Structural damage to meiotic chromosomes impairs DNA recombination and checkpoint control in mammalian oocytes

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Meiosis in human oocytes is a highly error-prone process with profound effects on germ cell and embryo development. The synaptonemal complex protein 3 (SYCP3) transiently supports the structural organization of the meiotic chromosome axis. Offspring derived from murine Sycep3−/− females die in utero as a result of aneuploidy. We studied the nature of the proximal chromosomal defects that give rise to aneuploidy in Sycep3−/− oocytes and how these errors evade meiotic quality control mechanisms. We show that DNA double-stranded breaks are inefficiently repaired in Sycep3−/− oocytes, thereby generating a temporal spectrum of recombination errors. This is indicated by a strong residual γH2AX labeling retained at late meiotic stages in mutant oocytes and an increased persistence of recombination-related proteins associated with meiotic chromosomes. Although a majority of the mutant oocytes are rapidly eliminated at early postnatal development, a subset with a small number of unfinished crossovers evades the DNA damage checkpoint, resulting in the formation of aneuploid gametes.

Introduction

A quarter of all conceived human embryos are aneuploid, i.e., they either have too many or too few chromosomes (Hassold and Hunt, 2001). The consequences of such chromosomal abnormalities are profound, affecting not only fertility, but also triggering spontaneous miscarriages. A few abnormal karyotypes are compatible with human life, including Down’s (trisomy 21), Turner (a single X chromosome), and Klinefelter’s (XXY) syndromes, but are also associated with developmental disabilities of variable penetrance. Analysis of human sperm and eggs has revealed that aneuploidy affecting embryos is primarily caused by an error-prone meiotic chromosome segregation mechanism in oocytes. Whereas ~1–2% of human sperm have an abnormal chromosomal content (the same level of aneuploidy is recorded in mouse haploid germ cells, including oocytes), an astonishing 20–25% of the human oocytes are aneuploid (Hassold and Hunt, 2001). The cause of this high error rate for the meiotic process in human female germ cells is unclear.

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Abbreviations used in this paper: AE, axial element; dpp, days postpartum; DSB, double-stranded break; E, embryonic day; SC, synaptonemal complex; SYCP, synaptonemal complex protein.

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the center of the transverse filament structure (Zickler and Kleckner, 1999; Page and Hawley, 2004). In mammalian male and female germ cells, several different meiosis-specific proteins have been defined as components of the SC, including the AE proteins SC protein 2 (SYCP2) and 3 (Dobson et al., 1994; Lammers et al., 1994; Schalk et al., 1998) and the transverse filament protein SYCP1 (de Vries et al., 2005). The AE proteins SYCP2 and -3 are found at the interchromatid domains of the sister chromatids, which is where they jointly form axial cores together with the cohesin complex proteins.

Several different error surveillance systems (checkpoints) have been characterized in meiotic cells (Lydall et al., 1996; Roeder and Bailis, 2000; Di Giacomo et al., 2005). A failure to repair DSBs that is caused by inactivation of DNA repair/recombination proteins such as DMC1, MSH4, and MSH5, or DNA damage checkpoint proteins such as ATM, will activate a DNA damage checkpoint that results in female germ cell death at early postnatal development (Di Giacomo et al., 2005). The mismatch repair protein MLH1 takes part in the conversion of crossovers into chiasmata at a late stage of the recombination pathway (Baker et al., 1996; Edelmann et al., 1996; Hunter and Borts, 1997). Surprisingly, inactivation of this protein in murine germ cells does not activate a DNA damage checkpoint. Instead, in mouse oocytes that are deficient for Mlh1, the resulting achiasmate mutant germ cells cannot establish a proper meiotic spindle and are eliminated at the metaphase I stage by the spindle checkpoint (Woods et al., 1999).

The absence of SYCP3 results in decompaction of the meiotic chromosome axis, premature loss of cohesin complexes from the meiotic chromosome axis, and irregular interruptions of the synaptic process as defined by SYCP1 (Yuan et al., 2002). Female Sycep3<sup>−/−</sup> mice are fertile, although one-third of their offspring die at an early stage of embryonic development as a result of aneuploidy (Yuan et al., 2002). We investigated the nature of the chromosomal errors introduced by the absence of SYCP3 and how these errors evade the meiotic quality assurance systems, thereby generating aneuploid offspring. Our results illustrate the importance of the axial element of the synaptonemal complex for efficient repair of recombination events.

