Heterochromatin and the Synaptonemal Complex Maintain Homologous Centromere Interactions in Mouse Spermatocytes

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Running Title: Heterochromatin connections during mouse meiosis

Summary

How achiasmate chromosomes become aligned and segregate during meiotic prophase is an important issue in eukaryotic genetics. We show that chromosomes are frequently transmitted properly in the absence of a chiasma in mouse spermatocytes, suggesting the existence of back-up systems aiding achiasmate chromosome segregation. Here, we report mechanisms that maintain the alignment of homologous pairs prior to disjunction, which may enable achiasmate chromosomes to segregate from each other in meiosis I. We present direct physical evidence showing centromere interactions provide a stable physical link between chromosome pairs in the absence of recombination and that pericentromeric heterochromatin tethers the centromeres of chromosome pairs after dissolution of synaptonemal complex-mediated centromere paring. Our results establish for the first time in a mammal a role for heterochromatin in maintaining the alignment of chromosomes in the absence of euchromatic synapsis and suggest that homologous recognition may lead to segregation, even in the absence of recombination.

Highlights

- Non-recombination based systems promote achiasmate chromosome disjunction in mice.
- Two novel mechanisms maintain homologous pairs alignment just before segregation.
- One is directed by SYCP1 at centromeres; the other by centromeric heterochromatin.
- A comprehensive analysis of pre- and post-synaptic centromere interactions.

Introduction

Faithful homologous chromosome segregation during meiosis depends upon the connections that tether chromosome pairs. The connections generate a force to oppose microtubules that pull the partners towards opposite poles of the spindle at anaphase I, creating tension that stabilizes metaphase kinetochore microtubule attachments. Chiasmata, products of recombination, often provide the physical link that holds the homologs in pairs (reviewed in (Bascom-Slack, 1997; Petronczki et al., 2003)). Consequently, mutations that reduce the level of recombination are invariably associated with increased errors during meiotic chromosome segregation. However, proper segregation of meiotic chromosomes can be achieved even in the absence of recombination. In yeast and Drosophila, a high proportion of non-exchange chromosomes partition correctly (Dernburg et al., 1996; Guacci and Kaback, 1991; Karpen et al., 1996; Mann and Davis, 1986; Ross et al., 1996); this also seems to be the case in mammals. In mice, the majority of chromosomes in oocytes from a recombination-deficient mutant appeared to be spatially balanced on the spindle (Woods et al., 1999), and in humans, while smaller chromosomes (21 and 22) fail to experience crossovers in about 5% of meioses (Cheng et al., 2009a; Fledel-Alon et al., 2009; Oliver et al., 2008), they are estimated to non-disjoin in only <1% of meioses (Fledel-Alon et al., 2009; Hassold et al., 1993a, b; Oliver et al., 2008; Tease et al., 2002). In this model, non-disjunction may occur as a result of dual failure: first, failure to generate a crossover, and second, failure of one or more back-up system(s) that promote proper segregation of achiasmate partners (Cheng et al., 2009a; Fledel-Alon et al., 2009; Oliver et al., 2008).

Specific mechanisms that compensate for the loss of chiasmata have been proposed in yeast and Drosophila, where pairing between centromeres (centromere pairing) promotes alignment of non-recombined meiotic chromosomes and may also significantly contribute to the segregation fidelity of recombined chromosomes (Gladstone et al., 2009; Kemp et al., 2004; Newnham et al., 2010; Takeo et al., 2011). The discovery of centromere pairing in mouse

spermatocytes (Bisig et al., 2012; Qiao et al., 2012) raises the possibility that in mammals centromere interactions provide an alternative mechanism for linking homolog pairs to promote proper segregation.

While centromere pairing has been observed in mice (Bisig et al., 2012; Qiao et al., 2012), the underlying interactions that promote pairing, and the impact of pairing on segregation have yet to be elucidated. Centromeric regions are largely heterochromatic, gene-poor and mainly composed of arrays of highly repetitive, simple sequences. In Drosophila, segregation of achiasmate partners depends on pairing of peri-centric heterochromatin (Dernburg et al., 1996; Hughes and Hawley, 2009). Indeed, chromosomes that fail to form chiasmata (*X* and *4*) are connected by heterochromatic threads during prometaphase I in oocytes; these threads are likely to be part of the mechanism by which heterochromatin facilitates non-recombinant chromosome segregation (Hughes et al., 2009). These studies prompted us to analyze whether in mouse spermatocytes chromosomes are transmitted properly in the absence of chiasmata and to examine the role of heterochromatin in meiotic chromosome alignment.

The central issue is whether heterochromatin and centromere pairing provide a physical association between chromosomes at any time prior to segregation at anaphase I. This may be particularly important for pairs of achiasmate chromosomes in which mechanisms of centromere connection may be the only means to establish physical linkage to maintain alignment prior segregation. To test this model, we looked for physical evidence of non chiasmate type associations between pairs of homologous chromosomes. We observed that after dissolution of the synaptonemal complex (SC) at centromeres, only pericentromeric heterochromatin maintains the centromeres homologously associated. This occurred in the absence of chiasma and in spite of desynapsis in euchromatic regions. Our results suggest that centromeric heterochromatic connections can maintain homolog alignment just before segregation.

We also report a number of meiotic achiasmate events that apparently segregate from each other at meiosis I. Alternative models of segregation, which explain faithful segregation of such chromosomes in absence of chiasmata, are considered in the Discussion.

Results

Chromosome Disjunction in the Absence of Chiasmata

If mechanisms exist for promoting achiasmate chromosome segregation, then the frequency of achiasmate chromosomes at the end of prophase should be higher than aneuploidy. In metaphase I chromosome spreads from wild-type C57BL/6 (Figure 1A), 8.4% of cells showed at least one achiasmate chromosome (*i.e.*, chromosomes separated from their partner). The majority of these nuclei (6.9%) exhibited only one pair of achiasmate chromosomes. The sex chromosomes represented nearly half of the achiasmate chromosomes (3.8% versus 4.6% for somatic chromosomes).

As expected given their haploid genotype after two meiotic divisions, the majority of cells in a large sample of elongating spermatids showed single FISH signals for chromosomes 8 (99.8%), 15 (99.6%), and 19 (99.5%; Figure 1B). Notably, we observe a high number of achiasmate sex chromosome pairs in metaphase I preparations. X and Y chromosome pairs normally form a short stretch of SC encompassing a small region of homology in the pseudo-autosomal region (PAR), which is very sensitive to reduced rates of recombination (Roig et al., 2010). We analysed the fate of X and Y chromosomes in diploid secondary spermatocytes and elongating spermatids; the majority of secondary spermatocytes (99.5%, Figure 1C) and elongating spermatids (Figure 1D) showed more than one FISH signal for the X or Y chromosomes.

We observed that in 4-8% of meioses at least one homologous chromosome pair is achiasmate; but, fewer than 1% of these cells' chromosomes mis-segregated. There are

multiple possible explanations for this discrepancy. It is possible that achiasmate cells might be eliminated via apoptosis during meiotic progression. To test this, we used the TUNEL assay to detect apoptotic cells in testis tissue sections and surface chromosome spreads (Figure 1E). In wild-type testis, fewer than 1% of prophase I diakinesis cells (1.13%), secondary spermatocytes (none detected), and rounded (0.2%) or elongated (0.1%) spermatids underwent apoptosis. We concluded that the lower number of aneuploid spermatocytes and spermatids in mice relative to the proportion of achiasmate diakinesis-stage cells is not the result of apoptotic elimination of achiasmate meiotic events. An alternate explanation would be a mechanism that promotes segregation of achiasmate chromosomes, analogous to the processes that involve pairing of centromere regions in budding yeast and Drosophila (Gladstone et al., 2009; Kemp et al., 2004; Newnham et al., 2010; Takeo et al., 2011).

