Mouse SyCop1 functions in synaptonemal complex assembly, meiotic recombination, and XY body formation

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In meiotic prophase, synaptonemal complexes (SCs) closely appose homologous chromosomes (homologs) along their length. SCs are assembled from two axial elements (AEs), one along each homolog, which are connected by numerous transverse filaments (TFs). We disrupted the mouse gene encoding TF protein SyCop1 to analyze the role of TFs in meiotic chromosome behavior and recombination. SyCop1−/− mice are infertile, but otherwise healthy. SyCop1−/− spermatocytes form normal AEs, which align homologously, but do not synapse. Most SyCop1−/− spermatocytes arrest in pachynema, whereas a small proportion reaches diplonema, or, exceptionally, metaphase I. In leptotene SyCop1−/− spermatocytes, γH2AX (indicative of DNA damage, including double-strand breaks) appears normal. In pachynema, SyCop1−/− spermatocytes display a number of discrete γH2AX domains along each chromosome, whereas γH2AX disappears from autosomes in wild-type spermatocytes. RAD51/DMC1, RPA, and MSH4 loci (which mark early and intermediate steps in pairing/recombination) appear in similar numbers as in wild type, but do not all disappear, and MLH1 and MLH3 loci (which mark late steps in crossing over) are not formed. Crossovers were rare in metaphase I of SyCop1−/− mice. We propose that SYCP1 has a coordinating role, and ensures formation of crossovers. Unexpectedly, SyCop1−/− spermatocytes did not form XY bodies.

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In meiosis, two rounds of chromosome segregation follow one round of replication. The first segregation, meiosis I, is reductional, as homologous chromosomes (homologs) move to opposite poles, whereas meiosis II is equational, because sister chromatids disjoin. The disjunction of homologs is prepared during the prophase of meiosis I, when homologs pair and nonsister chromatids of homologs recombine (for review, see Zickler and Kleckner 1999). The resulting crossovers and cohesion between the sister chromatids connect the homologs and ensure their proper disjunction at meiosis I. In most analyzed eukaryotes, meiotic recombination is accompanied by the close apposition of homologs by a zipper-like proteinaceous structure, the synaptonemal complex (SC). After premeiotic S-phase, the two sister chromatids of each chromosome develop a common axial structure, the axial element (AE), which consists of a linear array of protein complexes involved in sister chromatid cohesion (cohesin complexes), associated with various additional proteins (for review, see Page and Hawley 2004). Numerous transverse filaments (TFs) then connect the AEs of two homologs (synapsis) to form an SC. Within the SC, AEs are called lateral elements (LEs).

Genes encoding TF proteins have been identified in mammals (Sycp1, budding yeast (ZIP1), Drosophila (c3G), and Caenorhabditis (Syp-1 and Syp-2]). SYCP1, Zip1, and C3G are long coiled-coil proteins with globular domains at both ends. Within SCs, they form parallel coiled-coil homodimers, which are embedded with their C termini in the LEs, whereas the N termini of TF protein molecules from opposite LEs overlap in the narrow region between the LEs of the two homologs. Caenorhabditis Syp-1 and Syp-2 are two short coiled-coil proteins, which possibly take the place of a single longer coiled-coil protein in other species (for review, see Page and Hawley 2004).

In the three species in which it has been analyzed, Drosophila, Caenorhabditis, and yeast, TF-deficient mu-
tants still initiate meiotic recombination by induction of DNA double-strand breaks (DSBs) (Storlazzi et al. 1996, Colaiácovo et al. 2003, Jang et al. 2003), and align homologous chromosomes. However, they are deficient in crossover formation (for reviews, see Zickler and Kleckner 1999; Page and Hawley 2004). In Caenorhabditis and Drosophila, meiotic crossing over is abolished (Page and Hawley 2001, MacQueen et al. 2002, Colaiácovo et al. 2003), whereas in yeast zip1 null mutants, meiotic crossing over is reduced (Sym et al. 1993, Börner et al. 2004). Interestingly, Zip1 contributes to crossover formation even if no SC is assembled (Storlazzi et al. 1996), so not all functions of TF proteins in crossover formation require an intact SC structure. Storlazzi et al. (1996) proposed that Zip1 has a role in crossover designation before an SC is formed.

Börner et al. (2004) analyzed the crossover defect of yeast zip1 null mutants in detail. In wild-type yeast meiosis, DSB ends are resected so that 3' single-stranded tails arise (Sun et al. 1991). zip1 mutants show wild-type levels and kinetics of DSB formation and resection, but coordinate defects in later steps, namely, the formation of single end invasions (SEIs), double Holliday junctions [dHJs], and crossovers, which indicates that the progression from resected DSBs to SEIs is affected in zip1 mutants. Because in wild-type yeast SEIs appear in late zygonema (Hunter and Kleckner 2001), Zip1 fulfills its role in this step in crossover formation before an intact SC structure has been formed.

The relation between SC formation and recombination implies more than a requirement of TF proteins for crossing over. Synapsis and recombination are interdependent in most species, and the interdependency differs between species. In yeast and mouse, but not Drosophila, disruption of genes involved in meiotic DSB formation, resection, or strand invasion also affect synapsis, and most recombinational interactions in early meiosis probably serve to establish or stabilize homolog alignment and/or initiation of synapsis (for review, see Hunter 2003). Because of this interdependence it is important to analyze the localization of complexes of recombination-related proteins relative to the SCs/AEs in TF-deficient mutants. Such complexes can be recognized immunocytochemically (Anderson et al. 1997, Moens et al. 2002). Upon immunofluorescent labeling they are visible as foci by light microscopy. The composition of foci changes as meiotic prophase proceeds, which most likely directly or indirectly reflects successive steps in homolog alignment and recombination (for review, see Ashley and Plug 1998).

