The Hop2 Protein Has a Direct Role in Promoting Interhomolog Interactions during Mouse Meiosis

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Summary

The S. cerevisiae Hop2 protein and its fission yeast homolog Meu13 are required for proper homologous chromosome pairing and recombination during meiosis. The mechanism of this requirement is, however, not understood. The previous studies in Saccharomyces suggested that Hop2 is a guardian of meiotic chromosome synapsis with the ability to prevent or resolve deleterious associations between nonhomologous chromosomes. We have generated a Hop2 knockout mouse that shows profound meiotic defects with a distinct and novel phenotype. Hop2 is spermatocytes arrest at the stage of pachytene-like chromosome condensation. Axial elements are fully developed, but synopsis of any kind is very limited. Immunofluorescence analysis of meiotic chromosome spreads indicates that while meiotic double-stranded breaks are formed and processed in the Hop2 knockout, they fail to be repaired. In aggregate, the Hop2 phenotype is consistent with a direct role for the mouse Hop2 protein in promoting homologous chromosome synapsis.

Introduction

During meiosis, diploid precursor cells give rise to haploid gametes through the precisely controlled segregation of homologous chromosomes. In order for reductional division to occur, the homologs must first pair and engage in a transient physical association, required for the subsequent segregation to the different poles of the cell. This physical linkage is ensured by homologous recombination. Another important outcome of this event is the genetic exchange between parental chromosomes. Arguably the most puzzling question in the field is how homologs find each other in genomes of very high complexity, harboring up to thousands of megabases of genetic information. In most organisms, chromosome pairing culminates in the formation of the synaptonemal complex (SC). This proteinaceous ribbon-like structure holds homologs closely apposed along their entire length. Homologous chromosome pairing, recombination, and SC formation are closely interconnected in most organisms, but their precise role has been difficult to determine since distinct mechanisms to promote and/or stabilize these homologous interactions have developed in the course of evolution.

During meiosis, a type II topoisomerase Spo11 cleaves the chromosomal DNA at many sites (200 or more in mammals) in each and every nucleus (Keeney, 2001). The repair of these DNA double-stranded breaks (DSBs) is mediated by homologous recombination (HR). In most organisms, including S. cerevisiae (reviewed in Roeder, 1997; Zickler and Kleckner, 1999) and mice (Baudat et al., 2000; Romanienko and Camerini-Otero, 2000), but not in D. melanogaster and C. elegans (Dernburg et al., 1998; McKim et al., 1998), initiation of HR is required for proper synopsis of the homologs in the context of the SC. Flies and worms have developed alternative mechanisms to stabilize pairing interactions and promote SC formation (reviewed in Roeder, 1997; Villeneuve and Hillers, 2001; Walker and Hawley, 2000; Zickler and Kleckner, 1999; also see MacQueen et al., 2002). Curiously, the genes encoding three conservative meiosis-specific proteins—Dmc1, Hop2, and Mnd1—are only present in the genomes of organisms that require DSBs (hence Spo11) for meiotic chromosome synapsis (that is, they are absent from the genomes of flies and worms). At the same time, these three genes are among two dozen genes with the greatest changes in their level of expression in Spo11−/− mice (N. Smirnova and R.D.C.-O., unpublished).

The hop2 deletion mutant in S. cerevisiae fails to sporulate and undergoes a uniform arrest at the pachytene stage of meiosis I (Leu et al., 1998). SC formation is strongly delayed but ultimately proceeds to almost wild-type level of synopsis, albeit mostly between nonhomologous chromosomes. The mutant sustains wild-type levels of meiotic DSBs that are processed to expose single-stranded tails but not repaired. Formation of mature crossover recombinants drops to 0%-7% of the wild-type level, depending on the sensitivity of the assay (Leu et al., 1998; Tsubouchi and Roeder, 2002).

The MND1 gene was first discovered in a screen for meiotically expressed genes (Rabitsch et al., 2001) and was later identified as a multicopy suppressor of a hop2-ts allele (Tsubouchi and Roeder, 2002), and an mnd1 null mutant has a phenotype similar to that of the hop2 deletion (Gerton and DeRisi, 2002; Tsubouchi and Roeder, 2002). Co-immunoprecipitation of Mnd1 and Hop2 from meiotic extracts suggests the involvement of the two in the same meiotic process (Tsubouchi and Roeder, 2002).

