Meiotic cohesin complexes are essential for the formation of the axial element in mice

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Cohesin is a conserved multisubunit protein complex that participates in chromosome segregation, DNA damage repair, chromatin regulation, and synaptonemal complex (SC) formation. Yeast, but not mice, depleted of the cohesin subunit Rec8 are defective in the formation of the axial elements (AEs) of the SC, suggesting that, in mammals, this function is not conserved. In this paper, we show that spermatocytes from mice lacking the two meiosis-specific cohesin subunits RAD21L and REC8 were unable to initiate RAD51- but not DMC1-mediated double-strand break repair, were not able to assemble their AEs, and arrested as early as the leptotene stage of prophase I, demonstrating that cohesin plays an essential role in AE assembly that is conserved from yeast to mammals.

Introduction

In mammalian spermatogenesis, a subset of spermatogonia undergoes a terminal round of DNA replication and then enters meiosis. Meiosis is a specialized process in which two successive rounds of chromosome segregation and cell divisions occur without intervening DNA replication. This reduces the number of each chromosome from four copies in the meiocyte to one copy in haploid gametes. At the initiation of the prophase I, a proteinaceous structure called the axial element (AE) begins to form along replicated sister chromatids. Subsequently, the AEs of homologues become juxtaposed by transverse element proteins (e.g., SYCP1, beginning in zygonema), and the paired axes joined by transverse elements form the tripartite synaptonemal complexes (SCs) that connect all homologues at pachynema (Yang and Wang, 2009). This dynamic protein complex provides the structural framework in which homologous chromosomes undergo close juxtaposition and repair of double-strand breaks (DSBs) by recombination. Subsequently, but before the first meiotic division, homologues desynapse but retain stable connections formed by resolution of certain recombination events as crossovers (visible as chiasmata). In most organisms, chiasmata are required to enable proper orientation of homologues on the meiosis I spindle before the first meiotic division. Sister chromatid cohesion distal to crossovers maintains chiasmata at their initial positions until anaphase I.

During meiosis, sister chromatid cohesion is lost in two consecutive steps. Loss of chromosome arm cohesion in anaphase I releases the linkage between homologues, allowing them to segregate to opposite poles (Page and Hawley, 2003). However, maintenance of centromeric cohesion by the action of Shugoshin-like–2 in mammals ensures the generation of tension by the proper attachment of sister chromatids to the meiosis II spindle, enabling their proper segregation to opposite poles (Llano et al., 2008; Gutiérrez-Caballero et al., 2012).

During the mitotic cell cycle, sister chromatid cohesion is mediated by the multisubunit cohesin complex between S phase and anaphase (Gruber et al., 2003; Unal et al., 2007; Haering et al., 2008; Zhang et al., 2008). Structurally, the mitotic cohesin complex comprises four core proteins: SMC1α, SMC3, RAD21, and a HEAT repeat domain protein (STAG1, SMC1β, SMC3, RAD21, and a HEAT repeat domain protein (STAG1, starch-amylose-like 1). These proteins mediate sister chromatid cohesion, which is essential for the proper segregation of sister chromatids during mitosis, meiosis, and DNA replication. The cohesin complex is transiently linked to the mitotic spindle through Shugoshin-like–2 (Sgo2) proteins, which are required for the proper segregation of sister chromatids and centromeres. The cohesin complex is also involved in the assembly and maintenance of the synaptonemal complex (SC) during meiosis, which is essential for the proper segregation of homologous chromosomes.
Mice lacking RAD21L and REC8 develop normally but are infertile

Mice lacking either of two meiosis-specific cohesin subunits, REC8 or RAD21L, show similar defects in meiosis. To test for possible overlap in the functions of these two kleisin proteins, we generated kleisin double-knockout (dKO) mice. Rad21l−/− Rec8−/−, henceforth dKO-kls animals, were obtained from crosses of double heterozygotes (Rec8−/+ and Rad21l−/+), in the expected Mendelian ratios and analyzed. dKO-kls mice developed normally and displayed no overt defects besides infertility. As expected from the phenotypes of the single Rec8 and Rad21l mutants, all the dKO-kls mice (n = 11) were infertile (unpublished data; Bannister et al., 2004; Herrán et al., 2011). The testes from dKO-kls mice were reduced in size, weighing 30 ± 3 mg at 6–8 wk of age compared with 103 ± 8 mg in wild-type males (Fig. 1 A and Table S1). Furthermore, histological examination of testes from 6–8-wk-old dKO-kls males revealed seminiferous tubules that were always devoid of postmeiotic cell types, despite the presence of spermatogonia, and Sertoli and Leydig cells (Fig. 1 B and not depicted). Similar defects were also observed in the Rec8-deficient and Rad21L-deficient mice (Fig. 1, A and B; Bannister et al., 2004; Herrán et al., 2011).