Centromere Connections Link Homolog Pairs in Absence of Chiasmata

To elucidate how the faithful segregation of homologous chromosomes occurs in the absence of chiasmata, we investigated the physical basis for achiasmate segregation in mouse spermatocytes. Previous work showed the centromeres of exchange homologs persist in a paired configuration after SC disassembly in diplotene (Bisig et al., 2012; Qiao et al., 2012). Here, we show that the connections between pairs of homologous centromeres in mouse spermatocytes provide a stable physical link that maintains the association of achiasmate homologs in the later stages of prophase. We previously suggested a fraction of homologous chromosomes show no apparent chiasmata (non-exchange chromosomes) during late diplotene, and are only tethered by paired centromeres (Bisig et al., 2012). Here, we sought to more definitively prove the existence of these centromeric associations between achiasmate chromosomes using *Hfm1*$^{-/-}$, *Mlh3*$^{-/-}$, and *Hfm1*$^{-/-}$/*Mus81*$^{-/-}$ mice. In mice, the HFM1 and MLH3-dependent pathway gives rise to approximately 90% of crossovers (Class I crossovers), which exhibit interference and do not normally occur close to centromeres (Guiraldelli et al., 2013;

Lipkin et al., 2002). A second type of crossover (Class II), which can be randomly distributed, depends on the structure-specific endonuclease MUS81 that promotes the alternative MUS81-EME1 recombination pathway (Holloway et al., 2008). To evaluate SC-mediated centromere associations, we immunostained spermatocyte chromosome spreads for SYCP3 and SYCP1, components of the lateral and transverse filaments of the SC. SYCP3 immunostaining enabled visualization of chromosome cores and SYCP1 was used to monitor persistence of SC components at paired centromeres after SC disassembly (centromere pairing) (Bisig et al., 2012). DAPI was used to indicate pericentromeric heterochromatin. In agreement with previous reports (Guiraldelli et al., 2013; Lipkin et al., 2002), deletion of either *HFM1* or *MLH3* led to an increased numbers of achiasmate chromosomes (Figure 2A and B). Remarkably, most achiasmate chromosomes in *Hfm1*$^{-/-}$ and *Mlh3*$^{-/-}$ spermatocytes remained associated at their centromeres. This was reflected by the differing chiasmate and achiasmate chromosome distributions per cell in wild-type and *Mlh3*$^{-/-}$ and *Hfm1*$^{-/-}$ spermatocytes (Figure 2A and B). One explanation for the centromeric tether observed in pairs free of chiasma along their arms is that centromeric Class II crossovers provide a link between the partners. The fact that similar results were observed in *Hfm1*$^{-/-}$ and *Hfm1*$^{-/-}$/*Mus81*$^{-/-}$ spermatocytes disproved this possibility and pointed to a non-crossover basis for centromeric pairing between achiasmate partners.

The fact that chromosomes associate with their homologous partners at centromeres (Guiraldelli et al., 2013) regardless of whether they undergo exchange or not suggests homologous recognition can lead to chromosome alignment during meiosis, even if recombination is incomplete.

Centromeric Regions are the Last to Undergo Desynapsis

Our results suggest a model in which centromere connections play an important function during meiotic segregation by keeping homologous centromeres tightly connected throughout the later stages of prophase I, as reported previously in Drosophila and budding yeast (Dernburg et al.,

1996; Kemp et al., 2004; Newnham et al., 2010; Obeso and Dawson, 2010). To investigate this model, we evaluated whether centromere connections persist during the period of SC removal and homologous arm separation that precedes homolog disjunction at diplotene. We scored partially desynapsed chromosomes in diplotene spermatocytes immunostained for SYCP3 to visualize desynapsing chromosome axes, SYCP1 to evaluate SC dissolution and CREST to reveal centromere localization (Figure S1A). The analysis of 1,261 individual partially desynapsed chromosomes revealed several categories (Figure S1B). We observed chromosomes with a single stretch of SC at the centromeric end (35%). We also noted a proportion of chromosomes with near-centromeric desynapsis (29%); however, these invariably contained a remnant of SYCP1 at the centromere. Chromosomes desynapsed in a single internal stretch and in which both ends had not yet desynapsed accounted for 10%. Finally, chromosomes with separated centromeres represented 26% of the scored chromosomes.

Role for Pericentromeric Heterochromatin in Maintaining Homologous Chromosome Alignment

The existence of heterochromatic links between the centromeres of achiasmate Drosophila chromosomes (Dernburg et al., 1996) suggests these physical connections may be particularly important for promoting proper segregation of chromosome pairs that fail to undergo exchange. Using cytological methods, we investigated whether centromere pairing in mammals includes an association of heterochromatin (Figure 3A and B). We correlated nuclear morphology, as revealed by bright spots of pericentromeric heterochromatin (DAPI), with immunolocalization of SYCP3 and SYCP1 in spermatocyte chromosome spreads. All mouse chromosomes are sub telocentric, so that heterochromatin lies immediately adjacent to one of the two telomeres on each chromosome. Mice have 20 pairs of chromosomes (19 pairs of somatic chromosomes and a pair of heteromorphic sex chromosomes in males), thus we expected that complete pairing of homologous centromeres in pachytene spermatocytes would yield 21 centromeric signals, while

completely dispersed centromeres would yield 40 signals. We scored the number of heterochromatin signals and CREST foci per wild-type cell for type B spermatogonia (mean and standard deviation, 9.49±2.40, n=53 and 24.27±6.82, n=30) and spermatocytes at pre-meiotic S-phase (14.59±2.56, n=74 and 36.85±4.14, n=30), leptotene (4.39±1.69, n=23 and 40.67±1.88, n=24), zygotene (4.47±2.13, n=70 and 35.62±4.14, n=29), pachytene (9.16±1.63, n=36 and 23.87±2.82, n=30) and diplotene (13.34±1.83, n=47 and 27.72±3.31, n=25). During all stages of prophase I, DAPI staining revealed a lower number of heterochromatic foci per cell compared with the total number of individual centromeres (Figure 3A and B). These results indicate that pericentromeric heterochromatin tends to associate in discrete clusters regardless of whether chromosomes have established homologous contact.

Analysis of diplotene cells was particularly revealing. The SC persists at paired centromeres after SC disassembly during early diplotene and is removed prior to chromosome segregation (Bisig et al., 2012). In late diplotene cells many centromere pairs, though no longer joined by the SC, exhibited association of their peri-centric heterochromatin masses (Figure 3C). This could be clearly observed when heterochromatin was detected by staining for methylated histone 3 lysine 9 (Figure 3D and Figure S2). Centromeres associated by their heterochromatin but not SC occurred on both chiasmate (33% of 616 chromosomes counted in 116 cells) and achiasmate (4%) chromosomes (Figure 3C). Electron microscopy of silver-stained diplotene spermatocytes (Figure 3E) confirmed homologous pairs, regardless of whether they underwent exchange, remain in close proximity at their centromeres - apparently connected by highly electron-dense pericentromeric heterochromatin. In this analysis, different pairs of chromosomes had centromeres at different distances from each other (Figure 3E). In combination with epifluorescent images, we suggest pairs of centromeres further apart than 2 microns have lost SYCP1 but remain associated by heterochromatin.

These results suggest that after the final steps of SC disassembly at centromeres, associations persist between the homologous masses of peri-centric heterochromatin. We

propose that after removal of SYCP1 from centromeres, heterochromatin-mediated centromere connections hold homolog pairs until their eventual segregation in such a manner that favors their disjunction at meiosis I.