In budding yeast, dmc1 mutants arrest (or experience a substantial delay, depending on the genetic background) at the same stage as hop2 mutants (Bishop et al., 1992; Rockmill and Roeder, 1994; Rockmill et al., 1995). The chromosomes at this point are maximally condensed and correspond in length to the pachytene stage of wild-type meiosis (Roeder and Bailis, 2000). Overall, the recombination-defective phenotype of the hop2 null mutation is remarkably reminiscent of the dmc1 deletion, but with a more severe defect when both are compared in the same genetic background. However, a few important differences suggest distinct

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roles for these two proteins in the same pairing/recombination pathway (Leu et al., 1998; Roeder and Bailis, 2000; Shimada et al., 2002).

Dmc1 knockout mice revealed the conserved function of Dmc1 throughout evolution (Pittman et al., 1998; Yoshida et al., 1998). To define the function of the mammalian Hop2, we have generated a knockout mouse. The animals did not show any gross anomalies in somatic tissues but developed a profound deficiency in gametogenesis. Evaluation of the chromosomal spreads of knockout spermatocytes revealed striking meiotic defects, consistent with a failure in recombination. Our data strongly suggest that Hop2 has a direct role in promoting early steps of interhomolog interactions.

Results

Disruption and Inactivation of the Mouse Hop2 Gene

The mouse Hop2 gene (mTBPIP) was disrupted by gene targeting in embryonic stem (ES) cells. The targeting vector was designed to replace the first three exons of Hop2 with the Neo resistance gene (NEO) marker (see Supplemental Figure S1A at http://www.developmentalcell.com/cgi/content/full/5/6/927/DC1). A correctly targeted clone was injected into blastocysts, and the first progeny of chimeric mice was genotyped by Southern blot analysis (Supplemental Figure S1B). The ability to generate homozygous knockout mice indicates that Hop2 is not essential for mouse development. A polyclonal antibody raised against the intact mouse Hop2 protein revealed a band with an apparent size of 29 kDa on Western blots of wild-type and heterozygote but not knockout testes extracts (Supplemental Figure S1C, left panel). Even though the remaining four C-terminal exons could potentially code for three partial Hop2 polypeptides of 108, 92, or 39 amino acids, a polyclonal antibody raised against the C terminus peptide failed to detect any protein products in the knockout mouse (Supplemental Figure S1C, right panel). Thus, we have generated a mouse Hop2 knockout strain that produces no part of the Hop2 protein. Since the introduction of the NEO gene targeting marker could result in the silencing of adjacent promoters (Artelt et al., 1991), we examined the transcription of genes located up to 8 kb up- and downstream of the inserted NEO gene (GenBank mRNA accession numbers NM 026501 and NM 011550, respectively). We found no significant difference between wild-type and knockout mRNA levels for either of these genes using PCR (data not shown).

Defects in Gametogenesis of the Hop2−/− Mice Are Consistent with a Role for the Protein in Meiosis

In good agreement with the role of the yeast Hop2 protein in meiosis (Leu et al., 1998; Nabeshima et al., 2001), the Hop2 knockout mice are infertile. Males develop testicular hypoplasia with hyperplasia of interstitial cells and lack of spermatooza (Figure 1A). Knockout testes are approximately 30% of the size of the wild-type. This reduction in size is similar to that observed in other mouse knockouts of early meiotic genes such as Dmc1 and Spo11 (Baudat et al., 2000; Pittman et al., 1998; Romanienko and Camerini-Otero, 2000; Yoshida et al., 1998). The number of seminiferous tubules is not reduced, but the diameter is 25%–30% of normal. Primary spermatocytes represent the most advanced spermatogenic cells in the Hop2 knockout, indicating that spermatogenesis is blocked at prophase of meiosis I. Arrested cells undergo extensive apoptosis that could be detected in a fraction of the tubules (Figure 1C). Female Hop2 knockout shows ovarian tubulostromal hyperplasia with no follicles present (Figure 1B). The lack of mature gametes in both sexes of Hop2 knockout mice is consistent with a role for the Hop2 protein in meiosis. Heterozygous mice of both sexes are fertile with no obvious reproductive abnormalities. 6-month-old knockout mice do not show gross changes in any of the 32 somatic tissues examined (including thymus and spleen). Out of the first 374 pups born, 27.8% are homozygous, a number that is close to a normal Mendelian distribution and suggests that Hop2 is not an essential gene.