**Results**

**Loss of SYCP3 affects germ cell cyst survival and primordial follicle formation**

The absence of SYCP3 results in a complete elimination of male spermatocytes at the zygotene–pachytene transition of prophase I (Yuan et al., 2000). To investigate how the elimination of SYCP3 affects the oocyte maturation process, ovarian morphology and oocyte numbers were analyzed in pre- and postnatal animals from embryonic day (E) 16.5 to 8 d postpartum (dpp). Sequential sections of ovaries taken from wild-type or Sycep3<sup>−/−</sup> animals were either stained with hematoxylin and eosin or immunostained using antibodies against germ cell nuclear antigen (GCNA) or c-kit. GCNA and c-kit specifically stain the nuclei and the cytoplasm of oocytes, respectively (Manova et al., 1990; Enders and May, 1994). Histomorphometric analysis revealed no difference in the relative numbers of oocytes at E16.5, E18.5, or at birth when ovaries from wild-type or Sycep3<sup>−/−</sup> females were compared (Fig. 1 A and Fig. 2 A). This shows that germ cell development is not interrupted before the diplotene stage in Sycep3<sup>−/−</sup> females. A majority of the oocytes in 1-dpp mice are found in small clusters, called germ cell cysts, which are seen in both wild-type and mutant ovaries (Fig. 1, A and B). Starting at 2 dpp, we noted a distinct loss of germ cell cysts in the Sycep3<sup>−/−</sup> ovary, which was not seen in the wild-type ovary (Fig. 1, C and D, and Fig. 2 B). The relative loss of germ cell cysts in the Sycep3<sup>−/−</sup> ovary was further accentuated 4 dpp (Fig. 1, E and F; and Fig. 2 C). We also noted a reduction in the number of primordial oocytes in Sycep3<sup>−/−</sup> ovaries in 2- and 4-dpp mice.

**Figure 1.** Sycep3<sup>−/−</sup> oocytes are rapidly and selectively lost after birth. Oocytes were detected in ovary sections by anti-GCNA1 (A–F) and anti–c-kit (G and H) immunohistochemistry (brown stain). At 1 dpp, both germ cell cysts (arrowhead) and primordial follicles were found in wild-type (A), as well as in Sycep3<sup>−/−</sup> ovaries (B) in a similar number. The inset in B indicates germ cell cysts. (C) At 2 dpp, germ cell cysts (arrowhead) and primordial follicles were detected mostly in the periphery in wild-type. (D) A significant loss of germ cell cysts (arrowhead) and primordial follicles (inset) were observed in the Sycep3<sup>−/−</sup> ovary at 2 dpp. At 4 dpp, both primordial follicles (arrowhead) and primary follicles were detected in wild type (E). A further loss of primordial follicles (arrowhead) was found in the Sycep3<sup>−/−</sup> ovary (F), but no significant difference in the numbers of primary follicles (inset) was detected between wild-type and mutant (F). (G and H) At 8 dpp, primordial (arrowheads), primary, and secondary follicles (inset) were detected in wild-type (G) and the Sycep3<sup>−/−</sup> ovaries (H), but the number of primordial follicles was further reduced in the Sycep3<sup>−/−</sup> ovary. Bars, 30 μm.
compared with wild type (Fig. 1 and Fig. 2, B and C). It is likely that the loss of primordial oocytes is attributable to the rapid elimination of the germ cell cysts seen during early postnatal development, as the primordial oocytes develop from these cysts. We found that the collective loss of germ cell cysts and primordial oocytes in the mutant ovary amounts to 34% in 2-dpp mice and 52% in 4-dpp mice (Fig. 2 A). A further reduction in primordial follicle number occurs at 8 dpp (Fig. 1 and Fig. 2 D), suggesting that primordial follicles are also susceptible to elimination within the mutant ovaries. Notably, however, the residual fraction of primordial follicles in the Sycp3−/− ovary gives rise to both primary and secondary follicles in numbers that closely match the wild-type situation. We conclude that loss of SYCP3 function results in a drastic loss of germ cell numbers that sustain normal levels of fertility at this age (Yuan et al., 2002).

The loss of germ cell cysts and primordial follicles in the ovary of the mutant females could be caused by an apoptotic process that is introduced by the absence of SYCP3. TUNEL staining showed an increase in the number of apoptotic cells at 1 and 2 dpp in the Sycp3−/− ovary, compared with the wild-type counterpart (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200512077/DC1). The ovarian cells labeled by TUNEL staining in the Sycp3−/− females are predominantly localized at the cortex area (at the outer edge of the ovary sections), suggesting that the affected cells correspond to the germ cell cysts that are preferentially lost in the mutant background. The number of TUNEL-positive cells is low, relative to the total number of oocytes that are lost in the mutant ovary. This is most likely because of the transient nature of the TUNEL staining, which is also described in another mouse model monitoring female germ cell death during early postnatal development (Rajkovic et al., 2004).

