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Regulation of meiotic cohesion and chromosome core morphogenesis during pachytene in *Drosophila* oocytes

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Summary
During meiosis, cohesion between sister chromatids is required for normal levels of homologous recombination, maintenance of chiasmata and accurate chromosome segregation during both divisions. In *Drosophila*, null mutations in the ord gene abolish meiotic cohesion, although how ORD protein promotes cohesion has remained elusive. We show that SMC subunits of the cohesin complex colocalize with ORD at centromeres of ovarian germ-line cells. In addition, cohesin SMCs and ORD are visible along the length of meiotic chromosomes during pachytene and remain associated with chromosome cores following DNase I digestion. In flies lacking ORD activity, cohesin SMCs fail to accumulate at oocyte centromeres. Although SMC1 and SMC3 localization along chromosome cores appears normal during early pachytene in *ord* mutant oocytes, the cores disassemble as meiosis progresses. These data suggest that cohesin loading and/or accumulation at centromeres versus arms is under differential control during *Drosophila* meiosis. Our experiments also reveal that the α-kleisin C(2)M is required for the assembly of chromosome cores during pachytene but is not involved in recruitment of cohesin SMCs to the centromeres. We present a model for how chromosome cores are assembled during *Drosophila* meiosis and the role of ORD in meiotic cohesion, chromosome core maintenance and homologous recombination.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/120/17/3123/DC1

Key words: Meiosis, Cohesin, Synaptonemal complex, Recombination

Introduction
Accurate segregation of chromosomes during meiosis relies on a number of dynamic changes in chromosome morphology that take place within the context of sister-chromatid cohesion. Meiotic cohesion is not only required for the correct segregation of sisters during the second meiotic division, but also ensures that recombinant homologous chromosomes remain physically associated until anaphase I (Bickel et al., 2002; Buonomo et al., 2003; Hodges et al., 2005; Siomos et al., 2001). In addition, arm and centromeric cohesion must be regulated differently during meiosis. When the release of arm cohesion during meiosis I allows the segregation of homologues, centromeric cohesion must be protected and remain intact until anaphase II when sisters segregate to opposite poles (Kerrebrock et al., 1992; Kerrebrock et al., 1995; McGuinness et al., 2005; Wang and Dai, 2005; Watanabe and Kitajima, 2005).

Cohesion between meiotic sister chromatids plays an essential role in assembly of the synaptonemal complex (SC), a tripartite proteinaceous structure that forms between homologous chromosomes during prophase I (Cai et al., 2003; Klein et al., 1999; Pasierbek et al., 2001; Webber et al., 2004). During early prophase I, each pair of sister chromatids undergoes shortening along their longitudinal axes, resulting in the formation of ‘chromosome cores’ upon which the axial/lateral elements (AEs/LEs) of the SC assemble (Revenkova and Jessberger, 2006; Stack and Anderson, 2001). During pachytene, SC central element proteins join each set of homologous AEs/LEs along their entire length resulting in synopsis of homologues. In many species (yeast, mice, Arabidopsis), meiotic double-strand breaks (DSBs) are essential for homologue synapsis (Page and Hawley, 2003); however, chromosome core formation (axial shortening) does not depend on DSBs (Bhuiyan and Schmekel, 2004; James et al., 2002; Romanienko and Camerini-Otero, 2000). In addition, mutants that lack AE/LE components can still build chromosome cores (Couteau et al., 2004; Pelttari et al., 2001). Crossovers between homologous chromosomes, in conjunction with sister chromatid cohesion, are essential for correct chromosome segregation during meiosis I. In most organisms, recombination between homologues takes place in the context of the SC (Page and Hawley, 2003). Although EM studies indicate that the ultrastructure of the SC is highly conserved, SC components in different organisms show surprisingly little sequence homology (Page and Hawley, 2003). During both mitosis and meiosis, sister-chromatid cohesion is mediated by an evolutionarily conserved protein complex called cohesin that contains two SMC (structural maintenance of chromosomes) and two non-SMC subunits (Lee and Orr-Weaver, 2001; Petronczki et al., 2003). The α-kleisin subunit (Scc1/Mcd1/Rad21) bridges the two head domains of the SMC1-SMC3 dimer and thereby forms a ring that entraps DNA (Nasmyth, 2002; Shintomi and Hirano, 2007). Several meiosis-
specific cohesin subunits have been identified, including the α-kleisin Rec8, which has been shown to be crucial for meiotic cohesion and SC formation in all organisms examined (Klein et al., 1999; Molnar et al., 1995; Pasierbek et al., 2001; Petronczki et al., 2003).

In *Drosophila*, four cohesin subunits have been uncovered through sequence analysis (Adams et al., 2000; Hong and Genetzk, 1996; Warren et al., 2000a) and the localization and function of mitotic cohesin has been examined in *Drosophila* embryos and tissue culture cells (Valdeolmillos et al., 2004; Vass et al., 2003; Warren et al., 2000a; Warren et al., 2000b). However, little is known about the localization and dynamics of the cohesin complex during *Drosophila* meiosis.

*Drosophila* oogenesis is an excellent system to study meiosis, as each *Drosophila* ovary is composed of approximately 10-30 ovarioles (Fig. 1A) that contain a linear array of oocytes at progressive developmental stages from mitotic germ-line stem cells to metaphase-I-arrested oocytes (King, 1970). Meiosis initiates in the gerarium, the most anterior structure of each ovarirole (see Fig. 1A,B). Germ-line stem cells in region 1 of the gerarium (Fig. 1B) undergo four rounds of synchronous mitotic divisions resulting in 16 interconnected cells that comprise a ‘cyst’ (Spradling, 1993). As cysts mature, they move toward the posterior end of the ovariole. All germ cells within a 16-cell cyst undergo premeiotic S phase synchronously and prophase I of meiosis initiates in gerarial region 2A where up to four cells per cyst initiate SC assembly (Fig. 1B). In addition, meiotic DSBs are induced in region 2A (Jang et al., 2003; Mehrotra and McKim, 2006), but unlike several other organisms, synapsis in *Drosophila* does not depend on DSBs (McKim and Hayashi-Hagihara, 1998). As each cyst moves through the gerarium,

![Fig. 1. Cohesin SMC localization during early oogenesis.](image)