The mouse provides excellent opportunities for studying the role of TFs in chromosome pairing and recombination by an immunocytological approach, because the cytology of mouse meiosis is very well developed, and successive stages of meiosis can be determined precisely (for review, see Ashley 2004). Furthermore, several SC components, including TF protein SYCP1 (Meeuwen et al. 1992, Sage et al. 1995) and many recombination-related proteins have been identified in the mouse, and the localization of these proteins in mouse meiosis has been studied in great detail (Heyting and Dietrich 1991, Ashley and Plug 1998, Moens et al. 2002). We disrupted the Sycep1 gene and analyzed the effect of the disruption on male meiosis, following an immunocytochemical approach. We focused on the state of the AEs and the formation of foci containing recombination-related proteins in order to find out whether and how homolog alignment, meiotic recombination, and SC assembly are affected in Sycep1−/− mutants.

Results

Targeted inactivation of Sycep1

We disrupted the mouse Sycep1 gene, using a targeting vector in which exon 2 to exon 8 of the gene had been replaced by a neomycin selection marker. The replaced sequence includes the splice donor sequence of intron 1, the ATG start codon in exon 2, and ~20% of the Sycep1 ORF [Fig. 1A]. The targeting vector was linearized and electroporated into embryonic stem (ES) cells. We tested ~600 neomycin- and gancyclovir-resistant ES cell clones by PCR for correct targeting. About 2% of the clones

![Figure 1](image-url)
tested contained the disrupted SyCP1 allele. Correct targeting was confirmed by Southern blot analysis [Fig. 1B]. We injected targeted ES clones into C57BL/6 blastocysts and obtained germline transmitting chimeric animals. Intercrosses of SyCP1−/− animals yielded SyCP1+/−, SyCP1+/+, and SyCP1−/− offspring in the expected Mendelian ratio. The SyCP1−/− mice are viable and don’t display obvious developmental defects. Antibodies against peptides covering the N-terminal, middle, or C-terminal part of SCP1 [the rat protein homologous to SyCP1], or against nearly full-length SCP1, did not bind to any proteins in testis cell extracts from SyCP1−/− mice [Fig. 1C], indicating that these mice do not express truncated SYCP1. Most likely the SYCP1 disruption equals a null mutation.

**SyCP1−/− mice are infertile**

Whereas SyCP1−/− mice were fully fertile, repeated breeding attempts of SyCP1−/− males and females with wild-type animals did not yield any offspring. If the same wild-type animals were mated with heterozygous (SyCP1−/+ ) males or females, pregnancy was readily achieved. SyCP1−/− testes and ovaries were much smaller than those of SyCP1−/+ or wild-type mice [shown for testis in Fig. 2K], and SyCP1−/− testes weighed on average 70% less than wild-type testes. Spermatozoa were lacking in epididymides of SyCP1−/− knockout males [data not shown]. SYCP1 is thus required for correct development of the reproductive organs and for male and female fertility.

Histological analysis of the gonads revealed various abnormalities. As is explained in detail in the Supplemental Material, the mouse testis is organized in seminiferous tubules, in which cells differentiate coordinate and abnormally. The tubules from SyCP1−/− mice were much smaller than those from wild type [Fig. 2A–F]. They contained spermatogonia and spermatocytes, which appeared normal with respect to the presence of AE/LE proteins SYCP2 and SYCP3 [see below, Supplemental Material], but the morphology of their nuclei was often abnormal. Furthermore, spermatocyte stages beyond diplonema were rare in SyCP1−/− testes, and post-meiotic spermatogenic cells [spermatids and spermatozoa] were completely lacking. Apparently, spermatogenic differentiation is interrupted predominantly at the pachytene stage of SyCP1−/− spermatocytes, which most likely causes the sterility of SyCP1−/− males. However, as has been found in other mouse meiotic mutants, the organization of the seminiferous tubules was not disrupted and the residual spermatocytes in SyCP1−/− mice formed associations with similar cell types [except spermatids] as the corresponding spermatocytes in wild type (Supplemental Material).

SyCP1−/− ovaries weighed on average 35% less than SyCP1−/+ or SyCP1+/+ ovaries. Growing follicles and oocytes were lacking in sections of SyCP1−/− ovaries, which suggests a disruption of oocyte development during meiosis, followed by apoptosis.

**SYCP1-deficiency leads to increased apoptosis during pachynema**

One possible explanation for the lack of spermatids in SyCP1−/− testes is that spermatogenic cells enter apopto-
sis during meiotic prophase [for review, see de Rooij and de Boer 2003]. We tested this using TUNEL analysis of testis sections from ∼8-wk-old Sycep1+/− and Sycep1−/− mice. In Sycep1−/− testes, we found on average 0.7 apo-
ptotic nuclei per cross-sectioned tubule, which is slightly more than previously found in wild type (Baarends et al. 2003). In Sycp1<sup>−/−</sup> testes, the TUNEL-positive cells were most often in pachynema or metaphase/anaphase I [Fig. 2G–J]. In testis sections of Sycp1<sup>−/−</sup> but not of Sycp1<sup>+/+</sup> animals, certain tubules contained many [10 or more] apoptotic nuclei. Accordingly, the percentage of tubule sections with five or more apoptotic nuclei had almost doubled in Sycp1<sup>−/−</sup> compared with Sycp1<sup>+/−</sup> testes [20% vs. 11%]. Because the percentage of tubules without apoptotic nuclei had not changed, we think that apoptosis occurs at similar developmental steps in Sycp1<sup>−/−</sup> animals as in wild type, but with a highly increased incidence, resulting in Sycp1<sup>−/−</sup> tubules containing a whole layer of apoptotic nuclei. Thus, in Sycp1<sup>−/−</sup> males, spermatogonia enter meiotic prophase, but most spermatocytes die of apoptosis at pachynema, and exceptionally some get to metaphase I.