Hop2 Knockout Spermatocytes Arrest prior to Pachytene and Show a Marked Failure in Chromosome Synapsis

To investigate the defect in meiosis I in greater detail, we prepared chromosomal spreads from Hop2−/− spermatocytes (Figures 2A, 2B, and 3). The cells proceed normally through leptotene and early zygotene with 40 centromeres clearly identified by CREST antisera (Brenner et al., 1981). The correct number of centromeres and chromosome cores indicates that sister chromatid cohesion is not affected in the absence of Hop2 (Figure 2A). Immunostaining for the Scp3/Syn1 protein of axial/lateral elements (Dobson et al., 1994) revealed that homologous chromosomes are not assembled into the synaptonemal complex. Even though the chromosomes in arrested Hop2 spermatocytes look very compact and relatively stiff (pachytene-like), only limited synapsis could be seen in a fraction of cells (Figure 2B, panels II and III). Synapsed regions could be detected by staining for the Scep1/Syn1 protein, a component of the central element of the synaptonemal complex (Dobson et al., 1994). The most advanced Hop2 spermatocytes show short stretches of synapsed regions, mostly between nonhomologous chromosomes (Figure 2B, see below). However, chromosomes appear longer in the cells with maximal synapsis compared to those without it. The extent of nonhomologous synapsis varies from none (Figure 2A) to rather extensive synapsis (Figure 2B, panel III), but does not exceed 33% of the total length of a chromosome complement (Figure 2C). The extent of synapsis in the Hop2-arrested spermatocytes is significantly lower than in Spo11−/− mice (data not shown) or Dmc1−/− mice (Figure 2C). This difference could not be attributed to the different genetic background of the knockout mice, since pairs of littermates from double heterozygote crosses were used in this analysis. With respect to the extent of synapsis, the double Hop2−/−Dmc1−/− knockout mice display the Hop2−/− phenotype (Figure 2C). This indicates an epistatic relationship between these two genes and argues for an earlier role of Hop2 in meiosis compared to Dmc1. At earlier stages, Scp1 protein could be seen on the
Hop2 Promotes Meiotic Interhomolog Synapsis

Figure 1. Hop2 Knockout Mice Show Profound Defects in Gametogenesis
(A) Histological sections of wild-type (left) and knockout (right) testes. Note the reduced size of the seminiferous tubules in the mutant and the lack of cells beyond the primary spermatocyte stage.
(B) Histological sections of wild-type (left) and knockout (right) ovaries. Note the numerous follicles (F) at various development stages and the corpora lutea (CL) in the wild-type ovary. No follicles are present in the knockout ovary, and it is greatly reduced in size.
(C) TUNEL assay for apoptotic cells within the wild-type (left) and knockout (right) seminiferous tubules. Occasional apoptotic cells (arrowheads) are present in wild-type testes, whereas extensive apoptosis is seen in a fraction of the seminiferous tubules of knockout mice (arrows).

axial elements of the asynapsed Hop2 chromosomes (Figure 2B, panels I and II). This staining pattern is unusual for wild-type cells, although a limited number of transverse filaments (Scp1) has been previously reported in association with the partially synapsed chromosome cores in Locusta (Moens, 1969). Most likely, the binding of Scp1 to the axial elements promotes the fast assembly of the SC. This step may be too transient to detect in the normal course of mouse meiosis but is rather obvious in the arrested Hop2 mutant (Figure 2B).

A similar pattern of Scp1 staining could be observed in Dmc1 knockout spermatocytes (data not shown). However, reduced levels of Scp1 are also present on the unpaired regions of X and Y chromosomes in wild-type pachytene spermatocytes (Figure 2B, panel WT). It is possible that a significant accumulation of the Scp1 protein at asynapsed chromosome cores takes place only if synapsis is delayed or impossible.