(A) Diagram of a single ovarirole with the youngest stage at the top. Each ovarirole contains ‘cysts’ composed of 16 interconnected germ cells, one of which is the oocyte (red). Meiosis initiates at the anterior tip of the ovarirole in the gerarium. The remainder of the ovarirole is called the vitellarium. As cysts progress through oogenesis, they move toward the posterior end of the ovariole. In stage 14, the oldest egg chamber in an ovarirole, the oocyte is arrested at metaphase I. Passage through the oviduct triggers the resumption of the meiotic divisions. (B) The gerarium is made up of four regions: region 1, region 2A, region 2B and region 3 at the posterior end. Individual cysts are depicted in blue. On the far right is a diagram showing the assembly of SC (red) in a subset of cells within region 2A cysts. As cysts mature and move to the posterior end through the gerarium, the SC becomes restricted to the oocyte. (C) Bright foci as well as diffuse SMC1 signal (green) is visible within region 1. The fusome localization pattern (white) suggests this is either an 8-cell cyst or an early 16-cell cyst. Bar, 4 μm. (D) Simultaneous staining with antibodies against SMC1 and SMC3 shows localization of cohesin SMCs (green) coincident with the SC protein C(3)G (magenta) in different regions of the gerarium. In region 2A, two cysts are visible, with two to three cells per cyst containing thread-like SMC1/3 signal (arrow). In region 2B, SMC1/3 threads are restricted to two nuclei per cyst, and by region 3 long stretches of SMC1/3 signal are visible only within the oocyte. Bar, 5 μm. All panels represent projections of deconvolved Z-series using whole-mount gerarium.
the SC breaks down in all but one nucleus so that, by region 3, full-length SC is restricted to the oocyte, which lies at the posterior end of the rounded cyst (Fig. 1B). As cysts continue to grow and mature, they leave the gerarium and move into the ‘vitellarium’ (King et al., 1956). The oocyte remains in pachytene with full-length SC until vitellarial stage 6 (Carpenter, 1975; Page and Hawley, 2001); however, the remaining 15 cells within each cyst adopt a nurse cell fate and enter an endo cell cycle, during which multiple rounds of S phase in the absence of intervening M phase results in polyploid cells (Dej and Spradling, 1999).

Meiotic cohesion in Drosophila depends on the novel protein, Orientation Disruptor (ORD) (Bickel et al., 1996; Bickel et al., 1997; Mason, 1976; Miyazaki and Orr-Weaver, 1992). In mutants lacking ORD function, sister-chromatids segregate randomly through both meiotic divisions, consistent with complete absence of meiotic cohesion (Bickel et al., 1997). In addition, homologous recombination is severely reduced in ordnull females and SC assembly and maintenance are disrupted (Bickel et al., 1997; Webber et al., 2004). Immunolocalization studies have demonstrated that ORD is enriched at the centrromeres of meiotic chromosomes in both males and females (Balicky et al., 2002; Webber et al., 2004). In addition, ORD localization along the arms of female meiotic chromosomes coincides with that of the SC protein, C(3)G (Webber et al., 2004).

Here, we investigate the localization and dynamics of two cohesin subunits (dSMC1 and Cap/dSMC3) during early prophase I in Drosophila oogenesis. SMC1 and SMC3 localize along the arms and are enriched at the centrromeres of all 16 cells within each germ-line cyst. In nuclei that build SC, cohesin subunits coalesce into chromosome cores that provide the scaffold for SC assembly. We find that formation of chromosome cores depends on the α-kleisin C(2)M, and that the cohesion protein ORD is essential for cohesin loading at centrromeres and for maintenance of chromosome cores. Our data support the argument that during meiosis, the establishment of centrromeric cohesion is regulated differently than on the arms. Moreover, our results provide insight into the interconnected roles of meiotic cohesion, chromosome cores and homologous recombination.

Results

Accumulation of cohesin SMCs on chromosomes of pre-meiotic cells

To analyze the behavior of cohesin subunits during meiotic progression in the Drosophila ovary, we generated antibodies against Drosophila SMC1 and SMC3 peptides. Following affinity-purification, SMC1 and SMC3 antibodies each recognize a single predominant band at the predicted molecular mass in embryo extracts and a doublet/triplet in ovary extracts (see supplementary material Fig. S1a). In addition, when germ-line clones are generated that are homozygous for the smc1 excision allele, smc1exc46 (Dorsett et al., 2005), no SMC1 signal above background is observed (see supplementary material Fig. S1b). Because affinity-purified SMC1 and SMC3 antibodies display very similar staining patterns in Drosophila ovaries (see Fig. 6), they were combined for most of the experiments described below to maximize signal intensity (here referred to as SMC1/3 or SMC).

Fixation and staining of intact ovarioles (whole-mount preparations) revealed several distinct cohesin SMC staining patterns within the gerarium of wild-type females; multiple regions of each gerarium contained bright foci, as well as diffuse staining and nuclei with a thread-like SMC signal (see supplementary material Fig. S2). Although we do not detect cohesin SMC staining in gerarial stem cells and early cystoblasts in region 1 (see supplementary material Fig. S2, asterisk), bright foci as well as diffuse SMC localization are visible in the nuclei of germ-line cysts within region 1 (Fig. 1C). The bright SMC foci correspond to centrromeres as confirmed by co-staining with CID (see below, Fig. 4), a centrromere-specific histone H3 variant (Blower and Karpen, 2001; Henikoff et al., 2000). The diffuse staining in pre-meiotic cells most probably corresponds to cohesin localization along chromosome arms. This same localization pattern has been observed for the cohesion protein ORD in germ-line mitotic cysts (Webber et al., 2004).

Thread-like cohesin SMC signal coincides with the SC in pachytene nuclei

As 16-cell cysts enter region 2A of the gerarium, up to four nuclei in each cyst begin to assemble a SC and in these cells, SMC1/3 signal becomes visible as thread-like staining that coincides with the SC marker C(3)G (Fig. 1D). During the maturation of cysts and their progression through the gerarium, thread-like SMC1/3 staining mimics that of the SC (Fig. 1D). As cysts move through the gerarium, continuous linear SMC1/3 staining is visible in the two nuclei that contain full-length SC (pro-oocytes) but the SMC1/3 signal appears fragmented in the other C(3)G containing nuclei that will adopt a nurse cell fate (pro-nurse cells). Oocyte determination is complete by region 3 and, at this stage, the continuous thread-like SMC1/3 staining is restricted to the oocyte (Fig. 1D).

The oocyte nucleus will remain in pachytene for several hours as it progresses through the vitellarium (see Fig. 1A). Electron microscopy has shown that that full-length tripartite SC is present as late as stage 6 of vitellarial development (Carpenter, 1975) and these data have been supported by persistence of continuous threads of C(3)G immunostaining until the same stage (Page and Hawley, 2001). In vitellarial stages 2 to 6, we observe thread-like SMC1/3 staining in whole-mount preparations that is coincident with C(3)G signal. However, similar to C(3)G staining, the thread-like signal becomes weaker in these later stages and is accompanied by increased diffuse nuclear staining (see supplementary material Fig. S3).

Cohesin SMCs and ORD are present along chromosome cores during pachytene

Thread-like signals for cohesin SMCs as well as the cohesion protein ORD are restricted to germ-line cells that form SC (Fig. 1D) (Webber et al., 2004). One possibility is that, together, these proteins contribute to the proteinaceous ‘chromosome core’ that has been proposed to serve as a scaffold for SC formation (Revenkova and Jessberger, 2006; Stack and Anderson, 2001). If the cohesin complex and ORD are indeed part of the chromosome core, they should persist in the absence of DNA loops in SC-containing nuclei (Pearlman et al., 1992; Smith and Roeder, 1997). To test this hypothesis, we prepared chromosome spreads of germarial cells to visualize proteins bound to meiotic chromosomes. In
this procedure, soluble components are washed away (see supplementary material Fig. S4), leaving only chromosomes and their associated proteins attached to the slide. DNase I treatment of chromosome spread slides resulted in loss of histone and DAPI staining, confirming that DNA loops had been digested (Fig. 2). However, the thread-like SMC1/3 and ORD staining persisted in the absence of DNA loops, consistent with the model that ORD and the cohesin complex are components of the cores of meiotic chromosomes in SC-forming nuclei (Fig. 2).