**AEs are formed in the absence of SYCP1 and align homologously**

Sycp1<sup>−/−</sup> spermatocytes assemble morphologically normal AEs [Fig. 3], which align homologously [Fig. 3, Supplementary Fig. S1], but are not connected by TEs, and do not show a central element between them, i.e., they do not synapse [Fig. 3B]. In Sycp1<sup>−/−</sup> spermatocytes, AEs are only connected by a limited number of axial associations [AAs] [Fig. 3A,B], and are farther apart [211 ± 17 nm at AAs] than the LEs in pachytene spermatocytes of wild type [in agar filtrates: 79 ± 3 nm]. This resembles the yeast zip1 phenotype [Sym et al. 1993], including the size of the AAs, and is consistent with the idea that SYCP1 is a TF component. Beyond the most centromere proximal and distal AAs, the AEs tend to be somewhat wider apart, so the AAs are the only or at least the shortest connections between the AEs. All analyzed components of wild-type mouse AEs/LEs were also present in Sycp1<sup>−/−</sup> AEs [Fig. 3; for SYCP2, see Fig. 4].

In the Supplemental Material we present evidence that the order of Sycp1<sup>−/−</sup> and wild-type spermatocyte stages, as defined by AE morphology and extent of alignment/synapsis, is the same [Supplementary Figs. S2, S3], and that corresponding Sycp1<sup>−/−</sup> and wild-type stages have similar life spans, at least until the spermatocytes enter apoptosis. We use, therefore, AE morphology and alignment/synapsis as criteria for staging and comparing Sycp1<sup>−/−</sup> and wild-type spermatocytes. The assembly and alignment of AEs, as detected by REC8/SYCP3 double labeling, proceeds similarly in Sycp1<sup>−/−</sup> and wild type [Fig. 3K–T]. The pseudo-autosomal parts of the X and Y chromosome, however, were not aligned in 28% of the Sycp1<sup>−/−</sup> pachytene cells, whereas they were synapsed in 100% of the wild-type pachytene cells [examples shown in Figs. 3, 4]. Although most Sycp1<sup>−/−</sup> spermatocytes are lost during pachynema [above], some reach diplonema, 0%–3% [depending on the mouse] of the spermatocytes in spreads of Sycp1<sup>−/−</sup> testis cell suspensions were in diplonema, compared with 15% of the spermatocytes in spreads of wild-type testis cell suspensions [late meiotic prophase stages are overrepresented in cell suspensions]. Diploptene Sycp1<sup>−/−</sup> AEs resemble wild-type LEs/AEs, including the thickened ends and the apparent repulsion of the LEs/AEs of homologous chromosomes [Fig. 3T; Supplementary Fig. S2].

**Meiotic recombination is initiated in Sycp1<sup>−/−</sup> spermatocytes, but repair is not completed**

γH2AX is a phosphorylated form of histone variant H2AX, which marks chromatin domains with DNA damage, including DSBs [Rogakou et al. 1999]. γH2AX appeared throughout Sycp1<sup>−/−</sup> preleptotene and leptotene nuclei [Fig. 4F], as in wild type [Fig. 4A; Mahadevaiah et al. 2001]. However, whereas γH2AX becomes largely restricted to asynapsed portions of wild-type zygotene chromosomes [Fig. 4B; Mahadevaiah et al. 2001], it occurs all along the AEs, including the aligned portions, of Sycp1<sup>−/−</sup> zygotene chromosomes [Fig. 4G]. The intensity of γH2AX labeling along the Sycp1<sup>−/−</sup> bivalents varied somewhat, but in most zygotene cells we could not distinguish separate γH2AX-positive domains [Figs. 4C, S5]. This pattern changed in Sycp1<sup>−/+</sup> pachynema: Some Sycp1<sup>−/−</sup> pachytene cells displayed a mixture of long stretches of γH2AX and narrow, intense γH2AX-positive domains, and other pachytene cells (presumably of a later stage) showed only narrow, γH2AX-positive domains along otherwise γH2AX-negative bivalents [Fig. 4H]. Diploptene Sycp1<sup>−/−</sup> spermatocytes displayed only the latter pattern [Fig. 4I]. Late pachytene/early diplotene Sycp1<sup>−/−</sup> spermatocytes contained 110 ± 4.6 distinct, narrow γH2AX-positive domains per cell. In earlier spermatocyte stages the γH2AX-positive domains were too indistinct and heterogeneous to be counted. In wild type, we found only distinct, narrow γH2AX-positive domains along synapsed stretches in late zygonema and early pachynema [Fig. 4B,C], and these domains were weakly labeled and disappeared during the course of pachynema [Fig. 4D; Mahadevaiah et al. 2001]. Taken together, the γH2AX pattern suggests that meiotic DSBs are formed in Sycp1<sup>−/−</sup> meiosis, but that at least some DSBs are not repaired, or their repair gets stuck at some intermediate step that is still marked by γH2AX. Another abnormality in the γH2AX pattern was found on the sex chromosomes: Wild-type pachytene and diplotene spermatocytes have γH2AX throughout the chromatin of the XY body [Fig. 4C–E, Mahadevaiah et al. 2001] [a condensed chromatin structure containing the sex chromosomes formed during male meiotic prophase in mammals]. In striking contrast, the sex chromosomes of Sycp1<sup>−/−</sup> pachytene and diplotene spermatocytes displayed similar narrow γH2AX-positive domains as the autosomes [Fig. 4H].

