Progression of meiotic recombination requires structural maturation of the central element of the synaptonemal complex

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Summary
The synaptonemal complex is an elaborate meiosis-specific supramolecular protein assembly that promotes chromosome synapsis and meiotic recombination. We inactivated the meiosis-specific gene Tex12 and found that TEX12 is essential for progression of meiosis in both male and female germ cells. Structural analysis of the synaptonemal complex in Tex12–/– meiocytes revealed a disrupted central element structure, a dense structure residing between the synapsed homologous chromosomes. Chromosome synapsis is initiated at multiple positions along the paired homologous chromosomes in Tex12–/– meiotic cells, but fails to propagate along the chromosomes. Furthermore, although meiotic recombination is initiated in Tex12–/– meiotic cells, these early recombination events do not develop into meiotic crossovers. Hence, the mere initiation of synapsis is not sufficient to support meiotic crossing-over. Our results show that TEX12 is a component of the central element structure of the synaptonemal complex required for propagation of synapsis along the paired homologous chromosomes and maturation of early recombination events into crossovers.

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Key words: Chromosome synapsis, Meiosis, Meiotic recombination, Synaptonemal complex, TEX12

Introduction
During mammalian meiosis, one round of DNA replication is followed by two successive rounds of chromosome segregation: meiosis I (movement of the homologous chromosomes, each consisting of one pair of sister chromatids, to opposite poles) and meiosis II (separation of the sister chromatids into haploid cells). The prophase of meiosis I is highly regulated and can be subdivided in four cytological stages: leptotene (chromatin condensation), zygonema (synapsis of homologous chromosomes), pachynema (full synapsis) and diplonema (visible chiasmata) (Zickler and Kleckner, 1999). During these stages the sister chromatids are held together by cohesin complex proteins, whereas synapsis of the homologous chromosomes is achieved by formation of a large zipper-like protein complex called the synaptonemal complex (Page and Hawley, 2004; Petronczki et al., 2003).

Incorrect assembly of the synaptonemal complex leads to impaired recombination and cell death, which, in humans, causes infertility in males (Judis et al., 2004; Miyamoto et al., 2003) and a high aneuploidy rate in females (Hassold and Hunt, 2001; Hunt and Hassold, 2002).

Formation of the mammalian synaptonemal complex starts during leptotene when synaptonemal complex protein-2 and -3 (SYCP2 and SYCP3) initiate formation of axial elements, fibrous cores alongside the homologous chromosomes. In male mice, knockout of the Sycp3 gene, which also abolishes recruitment of SYCP2, leads to sterility; the Sycp3–/– spermatocytes fail to form visible axial elements and fail to develop beyond the zygotene stage (Liebe et al., 2004; Pelttari et al., 2001; Yuan et al., 2000). By contrast, the Sycp3–/– females are fertile. However, absence of SYCP3 in oocytes partially disrupts synapsis, impairs DNA damage repair and reduces meiotic recombination, which together eventually lead to a high aneuploidy rate and embryo death (Lightfoot et al., 2006; Wang and Hoog, 2006; Yuan et al., 2002). Similar phenotypes were also observed in Sycp2 mutant mice, probably due to the absence of SYCP3 on the residual axial elements in the Sycp2 mutant cells (Yang et al., 2006). Even though it is not clear how this works biochemically, both SYCP2 and SYCP3 are co-dependent and are required to initiate formation of the axial elements.

During the zygotene stage, synapsis is initiated and the axial elements of the homologous chromosomes (now referred to as lateral elements) are joined by transverse filaments shaped by SYCP1. SYCP1 molecules are long coiled-coil proteins with two globular heads that form parallel homodimers, C-terminal globular heads embedded in the lateral elements and N-terminal heads that interact in a dense region in the middle of the synaptonemal complex called the central element (Liu et al., 1996; Ollinger et al., 2005; Schmekel et al., 1996). Knockout of the mouse transverse filament gene Sycp1 leads to apoptosis of most meiotic cells during the pachyneme stage and subsequently to both male and female infertility (de Vries et al., 2005). In Sycp1–/– spermatocytes,
although axial element formation and alignment of the homologous chromosomes proceed normally, chromosome synapsis and the development of meiotic crossovers do not occur (de Vries et al., 2005).