Chromosome spread experiments also revealed that cohesin SMCs are associated with chromosome arms in all 16 nuclei of each germ-line cyst (Fig. 3A). However, in nuclei that do not build a SC, the SMC localization pattern is diffuse rather than thread-like (Fig. 3A, open arrows). In addition, we observed that during SC disassembly in non-oocyte nuclei, cohesin SMCs remain associated with chromosome arms and their staining pattern is indistinguishable from other pro-nurse cells (Fig. 3B). Association of ORD with chromosome arms in a pattern similar to SMC1/3 has been described previously (Webber et al., 2004). Confirmation that soluble nuclear proteins are removed during the spread preparation is shown in supplementary material Fig. S4. When transgenic flies expressing GFP-nls were used to generate spreads, diffuse SMC1 staining was visible in several nuclei but no corresponding GFP signal was detected (see supplementary material Fig. S4). Therefore, we conclude that the diffuse SMC1/3 staining we observe in chromosome spreads represents cohesin SMCs stably associated with the chromatin. These data support the model that cohesin SMCs are components of the cores of meiotic chromosomes (see supplementary material Fig. S4). Therefore, we conclude that the diffuse SMC1/3 staining we observe in chromosome spreads represents cohesin SMCs stably associated with the chromatin. These data support the model that cohesin SMCs are components of the cores of meiotic chromosomes.

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Enrichment of cohesin SMCs at the centromeres of meiotic chromosomes

In addition to the thread-like staining pattern in pachytene cells, cohesin SMCs are enriched at the centromeres of wild-type meiotic chromosomes (Fig. 4, top panels) as confirmed by colocalization of the SMC1/3 foci with CID, the centromere specific histone H3 variant (Blower et al., 2002; Henikoff et al., 2000). The centromeres of Drosophila chromosomes are usually clustered together into a single chromocenter (Carpenter, 1975) and each bright focus of SMC1/3 and ORD staining that is visible in the nuclei of whole-mount preparations corresponds to the chromocenter (Fig. 1D). In chromosome spreads, we frequently observe the chromocenter split into two or more regions. Interestingly, the increased resolution afforded by spread preparations indicates that the bright SMC1/3 signal at centromeres often extends beyond the area of CID staining (Fig. 4, see inset). Cohesin SMCs exhibit the same extensive centromeric localization pattern as ORD (Webber et al., 2004), consistent with enrichment of these cohesion proteins within pericentric as well as centromeric heterochromatin. Robust SMC1/3 and ORD signals in the vicinity of the centromere are not restricted to nuclei that build SC. Instead, centromeric enrichment of these proteins is visible in all 16 cells of germarial cysts whether they adopt a nurse cell or oocyte fate (Fig. 3 solid arrows) (Webber et al., 2004).

As egg chambers progress into the vitellarium, the SMC1/3 signal associated with the chromocenter in nurse cells begins to assume a very distinctive pattern that resembles a cluster of finger-like projections (Fig. 5 top, inset). Interestingly, the onset of this staining pattern coincides with the beginning of the endo-reduplication cell cycle in nurse cells, during which DNA replication occurs repeatedly in the absence of cell division. We observe these SMC1/3 finger-like projections during early vitellarial stages when the polytene nurse cell chromosomes exhibit polyteny, the precise alignment of multiple copies of sister chromatids (King et al., 1981). Unlike polytenic chromosomes in the Drosophila salivary gland, nurse cell polytenic chromosomes are short-lived. Around vitellarial stage 4, nurse cell chromosomes undergo a dramatic morphological change and no longer exhibit polyteny (Dej and Spradling, 1999). Although the SMC1/3 signal remains enriched at the pericentric heterochromatin as nurse cell chromosomes transition out of polyteny, the pattern becomes more diffuse and less structured in these later stages (data not shown).

ORD is required for centromeric localization of cohesin SMCs during meiosis

ORD protein is necessary for both arm and centromeric cohesion during Drosophila meiosis (Bickel et al., 1996; Bickel et al., 1997; Mason, 1976). In mutant flies lacking ORD activity, chromosomes segregate randomly through both meiotic divisions, indicating that cohesion is completely absent (Bickel et al., 1996; Bickel et al., 1997; Mason, 1976). The localization pattern of ORD protein during early oogenesis (Webber et al., 2004) closely mimics that of the cohesin SMC proteins. One possibility is that ORD controls the localization and/or function of the cohesin complex during meiosis. To study the localization dynamics of the cohesin complex in the absence of ORD, ovaries from ord<sup>5</sup>/Df (ord<sup>null</sup>) females were examined. The ord<sup>5</sup> mutation results in
premature truncation of the ORD open reading frame and genetically behaves like a null-allele (Bickel et al., 1996; Bickel et al., 1997).

When whole-mount ordnull germaria are stained with SMC1 and SMC3 antibodies, bright centromeric foci are conspicuously absent throughout the germaria even though continuous thread-like staining is visible in region 2A (Fig. 6 and supplementary material Figs S5 and S6). Within the germarium, SMC1/3 foci are undetectable in cells that form SC as well as the remaining cells of each cyst (see supplementary material Figs S5 and S6). ordnull oocytes also lack SMC1/3 centromeric foci after they exit the germarium (Fig. 5 bottom, open arrow). These data suggest that ORD is essential for normal accumulation of cohesin at oocyte centromeres and are consistent with the chromosome segregation defects observed in mutant flies (Bickel et al., 1996; Bickel et al., 1997; Miyazaki and Orr-Weaver, 1992). Absence of cohesin SMC localization at centromeres in the ord mutant was confirmed when chromosome spreads were immunostained for SMC1/3 and the centromere marker CID (Fig. 4). Cohesin SMCs do not colocalize with CID foci in ordnull germaria; the CID signal corresponds to gaps in the thread-like SMC1/3 signal (Fig. 4, bottom panels). These data suggest that ORD activity is required for loading and/or accumulation of centromeric cohesin during female meiosis.

Interestingly, although centromeric SMC1/3 staining is never visible in oocytes of ordnull flies, a distinct centromeric staining pattern becomes detectable in nurse cells as cysts progress into the vitellarium. Even in the absence of ORD, we observe finger-like projections of SMC1/3 staining in the vicinity of nurse cell centromeres in ordnull mutant egg chambers by vitellarial stage 3, presumably when polytene chromosomes are present (Fig. 5 bottom, solid arrow). Like wild type, this SMC1/3 staining becomes diffuse at later stages when polytene is absent. These data argue that loading of cohesin subunits onto centromeres is controlled differently in oocytes and nurse cells, and once germ-line cells adopt a nurse cell fate, accumulation of cohesin at centromeres is no longer dependent on ORD function.

ORD is necessary for maintenance of chromosome cores during early meiosis

Despite the centromeric defects that we observe in ordnull germaria, thread-like SMC1 and SMC3 staining along chromosome cores appear relatively normal during early pachytene even in the absence of ORD activity (Fig. 6). In early
region 2A (the anterior portion of region 2A), long continuous threads of SMC1 and SMC3 are visible in ordnull germaria, although SMC staining along cores is weaker than in wild type (Fig. 6, Tables 1, 2). However, both the intensity and integrity of cohesin thread-like staining deteriorates progressively as cysts mature and travel through the germarium (Fig. 6). A gradual loss of thread-like C(3)G staining as cysts mature has also been observed in ordnull germaria (Webber et al., 2004).