Meiotic kleisins are essential for the formation of AEs in spermatocytes

We staged and examined spermatocyte spreads by immunolocalization of SYCP3. In the absence of RAD21L and REC8, AE assembly and synapsis between homologues were disrupted very early (Fig. 1, C and D). dKO-kls spermatocytes arrested at a leptotene-like stage (100% of cells) were characterized by the punctate aggregates of SYCP3. In contrast, thin threads were observed in late leptotene spermatocytes from wild-type and single mutant mice (Fig. 1, C and D). SYCP2, another axial protein, colocalized with SYCP3 in these aggregates (Fig. 1 D). Antibodies against SYCP1 were used to determine whether transverse components of the SC assembled in dKO-kls mutant spermatocytes. The aggregates of AE proteins did not show reactivity with SYCP1 antibodies, indicating an absence of transverse filament assembly (n = 120; Fig. 1 D). In agreement with previous studies, spermatocytes of Rad21l and Rec8 single mutant mice arrested at a zygote-like stage, displaying several fragmented AEs and some partially synapsed lateral elements (LEs) that never progressed to the expected 19 fully synapsed autosomal bivalent chromosomes observed in wild-type pachynema (unpublished data; Bannister et al., 2004; Herrán et al., 2011).

Tubule section in wild-type mice can be categorized in stages running from I to XII according to the spectrum of germ cell types that are present (Russell et al., 1990). Following these criteria, dKO-kls, Rad21l, and Rec8 mutant mice appeared to be arrested at stage IV of the seminiferous epithelium cycle (Fig. 1 B; Herrán et al., 2011). In wild-type tubules, stage IV typically corresponds to midpachynema. At this stage, mutant spermatocytes that fail to complete recombination and/or chromosome synapsis will usually undergo apoptosis (de Rooij and de Boer, 2003). As examples, MSH5−, DMC1−, or SPO11-deficient spermatocytes...
Figure 1. **Absence of RAD21L and REC8 arrest mouse spermatogenesis in early prophase I.** (A) dKO-kls mice show a 70% reduction in testes size compared to wild type. Similar reductions are observed in Rad21l−/− and Rec8−/− males. (B) Mutation of both Rad21L and Rec8 elicits an arrest of spermatogenesis at stage IV characterized by intermediate spermatogonia (arrows) in a representative section of a seminiferous tubule. Massive apoptosis of spermatocytes (condensed nuclei indicated by asterisks) and absence of mature spermatozoa/spermatids are observed in the dKO-kls tubules. A similar arrest is observed in seminiferous tubules from singly mutant Rad21l and Rec8 mice. (C) Immunolabeling for SYCP3 in spermatocytes from a wild-type mouse at early and late leptotene and spermatocytes arrested at a leptotene-like stage from a dKO-kls mouse. (D) dKO-kls spermatocytes arrested at the leptotene-like stage show absence of chromosomal synapsis. Double immunolabeling for SYCP3 and SYCP2 or SYCP1 shows SYCP3/SYCP2 aggregates without synapsis as indicated by the lack of SYCP1 labeling in double mutant spermatocytes. Spermatocytes from Rad21l−/− (zygotene-like arrest), Rec8−/− (zygotene-like arrest), and wild-type (zygotene stage) mice show AEs and synapsed LEs with stretches of SYCP1. Bars: (A) 5 mm; (B) 25 µm; (C and D) 100 µm.
leads to absence of AEs, which elicits spermatocyte death after an extended leptotene-like stage. DSBs are formed but not repaired in dKO-kls male mice.