Moving from Heterologous to Homologous Centromere Associations at the Zygotene-Pachytene Transition

To determine when and how homologous heterochromatin connections are established, we characterized peri-centromeric heterochromatin associations between homologous and heterologous chromosomes in chromosome spreads containing pre-meiotic cells (type B spermatogonia) and primary spermatocytes at different stages of prophase I (Figure 4A-D). The cells were stained with DAPI to reveal peri-centromeric heterochromatin and SYCP3 for cell staging. FISH point probes specific for the centromeric regions of chromosome 8 and 15 were used to localize homologous centromeres. The FISH probes co-localized with DAPI in all cells (Figure 4A-D and Table S1), consistent with the specificity of the probes for centromeric heterochromatic regions. A FISH probe for a central region of chromosome 2 was used to monitor connections in a euchromatic region not involving peri-centromeric heterochromatin. We scored the number of cells with one FISH signal located in DAPI blob (conjoined FISH signal) and FISH signals associated with two different non-connected (no heterochromatin connection) or connected (heterochromatin connection) DAPI blobs. In S-phase through zygotene, the two copies of the chromosome 8 and 15 loci nearly always localized to different DAPI foci, demonstrating that, although they clustered with other heterochromatic loci, the chromosomes were not homologously paired. In contrast, at the beginning of meiosis (type B spermatogonia), both copies of the two loci were frequently associated with the same DAPI spot – consistent with homologous alignment in pre-meiotic cells (Boateng et al., 2013). Thus, it appears cells enter meiosis with a loose association of homologous sequences that breaks down as cells progress through prophase. As expected, most FISH signals for chromosome 2 (an interstitial

probe) were not associated with DAPI foci and were therefore not involved in heterochromatin clustering. This clustering of pericentric heterochromatic regions is reminiscent of the "centromere coupling" phenomena reported in several organisms (reviewed in (Obeso et al., 2014; Stewart and Dawson, 2008) including the mouse (Takada et al., 2011).

As an independent approach to determine the timing of centromeric associations, we scored the number of chromosomes from late zygotene, pachytene and diplotene spermatocytes containing homologous and heterologous pericentromeric connections. The cells were scored at the mid and late stages of prophase I, when chromosomes have fully developed lateral elements and are relatively condensed, which makes it easier to resolve individual chromosomes and visualize whether they synapsed with a partner. We observed a high number of non-homologous heterochromatin centromeric associations in late zygotene spermatocytes, in sharp contrast to chromosomes in pachytene and diplotene spermatocytes, in which homologous heterochromatin connections were prominent (Figure 5A and B).

In summary, pericentromeric heterochromatin is involved in non-homologous associations in pre-pachytene spermatocytes in mice. These heterologous interactions are apparently directed to pair-wise homologous interactions by synapsis, as we observed exclusively homologous centromeric interactions in chromosomes from pachytene and diplotene spermatocytes. These results support a model in which homologous connections involving pericentromeric heterochromatin are established at the late zygotene-pachytene transition as a result of SC assembly and the consequent close juxtaposition of homologous centromeres (Figure 5C).

Role for the SC in Establishing Homologous Pericentromeric Heterochromatin Connections

To explore the role of the SC in the conversion from non-homologous to homologous pericentromeric associations, we analyzed the effect of SYCP1 on establishment of centromere connections in mouse spermatocytes (Figure 6A). If SYCP1 promotes the conversion of non-

homologous to homologous pericentromeric associations, then we predicted that, in the absence of SYCP1, pericentromeric associations between non-homologous chromosomes will persist as prophase progresses. Homologous chromosomes in diplotene *Sycp1*$^{-/-}$ spermatocytes were aligned in prophase, as previously described (de Vries et al., 2005). However, in sharp contrast to wild-type (14 chromosomes with non-connected DAPI signal from a total of 500 chromosomes), we observed a strikingly high number of diplotene *Sycp1*$^{-/-}$ spermatocytes in which the pericentromeric heterochromatin from each homolog of a pair was engaged in non-homologous interactions (483 chromosomes from a total of 200 diplotene-like spermatocytes; Figure 6A). Our results indicate a role for SYCP1 in establishing homologous heterochromatin connections, likely related to its function in promoting close juxtaposition of homologous pairs.

We also tested whether precocious disruption of SYCP1-mediated centromere pairing at diplotene chromosomes affects heterochromatin homologous interactions. Treatment of short-term cultured wild-type spermatocytes (from one month-old mice) with Cantharidin, a specific PP2A inhibitor, or Hesperadin, which inhibits Aurora kinase activity at centromeres, disrupted centromere pairing coincidental with loss of SYCP1 from centromeres in late diplotene (Figure 6B). Importantly, even under these conditions, homologous centromeres remained connected via pericentromeric heterochromatin (Figure 6C). These results indicate that heterochromatin connections have been already established by mid-late diplotene, when SYCP1 remains at the centromere to mediate centromere pairing. This also indicates that heterochromatin connections between homolog centromeres are stable in the absence of a centromeric SC, suggesting that heterochromatin connections can support the alignment of homolog pairs independently of centromere pairing.

Discussion

Persistent Centromere Associations Promote Proper Homologous Chromosome Segregation

Chiasma formation via recombination is well-recognized to provide the predominant physical linkages between homologous chromosome pairs to facilitate correct orientation on the first meiotic spindle. Therefore, mutations in recombination genes result in a high rate of chromosome non-disjunction. However, proper segregation of meiotic chromosomes can be achieved even in the absence of recombination in a number of model organisms (reviewed in (Obeso et al., 2014; Wolf, 1994)). For example, in yeast and Drosophila, a high proportion of non-exchange chromosomes partition correctly (Dernburg et al., 1996; Guacci and Kaback, 1991; Karpen et al., 1996; Mann and Davis, 1986; Ross et al., 1996). Specialized mechanisms for segregation of non-exchange bivalents have also been proposed in mammals. A balanced orientation of univalent chromosomes has been observed in recombination-mutant mouse oocytes (Woods et al., 1999), and though approximately 5% of meiotic homologous chromosome pairs do not experience exchange in humans, less than 1% of these chromosomes mis-segregate (Cheng et al., 2009b; Fledel-Alon et al., 2009; Hassold et al., 1993a; Oliver et al., 2008; Tease et al., 2002). Here, we show chromosomes that fail to experience crossovers in mouse spermatocytes non-disjoin at much lower levels than would be expected if crossing-over alone supported homologous partner paring. This suggests mice possess non-recombination-based systems that ensure the accuracy of meiotic segregation. We propose centromere interactions promoted by the SC and heterochromatin connections serve as backup mechanisms to recombination, by enabling physical alignment between homologous chromosome pairs during prophase I. These recombination-independent but homology-mediated associations may improve chiasmata chromosome segregation, and most importantly, may provide the only means for proper disjunction of achiasmata chromosomes.

We and others recently identified components and structures that mediate centromere pairing in mice (Bisig et al., 2012; Qiao et al., 2012; Takeo and Hawley, 2012). The SC components SYCP1 and SYCP3 are retained at paired centromeres after the SC is removed from chromosome arms, and are required for persistent pairing of homologous centromeres

(Bisig et al., 2012; Qiao et al., 2012). The notion of centromere pairing connecting homologous chromosomes, even in the absence of chiasmata, is reinforced by the fact that diplotene chromosomes from recombination-deficient mutants have most homologous centromeres engaged in centromere pairing. Intriguingly, in late prophase I, coincidental with nuclear envelope dissolution and removal of SYCP1 from paired centromeres (Bisig et al., 2012), homolog pairs remain tethered by only pericentromeric heterochromatin. This, combined with the fact SYCP1 is essential for establishing centromere pairing, raises the possibility that SYCP1 enables centromere associations to be established. We propose a model (Figure 5C) in which, following removal of the SC at centromeres, pericentromeric heterochromatin connections maintain homolog pair alignment just prior to segregation, as reported in Drosophila (Dernburg et al., 1996). Our results also suggest heterochromatin connections improve segregation of meiotic chromosomes in the mouse and that homologous recognition can lead to segregation, even in the absence of chiasmata.

A high proportion (3.8%) of cells at diakinesis of prophase I contained unpaired X and Y chromosomes. However, only 0.5% of secondary spermatocytes and 0.2% of spermatids were aneuploid. The high fidelity of segregation for achiasmate X-Y pairs may not be facilitated by heterochromatin connections or SC-mediated centromere pairing as centromere interactions were not observed at any stage of prophase I for this chromosome pair, suggesting a specialized backup mechanism exists for achiasmata sex chromosomes.