To characterize the progressive deterioration of SMC1 and SMC3 thread-like staining in ordnull germaria, we scored the integrity and intensity of the threads in different regions of wild-type and mutant germaria (Tables 1 and 2). Careful analysis of the defects in several mutant germaria indicated that, by late region 2A (the posterior portion of region 2A), the intensity of the SMC1 and SMC3 thread-like staining was significantly reduced and fragmented threads were visible in a number of cells (Tables 1 and 2). For example, in late region 2A, a pronounced reduction in SMC1 signal intensity was observed in 43% of ordnull cysts but only 3% of wild-type cysts (Table 1). In older mutant cysts, loss of the thread-like SMC1 and SMC3 staining became more prominent (Fig. 6). By region 3, no mutant oocyte nuclei exhibited robust continuous thread-like SMC1 or SMC3 staining (Table 1, Fig. 6). SMC3 signal was undetectable in approximately 52% of region 3 ordnull oocyte nuclei and about 30% had short dim fragments (Table 2, Fig. 6). Interestingly, the anti-SMC1 and anti-SMC3 antibodies appear to have different affinities for their respective antigens in wild-type nuclei; SMC1 signal along chromosome cores was consistently more robust than that for SMC3 (Tables 1, 2). This difference may reflect variation in epitope accessibility for the two proteins and is most likely the cause for quantitative differences in the defects observed for SMC1 and SMC3 in mutant germaria (Tables 1, 2). However, deterioration of the thread-like signal followed the same trend for both proteins and reinforces the conclusion that ORD activity is required to maintain chromosome cores during early pachytene.

ORD is not required for stable association of cohesin SMCs with chromosome arms during pachytene. The loss of thread-like staining in whole-mount preparations of ordnull germaria initially suggested to us that cohesin...
dissociates from chromosome arms during pachytene in the absence of ORD activity, consistent with the essential role of ORD in arm cohesion. However, we reasoned that it was also possible that cohesin SMCs might remain associated with chromatid arms in the absence of ORD, but loss of thread-like staining might occur because the longitudinal compaction of meiotic chromosome cores depends on ORD function. If cohesin SMCs remain associated with chromosome arms during pachytene in \textit{ordnull} females but chromosome cores are unstable, the thread-like SMC1/3 signal would disappear. However, it is difficult to detect diffuse localization of cohesin SMCs along chromosome arms in whole-mount preparations. Therefore, we prepared chromosome spreads from \textit{ordnull} germaria and immunostained for SMC1/3 and C(3)G proteins (Fig. 7). Because in spread preparations, the temporal arrangement of individual cysts within each germaria is not maintained, we searched for semi-intact cysts that contained a maximum of one or two nuclei with C(3)G staining, reasoning that these most probably represent region 2B cysts. At this stage in \textit{ordnull} germaria, C(3)G thread-like signal has begun to fragment (Webber et al., 2004). As shown in Fig. 7, we found that diffuse SMC1/3 staining is readily evident in nuclei that also contain fragmented C(3)G and SMC1/3 threads. Moreover, the intensity of diffuse SMC1/3 signal in these nuclei is very similar to that of adjacent pro-nurse cell nuclei (Fig. 7, see circles). Because soluble nuclear protein is removed during the spread preparation (supplementary material Fig. S4), the diffuse SMC1/3 signal that we observe represents cohesin subunits that are associated with the chromatin but not organized into chromosome cores. These data argue that, in the absence of ORD activity, chromosome cores disassemble but cohesin SMCs remain associated with chromosome arms.

**Table 1. SMC1 localization in WT and \textit{ord} germaria**

<table>
<thead>
<tr>
<th>Localization pattern (%)*</th>
<th>Early region 2A (n=35)</th>
<th>Late region 2A (n=34)</th>
<th>Region 2B (n=19)</th>
<th>Region 3 (n=35)</th>
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<tr>
<td>Thread-like†</td>
<td>85.7</td>
<td>97.1</td>
<td>94.4</td>
<td>91.4</td>
</tr>
<tr>
<td>Dim threads</td>
<td>14.3</td>
<td>2.9</td>
<td>5.6</td>
<td>8.6</td>
</tr>
<tr>
<td>Fragments‡</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Blank§</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

\textit{ord null}¶ cysts

<table>
<thead>
<tr>
<th>Localization pattern (%)*</th>
<th>Early region 2A (n=64)</th>
<th>Late region 2A (n=63)</th>
<th>Region 2B (n=54)</th>
<th>Region 3 (n=64)</th>
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<tr>
<td>Thread-like†</td>
<td>70.3</td>
<td>42.6</td>
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<tr>
<td>Dim threads</td>
<td>29.7</td>
<td>42.6</td>
<td>22.2</td>
<td>1.6</td>
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<tr>
<td>Fragments‡</td>
<td>0.0</td>
<td>14.8</td>
<td>72.2</td>
<td>53.2</td>
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<tr>
<td>Blank§</td>
<td>0.0</td>
<td>0.0</td>
<td>5.6</td>
<td>45.2</td>
</tr>
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</table>

*Cysts exhibiting each localization pattern (in %). †Thread-like denotes long continuous staining. ‡Values include fragmented and/or punctate signal. §No visible staining. ¶\textit{ord5/Df} flies completely lack ORD activity.

**Table 2. SMC3 localization in WT and \textit{ord} germaria**

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<th>Localization pattern (%)*</th>
<th>Early region 2A (n=37)</th>
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<th>Region 2B (n=27)</th>
<th>Region 3 (n=36)</th>
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<tr>
<td>Thread-like†</td>
<td>40.0</td>
<td>40.5</td>
<td>3.6</td>
<td>42.9</td>
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<tr>
<td>Dim threads</td>
<td>60.0</td>
<td>59.5</td>
<td>82.1</td>
<td>51.4</td>
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<tr>
<td>Fragments‡</td>
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<td>0.0</td>
<td>14.3</td>
<td>5.7</td>
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<tr>
<td>Blank§</td>
<td>0.0</td>
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</table>

\textit{ord null}¶ cysts

<table>
<thead>
<tr>
<th>Localization pattern (%)*</th>
<th>Early region 2A (n=56)</th>
<th>Late region 2A (n=58)</th>
<th>Region 2B (n=48)</th>
<th>Region 3 (n=48)</th>
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<td>89.7</td>
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<td>Fragments‡</td>
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*Cysts exhibiting each localization pattern (in %). †Thread-like denotes long continuous staining. ‡Values include fragmented and/or punctate signal. §No visible staining. ¶\textit{ord5/Df} flies completely lack ORD activity.
disintegration of SMC1/3 threads. To test this hypothesis, SMC1/3 and C(3)G defects were examined simultaneously in individual nuclei at different stages in whole-mount preparations of intact germaria. As shown in Fig. 8B, continuous thread-like staining is evident for both C(3)G and SMC1/3 in early region 2A nuclei of germaria with extensive overlap between the two signals. However, by late region 2A, the SMC1/3 signal appears more fragmented than the C(3)G signal, with fewer SMC1/3 threads and more punctate staining. This difference is most obvious in region 3, where intact threads of C(3)G staining are still evident but the SMC1/3 pattern consists primarily of puncta. These data support our hypothesis that premature breakdown of chromosome cores induces the defects in C(3)G staining that we observe in germaria. However, the residual C(3)G staining that remain when SMC1/3 thread-like staining disappears raises the intriguing possibility that aligned C(3)G proteins might form polymers that remain transiently stable, even if they are no longer associated with chromosome cores.