Absence of SYCP3 affects the efficiency of the DSB repair process in meiotic cells

The temporal profile of the oocyte loss in Sycp3−/− females suggests the involvement of the DNA damage checkpoint, which is known to become active at an early stage of postnatal development in oocytes (Di Giacomo et al., 2005). Therefore, we monitored the progression of DNA repair of DSBs in meiotic cysts and primordial oocytes. The principles used to define the different meiotic stages in Sycp−/− oocytes are described in Fig. S1, Materials and methods, and Kouznetsova et al. (2005). In brief, both early zygote and zygote oocytes were derived from E16.5 embryos, whereas pachytene and diplotene oocytes were derived from E18.5 or E19.5 embryos. Formation of the axial cores was monitored by STAG3 staining, synopsis (transverse filament formation) was monitored by SYCP1 staining, and centromere morphology was monitored by CREST staining. Introduction of DSBs in meiotic DNA at the leptotene stage of prophase I results in the phosphorylation of H2AX (generating a modified form called γH2AX). γH2AX appears at leptotene in chromatin regions throughout the nucleus and generally form large, cloud-like patterns, suggesting that the majority of the affected H2AX molecules are found in chromatin loops that project out from the axial cores of the chromosomes (Mahadevaiah et al., 2001; Celeste et al., 2002). Subsequent repair of DSBs results in the disappearance of most of the γH2AX signal at the pachytene stage. A second and independent wave of γH2AX staining appears in late zygote and pachytene cells, which are associated specifically with the asynapsed axial cores of the meiotic chromosomes (de Vries et al., 2005; Turner et al., 2005). We found that γH2AX immunostaining of wild-type early zygote oocytes revealed dispersed, cloud-like signals throughout the nucleus (Fig. 3, C and D), whereas only a few patches of γH2AX signals associated with the remaining asynaptic axial cores were observed in pachytene nuclei (Fig. 3, G and H). We also consistently observed a few residual γH2AX patches in diplotene nuclei, the nature of which is not clear (Fig. 3, K and L).

We then analyzed the distribution of γH2AX signal at the pachytene stage. We found that γH2AX staining appears at late zygote and pachytene cells, which are associated specifically with the asynapsed axial cores of the meiotic chromosomes (de Vries et al., 2005; Turner et al., 2005). We found that γH2AX immunostaining of wild-type early zygote oocytes revealed dispersed, cloud-like signals throughout the nucleus (Fig. 3, C and D), whereas only a few patches of γH2AX signals associated with the remaining asynaptic axial cores were observed in pachytene nuclei (Fig. 3, G and H). We also consistently observed a few residual γH2AX patches in diplotene nuclei, the nature of which is not clear (Fig. 3, K and L).
Absence of SYCP3 strongly affects γH2AX distribution during meiotic prophase development. (A–D) γH2AX (green) staining is abundantly distributed throughout the nucleus in early zygotene wild-type and Sycp3−/− oocytes. Synapsed regions are labeled by SYCP1 (red) and centromeres (white) are visualized by CREST. (G and H) Only a few γH2AX patches remain in pachytene wild-type oocytes. The γH2AX staining is associated with the SYCP1-labeled structures of the SC and protrudes from these structures. (E and F) Pachytene Sycp3−/− oocytes retain a strong and ubiquitously distributed γH2AX staining pattern. (K and L) A few residual γH2AX patches were detected in diplotene wild-type oocytes. [I and J] Strong γH2AX staining similar to the pattern seen in early zygotene and pachytene oocytes is retained in diplotene Sycp3−/− oocytes. Bar, 10 μm.