We next sought to determine how recombination mechanisms are affected by the absence of RAD21L and REC8. Programmed DSBs that initiate meiotic recombination are normally generated by the nuclease SPO11 at the early leptotene stage (Keeney, 2001).

arrest at stage IV, but the terminal stage was described as zygonema, late zygonema, and midpachynema, respectively, based on the progression of synapsis (Yoshida et al., 1998; de Vries et al., 1999; Baudat et al., 2000). Interestingly, dKO-kls spermatocytes arrest at a stage best described as leptotene because they lack AEs yet remain viable up to stage IV. The absence of later stages indicates that these spermatocytes then undergo apoptosis, likely caused by activation of meiotic checkpoints. Thus, disruption of the meiotic cohesin complexes leads to absence of AEs, which elicits spermatocyte death after an extended leptotene-like stage.

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We analyzed break formation in mutant spermatocytes using antibodies against the γ-H2AX, a histone variant that is phosphorylated during early prophase I in response to SPO11-induced DSBs in an ATM-dependent manner (Mahadevaiah et al., 2001). As seen in Fig. 2 A, 100% of leptotene-like arrested spermatocytes from dKO-kls mice showed a positive staining that was similar to that observed in the wild-type mice (87 ± 30 vs. 92 ± 56; Table S1). This suggests that the formation of programmed DSBs is not markedly altered in dKO-kls mice. However, the presence of γ-H2AX staining in all the arrested spermatocytes from dKO-kls animals indicates that breaks are not repaired efficiently.

We further analyzed the recombination process to address why DSBs are not repaired in the double mutant spermatocytes. After DSBs are induced, the broken ends are resected, and the strand invasion enzymes RAD51 and DMC1 are recruited to the resulting single-strand DNA overhangs to promote homologue pairing and DNA repair (Symington and Gautier, 2011). In wild-type leptotene spermatocytes, RAD51 and DMC1 assemble on the AEs/LEs of bivalents and disappear toward pachynema, with the exception of the unsynapsed portions of the sex chromosome AEs (Tarsounas et al., 1999). As shown in Fig. 2 B, wild-type spermatocytes showed 70–150 RAD51 foci (111 ± 32). In contrast, the dKO-kls contained only a few RAD51 foci (14 ± 5, P < 0.05; Table S1). Next, we monitored the loading of DMC1, a meiosis-specific paralogue of RAD51. In wild-type spermatocytes, both recombinases colocalize extensively such that most recombination-associated foci contain both proteins (Tarsounas et al., 1999). Intriguingly, immunofluorescence with a DMC1-specific antibody revealed no detectable difference in numbers of DMC1 foci between wild type and dKO-kls in leptotena (52 ± 20 vs. 54 ± 19; Table S1 and Fig. 3 A). The presence of an equivalent number of DMC1 foci in double mutant leptotene spermatocytes implies that the γ-H2AX labeling in the dKO-kls spermatocytes is very likely generated by the meiotic SPO11 nuclease.

Next, we determined the distribution of the replication protein A (RPA) in dKO-kls spermatocytes. RPA is a single-stranded DNA-binding protein that enhances the formation of RAD51 and DMC1 filaments in vitro. RPA is first detected in few foci at leptonema. The initial binding of RPA to single-strand DNA at the resected ends of DSBs is supposed to be too transient to be cytologically detected by immunofluorescence because RPA is rapidly displaced by RAD51/DMC1 (Yang et al., 2008). Subsequently, after RAD51/DMC1 loading, abundant RPA foci are detected in the synapsed regions of the LEs at zygonema (Krogh and Symington, 2004; Moens et al., 2007). Intriguingly, despite failure to proceed beyond leptonema, RPA foci were increased threefold in the dKO-kls spermatocytes relative to wild type (55 ± 19 vs. 18 ± 10, P < 0.05; Table S1 and Fig. 3 B), which is very likely caused by the sharp reduction in the loading of RAD51 (Fig. 2 B; Roig et al., 2010). TRIP13 is the mammalian orthologue of the yeast Pch2 (pachytene checkpoint 2) gene, and its deletion leads to a block of spermatogenesis and oogenesis because of defects in DSB repair (Li and Schimenti, 2007; Roig et al., 2010). Interestingly, Trip13 mutant spermatocytes accumulate RPA foci in leptonema and also show...
can be genetically dissected. Collectively, our results indicate that meiotic cohesins are dispensable for the loading of DMC1 but are essential for normal loading of RAD51 (Xu et al., 2003; Sharan et al., 2004; Yang et al., 2008; Roig et al., 2010).

a sharply reduced number of RAD51 foci and an accumulation of DMC1 foci at leptonema (Roig et al., 2010). Thus, the RAD51 and DMC1 homologous recombination pathways, although very similar biochemically (Kagawa and Kurumizaka, 2010), can be genetically dissected. Collectively, our results indicate that meiotic cohesins are dispensable for the loading of DMC1 but are essential for normal loading of RAD51 (Xu et al., 2003; Sharan et al., 2004; Yang et al., 2008; Roig et al., 2010).