Role for Heterochromatin in Maintaining Meiotic Chromosome Alignment

Early work on chromosome segregation in female *Drosophila* demonstrated heterochromatic chromosomal connections are sufficient to support homologous achiasmate chromosome segregation (Dernburg et al., 1996; Karpen et al., 1996; Theurkauf and Hawley, 1992). Subsequent work demonstrated chromosomes that fail to undergo recombination (*X* and *4*) in oocytes are connected by heterochromatic threads during prometaphase I, and these threads

are likely to be part of the mechanism by which heterochromatin facilitates achiasmate chromosome segregation (Hughes et al., 2009). Additional evidence for the conservation of heterochromatic threads connecting chromosomes during meiosis comes from *Drosophila* and crane fly sperm (Hartl et al., 2008; LaFountain et al., 2002). The results presented here demonstrate heterochromatin also plays a role in promoting meiotic centromere interactions in the mouse, and that these interactions may promote proper achiasmate chromosome segregation.

Origin and Regulation of Heterochromatin-Mediated Centromere Clustering Early in Prophase

Observations in a wide range of organisms show that very early in the meiotic program, before homologous pairing occurs and independent of homology, centromeres associate in pairs or clusters (reviewed in (Obeso et al., 2014; Stewart and Dawson, 2008). This is termed centromere coupling (Tsubouchi and Roeder, 2005). Our work on centromeric heterochromatin associations early in prophase confirms previous observations of centromere clustering in the mouse (Takada et al., 2011) and advance our investigation to revealing the non-homologous nature of these interactions. Although the function of centromere coupling remains unknown, new information regarding the origin and nature of early centromere clustering has been obtained. Firstly, spermatocytes deficient for the histone H3 lysine 9 mono- and di-methyltransferase G9a and the heterochromatin-binding protein HP1ɣ exhibit an increase in the number of heterochromatin clusters of centromeres, with each cluster containing fewer centromeres (Takada et al., 2011). HP1ɣ appears to be required to localize G9a to pericentromeric heterochromatin and generate H3K9me2 at these sites. These observations suggest that the HP1ɣ/G9a axis is required to maintain clustering of pericentromeric heterochromatin and/or re-cluster pericentromeric heterochromatin early in prophase.

Origin and Regulation of Homologous Heterochromatin Connections

When and how are homologous heterochromatin connections established? Our results define a period of prophase I in which the SC promotes the stable homologous pericentromeric heterochromatin interactions observed between diplotene chromosomes. First, in contrast to late diplotene chromosomes in which most centromeres are homologously paired, leptotene to late zygotene spermatocytes displayed a strikingly high number of non-homologous centromeres connected by heterochromatin. This indicates chromosome synapsis at or near centromeric regions enforces a transition from heterologous to homologous heterochromatin interactions. Second, diplotene *Sycp1*$^{-/-}$ spermatocytes have abnormally high numbers of unpaired chromosomes and chromosomes engaged in non-homologous centromeric associations, suggesting the SC plays a role in establishment of homologous heterochromatin connections. However, precocious removal of the SC from paired centromeres of diplotene chromosomes disrupted the close juxtaposition of homologous centromeres (centromere pairing) but did not affect heterochromatin interactions between homologous pairs. This indicates the SC participates in establishment of pericentromeric associations but is not required to maintain these interactions, and that a specialized mechanism exists to stabilize heterochromatin connections between homolog pairs independently of the SC.

What is the nature of heterochromatin interactions and what activity could disrupt heterochromatin connections from homologous centromeres when they segregate? The tight physical association of heterochromatin observed in Drosophila oocytes during early meiosis suggests heterochromatin connections may be established during DNA replication (Dernburg et al., 1996). It has been suggested that linkages are established during stalled replication fork repair (Hughes et al., 2009), whereby catenation of DNA strands would hold homologs together via their heterochromatin until topoisomerase II or other enzymes resolve them. Consistent with this notion, topoisomerase or an associated protein (*i.e.*, BLM dissolvasome components) may resolve DNA catenanes that are part of the physical connections between sister-chromatids in

somatic cells. Indeed, upon challenge with a topoisomerase II inhibitor or topoisomerase IIα depletion, the number of DNA bridges between sister chromatids sharply increases, and immunostaining for PICH or BLM, helicases that may be involved in DNA decatenation, is commonly used to detect DNA bridges (reviewed in (Chan and Hickson, 2011)). Topoisomerase IIα may also be involved in chromatin decatenation in mouse spermatocytes, as this protein is detected at mouse prophase I spermatocyte chromocenters and stains DNA bridges in etoposide-treated anaphase I cells (Gomez et al., 2014). While this is an attractive model, the fact homologous heterochromatin connections are not detected in actively replicating meiocytes indicate DNA replication may not be the primary mechanism of establishing homologous heterochromatin connections in mouse spermatocytes.

Products of recombination are an alternative to DNA catenation products as the origin of pericentromeric heterochromatin interactions. Again, this is supported by work in mitotic cells, in which ultra-fine thread-like DNA bridges connecting centromeres (Chan et al., 2007) are marked by FANCD2/I DNA repair proteins (Chan and Hickson, 2009). Finally, it is possible that protein-protein or protein-DNA interactions of a different nature may promote post-pachytene stable homologous heterochromatin interactions. Centromeric regions are enriched for cohesion proteins and the roles of different types of meiotic cohesion complexes remain unclear. It is possible that cohesins act to form interhomolog cohesion that links centromeric heterochromatin or alternatively provide an environment in which catenation or other links between partner chromosomes are maintained until metaphase, consistent with the observation that most DNA bridges in mitotic cells are observed at centromeres. Further work in mouse spermatocytes should test these hypotheses.

Experimental Procedures

Mouse Strains

The Oklahoma Medical Research Foundation Animal Care and Use Committee approved all animal protocols. The following mice were used in this study: C57BL/6, *Hfm1*$^{-/-}$(Guiraldelli *et al.,* 2013*), Mlh3*$^{-/-}$ (Lipkin *et al.,* 2002), *Hfm1*$^{-/-}$*/Mus81*$^{-/-}$(Holloway *et al.,* 2008), *Kash5*$^{-/-}$(Horn *et al.,* 2013), *Sun1*$^{-/-}$ (Chi *et al.*, 2009*),* and *Sycp1*$^{-/-}$(de Vries *et al.,* 2005).

Cytology

Fixed spermatocyte images were analyzed using AxioVision software (Zeiss). Established approaches were employed for visualizing chromosomes in surface spreads [47]. Statistical tests were as described in the figure legends or text.

FISH combined with immunostaining

We used a combination of standard immunofluorescence and FISH protocols to assess homologous and heterologous centromeric associations. FISH was performed using specific fluorescent point probes for chromosomes 8, 15 and 2 (ID Labs Inc.).

Supplemental Information

Supplemental Information includes two figures and one table and can be found with this article online.

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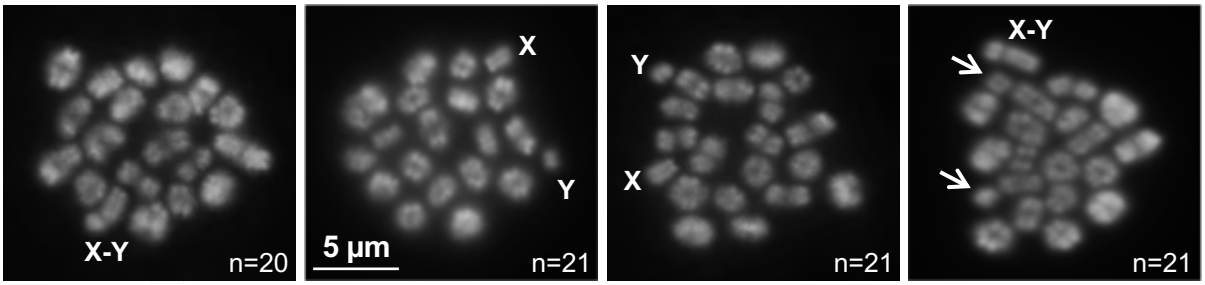
Figure 1