in Sycp3−/− oocytes at the early zygotene stage and noted that it was indistinguishable from the pattern observed in wild-type cells at this stage (Fig. 3, A and B). This suggests that neither SPO11-derived DSB formation nor phosphorylation of H2AX is dependent on SYCP3 expression. Su...
chromosomes. Despite a twofold increase in axial core length, approximately the same amount of MLH1 foci are observed in both wild-type and Sycp3−/− pachytene oocytes (Yuan et al., 2002). We have monitored the abundance of MLH1 foci in wild-type and Sycp3−/− oocytes at postpachytene meiotic stages to reveal if the temporal distribution of this protein is affected in the absence of SYCP3. We found that MLH1 foci persisted into the diplotene stage in wild-type oocytes, as previously described (Moens et al., 2002), but that these MLH1 foci disappeared at the end of diplotene (Fig. 5, C and D; 5, E and F; 5, G and H; and 5, I and J). However, a subset of the Sycp3−/− oocytes displayed fewer foci than the rest (a late diplotene stage oocyte is shown as an example in O). No MLH1 foci were observed in oocytes after birth (unpublished data). Many of the residual MLH1 foci that were retained at the very late diplotene stage were not associated with SYCP1 staining (Fig. 5, E and I). The most likely explanation for this is that the time course of the desynaptic process (i.e., the removal of SYCP1) is not affected in mutant oocytes (Fig. 5), whereas MLH1 foci persist for longer in these cells. The residual MLH1 foci, however, remain in association with the meiotic chromosome axis, as shown by the association of these foci with STAG3-staining regions in late diplotene mutant oocytes (Fig. 5, E and I). Synapsed regions were labeled by SYCP1 antibodies (red) and centromeres were visualized by CREST (white). Bars, 10 μm.
Figure 5. Absence of SYCP3 delays the removal of MLH1 during meiosis. The distribution of MLH1 foci (white) and association with synapsed regions (labeled by SYCP1; red) at different stages of meiosis are shown for Sycp3−/− oocytes (C, G, K, and M), whereas wild-type oocyte are shown as a control (A, E, and I). γH2AX (green) and centromeres visualized by CREST (blue) were added to these pictures for Sycp3−/− oocytes (D, H, L, and N) and wild-type oocytes (B, F, and J). A subset of the Sycp3−/− oocytes displays fewer foci than the rest (a very late diplotene stage oocytes is shown as an example [M and N]). A majority of the MLH1 foci in late diplotene Sycp3−/− oocytes was not associated with SYCP1 staining (O, arrows), but was associated with the asynapsed axial cores as defined by the cohesin complex protein STAG3 (P, arrows). (Q) The number of MLH1 foci was presented as the mean ± SEM of analyzed oocytes from five meiotic stages (n, counted wild-type oocytes; n, counted Sycp3−/− oocytes). The number of MLH1 foci shows no difference between wild-type and Sycp3−/− oocytes at the pachytene (middle to late; A and C; n = 50; n = 35) and early (n = 36; n = 41) to middle diplotene (n = 24; n = 29). An increased number of MLH1 foci relative to wild-type is seen in late (G) and very late diplotene (K) Sycp3−/− oocytes, [LD, n = 20 and n = 42; VLD, n = 20 and n = 24]. Bars, 10 μm.

For example, ~29% of the Sycp3−/− late diplotene oocytes displayed weak γH2AX staining. Furthermore, the same percentage of Sycp3−/− oocytes at late diplotene also contained relatively few RAD51/DMC1 and MLH1 foci (Table I, Fig. 4 O, and Fig. 5, M and N). Although the division of the oocytes into two groups is clearly arbitrary, it shows that the repair/recombination process in Sycp3−/− oocytes is impaired, not blocked, giving rise to a spectrum of mutant cells with different levels of damage.

Sycp3−/− ovaries at 2 dpp display a striking increase in oocytes that contain univalent chromosomes

A decreased efficiency of the DNA repair/recombination process should have consequences for completion of the crossing-over process between the homologous chromosomes. A failure to form or maintain chiasmata between the homologous chromosomes during meiosis will result in premature chromosome separation, giving rise to two separately labeled univalents (achiasmatic) chromosomes. To investigate this, chromosome-specific probes were labeled and used in FISH experiments. We found, as expected, that the chromosome-specific probes (19, 17, 12, 2, 1, and X) labeled single individual chromosome structures in wild-type oocytes (Fig. 6, A–D). In contrast, FISH analysis identified a large number of Sycp3−/− oocytes that contained univalent chromosomes (Fig. 6, E–H, and Table II). The incidence of univalency for each of the six different meiotic

Table I. Sycp3−/− oocytes display variable γH2AX staining and different numbers of DMC1/RAD51 and MLH1 foci.

| Sycp3−/− oocytes | L pachytene/E diplotene | L diplotene | VL diplotene |
|-----------------|------------------------|-------------|-------------|
| (n = 103; n = 20) | (n = 114; n = 38; n = 42) | (n = 41) |
| γH2AX           | 74% stronger           | 71% stronger|
| DMC1/RAD51      | 80% foci > 20          | 71% foci > 10| 66% foci > 5 |
|                 | 20% foci < 20          | 29% foci (0–10)| 34% foci (0–5) |
| MLH1            | 71% foci > 15          | 71% foci > 15| 67% foci > 5 |
|                 | 29% foci (0–15)| 29% foci (0–15)| 33% foci (0–5) |

Sycp3−/− oocytes were classified in groups depending on their level of γH2AX staining or the number of DMC1/RAD51 and MLH1 foci (n, counted oocytes for γH2AX; n, counted oocytes for DMC1/RAD51; and n, counted oocytes for MLH1) and shown as a relative percentage. E, early; L, late; VL, very late.