Careful analysis of the synapsed regions shows that synapsis in Hop2 knockout spermatocytes takes place mostly between nonhomologous chromosomes (Figure 2B, panel III). Some chromosomes are engaged in more than one interaction, leading to the formation of a large chromosomal network. To determine if any of the chromosomes are actually synapsed homologously, we used chromosome painting (Figure 2D). Hybridization of the Hop2 knockout spreads with the chromosome V-specific probe showed no significant alignment in the majority of the cells. In 88% of spermatocytes with a strong hybridization signal, two copies of chromosome V were clearly separated, and in 10% of the cells they partially overlapped (data not shown). Thus, the synapsis in Hop2 knockout spermatocytes is not only decreased, but also nonhomologous.

DSBs Are Formed but not Repaired in Hop2 knockout spermatocytes
Spo11-mediated DSBs initiate meiotic homologous recombination, and the repair of these breaks is required for chromosome synapsis in most organisms including the mouse (Keeney, 2001; Roeder, 1997; Villeneuve and Hillers, 2001; Zickler and Kleckner, 1999). The appearance of the breaks in leptotene is accompanied by the phosphorylation of histone H2ax over megabase-sized chromatin regions (Mahadevaiah et al., 2001). In wild-type mouse spermatocytes, this stage can be easily recognized by anti-\(\gamma\)-H2ax (phosphorylated H2ax) antibodies that produce a cloud-like staining pattern all over the chromatin. The Rad50/Mre11/Nbs1 protein complex processes the breaks in preparation for repair by the
Figure 2. Homologous Chromosomes Fail to Synapse in the Hop2 Knockout

Chromosomal spreads were prepared from adult mouse spermatocytes and immunostained (A and B) with anti-SCP3, anti-SCP1, or CREST antisera, as indicated.

(A) Left: Pachytene wild-type spermatocyte (WT) showing complete synapsis. Right: Arrested Hop2−/− spermatocytes showing no synapsis.

(B) Left column: A wild-type (WT) pachytene spermatocyte. Right columns: Hop2 knockout spermatocytes at different stages after arrest. (I) An early stage with fully developed axial elements but completely asynapsed chromosomes. (II) An intermediate stage. (III) A cell with maximum synapsis. SCP1 staining localizes to the asynapsed chromosome cores at the earlier stages and later relocates to the synapsed regions. Note the nonhomologous nature of the synapsis in panel III.

(C) Chromosomes in Hop2 knockout spermatocytes show less synapsis than in Dmc1 knockout spermatocytes. The cells with the indicated degree of synapsis were counted in populations of 100 randomly picked arrested spermatocytes from Hop2−/−, Dmc1−/−, or Hop2−/− Dmc1−/− mice. Total indicates the number of cells with significant (more than 10%) synapsis. The majority of the scored synaptic events involve nonhomologous chromosomes.

(D) Fluorescent in situ hybridization with a chromosome V-specific probe shows no homologous chromosome alignment in the Hop2 spermatocytes.
homologous recombination machinery. In wild-type spermatocytes, these proteins can be detected all over the nucleus up until early zygotene (Eijpe et al., 2000; and data not shown). By pachytene, Rad50/Mre11/Nbs1 and γH2ax staining remains only in the sex body region (Eijpe et al., 2000; Mahadevaiah et al., 2001). Current models (Allers and Lichten, 2001; Villeneuve and Hillers, 2001) suggest that the major event in meiotic recombination is the Rad51/Dmc1-mediated homology search followed by the single end invasion (Hunter and Kleckner, 2001) of the intact homologous chromosome. Rad51 and Dmc1 are believed to load onto the single-stranded ends of resected DSBs and can be observed as numerous foci on the chromosome cores. Replication Protein A (RPA) is known to assist Rad51 in the strand exchange reaction in vitro (Sigurdsson et al., 2001; Sung and Robberson, 1995). In mouse meiosis, RPA foci form on chromosomal cores shortly after Rad51 and Dmc1 are loaded and colocalizes with them (Moens et al., 2002; Plug et al., 1998). These foci disappear in pachytene, when the breaks are successfully repaired.