A

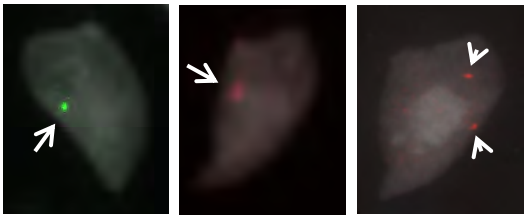
Metaphase I



Total cells	20 bivalents	21 bivalents	>21 bivalents	Achiasmata X Y	Achiasmata somatic
n=650	91.5%	6.9%	1.5%	3.8%	4.6%

B

Elongating spermatid

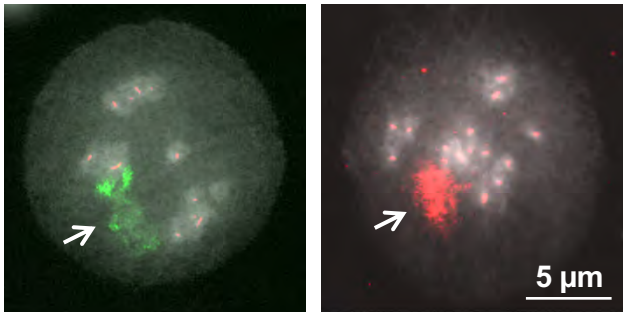


FISH8 FISH15 FISH8/19

Chromosome	1 FISH signal
8	99.8% n=400
15	99.6% n=718
19	99.5% n=546

C

Secondary spermatocyte

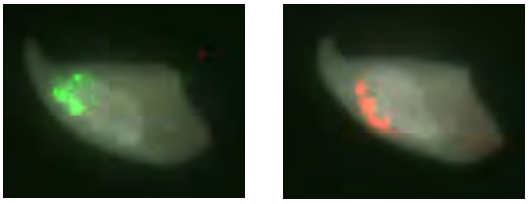


FISHX/SYCP3 FISHY/SYCP3

Total cells	>1 FISH signal	1 FISH X signal	1 FISH Y signal
n=300	0.5%	59.5%	40%

D

Elongating spermatid



FISHX FISHY

Total cells	>1 FISH signal	1 FISH X signal	1 FISH Y signal
n=1200	0.2%	55%	45%

E

Cell stage	TUNEL positive cells (%)
Metaphase I-telophase I	1.13 n=354
Secondary spermatocyte	0 n=350
Rounded spermatid	0.2 n=1515
Elongating spermatid	0.1 n=1991

Figure 1. Estimated numbers of achiasmate meiosis and aneuploid cells during late mouse spermatogenesis. A. Images and bivalent counts for cells at diakinesis of prophase I. X and Y indicate sex chromosomes; arrows indicate somatic univalent chromosomes. **B**. Examples of cells with one FISH signal (tailed arrows) for the indicated chromosomes and FISH signal counts in elongating spermatids. The definition of one or two FISH signals was based on whether the two signals touch each other or their centers were ≤1 μm apart. Arrowheads in the upper right image indicate two separate FISH signals for chromosome 8- and 19-specific probes. **C**. Examples of cells with one FISH signal for the indicated chromosomes and FISH signal count in secondary spermatocytes. **D**. Examples of cells with one FISH signal for the X and Y chromosomes and FISH signal count in elongating spermatids. **E**. Estimated numbers of apoptotic cells at different stages of mouse spermatogenesis.

Figure 2

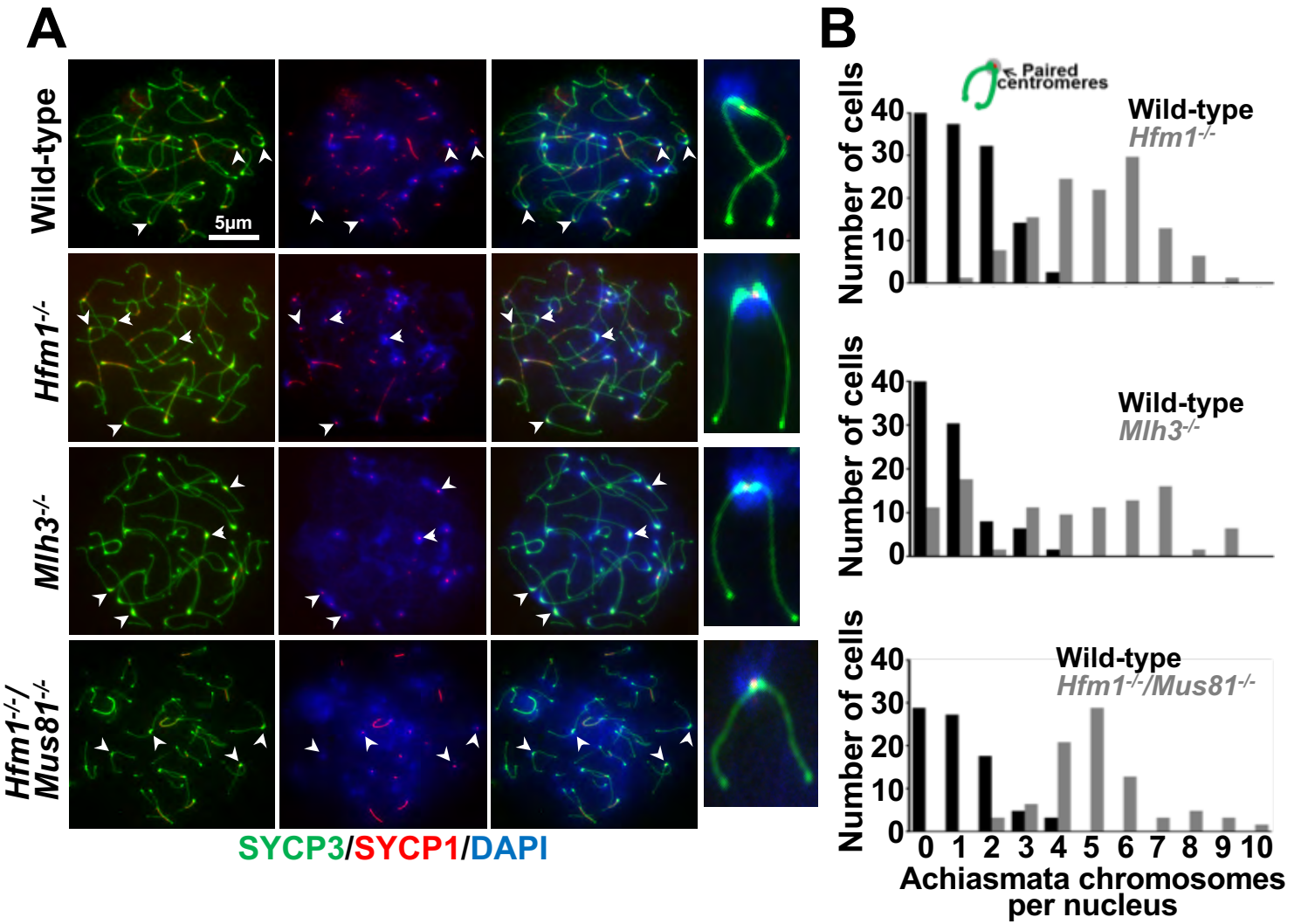

A
Wild-type
Hfm1-/-
Mlh3-/-
Hfm1-/-/ Mus81-/-
5μm
SYCP3/SYCP1/DAPI
B
Paired centromeres
Wild-type
Hfm1-/-
Wild-type
Mlh3-/-
Wild-type
Hfm1-/-/Mus81-/-
Number of cells
0
10
20
30
40
0 1 2 3 4 5 6 7 8 9 10
Achiasmata chromosomes per nucleus

Figure 2. Homologous centromere associations maintain homologous pair alignment in the absence of chiasmata. A. Examples of spermatocyte chromosome spreads from wild-type, *Hfm1*$^{-/-}$, *Mlh3*$^{-/-}$ and *Hfm1*$^{-/-}$/*Mus81*$^{-/-}$ mice. Note high numbers of homologous pairs only tethered by centromeres. Magnified chromosomes are examples of a single exchange wild-type chromosome with paired centromeres and achiasmate homologous chromosomes only tethered by centromeres. Arrows indicate some paired centromeres. Scale bar = 5 μm for all images except magnified images of individual chromosomes. **B.** Distribution of the number of homologous chromosomes connected by centromere pairing per spermatocyte in *Hfm1*$^{-/-}$, *Mlh3*$^{-/-}$, *Hfm1*$^{-/-}$/*Mus81*$^{-/-}$, and wild-type littermate mice; all mutants had increased numbers of chromosomes only tethered by centromere pairing.