*These oocytes (except for one cell) also showed a stronger γH2AX staining pattern by coimmunostaining with MLH1 (Fig. 6).
chromosomes analyzed in Sycep3−/− oocytes at 2 dpp varied considerably (Table II). The difference in univalency rate for the six different chromosomes in the analyzed mutant mouse oocytes is best explained in the context of their reported mean chiasmata frequency (Hulten et al., 1995; Lawrie et al., 1995; Broman et al., 2002). Chromosomes 1 and 2 display an almost twofold higher mean chiasmata frequency than reported for chromosomes 12, 17, and 19, strongly suggesting that homologous chromosomes connected with two or more chiasmata are more likely to retain at least one chiasma even in the absence of SYCP3. Analysis of mutant oocytes using two or three chromosome-specific FISH probes simultaneously showed that many Sycep3−/− oocytes contain multiple univalent chromosomes (Table II). We conclude that the reduced efficiency of the DSB repair process in Sycep3−/− oocytes give rise to achiasmatic chromosomes and results in a sharp increase in the number of oocytes that contain univalent chromosomes at 2 dpp.

Discussion

Absence of SYCP3 reduces the efficiency of the meiotic DNA DSB repair process and affects crossover formation

It has been proposed that structural changes in the organization of the axial cores of meiotic chromosomes could affect the maturation of DSBs into crossovers (Blat et al., 2002). We show that loss of SYCP3 impairs both the DNA DSB repair process and the formation of crossovers between homologous chromosomes. The time course and nuclear distribution of phosphorylated H2AX and several recombination-related proteins were monitored during meiosis in Sycep3−/− oocytes. We found that several aspects of meiotic progression were not affected in Sycep3−/− oocytes, including the temporal appearance of γH2AX, the recruitment of RAD51/DMC1, RPA, MSH4, and MLH1 to DNA DSBs, or the time course of meiotic markers such as STAG3, SYCP1, and CREST. In contrast, we found that the removal of the phosphorylated form of H2AX was severely delayed in Sycep3−/− oocytes. Similarly, RAD51/DMC1, RPA, MSH4, and MLH1 foci persisted for an extended time period at the late meiotic stages in the mutant oocytes. Such patterns were not observed in wild-type oocytes and likely reflect a failure to complete recombination within the temporal window provided by meiotic prophase I. In agreement with this, we found that ~75% of the Sycep3−/− oocytes contain univalents...
at 2 dpp. It has been shown that the inactivation of proteins that participate in the repair of meiotic DNA DSBs activates a DNA damage checkpoint during early postnatal development, resulting in the complete elimination of affected oocytes (Di Giacomo et al., 2005). In agreement with this, we found that a majority of the Sycp3−/− oocytes are eliminated beginning at 2 dpp. Furthermore, the increased loss of oocytes with univalent chromosomes during early postnatal development suggests that oocytes with incompletely repaired DNA are preferentially eliminated in Sycp3−/− females. Together, these results suggest that the absence of Sycp3 activates a DNA damage checkpoint in oocytes.

The delayed removal of MLH1 foci in very late diplotene oocytes also provides an explanation to a previously contradictory observation in Sycp3−/− oocytes (Yuan et al., 2002). It was shown that although the number of MLH1 foci at the pachytene stage in wild-type and Sycp3−/− oocytes were approximately the same (Fig. 5 Q), the number of chiasmata at the MI stage was reduced in Sycp3−/− oocytes compared with wild-type oocytes. Loss of MLH1 from meiotic chromosomes in wild-type meiotic cells normally precedes the removal of the SYCP1 protein, suggesting that the crossing-over process is completed in the context of an intact SC (Anderson et al., 1999; Moens et al., 2002). We found that some of the persistent MLH1 foci in very late diplotene Sycp3−/− oocytes do not colocalize with residual SYCP1 staining. We propose that the uncoupling of the recombination process from synopsis in Sycp3−/− oocytes affects the efficiency of the remaining MLH1 recombination complexes and that a subset of these fails to complete the crossing-over process.

A failure to establish chiasmata between homologous chromosomes could also be caused by an impaired positive genetic-interference mechanism (Jones, 1984; Novak et al., 2001). This mechanism ensures that crossovers are correctly distributed between chromosomes. A partially inactivated interference mechanism could lead to an unregulated distribution of a fixed number of chiasmata and result in a loss of obligatory chiasmata, thereby generating achiasmatic chromosomes. It has been proposed that the SC ensures a high level of interference (Zickler, 1999; Nabeshima et al., 2004, MacQueen et al., 2005; Carlton et al., 2006). We have studied if SYCP3 is required for interference by monitoring the number of MLH1 foci, which is a cytological marker for chiasmata distribution along SYCP1-labeled meiotic chromosomes (Baker et al., 1996; Edelmann et al., 1996; Hunter and Borts, 1997; Anderson et al., 1999). Sycp3 deficiency increases the length of the meiotic chromosome axes by twofold and introduces irregular gaps in SYCP1 staining along the axes (the meiotic axis in the SYCP1-negative gaps cannot be traced with certainty, as antisera against cohesin regions; Yuan et al., 2002; Kouznetsova et al., 2005). Therefore, it is impossible, using only cytological markers, to determine if individual meiotic chromosomes in Sycp3−/− oocytes lack associated MLH1 foci. Instead, we selected Sycp3−/− pachytene oocytes that displayed relatively intact SYCP1-labeled meiotic chromosomes and monitored the frequency of such structures.