Figure 4. Cohesin complexes in the absence of RAD21L and REC8. Double immunofluorescence of SYCP3 and either RAD21, SMC3, SMC1β, or STAG3. In wild-type leptotene spermatocytes, the cohesins RAD21, SMC3, SMC1β, and STAG3 colocalize with SYCP3 along the AEs of the chromosomes. In Rad21l and Rec8 single mutant leptotene spermatocytes, RAD21, SMC3, SMC1β, and STAG3 colocalize also with SYCP3 along the AEs/LEs of the chromosomes. In spermatocytes from dKO-kls, arrested at leptonema, SMC1β and STAG3 are not detected by immunofluorescence, whereas immunolabeling for RAD21 and SMC3 renders robust and very faint fluorescence signals, respectively. Bar, 100 µm.
Cohesin complexes in mutant spermatocytes

In the absence of SYCP3, the cohesin complex persists at a residual AE-like chromosomal core in mouse meiocytes (Pelttari et al., 2001; Kolas et al., 2004; Kouznetsova et al., 2005). We evaluated whether deficiency of both RAD21L and REC8 compromised the loading of other cohesin subunits by immunostaining for the cohesin subunits STAG3, SMC3, SMC1β, and RAD21 (Fig. 4). Surprisingly, we observed robust staining only for RAD21, which colocalized with SYCP3 labeling, and much fainter staining of SMC3 (Fig. 4). We did not detect either STAG3 or SMC1β (Fig. 4), suggesting that these cohesin subunits are complexed in vivo with REC8 and RAD21L kleisins. Collectively, these data suggest that the two α-kleisins, RAD21L and REC8, are not epistatic and sustain most, if not all, meiosis-specific cohesin complexes involved in AE formation and SC assembly. Our results are consistent with the observation that RAD21 is present on chromosome axes from leptonema to diplonema in mouse spermatocytes (this paper; Herrán et al., 2011) but contrast with one study in which RAD21 was first detected in the pachytene stage (Ishiguro et al., 2011). Because we observed RAD21 immunolabeling in the RAD21+/−, Rec8−/−, and dKO-kls spermatocytes (Fig. 4), cross-reactivity of our antibody with RAD21L and/or REC8 is not likely the reason for these contrasting results, as previously claimed (Ishiguro et al., 2011). This early localization of RAD21 would suggest a direct role of this kleisin in establishing cohesion in premeiotic S phase.

Sister chromatid cohesion in the absence of meiotic kleisins

In addition to their role in AE formation and DNA looping (Revenkova and Jessberger, 2006; Novak et al., 2008), cohesin complexes must tether the two sister chromatids from S phase until the onset of anaphase, when separase is activated, cleaves the cohesin subunits, and releases the sister chromatids (Hauf et al., 2001). Thus, a deficiency for two out of the three mammalian kleisins could partially affect chromosomal cohesion in premeiotic S phase. Our results are consistent with the prerequisite for SC formation. Our findings provide evidence for a crucial role for meiotic cohesins (REC8- and RAD21L-containing cohesin complexes) in the very early assembly of the AE-like chromosomal core in mouse meiotic prophase (Kouznetsova et al., 2011). Thus, the meiotic cohesin complexes are required to initiate AE/LE assembly in mammals (and not vice versa), which is a prerequisite for SC formation.