Hop2−/− spermatocytes show extensive γH2ax staining that persists to the most advanced stages with the maximum extent of synapsis (Figure 3A). Similarly, Nbs1 staining remains distributed all over the nuclei (Figure 3B). Rad51, Dmc1, and Rpa foci are highly abundant in Hop2−/− spermatocytes and localize to synapsed as well as asynapsed regions (Figures 3C–3H). These results indicate that DSBs are generated in the Hop2 knockout spermatocytes but are not repaired. Furthermore, the presence of the Rad51/Dmc1/Rpa foci also strongly suggests that the DNA ends are already processed and prepared for strand invasion but that in the absence of the Hop2 protein the repair cannot proceed. Thus, the chromosomes have DSBs, and the proper RecA homologs are loaded onto them as if they are primed for and on the cusp of synapsis but can’t initiate this process. These findings suggest that the Hop2 protein might play a heretofore-unrecognized central role in bringing meiotic chromosomes together.

Discussion

The Meiotic Function of the Hop2 Protein

Is Conserved in Yeast and Mammals

Genetic studies in yeast suggested a role for the Hop2 protein in homologous pairing and recombination (Leu et al., 1998; Nabeshima et al., 2001; Shimada et al., 2002; Tsubouchi and Roeder, 2002). In Hop2 knockout male mice, meiosis is arrested at the stage that could be defined as pachytene with respect to the level of chromosome compaction. Axial elements are complete, but no synopsis takes place at the appropriate time: on the 15th day after birth, when 60% of wild-type spermatocytes are in pachytene with complete synopsis, no Hop2 spermatocytes show any degree of synopsis. Arrested spermatocytes eventually accumulate Scp1/Cor1 on asynapsed chromosome cores and undergo nonhomologous synopsis that could reach as much as 33%. Since meiosis in mammals is not synchronized except for the first wave of spermatogenesis in juvenile mice, adult animals have a mixed population of arrested spermatocytes with variable degrees of synopsis. Nonhomologous synopsis seems to be a common feature among yeast mutants with delayed synopsis (Zickler and Kleckner, 1999). The S. cerevisiae hop2 null mutation is rather unique in this regard because of the very high degree (up to wild-type levels) of SC assembly between nonhomologous chromosomes (Leu et al., 1998). We did not, however, observe such a phenomenon in mice. In fact, we see noticeably less synopsis in Hop2 knockout spermatocytes and immunostained with specific antibodies as indicated. See text for discussion.
synapsis in a hop2 mutant is not as striking as was described for later time points (Tsuzuki and Roeder, 2002). Thus, the synaptic defect in Hop2−/− mice is similar to that in budding yeast hop2: SC formation is delayed and aberrant.

Phosphorylation of the H2ax histone (Mahadevaiah et al., 2001) in Hop2 knockout spermatocytes indicates that meiotic DSB formation does not require Hop2 function. However, the repair of the breaks must be postponed, since γH2ax staining persists until the latest stages in arrested spermatocytes. Similarly, since numerous Rad51/Dmc1 foci form and stay along the chromosomal cores, the removal of Spo11 and resection of the DSB ends must not be affected. The accumulation of Rad51/Dmc1 foci in knockout spermatocytes has been shown for a number of genes involved in homologous pairing and recombination (see for example Pittman et al., 1998, for Dmc1−/−; Edelmann et al., 1999, for Msh5−/−; Kneitz et al., 2000, for Msh4−/−; or Yuan et al., 2000, for Scp3−/−). The striking difference in the Hop2 knockout is the very high degree of chromosome compaction combined with the complete lack of even nonhomologous synapsis in a large fraction of cells.

What could be the role of Hop2 that a lack of this protein disrupts meiosis in such a profound and unique manner? In principle, there are several possibilities. First, mouse Hop2 could be a component of the synaptonemal complex. Since our numerous attempts to raise anti-Hop2 antibody suitable for immunofluorescence studies were not fruitful, the localization of the protein in spermatocytes is still unknown. Nevertheless, the following two reasons make the structural role of Hop2 in SC formation highly unlikely: (1) in S. cerevisiae, the protein localizes to the meiotic chromatin, rather than chromosome cores, as would be expected for an SC component; and (2) the meu13 (hop2) mutation results in a recombination deficient phenotype even in S. pombe, where SC does not form in the normal course of meiosis. Second, Hop2 could be involved in HR indirectly, by regulating the expression of gene(s) involved in homologous pairing or recombination. The function of Hop2 as a transcriptional modulator has been reported (Ijichi et al., 2000; Ko et al., 2002; Tanaka et al., 1997). However, as discussed in the next section, the phenotype of the Hop2−/− mice could not be easily explained by the absence of this activity. Thus, we favor the third possibility, that Hop2 is directly involved in meiotic homologous recombination (see below).