Following the association of SYCP1 with the lateral elements, further progression of synapsis gives rise to the central element structure in the middle of the synaptonemal complex. The morphology of the central element has been studied in detail by electron microscopy (Schmekel and Daneholt, 1995; Solari and Moses, 1973) and consists of three to four layers of transverse filament components that are longitudinally connected by pillar-shaped protein structures (Schmekel and Daneholt, 1995). In mammals, two central element proteins have been identified: synaptonemal complex central element protein-1 and -2 (SYCE1 and SYCE2) (Costa et al., 2005), of which SYCE2 recently has been shown to be required for completion of synapsis (Bolcun-Filas et al., 2007). We previously identified a novel meiosis specific protein, testis-expressed protein 12 (TEX12), and showed that this protein localizes to the central element of the synaptonemal complex (Hamer et al., 2006), suggesting that TEX12 is a component of the central element. However, even though TEX12 is conserved in vertebrates, its sequence does not tell us anything about its possible function.

To gain more insight in the function of TEX12 and its putative role in central element organization, we have now analyzed Tex12−/− mice. We found that TEX12 is essential for recruitment of SYCE2 to the central element region of the meiotic chromosomes and for the formation of the central element structure. In Tex12−/− cells, synapsis is initiated and visible as small stretches of SYCP1. However, SYCP1 fails to polymerize along the axial elements in the absence of TEX12. Recruitment of the early recombination proteins DMC1 and RPA to the meiotic chromosomes occurs independently of TEX12, but meiotic crossovers do not occur in Tex12−/− meiocytes. Hence, initiation of synapsis is insufficient to support meiotic crossovers in the mouse.

Fig. 1. Generation of Tex12−/− mice. (A) Exon 2 to exon 5 of the Tex12 gene were replaced by a neomycin cassette to generate Tex12−/− mice. (B,C) The mice were genotyped using PCR (black primers in A, two in the gene, one in the neomycin cassette) and did not contain the Tex12 RNA (RT-PCR, grey primers in A, positive control using Trip13) or protein (western blot, WB, positive control α-tubulin).

Fig. 2. Histological analysis of wild-type and Tex12−/− testes and ovaries. (A) Spermatogenesis is halted at epithelial stage IV in the Tex12−/− testes leading to a total absence of round and elongated spermatids. Stages of the seminiferous epithelium are shown as roman numerals. Different testicular cell types depicted are: Int, intermediate spermatogonia; Ls, leptotene spermatocytes; Ps, pachytene spermatocytes; Ps*, apoptotic spermatocytes; Ds, diplotene spermatocytes; M, meiotic cell division; Rs, round spermatids; Es, elongated spermatids; Ser, Sertoli cells. Oogenesis proceeds until the dictyate arrest stage, even though 30% of the oocytes are lost during early embryonic development. After birth, the Tex12−/− oocytes degenerate rapidly and virtually no oocytes can be found in ovaries more than 1 week after birth. Oocytes are labeled with anti-GCNA (brown). Bars: 20 μm (testes) and 5 μm (ovaries). (B) Average number of oocytes (± s.e.m.) per ovary in wild-type and Tex12−/− ovaries at embryonic stage E16.5 and day 1 after birth (n=3).
Results

Loss of TEHX2 results in infertility

TEX12 has been shown to localize to the central element of the synaptonemal complex (Hamer et al., 2006). To gain more insight in the function of this protein during meiosis, we generated Tex12 knockout mice. To inactivate the Tex12 gene, a targeting vector, in which a NEO cassette replaced exon 2 to exon 5 of the gene, was electroporated into embryonic stem (ES) cells. Successfully targeted ES cells were used for generation of chimeric mice that transmitted the Tex12<sup>–/–</sup> allele to the germline. The Tex12<sup>–/–</sup> allele transmitted in a mendelian fashion in Tex12<sup>+/–</sup> intercrosses and the wild-type Tex12 gene, gene transcript or encoded protein could not be detected in the Tex12<sup>–/–</sup> mice (Fig. 1).

Interruption of Tex12 leads to complete elimination of spermatocytes at epithelial stage IV of spermatogenesis and subsequent infertility (Fig. 2). Analysis of Tex12<sup>–/–</sup> females showed that, in comparison to the wild type, only ~30% of the oocytes are present at embryonic stage E16.5 in Tex12<sup>–/–</sup> ovaries (Fig. 2). Notably, as the Tex12<sup>–/–</sup> ovary size is proportionally smaller than comparable wild-type ovaries, the density of oocytes in Tex12<sup>–/–</sup> ovaries is similar to the wild-type situation. The surviving Tex12<sup>–/–</sup> oocytes reach the dictyate arrest stage and 1 day after birth ~30% of the oocytes are still present (Fig. 2). However, Tex12<sup>–/–</sup> oocytes are not able to form healthy primordial follicles and eventually degenerate. As a consequence, hardly any follicles could be observed in Tex12<sup>–/–</sup> ovaries 1 week after birth (Fig. 2).