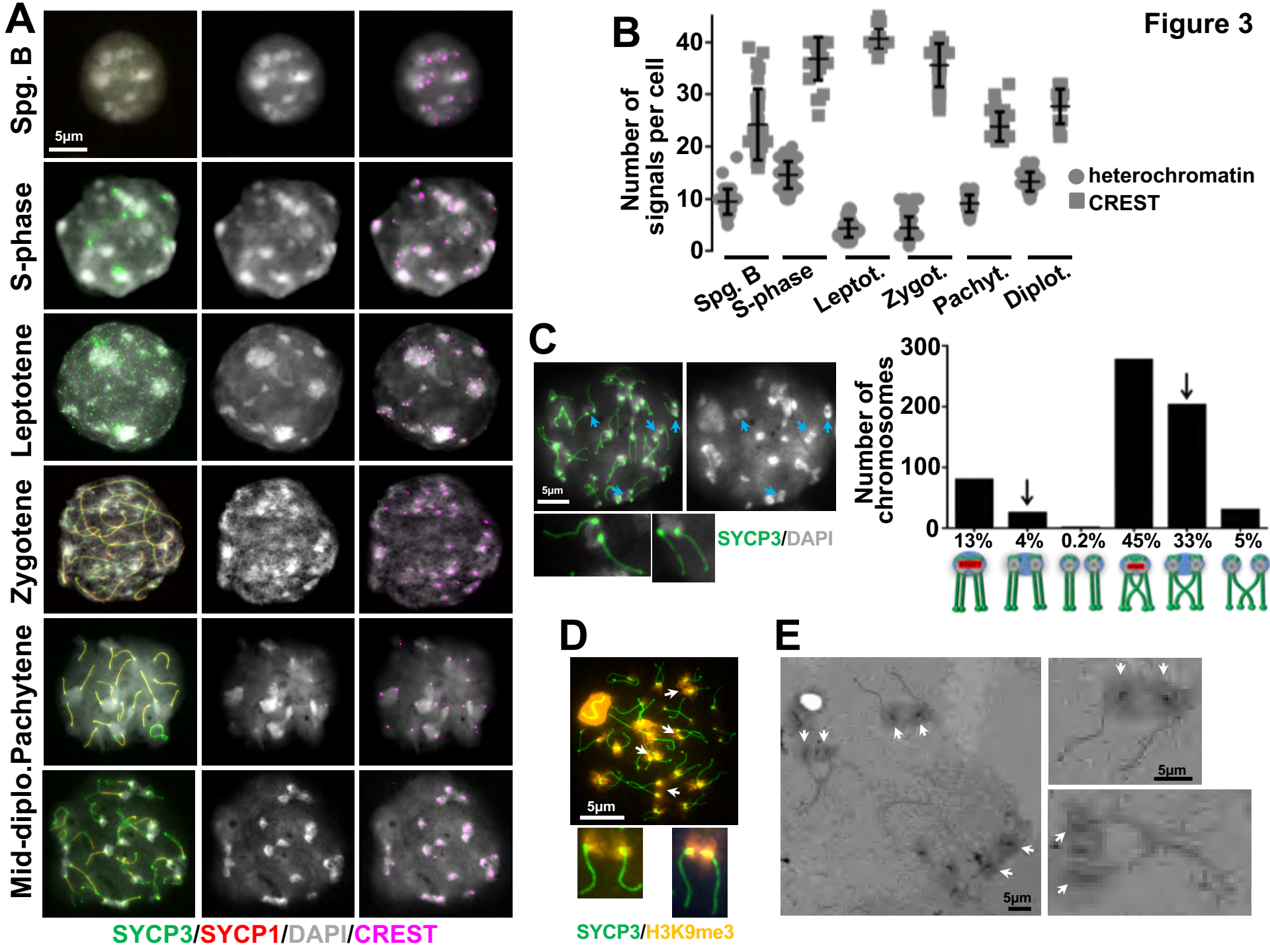
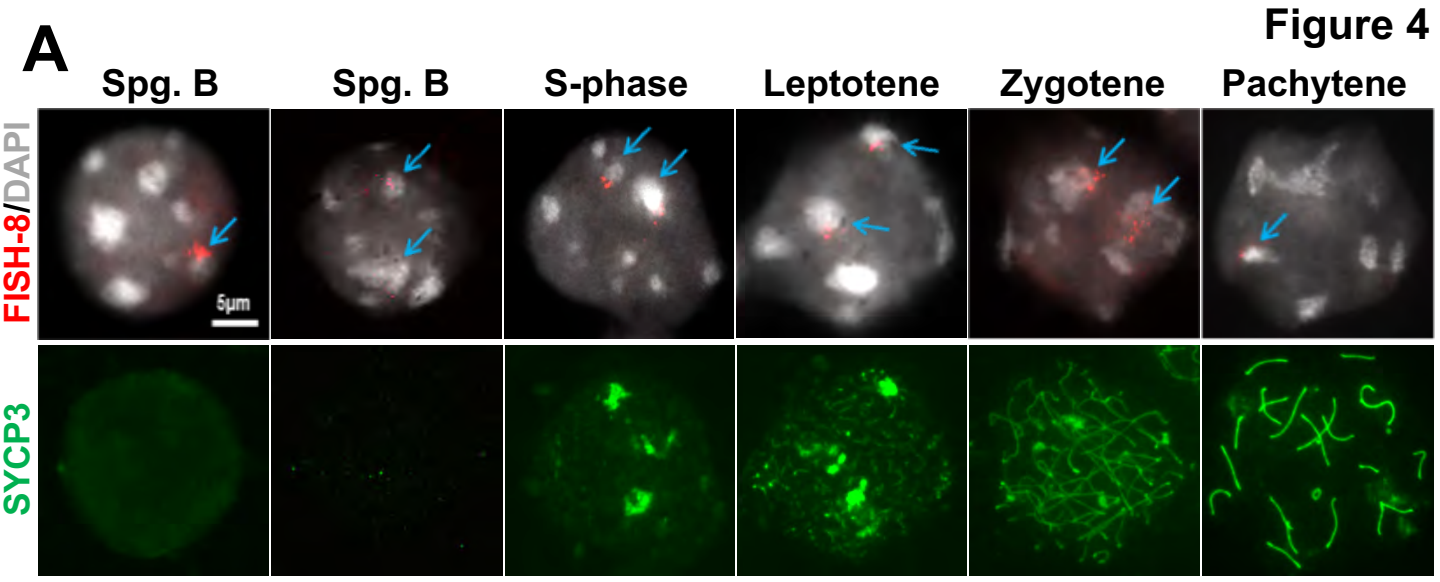
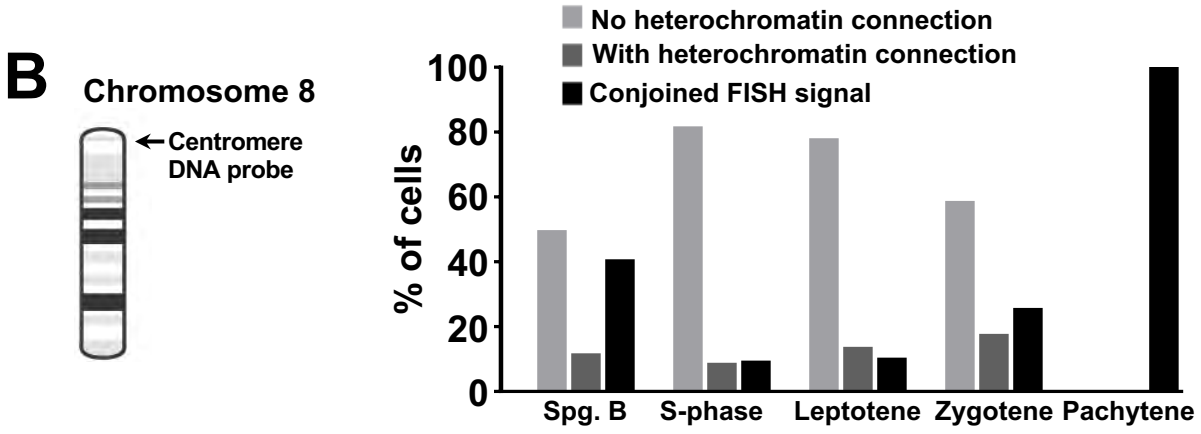