We also analyzed the putative H2AX phosphorylating kinase ATR [Turner et al. 2004]. In leptonema of wild-type mouse, ATR forms foci in association with AE segments [Fig. 4I], and in early zygonema, ATR foci occur along synapsed and asynapsed portions of LEs/AEs [Fig. 4K]. From mid-zygonema to early pachynema, ATR dis-
appears from synapsed portions of SCs and accumulates along the nonautosomal parts of the XY bivalent and late pairing (“laggard”) portions of autosomal LEs/AEs [Fig. 4L; Turner et al. 2004 and references therein]. In Sycep1−/− leptonema and early zygonema, the ATR pattern was indistinguishable from wild type [Fig. 4N]. However, whereas the ATR signals disappeared from the synapsed portions of AEs in wild type, they were present along the aligned AEs in Sycep1−/− spermatocytes, usually in AE-associated foci, or incidentally in distinct domains that were reminiscent of the γH2AX domains [Fig. 4P,Q]. The dense ATR coating of laggard asynapsed portions of AEs as is found in wild type [Fig. 4L] was not found in Sycep1−/− spermatocytes. Strikingly, ATR shows the same aberrant pattern on the X and Y chromosome in Sycep1−/− pachynema as γH2AX: It forms few, discrete foci, or occasionally domains, on the AEs of the X and Y chromosome rather than covering all non-pseudo-autosomal parts of the AEs of the sex chromosomes [Fig. 4P,Q]. In short, the ATR pattern in Sycep1−/− spermatocytes differs in various respects from that in wild type, but the similarity of the ATR and γH2AX patterns found in wild type [Turner et al. 2004] is also found in Sycep1−/− spermatocytes [Fig. 4].
(Figure 5 legend on facing page.)
**Symp1^-/- spermatocytes barely form crossovers**

To find out which step in meiotic recombination could be blocked in *Symp1^-/-*, we analyzed proteins involved in later steps of meiotic recombination. RAD51 and Dmc1 are RecA homologs required for heteroduplex formation in meiosis, probably by assembling on 3' tails of resected DSB ends and initiating the strand invasion step [for review, see Shinohara and Shinohara 2004]. In wild-type mouse, RAD51/DMC1 foci are formed along the AEs from leptonema on. In zygonema, they are located along synapsed and asynapsed portions of SCs, and in pachynema they gradually disappear [Fig. 5A,B, Ashley and Plug 1998]. In *Symp1^-/-* leptonema, RAD51/DMC1 foci appeared in similar numbers as in wild-type, but their number decreased more slowly [Fig. 5C,D, Supplementary Fig. S4]. Even late pachytene/early diplonete *Symp1^-/-* spermatocytes displayed appreciable numbers of RAD51/DMC1 foci [Fig. 5D,S]. Thirty percent to 50% of the RAD51/DMC1 foci were between the aligned AEs in *Symp1^-/-* late zygonema and late pachynema/early diploneta [Fig. 5D; Supplementary Fig. S4]. RAD51/DMC1 foci between aligned AEs occur also in wild-type mouse, maize, and *Sordaria* [for review, see Zickler and Kleckner 1999; Tessé et al. 2003]. Because homolog alignment requires DSBs [Tessé et al. 2003], the RAD51/DMC1 foci between aligned AEs might mark recombinational interactions between homologs. Part of the RAD51/DMC1 foci in *Symp1^-/-* pachynema cells colocalize with γH2AX domains [Fig. 5Q,R].

RFA binds to single-stranded DNA, and in vitro it enhances nucleoprotein formation by RAD51 if added to the reaction mixture after RAD51 [Paques and Haber 1999]. In wild-type spermatocytes, RFA foci appear and disappear on average later than RAD51/DMC1 foci [Fig. 5E,F, Supplementary Fig. S4; Moens et al. 2002]. In *Symp1^-/-* leptonete and late zygotene spermatocytes, the number of RFA foci and their time of appearance in relation to alignment/synapsis were similar as in wild-type zygonema [Fig. 5G,S; Supplementary Fig. S4]. However, *Symp1^-/-* diplonete spermatocytes still have appreciable numbers of RFA foci [Fig. 5H,S; Supplementary Fig. S4]. About 80% of the RFA foci were located between two aligned AEs of *Symp1^-/-* spermatocytes [Fig. 5G; Supplementary Fig. S4]. RFA foci between aligned AEs occur also in wild-type zygonema [Fig. 5G] and between homologously aligned but not synapsed LE/AE segments between translocation breakpoints in *Symp1^-/-* pachynema [Plug et al. 1997].