Materials and methods

Immunocytology and antibodies

Tests were detergent- and formaldehyde-fixed and processed for spreading using the “dry-down” technique (Peters et al., 1997). The slides were rinsed three times for 5 min in PBS and incubated overnight at room temperature with primary antibodies diluted in PBS. The primary antibodies used for immunofluorescence were rabbit αSMC3 serum K987 (1:20), rabbit αSMC1β serum K974 (1:20; Prieto et al., 2004), rabbit αSTAG3 serum K403 (1:20; Prieto et al., 2001), rabbit αRAD21 IgG K854 (1:5; Prieto et al., 2002), rabbit αSYCP2 serum K1035 (1:20), mouse αSYCP3 IgG sc-74569 (1:100), rabbit αRAD51 sc-8349 (1:30), and PC130 (1:5; EMD; provided by J. Page, Universidad Autonoma de Madrid, Madrid, Spain), rabbit αMC1 sc-22768 (1:20; Santa Cruz Biotechnology, Inc.), rabbit αSYCP1 IgG ab15090 (1:200; Abcam), rabbit anti–γH2AX (ser139) IgG (#07-164; 1:200; Millipore), and rabbit αRPA IgG (1:300; provided by E. Marcon, Toronto University, Toronto, Canada). The secondary antibodies used were TRITC α-rabbit 115–095-146/α-rabbit 111–025-144 and FITC α-mouse 115–095-146/α-rabbit 111–095-045 (Jackson ImmunoResearch Laboratories, West Grove, PA).
Mice
The mutation at the Rad21l<sup>-/-</sup> locus is a null allele generated by gene targeting using an insertional targeting vector (Herren et al., 2011). Mice were genotyped by genomic Southern blot analysis of tail genomic DNA digested with SpeI and hybridized with a 5' external probe. The probe (1 kb) was generated by PCR using the primers 5'-ACTAGTCTAATAAAGGTCTT-3' and 5'-GATTTAAGCTGAAAGAAGTAA-3'. The mutation at the Rec8 locus is a null allele (premature stop codon at exon 6) isolated in a forward genetic screen for mouse infertility (Bannister et al., 2004). Mice were genotyped by direct sequencing of the PCR-amplified exon 6 from genomic DNA using the primers 5'-CTCTTACAATCCGTTCTC-3' and 5'-ACAGGAACACCAACTAACC-3'. dKO-klis mice were obtained by genetic crossing of double heterozygote mice (Rec8<sup>-/-</sup> Rad21l<sup>-/-</sup>) and compared with single mutant and wild-type littermates. All animal experiments were reviewed and approved by the Consejo Superior de Investigaciones Científicas (Spanish Research Council) and the Universidad de Salamanca National Committee on Bioethics.

FISH analysis
The mouse X-specific probes were obtained by PCR using the following three set of primers: 1S, 5'-TAGGATGATGGAACCCACATGC-3'; 1A, 5'-TGGTTGTAATGTTTGG-3'; 2S, 5'-CATGCCCTTGAGCTAGC-3'; 2A, 5'-CTTTTTTTTTCTTCTCT-3'; 3S, 5'-CTCTTCTGGACAAGGAGCA-3'; and 3AS, 5'-CTCTCCGCTCACAATCTATCA-3' (Navin et al., 1996). The X-specific DNA probe is a pericentromeric DNA fragment cloned in a plasmid (Disteche et al., 1985). Both probes were labeled by Nick translation with Dig-11-deoxy-UTP and Bio-16-deoxy-UTP and hybridized to spermatocyte spreads following standard procedures. In brief, slides were pre-treated with pepsin (0.005% in 0.01 N HCl for 5 min at 37°C), dehydrated, and RNase treated (0.1 mg/ml for 1 h at 37°C). After denaturation in 70% formamide during 3 min at 75°C, slides were dehydrated in ice-cold ethanol and hybridized overnight at 37°C to a denatured DNA probe. After washing (50% formamide and 2× SSC at 42°C), biotin- and digoxigenin-labeled probes were immunodetected using streptavidin-Cy3/goat anti-mouse/rabbit anti-mouse-FITC (Roche), respectively (Pendás et al., 1994).

FACS analysis
Wild-type, Rad21l<sup>-/-</sup>, and dKO-klis testicular cell preparation and their DNA content measurement were performed as previously described (Malkov et al., 1998). In brief, tests were dissected in separation medium, diluted 1:1 with propidium iodide solution (10 mM Tris, pH 8, 1 mM NaCl, 0.1% Nonidet P-40, 0.7 mg/ml RNase A, and 0.05 mg/ml propidium iodide), and freshly analyzed by a cell-sorting instrument (FACSort; BD).

Online supplemental material
Fig. S1 shows that the absence of RAD21L and REC8 does not impair DNA replication and does not provoke loss of chromatin cohesion in dKO-klis spermatocytes. Table S1 shows the number of early recombination-associated foci in spermatocytes and the weight of testis from wild-type and dKO-kls mice. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201201100/DC1.

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