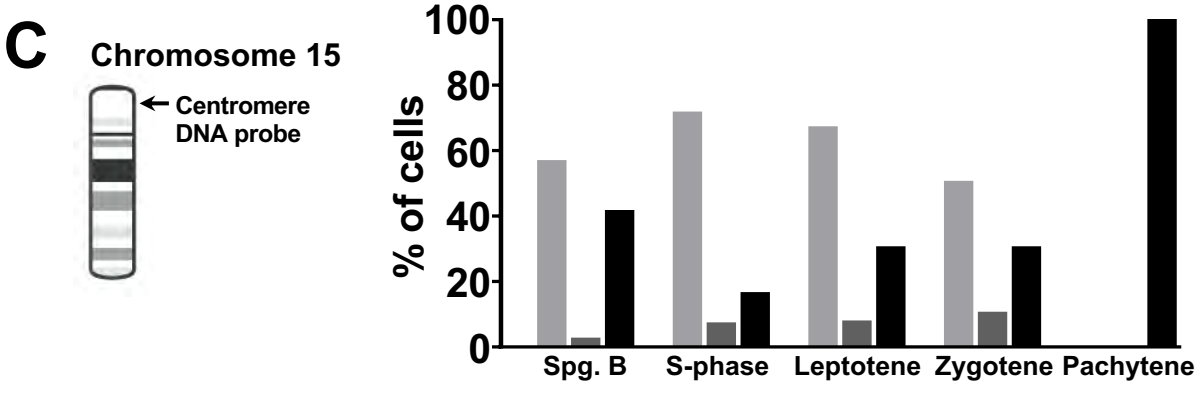
Figure 3
A
Spg. B
S-phase
Leptotene
Zygotene
Mid-diplo.Pachytene
5µm
SYCP3/SYCP1/DAPI/CREST
B
Number of signals per cell
0
10
20
30
40
heterochromatin
CREST
Spg. B
S-phase
Leptot.
Zygot.
Pachyt.
Diplot.
C
5µm
SYCP3/DAPI
Number of chromosomes
0
100
200
300
13%
4%
0.2%
45%
33%
5%
D
5µm
SYCP3/H3K9me3
E
5µm
5µm

Figure 3. Dynamics of centromeric heterochromatin configuration during prophase in mouse spermatocytes. A. Examples of wild-type spermatocytes at different stages of meiotic prophase I. Heterochromatin was visualized using DAPI, and SYCP3 and SYCP1 immunostaining were used to stage spermatocytes and visualize the SC in paired centromeres. CREST served as a marker for centromeres. **B**. Quantitation of heterochromatin and centromere association in mouse spermatocytes. Note the number of individual DAPI-stained heterochromatin bodies increased as spermatocytes transition to the later stages of prophase, consistent with individualization of homologous chromosome pairs in preparation for segregation. Horizontal lines denote means; vertical lines, standard deviations. **C**. Example of a diplotene spermatocyte showing chromosomes apparently only tethered by heterochromatin. Magnified chromosomes show details of pairs of homologs. Scale bar = 5 μm except for magnified images of individual chromosomes. Quantification of chromosomes only tethered by SC-mediated centromere pairing and pericentromeric heterochromatin at diplotene in spermatocyte spreads; non-exchange and chiasmata chromosomes experiencing SYPC1-mediated paired centromeres, centromeres only tethered by heterochromatin, and non-connected centromeres were scored. **D**. Example of a diplotene spermatocyte showing chromosomes connected by pericentromeric H3K9me3-immunopositive heterochromatin. Magnified images show details of homolog pairs. **E**. Electron microscopy of silver-stained wild-type diplotene spermatocytes showing chromosomes connected by electron-dense pericentromeric heterochromatin. Magnified chromosomes show details of chiasmata and apparent achiasmata homologous pairs.

Figure 4


A
Spg. B
Spg. B
S-phase
Leptotene
Zygotene
Pachytene
FISH-8/DAPI
SYCP3
5μm


B
Chromosome 8
Centromere DNA probe
No heterochromatin connection
With heterochromatin connection
Conjoined FISH signal
% of cells
100
80
60
40
20
0
Spg. B
S-phase
Leptotene
Zygotene
Pachytene


C
Chromosome 15
Centromere DNA probe
% of cells
100
80
60
40
20
0
Spg. B
S-phase
Leptotene
Zygotene
Pachytene

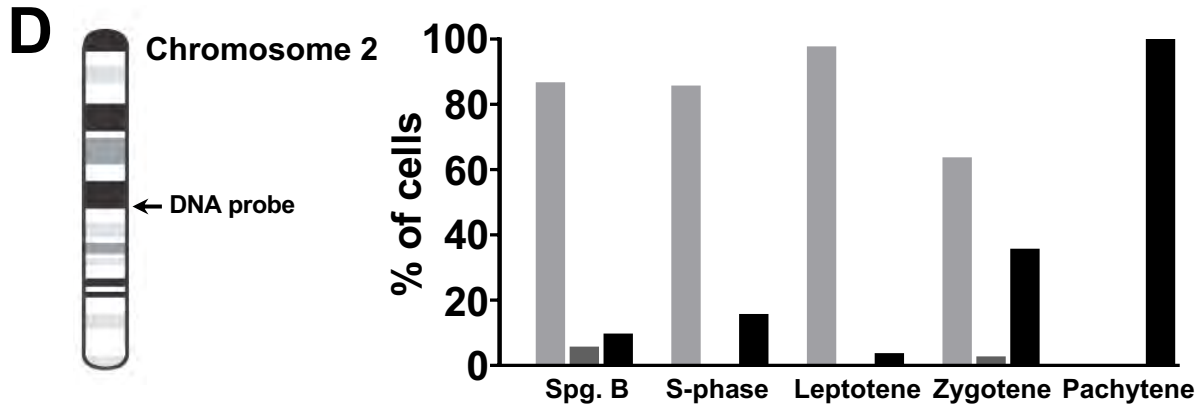
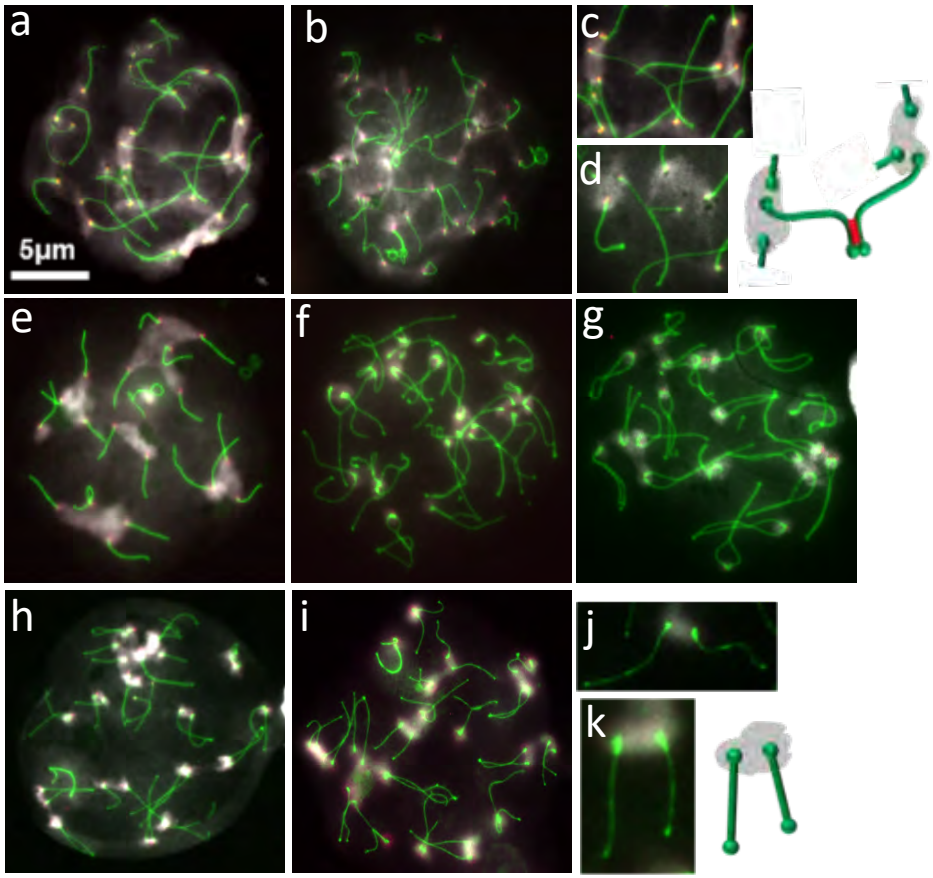