The Meiotic Phenotype of the Hop2−/− Mice Is Consistent with a Failure in Recombination

The human counterpart of the Hop2 protein, GT198, was found to interact with a number of nuclear hormone receptors and to stimulate the transcription mediated by some of these receptors (Ko et al., 2002). However, mice with targeted disruptions of nuclear receptor genes show phenotypes that are very different from that of the Hop2−/− mice (Cooke and Saunders, 2002; Couse and Korach, 1998; Gothe et al., 1999; Reichardt et al., 1998). This indicates that there is a different basis for the fertility defects in the Hop2 knockout. So far, we have been unable to discern any somatic defects in the Hop2 knockout mice. In addition, the meiotic defects of the Hop2 disruption in unicellular organisms like yeast are similar to the mouse knockout phenotype (see Results and Discussion). Thus, the meiosis-specific phenotype observed in the Hop2 knockout mouse is most likely the consequence of a lack of a recombinational Hop2 function rather than its role as a transcription factor. Curiously, the expression of GT198 was reported to be restricted to the tissues with developmentally regulated recombination events (primarily testis, but also thymus, spleen, and ovaries) (Ko et al., 2002).

Essential Role of Hop2 in Meiotic Recombination

As opposed to only one RecA protein in bacteria, unicellular eukaryotes have four RecA homologs (Rad51, Rad55, Rad57, and Dmc1 in S. cerevisiae) and mammals have seven (Rad51, Rad51B, Rad51C, Rad51D, Xrc2, Xrc5, and Dmc1 proteins). Still, E. coli RecA remains the most robust recombinase in the family, whereas its eukaryotic homologs either do not possess canonical recombinational activities in vitro or rely on a number of accessory proteins for their function (Sung et al., 2000; Symington, 2002). Most of these factors act in mitotic as well as meiotic recombination. The meiosis-specific RecA homolog, Dmc1 protein, is a rather inefficient recombinase, and no proteins so far have been found to stimulate its activity in vitro (Hong et al., 2001; Li et al., 1997; Masson et al., 1999). Rad51 protein requires RPA, Rad52, and Rad55/Rad57 proteins to carry out efficient strand exchange, whereas for D-loop formation, there is an additional requirement for the Rad54 or Radh54 proteins (reviewed in Raoul Tan et al., 2003; Sung et al., 2000). Whereas single deletions of these last two proteins in yeast cause only mild sporulation defects (Symington, 2002), sporulation in the rad54/radh54 double mutants is severely impeded (Klein, 1997; Shinohara et al., 1997). On the other hand, single knockouts of both mouse Rad54 homologs (Rad54 and Rad54B) as well as the double Rad54/Rad54B knockout mice are not sterile (Essers et al., 1997; and R. Kanaar, personal communication). Since neither Dmc1 nor Rad51 can promote efficient strand invasion by itself, this implies that there might be additional factor(s) playing a critical role in meiotic recombination in mammals. Rad52 protein is the only of the Rad51 partners shown to promote D-loop formation on its own (Kagawa et al., 2001). Nevertheless, while rad52 deletion yeast produce inviable spores (Game et al., 1980; Prakash et al., 1980), the knockout mice are fertile (Rijkers et al., 1998). This again suggests a missing essential player in mammalian meiotic recombination. We propose that Hop2 is the heretofore-unrecognized recombinase that either initiates the Rad51- and/or Dmc1-dependent heteroduplex formation by promoting single strand invasion or is an accessory factor for one or both these proteins.