We also asked whether C(3)G is required to maintain chromosome core integrity and found that thread-like SMC1/3 staining is still visible in c(3)G0/Df mutant germaria in which the SC fails to form (see supplementary material Fig. S7). These data indicate that intact SC is not necessary for chromosome core formation or maintenance. Interestingly, SMC1/3 threads appear more numerous in c(3)G mutant nuclei than in wild type, consistent with the inability of homologues to synapse in the absence of C(3)G. In the absence of synapsis, the homologous cores would not be intimately associated and cohesin staining would be visible along individual chromosome cores. A similar staining pattern has been reported for the putative lateral element component C(2)M in c(3)G mutants (Manheim and McKim, 2003) providing further support for this model.

C(2)M is required for chromosome core formation during pachytene

To explore the mechanistic interplay between cohesion proteins (ORD, cohesin SMCs), and an α-kleisin involved in SC assembly (C(2)M), we examined the localization of SMC1/3 and GFP-ORD in females homozygous for the c(2)M-null allele, c(2)M<sup>Ep[2115]</sup> (Manheim and McKim, 2003). To observe ORD localization, we generated a c(2)M<sup>Ep[2115]</sup> stock homozygous for a functional GFP-ORD transgene (Balicky et al., 2002) and confirmed that X-chromosome meiotic nondisjunction in c(2)M<sup>Ep[2115],GFP-ORD</sup> females (24.44%, n=753) was similar to that previously reported for c(2)M<sup>Ep[2115]</sup> homozygotes (29.3%) (Manheim and McKim, 2003).

At first glance, the staining pattern for SMC1/3 and GFP-ORD in c(2)M<sup>Ep[2115]</sup> germaria appeared very similar to that previously observed for C(3)G in this mutant (Manheim and
McKim, 2003). Thread-like SMC1/3 staining is completely absent in the germaria of whole-mount ovaries (see supplementary material Fig. S8). Instead, SMC1/3 staining is restricted to patches and foci.

In c(2)M[EP2115] females, the chromosome segregation defects are severe in meiosis I, but negligible during meiosis II (Manheim and McKim, 2003). This suggests that centromeric cohesion in these mutants is intact. To test whether the patches of SMC1/3 and GFP-ORD staining correspond to centromeres, c(2)M[EP2115] ovaries were co-immunostained with anti-CID antibodies. In these whole-mount preparations, CID foci largely coincide with ORD and SMC1/3 patches in pro-oocyte and oocyte nuclei in all regions of c(2)M-mutant germaria (Fig. 9A). These data argue that neither loading nor maintenance of cohesin SMCs at centromeres depends on the activity of C(2)M protein, consistent with low levels of meiosis II segregation defects in c(2)M-mutant females (Manheim and McKim, 2003).

Absence of thread-like SMC1/3 signal in whole-mount preparations of c(2)M-mutant germaria raises the possibility that C(2)M activity is required for loading cohesin subunits onto chromosome arms. Alternatively, cohesin subunits may localize normally to the arms, but fail to coalesce into chromosome cores in the absence of C(2)M. In this case, diffuse chromatin-bound cohesin signal would probably go undetected in whole-mount ovary preparations. To address this possibility, we examined chromosome spreads prepared from c(2)M[EP2115];P[GFP-ORD] germaria (Fig. 9B). In the c(2)M mutant, each nucleus contained one to four centromeric foci in which SMC1/3 and GFP-ORD always colocalized. These foci also coincided with C(3)G foci in the subset of cells that contained C(3)G signal. This staining pattern is consistent with that observed in whole-mount preparations of c(2)M germaria. However, in the chromosome spreads, diffuse chromosomal ORD and SMC1/3 staining also was observed in all mutant c(2)M pro-oocytes and pro-nurse cells (Fig. 9B). Moreover, the intensity of diffuse SMC1/3 and ORD signal is comparable in nuclei with and without C(3)G patches/foci (pro-oocytes and pro-nurse cells, respectively).

The data for chromosome spread localization indicate that in the absence of C(2)M, ORD and cohesin SMCs are loaded and maintained on both the arms and centromeres of meiotic chromosomes. However, absence of thread-like ORD and SMC1/3 staining argues that assembly of chromosome cores requires C(2)M activity. Because stable chromosome cores and lateral elements are a prerequisite for SC formation, their absence most probably explains the lack of thread-like C(3)G signal in c(2)M mutant germaria.

Discussion
Here, we describe temporal and spatial changes in cohesin localization during early prophase in wild-type Drosophila ovaries as well as in mutants with compromised cohesion and/or homologous recombination. Drosophila oogenesis provides a unique opportunity to examine important changes in chromosome morphology that occur during meiotic prophase. Because not all germ-line cells adopt an oocyte fate, the 16-cell cyst allows direct comparison of nuclei that assemble meiotic chromosome cores (SC) with those that do not. Importantly, this dynamic transformation in chromosome structure depends upon and must occur within the context of functional sister-chromatid cohesion. In addition, these events are crucial for homologous recombination and, therefore, accurate segregation of meiotic chromosomes.
The formation and maintenance of meiotic chromosome cores

Our analysis of chromosome spread preparations from wild-type ovaries indicates that the cohesin subunits SMC1 and SMC3 localize along the arms of chromosomes in all 16 cells of each cyst. However, thread-like cohesin SMC staining is only observed in the nuclei that build a SC. The simplest model to explain the differences we observe in pro-nurse cells and pro-oocytes is that multiple cohesin complexes come together to form long continuous threads of cohesin staining in nuclei that build SC (Fig. 10). We propose that chromosomes in pro-nurse cells maintain an extended interphase-like organization in which cohesin complexes localize along the arms but fail to assemble into this higher order structure. By contrast, the formation of chromosome cores in a subset of nuclei occurs when multiple cohesin complexes along the arms coalesce into threads and, thereby, bring about the shortening of the longitudinal axes of meiotic chromosomes (Fig. 10).