Msh4 is a MutS homolog, which forms a heterodimeric complex with another MutS homolog, Msh5. The Msh4/Msh5 heterodimer probably recognizes and stabilizes meiotic recombination intermediates [Ross-Macdonald and Roeder 1994, Snowden et al. 2004]. Yeast Msh4 localizes to sites of synopsis inactivation. msh4 mutants show partial and delayed synopsis and 30%-50% of the wild-type level of crossing over, and msh4 mutations affect the same subset of crossovers as zip1 mutations [Novak et al. 2001]. In mouse, MSH4 foci colocalize extensively with RFA foci, but appear and disappear slightly later [Moens et al. 2002]. Otherwise than in yeast, the number of MSH4 foci in mouse far exceeds the number of chiasmata that will be formed [Fig. 5I,S, Supplementary Fig. S4]. Neyton et al. [2004] proposed that in mouse meiosis, MSH4 cooperates first in zygonema with RAD51/DMC1 in homolog alignment, synopsis inactivation, and/or in resolution of early DNA–DNA interactions, and subsequently, in pachynema, with MLH1 and MLH3 in crossover formation. In Caenorhabditis, MSH-4 and MSH-5 appear to fulfill only this second role: msh-4 or msh-5 mutants align homologs and assemble SC, but RAD-51 foci persist and crossovers are not formed, which suggests a role for MSH-4/5 downstream RAD-51 in crossover formation in Caenorhabditis [Colaiácovo et al. 2003]. In mouse, *Symp1^-/-* leptotene and zygotene spermatocytes display similar numbers of MSH4 foci as wild-type spermatocytes [Fig. 5K,M,S; Supplementary Fig. S4]. Most *Symp1^-/-* MSH4 foci are between aligned AEs, indicating that they mark DNA interactions between homologs. However, in *Symp1^-/-* late pachynema/early diploneta, the number of MSH4 foci is still 70% of that in late zygonema [Fig. 5L,S, Supplementary Fig. S4]. This might suggest that the DNA–DNA interactions to which MSH4 binds are formed normally in *Symp1^-/-*, but that most of these cannot be processed.

MLH1 is essential for crossover formation, both in mammals and yeast [Baker et al. 1996; Hunter and Borts 1997]. In mouse, MLH1 foci appear in mid-pachynema, and their position and number closely correlate with those of chiasmata [Froenicke et al. 2002]. MLH3 foci largely colocalize with MLH1 foci in the mouse [Svetlanov and Cohen 2004], and MLH3 most probably cooperates with MLH1 in crossover formation [Wang et al. 1999, Lipkin et al. 2002]. *Symp1^-/-* spermatocytes do not form MLH1 and MLH3 foci [Fig. 6], which indicates that SYCP1 is required for crossover formation. Accordingly, we observed only univalents in the two natural meta-
phases I that we found among spread spermatocytes of Sycp1−/− mice [Fig. 6E]. If we forced pachytene or diplotene Sycp1−/− spermatocytes to condense their chromosomes, using okadaic acid [OA], most chromosomes formed univalents [Fig. 6F]. Chromosomal fragments were rare in natural metaphase I or OA-induced metaphase I-like configurations of Sycp1−/− [Fig. 6E,F].

Taken together, the immunofluorescence labeling of foci suggests that Sycp1−/− spermatocytes can initiate meiotic recombination at wild-type level and establish stable homologous alignment of autosomes. However, many repair/recombination intermediates are not repaired and crossovers are not formed.

Sycp1−/− spermatocytes do not form XY bodies

In 28% of the Sycp1−/− pachytene spermatocytes, the X and Y chromosome were associated, but this did not ensure formation of an XY body. In Sycp1−/− pachynema, γH2AX and ATR occurred in a similar discrete pattern along the AEs of the XY bivalent as along autosomal AEs [Fig. 4I,P,R] (rather than covering the nonautosomal parts of the sex chromosomes), the characteristic DAPI-intense domain of the XY body was not formed, and the AEs of the X and Y chromosomes were not curled or bent, as is usually seen in wild-type XY bodies [Fig. 4, cf. D,E and H,I,Q]. We will analyze the XY bivalent in Sycp1−/− spermatocytes in more detail in a separate study.

Discussion

In this study we disrupted the mouse SYCP1 gene and analyzed the effect on meiotic recombination and chromosome behavior by an immunocytological approach. We will assume that immunofluorescence signals represent functional protein complexes and that orthologous proteins fulfill similar roles in mouse and yeast meiosis, unless there are indications that this is not so. In addition, we will have to make assumptions when and how the proteins act that we detect by immunofluorescence, to link the cytological observations in Sycp1−/− mice to studies at the DNA and cytological level in other organisms.

Early meiosis in Sycp1−/− spermatocytes

We used AE morphology as detected by SYCP2 or SYCP3 labeling and alignment/synapsis as a basis for staging [Fig. 3; Supplementary Fig. S3]. Sycp1−/− and wild-type spermatocyte stages that correspond by these criteria also show similar patterns of cohesins [shown for REC8 in Fig. 3K–T], but display largely different patterns of recombination-related proteins other than cohesins. Leptonema is the only analyzed stage in which Sycp1−/− and wild-type spermatocytes show similar immunofluorescence patterns of all analyzed proteins: The γH2AX and ATR patterns are similar, and RAD51/DMC1, RPA, and MSH4 foci occur in similar numbers in wild-type and Sycp1−/− leptotene, indicating that DSBs are induced and that some post-DSB step(s), presumably at least resection of DSB ends [Xu et al. 1997], take place at wild-type levels in Sycp1−/− leptotene. However, differences (other than synapsis) between Sycp1−/− and wild type become apparent between leptotene and zygonema, in particular with respect to γH2AX and ATR [Fig. 4K]. Furthermore, the number of RAD51/DMC1 foci has decreased between leptotene and zygonema, but less so in Sycp1−/− than in wild type [Fig. 5S; Supplementary Fig. S4]. The numbers of RPA and MSH4 foci on the other hand are similar in late zygonema in mutant and wild type [Fig. 5S; Supplementary Fig. S4]. In wild type, RPA and MSH4 foci most likely arise from RAD51/DMC1 foci, and then lose RAD51/DMC1 [Moens et al. 2002]. Although the RAD51/DMC1 foci occur in similar numbers per cell as MSH4 and RPA foci in Sycp1−/− late zygonema [Fig. 5S], they cannot completely overlap with these foci, because >80% of the MSH4 and RPA foci are between the aligned AEs, but only 46% of the RAD51/DMC1 foci [Supplementary Fig. S4]. Possibly MSH4 foci
are normal in Sycp1−/− late zygonema, but some repair pathway is affected that is marked by RAD51/DMC1 but not by MSH4 foci. However, as the turnover of MSH4 and RAD51/DMC1 foci is not known, other explanations are conceivable.