Rad51 and Dmc1 form overlapping foci on the late leptotene-zygotene chromosome cores and are believed to act at the same stage of meiosis. We propose that Hop2 acts upstream of Rad51 and Dmc1 for the following reasons. First, epistatic analysis suggests an earlier role of Hop2 protein compared to Dmc1 (Figure 2C). A Rad51 deletion is lethal in mice (Tsuzuki et al., 1996), but both S. cerevisiae rad51 and dmc1 mutants have a less severe meiotic defect than a hop2 mutant.
Thus, in mammals DSB-independent pairing should occur in the same time frame as meiotic DSB repair. If Hop2 is involved in such interactions, this activity could coincide with the recombinational function of the protein described above. It would be interesting to see if Hop2 alone or in combination with other proteins could promote duplex-duplex pairing interactions that would contribute to the pairing process in the absence of DSBs.

Based on our findings and data from studies in yeast, we propose the following model for Hop2 function in meiosis (Figure 4). The protein can bind to chromosomes regardless of meiotic DSB formation. In the normal course of meiosis Hop2 binds to chromosomes before the loading of Rad51/Dmc1 proteins onto the single-stranded tails of the resected breaks (Leu et al., 1998; Tsubouchi and Roeder, 2002). Since there is an indication for a role of Hop2 in DSB-independent homologous pairing (Leu et al., 1998; Nabeshima et al., 2001), the protein could ensure such associations by homologous duplex-duplex interactions or otherwise. Regardless of this, Hop2 may promote strand invasion of the intact chromosome by a broken homolog. This initial interaction could be stabilized by strand exchange promoted by Rad51/Dmc1 and other proteins required for formation and further processing of heteroduplexes.

**Experimental Procedures**

**Generation and Characterization of the Knockout Mice**

The targeting construct was based on the ploxPneo vector (Yang et al., 1998) (a gift from Dr. Chu-Xia Deng). A 5.7 kb BsaBI/NotI fragment containing the 5’ region of the mouse Hop2 gene was cloned in the ploxPneo plasmid between the TK and neo genes in an opposite orientation to the neo gene. A 6.9 kb PacI/Eco47III fragment containing introns 4–8 of the Hop2 gene was cloned on the other side of the neo cassette. The plasmid was linearized and transfected into 1.8 × 10⁶ TC1 ES cells (Deng et al., 1998) (a gift from Dr. Chu-Xia Deng) by electroporation. The cells were seeded onto mitomycin C-treated mouse embryonic feeder cells (Incyte Genomics), and the colonies were selected in media containing LI F (GIBCO-BRL), 350 μg/ml G418, and 5 μM gancyclovir. Colonies were picked and expanded, and genomic DNA was extracted for Southern blot analysis. The DNA was digested with EcoRI and XhoI restriction enzymes and hybridized with either 5’ or 3’ probes (see Supplemental Figure S1A at http://www.developmentalcell.com/cgi/content/full/5/6/927/DC1). Cells from one of the correctly targeted ES clones were injected into C57/B6 blastocysts to obtain germline transmission. For genotyping the tips of the tails, 1–1.5 mm was incubated in 200 μl of lysis buffer (20 mM TrisHCl [pH 8.0], 2.5 mM MgCl₂, 50 mM KCl, 0.45% NP-40, 0.45% Tween 20, and 1.2 mg/ml Proteinase K) for at least 4 hr. The protease was inactivated for 15 min at 95°C, and 1 μl of the DNA mixture was used for further analysis. The genotyping was done by PCR using primers 1 (5’-CCGTCATTTCTGGGCGTCAAATG) and 2 (5’-CTAGTACGGGCTGTCGAAGATCG) to amplify the wild-type allele (a 410 bp fragment) and primers 1 and 3 (5’-CAGGCTTCCTTCTCCCACTCATGATC) to amplify the knockout allele (a 770 bp fragment). The Advantage 2 DNA Polymerase Mix (Clontech) was used in 20 μl reactions. Tissues for histological examination were removed and fixed overnight in 10% neutral-buffered formalin (Sigma). Tissues were embedded in paraffin and 6 μm sections were cut (American Histolabs). Gross and histopathologic diagnoses of the knockout mice were carried out by the NIH ORS/VRP Mouse Phenotyping Service.