TEX12 is essential for elongation of synapsis between the homologous chromosomes

To study the role of TEX12 in synaptonemal complex formation and synapsis, we analyzed meiotic cells from Tex12<sup>–/–</sup> and wild-type testes and ovaries using cell spread preparations and immunofluorescence microscopy. Because staining with antibodies against SYCP2 or SYCP3 interfered more with co-staining of other proteins, we used an antibody against the protein STAG3 to visualize the axial elements. STAG3 is part of the meiotic cohesin core, which keeps the sister chromatids together during meiosis, and colocalizes perfectly with the axial elements in both Tex12<sup>+/+</sup> and wild-type cells. By studying staining of SYCP3 (data not shown) and STAG3, we observed that the axial elements form normally and that the meiotic chromosomes align in cells without TEX12 (Fig. 3A). However, progression of synapsis was affected in Tex12<sup>–/–</sup> cells, resulting in only partially synapsed meiotic chromosomes (Fig. 3A). In wild-type cells, synapsis of the homologous chromosomes is marked by staining of the transversal filament protein SYCP1 and the central element protein SYCE1 along the axial elements. Tex12<sup>–/–</sup> meiotic cells also showed staining for SYCP1 and SYCE1, which overlapped with STAG3 on the axial elements (Fig. 3A). However, instead of covering the complete meiotic axes as observed in the wild type, SYCP1 and SYCE1 only formed foci and small stretches in Tex12<sup>–/–</sup> meiotic cells (Fig. 3A). Importantly, the central element protein SYCE2 was completely absent from the Tex12<sup>–/–</sup> chromosome cores (Fig. 3B).

We also studied the structure of the meiotic chromosomes using electron microscopy (EM) and compared wild-type (full synapsis), Tex12<sup>–/–</sup> (partial synapsis) and Sycp1<sup>–/–</sup> (no synapsis) testes and ovaries (Fig. 4). In line with the immunofluorescence experiments, short synapsed axial element structures in Tex12<sup>–/–</sup> cells could be confirmed at the EM level in both spermatocytes and oocytes. Although partially synapsed, the Tex12<sup>–/–</sup> synaptonemal complex showed a weakly stained and disrupted central-element-like structure. We also observed very small areas of chromosome convergence in Sycp1<sup>–/–</sup> spermatocytes. However, these areas of convergence completely lacked any obvious central-element-like structure (Fig. 4).
DNA double-strand breaks fail to develop into meiotic crossovers
The absence of TEX12 results in a disrupted central element and only partial synapsis of the meiotic chromosomes, which could have consequences for the progression of meiotic recombination. Because the Tex12−/− spermatocytes are eliminated from the testis as early as epithelial stage IV, we monitored the effects of these structural deficiencies on the progression of meiotic recombination in Tex12−/− and wild-type oocytes, which survive until the dictyate arrest stage. As described for the Syce2−/− spermatocytes (Bolcun-Filas et al., 2007), the Tex12−/− spermatocytes show normal loading of early recombination markers but do not form an XY body marked by BRCA1 or γ-H2AX as observed in wild-type spermatocytes (supplementary material Fig. S1). We stained Tex12−/− and wild-type oocytes for proteins that mark defined stages of meiotic DNA double-strand break (DSB) processing: presence of DSBs and asynapsis (γ-H2AX, BRCA1), homologous recombination (DMC1, RPA) and sites of crossing-over (MLH1) (Mahadevaiah et al., 2001; Moens et al., 2002; Turner et al., 2004).

At the leptotene stage, meiotic DSBs are formed by SPO11 and marked by phosphorylation of H2AX (then referred to as γ-H2AX) (Mahadevaiah et al., 2001). These meiotic DSBs will be resolved by homologous recombination, which can be visualized as early recombination nodules (DMC1 foci), transformed recombination nodules (RPA foci) and sites of crossovers (MLH1 foci) (Moens et al., 2002).

In early oocytes from stage E16.5 embryos, we observed wild-type levels of DMC1 foci in both wild-type and Tex12−/− oocytes (Fig. 5). However, at a later stage (E18.5), high levels of DMC1 foci were still present in the Tex12−/− oocytes, whereas these foci were lost in wild-type oocytes (Fig. 5). Later during meiosis, the DMC1 foci are gradually replaced by RPA (Moens et al., 2002) and, in accordance, we observed high levels of RPA in both wild-type and Tex12−/− oocytes at stage E17.5 (Fig. 5). However, these RPA foci failed to be removed in the Tex12−/− oocytes and high RPA levels were still present at stage E18.5 in the Tex12−/− oocytes (Fig. 5). Hence, the Tex12−/− oocytes never complete the transition from transformed recombination nodules to meiotic crossovers. In accordance with the impaired processing of DMC1 and RPA foci, we could not observe any MLH1 foci in Tex12−/− oocytes (Fig. 5).