Formation of chromosome cores represents the first step in the organized assembly of the SC. Although DSBs are required for synapsis of homologues in a number of species (Giroux et al., 1989; Grelon et al., 2001; Mahadevaiah et al., 2001), lateral elements are still visible in mutants that fail to make DSBs and therefore lack tripartite SC (Bhuiyan and Schmekel, 2004; Romanienko and Camerini-Otero, 2000). Moreover, chromosome cores have been proposed to serve as the scaffold upon which SC components organize and assemble (Revenkova and Jessberger, 2006; Stack and Anderson, 2001). Genetic and cytological analyses in a number of organisms have confirmed that the cohesin complex plays an integral role in SC assembly (Cai et al., 2003; Klein et al., 1999; Molnar et al., 1995; Pasierbek et al., 2001; Petronczki et al., 2003; Revenkova et al., 2004). Our work argues that cohesin SMCs as well as the cohesion protein ORD are stable components of meiotic chromosome cores, which remain intact when DNase I treatment removes chromatin loops (Pearlman et al., 1992; Smith and Roeder, 1997). Interestingly, in chromosome spread preparations, localization of cohesin subunits and ORD appears to be restricted to chromosome cores; diffuse staining is not detectable in the areas between threads. These data are consistent with the model that, in Drosophila meiotic cells, cohesin proteins localize predominantly along the chromatid axes and are not found decorating the loops (Fig. 10). Similar arguments have been made for cohesin localization in S. cerevisiae and mouse meiotic cells (Ding et al., 2006; Prieto et al., 2001; Revenkova and Jessberger, 2005).

Here, we provide evidence that the meiosis-specific protein, C(2)M, is required for chromosome core formation in Drosophila oocytes. Chromosome spread preparations indicate that in the absence of C(2)M activity, SMC1 and SMC3 diffuse staining is visible throughout the nuclei of all 16 cells within each cyst, indicative of the association of cohesin subunits with chromosome arms. However, no thread-like SMC1/3 or ORD staining is observed in c(2)M-mutant germaria (this study). These data indicate that C(2)M protein controls an early step in the formation of chromosome cores. We propose that, by virtue of its ability to interact with cohesin SMC proteins (Heidmann et al., 2004), C(2)M drives the association of cohesin complexes to form meiotic chromosome cores (Fig. 10). Failure in this process would prohibit subsequent assembly of the SC in c(2)M mutant germaria as evidenced by lack of thread-like immunostaining for the transverse filament protein C(3)G (Manheim and McKim, 2003). These results also are consistent with EM localization of C(2)M protein along the lateral elements of the wild-type Drosophila SC (Anderson et al., 2005).

Surprisingly, we found that the cohesion protein ORD is required for the maintenance of chromosome cores during early pachytene in Drosophila (Fig. 10). In the absence of ORD activity, thread-like SMC1/3 staining is visible in region 2A of the germarium; however, the intensity and integrity of SMC1/3 threads deteriorate as pachytene progresses within the germarium. Analysis of chromosome spread preparations indicates that, although chromosome cores disassemble, the cohesin subunits SMC1 and SMC3 remain associated with the chromosome arms in ordnull germaria.

Our quantitative analyses of temporal progression of SMC1/3 and C(3)G defects in ord mutant germaria (this study) (Webber et al., 2004), as well as co-immunostaining experiments that simultaneously monitored cohesin subunits and C(3)G in individual ordnull nuclei, argue that the onset of chromosome core dissolution precedes fragmentation of thread-like epifluorescent signal for the SC marker, C(3)G. These data demonstrate that initial assembly of cores is not sufficient for stable SC; instead, maintenance of chromosome cores is an ongoing requirement to preserve SC integrity.

![Diagram of chromosome core assembly and maintenance](image_url)
Why do cores disassemble in the absence of ORD activity? By immunofluorescence, continuous thread-like C(2)M and C(3)G staining is transiently present during early pachytene in ordnull germlia; however, at the same stage, normal tripartite SC is not detectable by EM (Webber et al., 2004). Therefore, although the highly organized SC ultrastructure is absent, some aspects of SC assembly still occur in the absence of ORD function [namely recruitment of C(2)M and C(3)G]. These data suggest that ORD is required to recruit additional proteins along the chromosome cores and/or lateral/axial elements that are required for core integrity. Alternatively, ORD itself might be required to maintain C(2)M-mediated organization of the cores into stable structures.

Programmed cycles of stress and relaxation along meiotic chromosomes have been proposed to govern several critical events during prophase (Kleckner et al., 2004). One possibility is that in the absence of ORD function — although chromosome cores assemble — they are unable to withstand normal changes in compression and/or relaxation, and subsequently buckle. Interestingly, Carpenter (Carpenter, 1975) has reported that, during wild-type pachytene, the SC shortens significantly as cysts move through the Drosophila germlia. If chromosome cores that assemble in the absence of ORD are inherently unstable, programmed shortening of the SC could cause additional stress that results in fragmentation of the cores. Curiously, we have observed that in later stages (stages 3-6 of the vitellarium), continuous thread-like SMC1/3 staining often reappears during early pachytene in ordnull germlia. If chromosome cores that assemble in the absence of ORD are inherently unstable, programmed shortening of the SC could cause additional stress that results in fragmentation of the cores. Curiously, we have observed that in later stages (stages 3-6 of the vitellarium), continuous thread-like SMC1/3 staining often reappears in ordnull oocytes (see Fig. 5). These stages loosely correspond to the time after which the SC reaches its shortest length and starts to expand in wild-type. Chromosome cores might be able to reassemble at these later stages in ordnull ovarioles if decompaction of the chromosome axes reduces stress.

Breakdown of chromosome cores in ordnull germlia is also temporally linked to the onset of DSBs. Both the timing (early 2A) and the number of DSBs are normal in ordnull germlia (Webber et al., 2004). However, we begin to detect fragmentation of SMC1/3 thread-like staining in late region 2A, after the onset of DSBs. Therefore, induction of DSBs might contribute to destabilization of chromosome cores that are compromised due to lack of ORD protein. A similar model has been proposed to explain the phenotypes associated with disruption of the Pds5 orthologue (Spo76) in Sordaria (Storlazzi et al., 2003). Although AEs are continuous during early prophase I in spo76 mutants, they fragment prematurely in a DSB-dependent fashion (Storlazzi et al., 2003).

The disassembly of chromosome cores in ordnull germlia is most probably responsible for the severe reduction in crossovers between homologues in mutant females. ORD activity is essential for the crucial decision each chromatid must make after the induction of DSBs — namely whether the broken chromatid will choose its sister or its homologue for repair. Strand invasion and crossovers are biased towards the homologue during meiosis, resulting in stable chias mata that keep homologous chromosomes physically associated until anaphase I (Schwacha and Kleckner, 1997; Zickler and Kleckner, 1999). Previous experiments have shown that ORD activity is required for homologue bias during meiotic recombination in Drosophila. In ordnull females, the frequency of crossovers between homologues is decreased, while that between sister chromatids is significantly increased (Webber et al., 2004). These data combined with our current analyses suggest that chromosome cores are necessary for homologue bias during meiosis, and partner choice takes place in late region 2A or region 2B of the Drosophila germlia.