Important questions about the immunofluorescence signals in zygonema are, What does the γH2AX signal throughout the Sycp1−/− nuclei indicate? And what do the MSH4 foci represent? In yeast, the number of Msh4 foci per cell roughly equals the number of Zip1-dependent crossovers per cell [Novak et al. 2001], and it seems likely that most or all MSH4 foci in yeast mark sites of future crossovers. In mammals and Arabidopsis, the number of MSH4 foci exceeds the number of crossovers by far, so in these organisms most MSH4 foci will not become crossovers, but mark possibly recombinational interactions that serve homolog alignment [Higgins et al. 2004; Neyton et al. 2004], the MSH4 images of late zygotene cells [Fig. 5K,M, Supplementary Fig. S4] suggest that such interactions occur normally in Sycp1−/−. An ensuing question is whether there are any crossover-designated MSH4 foci at all among the MSH4 foci in mouse zygonema. In wild-type mouse this might be the case, because some MSH4 foci colocalize with MLH1 in pachynema [Santucci et al. 2000], but the question remains whether these colocalizing MSH4 foci were already present in zygonema. Based on work in yeast [Börner et al. 2004] we would expect so. If so, then they would comprise <10% of all late zygotene MSH4 foci in wild type. It would probably have escaped us if Sycp1−/− would lack this type of MSH4 foci (if any) in zygonema. So we do not know whether crossover-designated MSH4 foci are missing from Sycp1−/− zygotene spermatocytes (either because crossovers are not designated, or because crossover-designated intermediates fail to form MSH4 foci), or whether crossover-designated MSH4 foci are assembled in Sycp1−/− zygonema, but fail to become crossovers in a later stage.

Similar questions arise with respect to γH2AX. γH2AX is not restricted to AEs, but occurs throughout chromatin loops. Possibly, a single meiotic DSB causes H2AX phosphorylation on megabases of DNA (which corresponds to tens of loops), like DSBs in somatic cells [Rogakou et al. 1999]. Therefore γH2AX-positive domains might contain only one or a few DSBs (or other γH2AX-marked recombination intermediates) associated with the AEs [Fig. 5O–R], but do not necessarily also have DSBs in the loops. However, even if we assume this, it is not clear which lesions γH2AX might mark in Sycp1−/− zygonema. Late zygotene spermatocytes of wild type and Sycp1−/− have similar numbers of RPA and MSH4 foci [Fig. 5S, Supplementary Fig. S4], yet γH2AX is restricted to asynapsed AEs and some weak domains in synapsed SC segments in wild type [Fig. 4B], but covers all the chromatin in Sycp1−/− [Figs. 4C, 5M]. Possible explanations for this difference are (1) MSH4, RPA, and/or RAD51/DMC1 foci in wild type and Sycp1−/− look similar but contain different recombination intermediates; only those present in Sycp1−/− are marked by γH2AX. [2] The 70% “extra” RAD51/DMC1 foci in Sycp1−/− late zygonema [Fig. 5S] bring about the overall γH2AX labeling. We doubt whether this relatively small number of RAD51/DMC1 marked DSBs or recombination intermediates [60–70 per cell] could cause this. (3) Late zygotene Sycp1−/− spermatocytes contain, besides the RAD51/DMC1, RPA, and MSH4 marked DNA structures, other DNA lesions that are not marked by any of these proteins, but are marked by γH2AX, e.g., unresected DSBs. This seems unlikely: If yeast zip1 mutants are similar to Sycp1−/− in this respect, it would predict an elevated level of that type of DNA-lesions in zip1, there are no indications for this [Börner et al. 2004]. (4) The γH2AX labeling in late zygotene Sycp1−/− spermatocytes reflects some disorganization in the Sycp1−/− cell that is not related to the presence of DSBs. (5) Loss of DSBs or recombination intermediates (due to repair) is uncoupled from loss of γH2AX staining in Sycp1−/−. This may result in the persistence of γH2AX labeling at sites where there are no breaks (anymore). Although there are no conclusive arguments against the other possible explanations, we prefer the last one, because it accounts for the close correlation between asynapsis and the presence of γH2AX in wild type. A similar correlation exists between asynapsis and the presence of ATR [Turner et al. 2004] and RAD50 and MRE11 [Eijpe et al. 2000]. This correlation could either mean that synopsis can only occur in chromosomal regions where these proteins have been lost, or that these proteins are lost from chromatin loops upon synopsis (or some local SYCP1-dependent event preceding synopsis). The presence of γH2AX all over the chromatin in Sycp1−/− zygotene nuclei argues for the second interpretation: Perhaps synopsis/SYCPI causes first the loss of ATR, which is then followed by loss of γH2AX. In Sycp1−/− mid- to late pachynema, most γH2AX eventually disappears from the chromatin, except from a number of distinct domains [Fig. 4H]. Since most of these domains have RAD51/DMC1 [Fig. 5Q,R] or MSH4 [Fig. 5O,P] foci at their bases, they probably represent loops in which repair has not been completed.