When the DSBs become properly resolved at the pachytene stage of meiosis, γ-H2AX is only retained on unsynapsed chromosomal regions, a process dependent on BRCA1 (Baart et al., 2000; Mahadevaiah et al., 2001; Turner et al., 2004; Turner et al., 2005). In accordance, we found γ-H2AX and BRCA1 to persist on the unsynapsed chromosomal regions in Tex12−/− oocytes (Fig. 6).

Discussion
TEX12 has been shown to localize to the central element of the synaptonemal complex (Hamer et al., 2006). To study the role of this protein in synaptonemal complex formation and function, we
analyzed meiotic progression in Tex12+/− spermatocytes and oocytes. We found that without TEX12, the central element protein SYCE2 is not recruited to the meiotic chromosomes but small areas and foci containing SYCP1 and SYCE1 remain localized to the Tex12−/− meiotic chromosomes. Chromosome synapsis is therefore initiated without TEX12 but the Tex12−/− synaptonemal complex lacks a clear central element structure and synapsis is not propagated along the chromosome cores. Also, meiotic recombination is initiated in the Tex12−/− meiotic cells, but the recombination proteins DMC1 and RPA are not properly removed from the recombination sites and crossing-over does not occur.

Progression of synopsis requires an intact central element structure

The transverse filament protein SYCP1 is required for recruitment of the central element proteins to the homologous chromosomes (Hamer et al., 2006). In cultured mammalian cells, initiation of SYCP1 fiber formation completely depends on the presence of the C-terminus (axial region) of SYCP1, whereas absence of the N-terminus (central region) only decreases the efficiency of fiber formation and elongation (Ollinger et al., 2005). Together, these results indicate that initiation of synopsis starts with the interaction of the C-terminus of SYCP1 with the axial elements. It has been proposed that the central element protein SYCE1 stabilizes the interaction between the two opposing N-termini of SYCP1 in the center of the synaptonemal complex, whereas SYCE2 would longitudinally connect these SYCP1 dimers to shape a complete central element (Bolcun-Filas et al., 2007). The central element consists of multiple layers of columns and pillars that shape an almost crystalline 3D structure in the center of the synaptonemal complex (Schmekel and Daneholt, 1995). We found that TEX12 is essential for the inclusion of SYCE2 in the central element and confirm the notion that these proteins form an independent complex (Hamer et al., 2006), possibly corresponding to the columns and pillars observed in the structural studies of the central element (Schmekel and Daneholt, 1995). Without TEX12, this structure is not properly assembled. As a consequence, the synaptonemal process does not proceed in Tex12+/− meiotic cells and the synapsed stretches containing SYCP1 are not elongated along the axial elements.

Progression of meiotic recombination requires structural maturation of the central element of the synaptonemal complex

In Saccharomyces cerevisiae, initiation and elongation of the synaptonemal complex depend on a protein complex called the synopsis initiation complex, consisting of the ZMM proteins (Zip1, Zip2, Zip3, Zip4, Mer3 and Msh4) (Fung et al., 2004; Lynn et al., 2007). Of these proteins Zip2, Zip3 and Zip4 appear functionally related to the mammalian central element proteins: they are essential for synaptonemal complex formation, they are located at the center of the synaptonemal complex and without
them 80% of all crossovers (Class I crossovers) do not occur (Agarwal and Roeder, 2000; Chua and Roeder, 1998; Lynn et al., 2007; Tsuouchi et al., 2006). Binding of Zip3 to synopsis initiation sites recruits Zip2 and Zip4, which in turn are responsible for polymerization of Zip1 (Agarwal and Roeder, 2000; Tsuouchi et al., 2006). The fact that these proteins are necessary for class I crossovers and that the number of synopsis initiation sites corresponds with the number of these crossovers has led to the proposal that initiation of synopsis determines the sites of future crossovers during meiosis (Henderson and Keeney, 2004; Henderson and Keeney, 2005; Zickler, 2006).