Different requirements for arm and centromeric cohesin loading
In ordnull germlia, SMC1 and SMC3 fail to accumulate at centromeres, but appear to localize normally along chromosome arms within all 16 cells of each germ-line cyst. These results suggest that distinct pathways mediate cohesin loading on the arms and centromeres during Drosophila meiosis. We cannot differentiate whether centromeric loading of SMC1 and SMC3 is completely ablated or whether cohesin SMCs are able to load at centromeres but are quickly removed when ORD activity is absent. Regardless, accumulation of cohesin subunits at the centromeres of meiotic chromosomes appears to depend on ORD function. By contrast, ORD activity is not required for stable association of SMC1 and SMC3 along chromosome arms. Curiously, after germ-line cells adopt a nurse cell fate, ORD is no longer necessary for centromeric accumulation of cohesin; the clustered finger-like projections of SMC1/3 staining at nurse cell centromeres in ordnull ovarioles (stages 3-4) are indistinguishable from that in wild type. However, even at these later stages when cohesin subunits are visible at nurse cell centromeres, we never detect SMC1 or SMC3 at the centromeres of oocytes in ordnull ovarioles. Therefore, our data implicate ORD in a meiosis-specific pathway for cohesin loading and/or accumulation at centromeres.

Co-immunostaining experiments with CID and SMC1/3 antibodies indicate that absence of cohesin subunits appears to be restricted to the centromeres in ordnull germlia; within the resolution limits of our chromosome spread images, the area lacking SMC1/3 signal is approximately the same size as the CID staining. These data suggest that cohesin loading and/or accumulation at pericentromeric heterochromatin occurs in the absence of ORD function. However, we do not observe the striking enrichment of SMC1/3 in pericentromeric heterochromatin prominent in wild type. Therefore, our analysis of defects of cohesin localization in ordnull germlia suggest that normal loading and/or accumulation of cohesin is regulated differently even within the different domains of heterochromatin in and around centromeres. In addition, the chromocenter appears to be less stable in ordnull oocytes (see Fig. 4), raising the possibility that changes in heterochromatin structure in the absence of ORD activity diminishes the ability of centromeres to associate.

Our chromosome spread experiments clearly indicate that SMC1 and SMC3 are stably associated with the chromosome arms of both pro-nurse cells and pro-oocytes within ordnull germlia. However, whether the localization of cohesin subunits represents functional cohesin is not clear. From genetic and cytological studies, we know that meiotic cohesion is completely absent in ordnull oocytes by the time that meiotic chromosomes make microtubule attachments (Bickel et al., 2002; Bickel et al., 1997). Separated sister chromatids have not been detected during early pachytene in ordnull germlia by FISH (Webber et al., 2004). However, catenation might hold sisters together at this time, thereby masking defects arising.
from the absence of cohesin-mediated cohesion (Toyoda and Yanagida, 2006).

Stepwise loading of cohesin subunits during meiotic prophase has been described for a number of organisms (Chan et al., 2003; Eijpe et al., 2003; Valdeolmillos et al., 2007). In worms and grasshoppers, stable association of cohesin SMCs in the absence of non-SMC subunits has been reported for meiotic chromosomes (Chan et al., 2003; Valdeolmillos et al., 2007). One possibility is that, in the absence of ORD, cohesin SMCs load without their non-SMC partners. Another possibility is that the entire cohesin complex loads in ord null ovaries but cohesin-mediated cohesion is not established. At least two reports have indicated that, in S. cerevisiae, the binding of cohesin to specific genomic locations is insufficient for cohesin-mediated cohesion (Chang et al., 2005; Lam et al., 2006). In addition, recent work by Ellenberg and colleagues (Gerlich et al., 2006) it has been elegantly demonstrated that different populations of chromatin-bound mitotic cohesin exist within Hela cells. This work suggests that, during replication and the establishment of cohesion, a subset of chromatin-bound cohesin complexes is converted from ‘dynamically associated’ to ‘irreversibly bound’. ORD might be necessary for the establishment of meiotic sister-chromatid cohesion but not for association of cohesin subunits with the chromosomes. Alternatively, cohesion might be established in the absence of ORD activity but not maintained.

The identity of Drosophila Rec8?

In most species, a meiosis-specific α-kleisin subunit (Rec8) promotes meiotic cohesion by interacting with the heads of the SMC subunits and closing the cohesin ring. Surprisingly, an obvious Rec8 orthologue has not been identified in the Drosophila genome. Although its limited sequence homology to Rec8 in other organisms has led to the proposal that C(2)M functions as the meiosis-specific α-kleisin subunit of the cohesin complex in Drosophila (Schleiffer et al., 2003), phenotypic analysis of c(2)M mutant flies is inconsistent with this hypothesis (Manheim and McKim, 2003). In contrast to other Drosophila mutations that disrupt meiotic cohesion (Bickel et al., 1997; Kerrebrock et al., 1992; Miyazaki and Orr-Weaver, 1992), defects of meiosis II segregation are negligible in c(2)M females and accurate chromosome segregation during male meiosis also does not depend on C(2)M function (Manheim and McKim, 2003). Moreover, female germline expression of a mutated C(2)M protein, C(2)M (Schleiffer et al., 2003), may be too small to bridge the heads of the Drosophila SMC1/3 dimer. In addition, ORD does not share obvious sequence homology with Rec8, Scc3/SA or regulators of cohesin (Pds5, Scc4, Scc2). One possibility is that during Drosophila meiosis, two proteins collaborate to provide Rec8 function. Such is the case for Drosophila separase, which is composed of two subunits encoded by separate genes (Hervig et al., 2002; Jager et al., 2001; Leisemann et al., 2000). ORD may cooperate with Rad21 or another unidentified protein to provide Rec8 function during meiosis.

Why flies would use an altered mechanism to accomplish such a highly conserved activity is an enigma. However, further analysis of the regulation of meiotic cohesion in Drosophila should provide important evolutionary insights into fundamental aspects of recombination and chromosome segregation during meiosis.

Materials and Methods

Fly strains

Flies were reared at 25°C on standard cornmeal molasses medium. y w; ord+/bw females containing a functional P[acman::ord] transgene (Balicky et al., 2002) were used for immunolocalization of GFP-ORD. For all other ord experiments, y; cv bw sp females were used. ord+/bw (ord+/Df) females were obtained by crossing y w; ord+/bw; SM1; P[acman::ord] to y; ord−/bw. y w; ord+/bw; SM1; P[acman::ord] females were obtained by crossing y w; ord−/bw females to y; c(2)M 2215/CyO females (Balicky et al., 2002). For experiments with c(2)M mutants, homozygous c(2)M females from the stock y w; y w; c(2)M 2215/SIM1; P[acman::ord] were used. The c(2)M 2215 mutant stock expressing GFP-tagged ORD was generated by crossing y w; P[acman::ord] females (Balicky et al., 2002) to y w; y w; c(2)M 2215/CyO females (Manheim and McKim, 2003). For experiments with c(3)G mutants, y w; y w; c(3)G 1031 ess ess ess es ess Sp (Nelson and Sturtevant, 1920), smc1+/bw (Dorsett et al., 2005) germ-line clones were generated by crossing y w; FRT 82B lyrv w; P{acman::ord} to y w; P{acman::ord}; FRT 82B lyrv w; P{acman::ord} to y w; P{acman::ord}. y w; P{acman::ord} to y w; P{acman::ord}

Fixation and immunolocalization of whole-mount ovaries

Most immunolocalization experiments were performed as previously described (Page and Hawley, 2001; Weber et al., 2004). However, ovary images were fixed in unbuffered formaldehyde/acetone (200 μl of unbuffered 2% EM-grade formaldehyde containing 0.5% Nonidet P-40 and mixed with 600 μl heptane). Ovaries were incubated in primary antibodies for 2 hours and the antibodies (primary and secondary) were diluted in PBS containing 0.5% BSA and 0.01% Tween-20.