**Antibodies**

Anti-mHop2 rabbit polyclonal antibodies were raised against the full-length protein or the 40 amino acid C-terminal peptide. Anti-Scp3 mouse polyclonal antibodies were raised against a GST fusion of the full-length rat Scp3 protein. Commercial primary antibodies...
against γH2AX (Trevigen), Rad51, and Dmc1 (Santa Cruz Biotechnology, Heidelberg, and C-20, respectively) were used for immunofluorescence. Rabbit anti-Rpa was a gift from P. Moens (Moens et al., 2002), mouse anti-Scp1 antibodies was a gift from C. Heyting (Eijpe et al., 2003), rabbit anti-Scp1 antibody was a gift from C. Hoog (Liu et al., 1996), anti-Nbs1 antibody was a gift from J. Petriti (Williams et al., 2002), and CREST antiserum was a gift from B. Brinkley (Brenner et al., 1981). Secondary antibodies were from Jackson IR laboratories. The primary antibodies were used at the following dilutions: anti-Hop2, 1:5000 (Westerns); anti-SCP3, 1:800; anti-γH2AX, 1:100; anti-Rad51, 1:50; anti-Dmc1, 1:100; anti-Rpa, 1:200; mouse anti-Scp1, 1:20; rabbit anti-Scp1, 1:100; anti-Nbs1, 1:100; and CREST antiserum, 1:500. Rad51 and Dmc1 antibodies did not cross-react on a Western blot prepared from testis extracts (1:100–1:200 dilutions). Though unlikely, we cannot, however, exclude the possibility that the Rad51 antibody could recognize Dmc1 protein on the chromosomal spreads. Since the anti-Dmc1 antibody did not generate a signal in the Dmc1 knockout mice, the Dmc1 antibodies can be considered specific.

Meiotic Chromosome Spreads and Immunofluorescence

The protocol was modified from the procedure described by Moens and coauthors (Moens et al., 1998). One wild-type testis was dissected and the capsule was removed. Seminiferous tubules were finely chopped with a scalpel in a Petri dish containing 10 ml of RPMI 1640 high-glucose media ( Gibco-BRL). The cells were released from the tubules by gently pipetting in and out and filtered through a 40 μm cell strainer (Falcon). The suspension was centrifuged for 8 min at 800 × g, and the cells were resuspended in 10 ml of RPMI and pelleted again. The resulting pellet was resuspended in 1 ml of 0.5% NaCl after any residual media had been removed. 15 μl of the cell suspension was added to the glass slides with hydrophobic rings (Becton Dickinson), and the cells were allowed to settle for 10–15 min. The slides were fixed on a Western blot prepared from testis extracts (1:100–1:200 dilutions). Though unlikely, we cannot, however, exclude the possibility that the Rad51 antibody could recognize Dmc1 protein on the chromosomal spreads. Since the anti-Dmc1 antibody did not generate a signal in the Dmc1 knockout mice, the Dmc1 antibodies can be considered specific.

For chromosome painting, the cell pellet was prepared as described above and the cells were resuspended in 10 ml of 0.075 M KCl. The suspension was incubated at 37°C for 15 min followed by addition of a few drops of freshly prepared fixative (methanol/glacial acetic acid, 3:1). The cells were pelleted, resuspended in 10 ml of fixative solution, and pelleted again. The last step was repeated once more time and the resulting pellet was resuspended in 1 ml of fixative solution. 10 μl of the suspension was dropped on the glass slides and let air dry.

For immunostaining, the slides were incubated with blocking solution (10% goat or donkey serum, 3% BSA, and 0.05% Triton X-100 in PBS) for 1 hr at room temperature. Primary antibodies were diluted in blocking buffer and incubated under the same conditions for 1–2 hr. After 2 min washes in 0.4% Photo-Flo/PBS solution, slides were blocked for an additional 5 min and incubated with secondary antibodies for 20 min at 37°C. The slides were washed twice with 0.4% Photo-Flo in PBS, rinsed twice with 0.4% Photo-Flo, and allowed to air dry. Vectashield Mounting Medium with DAPI (Vector Laboratories) was added and the slides were covered with cover slips and viewed with a Leica fluorescence microscope. The images were captured at 1000× magnification with OpenLab software and processed using Adobe Photoshop.

Chromosome Painting

The chromosome V-specific probe was purchased from Applied Genetics Laboratories, Inc., and hybridization was carried out according to the protocol provided.

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