### Table II. Sycp3−/− oocytes that contain univalent chromosomes are preferentially eliminated during early postnatal development

| Chromosome | 2 dpp WT | 2 dpp Sycp3−/− | 8 dpp WT | 8 dpp Sycp3−/− |
|------------|---------|----------------|---------|---------------|
|            | Univalents/Total | % | Univalents/Total | % | Univalents/Total | % | Univalents/Total | % |
| ChrX       | 22/1,554  1.42 | 238/1,672 14.23 | 1/529 0.19 | 55/591 9.31 |
| Chr1       | 13/1,025 1.27 | 11/350 3.14  | 2/888 0.23 | 2/304 0.66 |
| Chr2       | 7/642 1.09  | 10/312 3.21  | 1/607 0.20 | 2/348 0.60 |
| Chr12      | 22/1,865 1.18 | 113/1,350 8.37 | 3/1,240 0.24 | 15/808 1.86 |
| Chr17      | 18/1,540 1.17 | 126/1,443 8.66 | 7/1,625 0.43 | 23/777 2.96 |
| Chr19      | 23/1,939 1.19 | 191/1,414 13.51 | 5/1,339 0.37 | 48/857 5.60 |
| Chr12 + 19 | 1/1,093 0.09 | 17/444 3.83 | 0/569 0.00 | 2/651 0.31 |
| Chr17 + 19 | 0/930 0.00  | 17/517 3.29 | 0/723 0.00 | 2/666 0.30 |
| Chr12 + 17 | 0/496 0.00  | 9/439 2.05 | 0/569 0.00 | 1/651 0.15 |
| Chr12 + 17 + 19 | 0/496 0.00 | 5/439 1.14 | 0/569 0.00 | 0/651 0.00 |
| ChrX + 19  | 0/338 0.00  | 11/251 4.38 | 0/187 0.00 | 2/142 1.42 |
| ChrX + 17  | 2/338 0.59  | 11/251 4.38 | 0/187 0.00 | 2/184 1.09 |
| ChrX + 17 + 19 | 0/338 0.00 | 4/251 1.59 | 0/187 0.00 | 0/142 0.00 |

Six individual chromosomes, and combinations of either two or three of these chromosomes, were analyzed by immunofluorescence FISH, and single or double FISH signals were scored. The number of oocytes that contained univalent chromosomes (separated FISH signals) versus the total numbers of oocytes was determined and the estimated aneuploidy rate observed for chromosomes 1–18 and, therefore, was added separately. A summary of the estimated univalency for all 20 chromosomes that was calculated as described above produced an outcome of 135%. However, as shown in Table II, many Sycp3−/− oocytes contain multiple univalent chromosomes. We estimated this redundancy factor for all 20 chromosomes to affect 44% of the oocytes. Whereas 45% of the oocytes analyzed for chromosomes 12, 17, 19, and X displayed univalency for one of the measured chromosomes, 20% of the oocytes analyzed using chromosomes 12, 17, 19, and X contained multiple univalent chromosomes. By dividing the 20% by 45%, we got a percentage of 44. Therefore, the percentage of Sycp3−/− oocytes affected by univalency should amount to ~75% (1.35 × [1 – 0.44]). A similar calculation used to estimate the percentage of univalent cells at 8 dpp in Sycp3−/− oocytes suggests that 36% of them contain achiasmatic chromosomes. The latter figure is in close agreement with the estimated aneuploidy rate observed for Sycp3−/− females (Yuan et al., 2002).
which had two MLH foci associated to them. Analysis of 24 Sycp3−/− oocytes and 31 wild-type oocytes produced a very similar result, where both groups showed an average of ~3.5 intact SYCP1-labeled structures each having two MLH1 foci per cell. This result, therefore, does not support a model where the loss of SYCP3 negatively influences the impact of positive genetic interference in Sycp3−/− oocytes. It is important to note that in cases in Sycp3−/− oocytes where the TF structure as labeled by SYCP1 is severely fragmented, making it impossible to trace the meiotic chromosome axis, we cannot analyze if the MLH1 distribution pattern is affected. However, we only rarely identify entirely asynapsed homologous chromosomes (FISH studies suggest that ~1–2% of the pachytene Sycp3−/− oocytes contain asynapsed configurations of chromosome 19; unpublished data), excluding this as an important mechanism to explain the univalency statistics observed at 2 dpp in mutant oocytes.