Role of SYCP1 in later steps of meiotic recombination

In wild-type late pachynema/early diplonema, most or all RAD51/DMC1, RPA, and MSH4 foci have disappeared, whereas Sycp1−/− late pachytene/early diplotene cells still have 50–70% of the number of foci found in zygonema [Fig. 5S; Supplementary Fig. S4]. Similar observations have been made in TF mutants of Caenorhabditis [Alpi et al. 2003; Colaiacovo et al. 2003] and yeast [Novak et al. 2001]. Apparently meiotic recombination is blocked or impeded at a step where these proteins act, possibly single end invasion, because yeast Rad51 and Dmc1 are required for strand invasion [Hunter and Kleckner 2001]. Thirty percent to 50% of the RAD51/DMC1, RPA, and MSH4 foci disappear between zygonema (Fig. 5S; Supplementary Fig. S4). Similar observations have been made in TF mutants of Caenorhabditis [Alpi et al. 2003; Colaiacovo et al. 2003] and yeast [Novak et al. 2001]. Apparently meiotic recombination is blocked or impeded at a step where these proteins act, possibly single end invasion, because yeast Rad51 and Dmc1 are required for strand invasion [Hunter and Kleckner 2001]. Thirty percent to 50% of the RAD51/DMC1, RPA, and MSH4 foci disappear between zygonema (Fig. 5S; Supplementary Fig. S4). Similar observations have been made in TF mutants of Caenorhabditis [Alpi et al. 2003; Colaiacovo et al. 2003] and yeast [Novak et al. 2001].
mutants in other species that crossover formation is affected. More than 90% of the crossovers in the mouse depend on synapsis/SYCP1 [Fig. 6E,F]. However, the number of RAD51/DMC1, RPA, and MSH4 foci and γH2AX signals that are still present in Sycp1−/− diploids exceeds the number of crossovers in wild type about fivefold. We counted 117 ± 17 MSH4 foci per diplo- tote Sycp1−/− spermatocyte, whereas there are on aver- age 21–25 exchanges per cell in male mouse meiosis [Koehler et al. 2002]. SYCP1 is therefore not only required for crossover formation, but also for repair of DSBs that will not become crossovers, at least if persisting MSH4 foci in Sycp1−/− still mark DNA lesions. Upon exposure to OA, Sycp1−/− spermatocytes repair the recombination intermediates [if any] that underlie the RAD51/DMC1, RPA, and MSH4 foci, because chromatid breaks are rare in OA-induced metaphases I [Fig. 6F]. Possibly, OA opens up a DNA repair pathway that is not normally used in wild type, for instance, by releasing the sister chromatid as template for repair. However, exposure to OA reveals little or no crossing over in Sycp1−/−, whereas it reveals crossover formation in wild type. Therefore, SYCP1 must have a role in crossover formation besides its proposed role in the repair of breaks that will not become crossovers.

To summarize the role of SYCP1 in recombination: A substantial fraction of meiotic DSBs does not require SYCP1 for repair [Fig. 5B]; it is not known whether these breaks are a random sample or a specific subset of breaks. One hundred to 200 breaks per cell [as estimated from the number of foci and γH2AX signals in late pachy- nema] require SYCP1 and/or synapsis for repair. And the formation of >90% of the crossovers depends on SYCP1 and/or synapsis. This resembles the yeast zip1 phenotype. The role of SYCP1 in crossover formation is a con- served TF function in all species analyzed thus far. Possibly SYCP1/the SC serve as support for the assembly of MLH1 foci and/or enhance crossing over by providing a close apposition of homologs. Alternatively, or in addition, TF proteins/synapsis might ensure certain overall structural alterations in the bivalents that lead to cross- over formation [Börner et al. 2004].

Role of SYCP1 in XY body formation

This study revealed an unexpected role of SYCP1 in the formation of the XY body. Turner et al. [2004] presented recent evidence that coating of asynapsed portions of AEs with BRCA1 and ATR was correlated with H2AX phosphorylation and transcriptional inactivation. In the XY bivalent this would ultimately result in the forma- tion of an XY body. The aberrant distribution of ATR and γH2AX in Sycp1−/− pachytene spermatocytes provides therefore an obvious explanation for the failure to form XY bodies. However, the question remains why ATR is distributed aberrantly. Perhaps ATR relocates to asynap- sed portions of AEs after it has disappeared from synap- sed portions of AEs, this might explain the dense coating (rather than discrete foci) of ATR along the last asynap- sed portions of AEs, including those of the sex chromosomes. Possibly ATR does not relocate in Sycp1−/−, because it is sequestered at unrepair DNA breaks, and/or because SYCP1/synapsis is directly or indirectly required for relocation of ATR.

Comparison with other meiotic recombination-deficient mouse mutants

Besides Sycp1, other mouse genes homologous to yeast genes involved in the Zip1-dependent pathway of cross- over formation have been knocked out, namely, Msh4 [Kneitz et al. 2000], Msh5 [de Vries et al. 1999; Edelmann et al. 1999], and Dmc1 [Pittman et al. 1998; Yoshida et al. 1998]. Contrary to Sycp1−/− mice, these knockouts display partial and nonhomologous alignment/synapsis rather than full-length homologous alignment. Presump- tively, MSH4, MSH5, and DMC1 are indispensable for estab- lishment of stable recombinational interactions be- tween homologs in the mouse, whereas SYCP1 contrib- utes only to a minor extent to the stability of such interactions, at least in leptonema till pachynema. Msh4, Msh5, and perhaps Dmc1 knockouts enter apo- ptosis when the spermatogenic epithelium is in develop- mental stage IV and the spermatocytes should be in early/mid-pachynema [de Vries et al. 1999, de Rooij and de Boer 2003]. At least a small proportion of Sycp1−/− spermatocytes progresses further and reaches diplonema or exceptionally metaphase I. This could be related to the ability of Sycp1−/− spermatocytes to establish reason- ably stable homologous alignment. Among other mutant mice with a less defined but on average later arrest in meiosis than early/mid pachytene [stage IV], there are several that can align or synapse chromosomes homologously, including Mlh1, Mlh3, and Brca1 mutants [for review, see de Rooij and de Boer 2003].

