EDTA solution at 37 °C for 5 min and then washed briefly in PBS. To obtain single cells, these trypsin-digested gonads were pipetted repeatedly and then centrifuged, followed by resuspension in 30 μl of hypotonic solution (0.5% sodium chloride in PBS). Cell suspensions were placed on poly-L-lysine-coated slides and kept in a humid chamber at room temperature (22 °C) until the cells had settled. The slides were then fixed in 2% paraformaldehyde and 0.03% SDS for 1 h at room temperature, washed three times in 0.4% Photoflo (Kodak) for 1 min and air dried. These slides were stored at –80 °C before use.

Cell-spread immunohistochemistry. For fluorescence immunostaining, slides were removed from storage at –80 °C, washed twice in PBS and treated with blocking buffer (3% bovine serum albumin, 0.05% Tween-20 and 0.05% Triton X-100 in PBS). Slides were then incubated with a 1:500 dilution of rabbit α-SCP3 or α-REC8 overnight at 4 °C, washed with PBS and incubated with Texas red– or fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:200 dilutions; Jackson ImmunoResearch Laboratories) for 1 h at room temperature. After extensive washing with PBS, slides were mounted using VECTASHIELD medium with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Images were taken using an Olympus BX51TF Fluorescence Microscope, a Spot RT digital camera and Spot 4.1 software (Diagnostic Instruments).

Whole-mount immunohistochemistry. Whole-mount indirect fluorescent immunohistochemistry was performed as previously described[30]. Mouse monoclonal γ-H2AX antibody (Upstate) was used at a 1:1,000 dilution, and donkey anti-mouse Texas red–conjugated secondary antibody (Jackson) was used at a 1:200 dilution. Images were merged *z*-stacks taken using a Zeiss LSM 510 meta confocal microscope.

Whole-mount *in situ* hybridization. Gonads for *in situ* hybridization were collected at E15.5 and fixed in 4% paraformaldehyde at 4 °C overnight. Tissues were dehydrated in 100% methanol and stored at –20 °C before use. Digoxigenin whole-mount *in situ* hybridization for *Dmc1* was performed as previously reported[2].

RT-PCR. Total RNA was isolated from gonads using an RNA miniprep kit (Qiagen). Total RNA (1 μg) was reverse transcribed with oligo-d(T)18N using a First-Strand cDNA Synthesis Kit for RT-PCR (Roche) in a total reaction volume of 20 μl, of which 1 μl was used as template for PCR with primer sets for *Dmc1*, *Spo11* and β-actin, listed in **Supplementary Table 1**.

DNA content analysis of ovarian cells. Cell spreads were prepared as described above for meiotic chromosome analysis except that cells were not treated with hypotonic solution. Immunostaining with rabbit antibody to MVH (1:1,000 dilution) was performed as described above. DAPI (blue) and MVH (red) images were taken using an Olympus BX51TF Fluorescence Microscope, a Spot RT digital camera and Spot 4.1 software (Diagnostic Instruments). For each

slide, many nonoverlapping fields were imaged to enable analysis of as many independent cells as possible.

All image sets were processed automatically using the software program CellProfiler (http://www.cellprofiler.org) to ensure consistent, unbiased results. Before processing, clumped cells and noncellular artifacts were manually removed using Adobe Photoshop 7.0. For purposes of analysis, background fluorescence in MVH images below an absolute threshold of 0.1 was set to 0. (The dynamic range of incoming images is 0–1.) DAPI and MVH images were aligned, and nuclei were identified in DAPI images using local intensity maxima with a maxima suppression neighborhood of 7 and a blur radius of 3. Edges of nuclei were identified using a threshold calculated using Otsu's method and an adjustment factor of 0.7. In this analysis, objects touching the image border or those with area smaller than 35 pixels were discarded. As MVH is a cytoplasmic protein, MVH-positive cells were identified using nuclei as seeds for the propagate function, with a regularization factor of 0.05. For each cell, the perimeter of the MVH-positive region was determined using a threshold calculated using Otsu's method and an adjustment factor of 0.8.

Intensities of DAPI and MVH signals were then calculated for each cell. As the majority of embryonic gonadal cells are nonreplicating somatic cells (with 2C DNA content), we normalized the integrated intensity of DAPI (directly proportional to DNA content) of each cell to the median integrated intensity of DAPI (set to 2C) across the field. Cell data from several gonads at each time point and genotype were pooled, and histograms were prepared with cells sorted by DNA content into 40 bins ranging from 0C to 8C at 0.2C increments. Additionally, histograms were generated for germ cell–enriched populations by binning only those cells with an integrated intensity of MVH above a threshold determined empirically for each data set.

Mice. All experiments involving mice were approved by the Committee on Animal Care at the Massachusetts Institute of Technology.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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