Absence of SYCP3 generates oocytes with different levels of DNA damage, a subset of which evades two meiotic checkpoints

Our experiments show that loss of SYCP3 affects the efficiency of the DNA repair/recombination process. However, in contrast to the situation in mouse models, where components of the repair machinery have been inactivated (Di Giacomo et al., 2005), the repair/recombination process in Sycp3−/− oocytes is impaired, not blocked. We provide two sets of evidence for this; we found that ~34% of the oocyte pool remains at 8 dpp and of those that remain only approximately one-third contain univalent chromosomes. We also found a large diversity in the γH2AX staining pattern and the number of foci corresponding to RAD51/DMC1 and MLH1 in individual Sycp3−/− oocytes, strongly suggesting that loss of SYCP3 generates a temporal spectrum of recombination intermediates.

A fascinating aspect of the Sycp3−/− mouse model is the effectiveness with which it contributes to the formation of aneuploid offspring (Yuan et al., 2002). We have shown that Sycp3−/− oocytes that contain univalent chromosomes can bypass the DNA damage checkpoint at early postnatal development. A similar situation has been noted in mice that are deficient for the spindle checkpoint at the first meiotic cell division and give rise to aneuploid offspring (Yuan et al., 2002). Our results for Sycp3−/− oocytes are in agreement with studies of human oocytes that suggest that a reduced level of recombination is linked to an increase in aneuploidy (Hassold and Hunt, 2001). Interestingly, it has been observed that γH2AX signals are more slowly removed during meiosis in human oocytes compared with sperm, suggesting that progression of DSB repair is slower in oocytes (Roig et al., 2004).

We have shown that loss of Sycp3−/− oocytes does not occur until the diplotene stage. In contrast, Sycp3−/− spermatoocytes are already eliminated at the zygote/pachytene stage of meiosis (Yuan et al., 2000). A similar temporal difference in the loss of damaged male and female germ cells has been noted for a large number of gene deficiencies (Hunt and Hassold, 2002).

We propose that the relative incidence of aneuploidy observed for male and female gametes can be partly explained by a temporal difference in the activation of the DNA damage checkpoint during meiosis. In cases where a mutation generates a temporal spectrum of recombination deficiencies, the timing of the activation of the DNA damage checkpoint becomes crucial. The late activation of the female DNA damage checkpoint during meiosis, relative to the temporal activation of the same checkpoint in male germ cells, provides additional time for the formation of advanced recombination intermediates that can no longer be detected by this checkpoint in oocytes. This increases the risk that such recombination intermediates will contribute to the formation of univalent chromosomes.

Materials and methods

Generation of Sycp3−/− mice

Derivation of the Sycp3 knockout mouse has been previously described (Yuan et al., 2000). In brief, C57BL/6NCrRb wild-type males were mated with Sycp3−/− females to generate Sycp3−/− offspring. Nonsibling Sycp3−/− males and females were then mated to produce Sycp3−/− (wild-type) and Sycp3−/− mice. To detect the pregnancy, two females were caged with one male after 16:00 (4:00 pm). The vaginal plugs were examined daily between 8:00 and 9:00 (am). The day that the plug was found was defined as E0.5. For ovary collection at embryonic stages, pregnant mice were killed at E16.5, E17.5, and E18.5. For ovary collection at postnatal stages, the pups were killed after birth at days 1, 2, 4, and 8, which were referred to as 1, 2, 4, and 8 dpp. Ovaries from adult mice were also collected at 8 wk.

Histomorphometry

Ovaries were fixed in 4% paraformaldehyde for 4 h before paraffin embedding. The entire ovary embedded in the paraffin was sequentially sectioned at 5 μm. Every tenth section was stained either by hematoxylin and eosin or immunostained for GCNA, which is a germ cell marker (Enders and May, 1994), or c-kit, which is an oocyte marker (Manova et al., 1990). These sections were then used for estimation of oocyte numbers. In embryonic and newborn ovaries, oocytes can be clearly distinguished from somatic cells by GCNA staining. Immunohistochemistry was performed with a rat anti–GCNA-1 (a gift from G.C. Enders, University of Kansas Medical Center, Kansas City, KS) and a polyclonal rabbit anti–c-kit (PC34; Oncogene Research Products), using the Vectastain Elite ABC kit (SK 4100; Vector Laboratories), according to the manufacturer’s instructions. The peroxidase substrate DAB (DakoCytomation) was used to visualize the immunostaining reaction and hematoxylin was used for counterstaining. For the postnatal mouse ovaries, primordial and primary follicles were defined by their morphology and by c-kit immunostaining. Oocyte counts were first determined individually for germ cell cysts (germ cells that were not individually separated by stromal cells), primordial follicles (small oocytes surrounded by a few flattened pregranulosa cells), primary follicles (oocytes with a visible nucleolus surrounded by a single layer of cuboidal granulosa cells, ranging from five to nine cells), and secondary follicles (an oocyte with a visible nucleolus surrounded by two layers of cuboidal granulosa cells made up of more than eight granulosa cells). Only follicles with a visible nucleus were counted to avoid double counting. The total oocyte numbers for each ovary were summarized from different follicle stages by using five sections/ovary (6 sections/ovary in 8-dpp mouse and 15 sections/ovary in 8 wk-old mice). Three to seven ovaries per genotype (null and wild-type mice were from the same litter) were included in each group.