Materials and methods

Construction of the targeting vector

To inactivate the Sycp1 gene, we designed a targeting construct to replace exons 2–8 by a neomycin gene, using pKO Scrambler V905 as a vector [Lexicon Genetics, Incorporated]. The neomycin phosphotransferase gene was isolated as an AscI fragment from pKO Select Neo [Lexicon Genetics] and inserted at the unique Ascl site of pKO V905. The thymidine kinase gene was derived from pKO Select TK [Lexicon Genetics] by RsaI digestion and subcloned at the unique RsaI site of pKO V905. Ge- nomic fragments were isolated after screening of a λ Fixll library derived from 129/Ola E14 cells [a gift of B. Vennström, Mouse Camp Transgene Facility, Karolinska Institute, Stockholm, Sweden]. A 2.4-kb Sall [vector derived]–SacII fragment was used as a left arm. This fragment was first cloned in pGEM-T Easy [Promega], excised as a Sall–NotI fragment, and cloned as a blunt fragment on the Hpal site of pKO V905. A 6-kb EcoRI fragment was used as the right arm and inserted in the EcoRI site of the targeting vector [Fig. 1]. The final pKO plasmid men- tioned above, containing all four elements, was linearized with Sall before electroporation.

Targeted inactivation of the Sycp1 gene

129/Ola-derived IB10 ES cells [a subclone from E14 ES cells] were cultured on lethally irradiated mouse embryonic fibro-
We prepared cell suspensions from testes of 72 °C for 35 cycles. 1 mM CaCl2, 0.1 mM dithiothreitol at pH 6.8) for 5 min (Goral, 1992). Slides were subsequently washed in terminal deoxynucleotidyl transferase [TdT] buffer [100 mM cacydolate buffer, 1 mM CaCl2, 0.1 mM dithiothreitol at pH 6.8] for 5 min (Gorczyca et al. 1993) and incubated for at least 30 min at 25 °C in TdT buffer containing 0.01 mM Biotin-16-DUTP [Roche Diagnostics] and 0.4 U/µL TdT enzyme [Promega]. The enzymatic reaction was stopped in TB buffer [300 mM NaCl, 30 mM Na-citrate at pH 7.0], and the sections were washed [Gavrieli et al. 1992]. Slides were then incubated with streptABCComplex/horseradish peroxidase conjugate [Dako] for 30 min and washed in PBS. DUTP-biotin labeled cells were visualized with 3,3′-diaminobenzidine tetrahydrochloride [DAB]/metal concentrate [Pierce]. Then we counterstained the sections with hematoxylin and counted the number of TUNEL-positive cells per cross-sectioned tubule. However, for Sycp1−/− mice this was not possible, because numerous TUNEL-positive nuclei were clustered in single cross-sectioned tubules. Therefore we counted the number of cross-sectioned tubules with no, one to five, or more than five apoptotic nuclei. Tubules without germ cell development up to meiotic prophase were excluded from the analysis. We performed this analysis on two Sycp1−/− and two Sycp1+/− mice, classifying a minimum of 150 tubule sections for each genotype.

**Cytology, immunocytochemistry, and chromosome painting**

The antibodies used in this study are listed in the Supplemental Material. Paraffin and frozen sections of mouse testes [Meuwissen et al. 1992], and dry-down [Peters et al. 1997] or squash [Page et al. 1998] preparations of testis cell suspensions were prepared, incubated for immunocytochemistry, and analyzed as described [Meuwissen et al. 1992; Eijpe et al. 2003]. In some experiments, we exposed the cells to 1.25 µM OA for 5 h [Wilshire et al. 1995] before spreading. For ultrastructural analysis we prepared uranyl-acetate-stained agar filtrates of lysed spermatocytes and analyzed them as described [Heyting and Dietrich 1991].

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**Western blot analysis**

We prepared cell suspensions from testes of Sycp1−/− and Sycp1+/− mice [Heyting and Dietrich 1991] and lysed the cells in Laemmli sample buffer. We loaded 5 × 10^5 lysed cells per 0.8-cm-wide slot of a 10% polyacrylamide gel and separated the proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After transfer of the proteins to nitrocellulose [Schleicher & Schuell] by electroblotting, we stained the resulting blots with Ponceau S and scanned them using an Agfa Snapscan 1212 flatbed scanner before we probed them with antibodies. From each lane, four strips were cut, which were each incubated in one of the anti-Sycp1 antisera, and then in secondary (anti-rabbit) antibodies conjugated to alkaline phosphatase [AP; Roche Diagnostics], as described (Offenberg et al. 1998).

**Histological analysis and TUNEL assay**

Animals were killed by cervical dislocation. Testes, epididymides, and seminal vesicles, or ovaries and uterus were examined and weighed. From each male, we fixed one testis and epididymis in Bouin's fixative for 24 h at room temperature, and the other testis in phosphate-buffered formalin for 24 h at 4 °C, deparaffinized, and stained with hematoxylin and eosin. For TUNEL analyses, formalin-fixed sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin. Sections were analyzed as described (Heyting and Dietrich 1991).
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Mouse *Syct1* functions in synaptonemal complex assembly, meiotic recombination, and XY body formation

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