To analyze smc1+/− germ-line clones (see supplementary material Fig. S1b), fixation of ovaries and staining with LacZ, SMC1 and C(3)G antibodies was performed using a protocol adapted from Song et al. (Song et al., 2002). Ovaries were incubated in primary antibodies for 2 hours and the antibodies (primary and secondary) were diluted in PBS containing 0.5% BSA and 0.01% Tween-20.
were fixed in 4% formaldehyde in 1× PBS for 15 minutes, followed by four washes in 1× PBS. Tissue was then rehydrated in PBS for 20 mins. After three rinses and one wash in 1× PBS containing 0.1% Triton X-100, the ovaries were placed in a closed humid chamber overnight and allowed to dry slowly. The humid chamber was opened slightly the next day and slides were allowed to dry completely. Dry slides were dipped in a 0.4% solution of Kodak PhotoFlo 200 for 2 minutes and allowed to air dry at room temperature.

Antibodies

Gluten pig polyclonal antibodies were generated against SMC1 and SMC3 peptides at Alpha Diagnostic International Inc., San Antonio, TX. The SMC1 peptide MTEEDDDV AQRV ATAPVRKP corresponds to the N-terminus of the predicted amino acid sequence of SMC1 (see supplementary material Fig. S4), and the SMC3 peptide SLK19 corresponds to the N-terminus of the predicted amino acid sequence of SMC3 (Webber et al., 2004). The procedure for preparing Drosophila ovary chromosome spreads (Webber et al., 2004) was adapted from a protocol developed for mammalian meiotic cells (Peters et al., 1997). Twelve female flies of the appropriate genotype were fattened overnight with yeast and males, and their ovaries were isolated in PBS. Ovaries were rinsed once in freshly prepared hypotonic buffer (50 mM sucrose, 17 mM trisodium citrate dihydrate, 5 mM EDTA, 0.5 mM DTT, 30 mM Tris pH 8.2 and 0.5 mM Pefabloc) and incubated for 20-30 minutes in hypotonic buffer. Tungsten needles were used to isolate the transparent tips of the ovaries in hypotonic buffer. Slides were rinsed and three washes in 1× PBS containing 0.1% Triton X-100, followed by one wash in 1× PBS, followed by another wash in 1× PBS containing 0.1% Triton X-100. Ovaries were then fixed in 4% formaldehyde in 1× PBS containing 0.5% BSA and 0.01% Tween-20 (antibody buffer) and incubated overnight in SMC1 and C(3)G antibodies diluted in antibody buffer. After three rinses and three washes in 1× PBS containing 0.2% Tween-20, the ovaries were incubated in appropriate secondary antibodies. Following three rinses and three washes in 1× PBS containing 0.1% Triton X-100, the ovaries were incubated overnight in β-galactosidase antibodies diluted in antibody buffer. For DNase treatment of chromosome spreads, tissue was rehydrated in PBS for 15 minutes and blocked for 1 hour in 5% normal donkey serum, 2% BSA, 0.1% Triton X-100, PBS. Slides were rinsed three times with antibody buffer (0.1% BSA, 0.1% Triton X-100, PBS) and 100 μl of antibodies diluted in the antibody buffer were added to the slides and a piece of paraffin was used to cover the liquid on the slide. The primary antibody incubation was performed for 2-4 hours in a humid chamber. Slides were rinsed three times and washed three times for 10 minutes each with wash buffer (0.1% Triton X-100, PBS). Secondary antibody incubations were performed for 1 hour and after rinsing and washing slides with wash buffer the tissue was stained with DAPI at 1 µg/ml in PBS. After an additional rinse in PBS, the slides were mounted in 40-50 µl of Prolong (Molecular Probes) or Vectashield (Vector Laboratories) and incubated 3-4 hours at room temperature. Once dry, they were stored at –20°C and used for immunofluorescence within 1-2 days.

Primary immunolocalization, the spreads were rehydrated in PBS for 15 minutes and blocked for 1 hour in 5% normal donkey serum, 2% BSA, 0.1% Triton X-100, PBS. Slides were rinsed three times with antibody buffer (0.1% BSA, 0.1% Triton X-100, PBS) and 100 μl of antibodies diluted in the antibody buffer were added to the slides and a piece of paraffin was used to cover the liquid on the slide. The primary antibody incubation was performed for 2-4 hours in a humid chamber. Slides were rinsed three times and washed three times for 10 minutes each with wash buffer (0.1% Triton X-100, PBS). Secondary antibody incubations were performed for 1 hour and after rinsing and washing slides with wash buffer the tissue was stained with DAPI at 1 µg/ml in PBS. After an additional rinse in PBS, the slides were mounted in 40-50 µl of Prolong (Molecular Probes) under parafilm.

Antibody incubations

SMC1 antibodies were diluted 1:2000 for whole-mount experiments and 1:1000 for chromosome spreads. SMC1 and SMC3 antigens were reacted with polyclonal antibodies (supernatant from line 1A8-1G2) (Anderson et al., 2005) or were used at 1:500 followed by either Alexa Fluor 488-conjugated anti-mouse secondary or Cy5-conjugated anti-mouse secondary. For detection of GFP-ORD, rabbit anti-GFP antibodies (Molecular Probes) were diluted 1:2000 for whole-mount experiments and 1:1000 for chromosome spreads followed by Alexa Fluor 488-conjugated anti-rabbit secondary. Affinity-purified chicken anti-CID antibodies (Blower and Karpen, 2001) were used at 1:100 followed by Cy5 anti-chicken secondary. Anti-CID rabbit antibodies (Abcam) were used at 1:1000 followed by either Alexa Fluor 488-conjugated anti-rabbit secondary or Cy5-conjugated anti-rabbit secondary. IB1 mouse monoclonal antibodies (Zaccai and Lipshitz, 1996) were used at 1:20 to detect the fusome with Cy5-conjugated anti-mouse secondary. Anti β-galactosidase chicken antibodies (Abcam) were used at 1:2000. Secondary antibodies conjugated to Cy3 and Cy5 were obtained from Jackson ImmunoResearch Laboratories, and the Alexa Fluor 488-conjugated secondary antibodies were obtained from Molecular Probes. All secondary antibodies were used at a final dilution of 1:400.

Microscopy and image analysis

Epifluorescence microscopy was performed using a Zeiss AxioImager M1 microscope equipped with a Hamamatsu ORCA-ER camera. 100× Plan-Apochromat (NA 1.4) and 63× Plan-Apochromat (NA 1.4) objectives were used for imaging whole mounts and spreads, respectively. Images were captured using Openlab software (Improvement, version 3.1.5 and higher). Registration of images for colocalization experiments was performed using Tetraspeck fluorescent beads (Molecular Probes) and the Openlab software registration module. Openlab or Velocity (Improvement) was used to crop and pseudo-color images. Deconvolution of image stacks (0.1 µm step size) was performed using Velocity (version 2.5 and higher). To preserve relative intensities, mutant and wild-type images for each stage were captured using the same exposure time, and deconvolved simultaneously as different ‘timepoints’ within a single ‘image sequence’.

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References