TUNEL assay

Apoptotic cells in paraffin-embedded sections of ovaries were identified using a TUNEL staining kit (Serologicals Corp.), following the manufacturer’s instructions. The sections were counterstained with methyl green. Every tenth section from the same ovary used for oocyte counting was also used for TUNEL staining. The relative number of apoptotic cell was summarized...
from five sections/ovary for each study group, with the exception of six sections taken from ovaries derived from 8-dpp mice.

**Statistics**

Statistical calculations of oocyte numbers were performed by one-way analysis of variance, using the SigmaStat program (SPSS, Inc.). *P* ≤ 0.05 indicates a significant difference.

**Immunofluorescence microscopy**

Wild-Type and Sycp3−/− oocytes were obtained using a “dry-down” technique (Peters et al., 1997) from ovaries at E16.5 [early and late zygote oocyte], E18.5, and E19.5 [pachytene and diplotene oocytes]. RAD51/DMC1, RPA, and MSH4 loci counting was performed at five different meiotic stages in wild-type and Sycp3−/− oocytes. Staging of oocytes was performed using several markers, including SYCP1, STAG3, and CREST, as well as DAPI (Fig. S2; Kouznetsova et al., 2005). In early zygote, SYCP1 expression has started and short SYCP1 fibers are visible, but centromeres are not yet paired (around 40 CREST foci). In late diplotene, mouse anti-RAD51 at 1:100 and rabbit anti-RPA at 1:500 (gifts from P. Moens, York University, Toronto, Canada), mouse anti-human MSH4 (a gift from J. Hunter, Washington State University, Pullman, WA) at 1:100, and mouse anti-human MLH1 (BD Biosciences). All primary antibody incubations were performed overnight at 4°C or 37°C. Secondary antibodies were swine anti-rabbit conjugated to FITC (DakoCytomation) at 1:100, donkey anti-guinea pig conjugated to Cy3 (Jackson ImmunoResearch Laboratories) at 1:1,000, goat anti-human conjugated to Cy5 (GE Healthcare), goat anti-mouse Alexa Fluor 488 (Invitrogen) at 1:1,000, and goat anti-rabbit Alexa Fluor 350 (Invitrogen) at 1:100. All secondary antibodies were incubated for 1 h at room temperature. DNA was stained with DAPI. Slides were mounted with antifade medium before being analyzed. Slides were viewed at room temperature using fluorescence microscopes (DMRA2 and DMRXA; Leica) and 100× objectives (Leica) with an aperture of 1.4 producing epifluorescence. Images were captured with a digital charge-coupled device camera (model C4742-95; Hamamatsu) and the Openlab version 9 (Adobe).

**Identification of univalent chromosomes by immunofluorescence FISH**

Oocytes were obtained from 2- and 8-dpp female mice ovaries. To increase the number of CREST foci, we performed overnight at 4°C or 37°C. Oocytes were obtained from 2- and 8-dpp female mice ovaries. To increase the number of CREST foci, we performed overnight at 4°C or 37°C. Oocytes were also distinguished from somatic cells on the basis of their size, 0.15% Triton X-100. Oocytes were detected by GCNA staining. The oo- tides were isolated by pipetting and fixed by using 1% paraformaldehyde and 0.15% Triton X-100. Oocytes were detected by GCNA staining. The oocytes were also distinguished from somatic cells on the basis of their size, the dispersed nature of their chromatin, and a characteristic congregation of oocytes at several distinct locations within the nucleus [Hodges et al., 2001]. After immunostaining, the slides were washed and air dried, and then denatured in 70% formamide and 2× SSC at 70°C for 2–4 min. Hybridization with specific chromosome probes was performed for 40 h at 37°C. The Cy3-labeled chromosomal probes (Chrombios GmbH) were used to identify chromosomes 1, 2, 12, 17, 19, and X in the oocyte by using FISH. Double- and triple-color FISH probes were labeled with Chr19: Cy3, Chr17: Cy5, and Chr12 (for Chr12DEAC). The washing step followed the manufacturer’s protocols [Chrombios GmbH]. DAPI was used as a DNA counterstain, and slides were mounted with antifade before analysis.

**Online supplemental material**

Fig. S1 shows that an increased number of oocytes are TUNEL positive in the Sycp3−/− ovaries. Fig. S2 shows the classification of zygote and diplotene stage meiotic cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200512077/DC1.

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