Also in mammals, synopsis and meiotic recombination are two highly intertwined events. In the mouse, although meiotic recombination initiates prior to and independently of synopsis (Baudat et al., 2000; Mahadeviah et al., 2001), synopsis is required for recombination sites to develop into meiotic crossovers (de Vries et al., 2005). Furthermore, synopsis of the homologous chromosomes is completely dependent on the initiation of meiotic recombination (Baudat et al., 2000). However, in Tex12−/− oocytes, even though synopsis is initiated, crossing-over still does not occur. Hence, merely initiating synopsis is not sufficient to generate meiotic crossovers in the mouse.

How synopsis, including a fully functional central element, could promote the formation of meiotic crossovers remains unclear. The lack of TEX12 or incomplete synopsis might trigger an unknown checkpoint in the Tex12−/− oocytes. Moreover, TEX12 could even be part of the recombination or checkpoint machinery itself. Activation of a checkpoint in the Tex12−/− oocytes could possibly lead to a cell cycle arrest that would prevent the meiotic prophase to progress to the pachytene stage during which crossovers would normally occur. It has also been postulated that the mechanical properties (such as robustness or flexibility) of the synaptonemal complex affect how meiotic DSBs are resolved (Blat et al., 2002; Borner et al., 2004; Moens, 1978). The lack of crossovers in Tex12−/− mice could then be explained by a structural defect caused by the absence of central element proteins from the synaptonemal complex. Either way, without full synopsis along the meiotic cores and a functional central element, the limited synapsed areas on the Tex12−/− chromosomes fail to support the development of meiotic crossovers.

Materials and Methods

Animals

The Tex12 mutant mouse line was established at the MCI/ICS (Mouse Clinical Institute/Institut Clinique de la Souris, Illkirch, France; http://www-mci.u-strasbg.fr). The targeting vector was constructed as follows. A 4.4 kb fragment encompassing Tex12 exon 1 was amplified by PCR (129S2/SvPas) and subcloned in an MCI proprietary vector, resulting in a step 1 plasmid. This MCI vector has a floxed neomycin-resistance cassette. A 4.1 kb fragment was amplified by PCR and subcloned in the step1 plasmid to generate the final targeting construct (Fig. 1). The linearized construct was electroporated in 129S2/SvPas mouse embryonic stem cells. After selection, targeted clones were identified by PCR using external primers and further confirmed by Southern blot with neomycin and external probes. Two positive ES cell clones were injected into C57BL/6J blastocysts, and male chimaeras derived from these were then used for germline transmission. The Tex12 mice were further analyzed by PCR, RT-PCR and western blot using guinea pig anti-TEX12 (Hamer et al., 2006) and mouse anti-α-tubulin (SIGMA).

Wild-type, Tex12 and Sycp1 (de Vries et al., 2005) mice were used and maintained according to regulations provided by the animal ethical committee of the Karolinska Institute who also approved of the experiments.

Immunohistochemistry, immunocytochemistry and electron microscopy

Histology and immunohistochemistry were performed as described (Hamer et al., 2001; Wang and Hoog, 2006). Immunohistochemistry was performed as described (Kouznetsova et al., 2005) using a ‘drying-down’ technique (Peters et al., 1997) and the following antibodies: rabbit anti-SYCP1 (1:50) (Liu et al., 1996), mouse anti-SYCP1 (1:200) (gift from C. Heyting, Wageningen, The Netherlands), rabbit anti-SYCP3 (1:200) (Liu et al., 1996), human anti-CREST (1:1500) (Hladzcky et al., 1986), rabbit anti-STAG3 (1:400) (Pezzi et al., 2000), guinea pig anti-STAG3 (1:200) (Kouznetsova et al., 2005), guinea pig anti-SYCE1 (1:1500) (Hamer et al., 2006), guinea pig anti-SYCE2 (1:400) (Hamer et al., 2006), rabbit anti-γH2AX (Upstate Biotechnology) (1:100), rabbit anti-BRCA1 (gift from J. M. A. Turner, National Institute for Medical Research, London, UK) (1:1000), rabbit anti-DMC1 (1:100) and rabbit anti-RPA (1:500) (gifts from P. Moens, York University, Toronto, Canada) and mouse anti-MLH1 (1:50) (BD Biosciences). Secondary antibodies were applied as described (Kouznetsova et al., 2005; Wang and Hoog, 2006). Electron microscopy was performed on ultra thin sections of testis or ovary tissue fixed in 2.5% glutaraldehyde and 1% OsO4 as described previously and according to standard protocols (Liebe et al., 2004).

Microscopy and imaging

Slides were viewed at room temperature using Leica DMRA2 and DMRXA microscopes and 100× objectives with epifluorescence. Images were captured with a Hamamatsu digital charge-coupled device camera C4742-95 and OpenlabTM software version 3.1.4. Images were processed using Adobe Photoshop version 9.0.

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