D
Chromosome 2
DNA probe
% of cells
100
80
60
40
20
0
Spg. B
S-phase
Leptotene
Zygotene
Pachytene

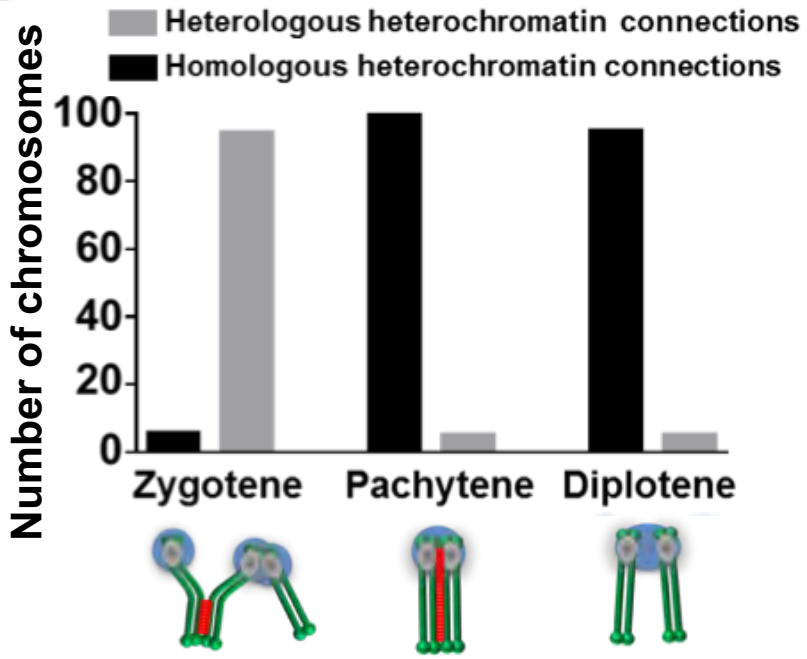
Figure 4. Pre-meiotic cells and early prophase I spermatocytes show no signals for homologous pericentromeric heterochromatin connections. A. Examples of spermatocytes showing FISH signals for a chromosome 8 region-specific probe (arrows). SYCP3 immunostaining was used for spermatocyte staging; DAPI indicates pericentromeric heterochromatin. (**B**, **C** and **D**) Diagrams of chromosomes 8, 15 and 2 showing the FISH probe DNA target sites. Quantitation of pre-meiotic cells (Spg. B, type B spermatogonia) and spermatocytes at different stages of prophase I with and without heterochromatin connections between homologous centromere pairs. Heterochromatin connections were defined as two discreet FISH signals separated by a gap; heterochromatin connections, discreet FISH signals bridged by the DAPI signal.

Figure 5

A


a
b
c
d
e
f
g
h
i
j
k
5μm
SYCP3/CREST/DAPI

B


Heterologous heterochromatin connections
Homologous heterochromatin connections
Number of chromosomes
100
80
60
40
20
0
Zygotene
Pachytene
Diplotene

C

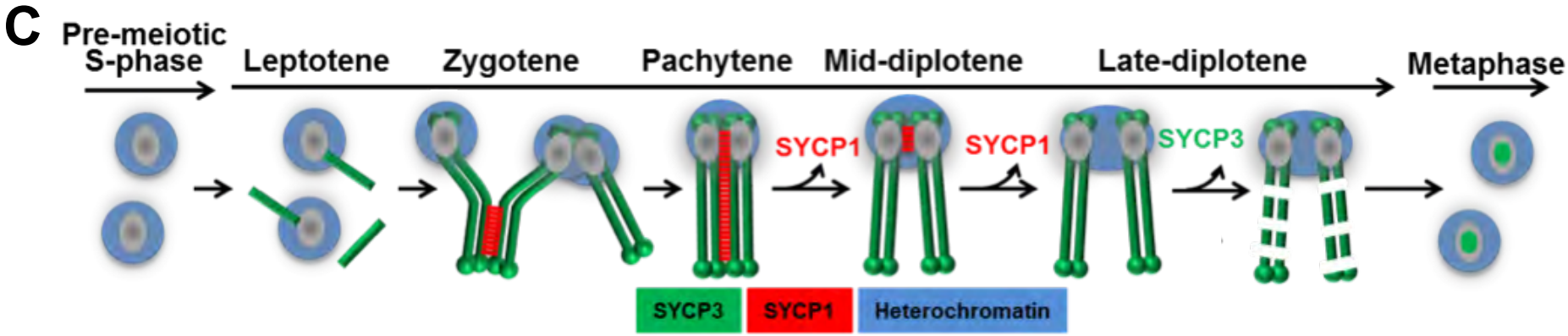
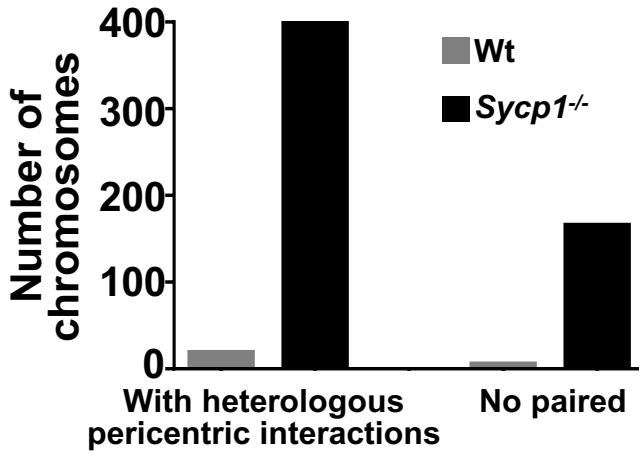

Pre-meiotic S-phase
Leptotene
Zygotene
Pachytene
Mid-diplotene
Late-diplotene
Metaphase
SYCP1
SYCP1
SYCP3
SYCP3
SYCP1
Heterochromatin

Figure 5. Two mechanisms of centromere associations exist in mouse spermatocytes: one directed by SYCP1 at centromeres and the other by centromeric heterochromatin. A. Representative zygotene (panels a-d), pachytene (panel e), mid-diplotene (panels f and g) and late diplotene (panels h-k) spermatocytes. Note the high number of chromosomes in zygotene spermatocytes in which heterologous heterochromatin-mediated centromeric interactions are preferred over homologous connections. Note the high frequency of homolog pairs in late diplotene cells with centromeres solely connected by heterochromatin. Scale bar = 5 μm for all images except magnified images of individual chromosomes. **B**. Quantitation of heterologous and homologous pericentromeric heterochromatin associations in zygotene, pachytene and diplotene nuclei. **C**. Heterochromatin association and SC-mediated centromere pairing events in mouse spermatocytes. The proposed series of events for heterologous and homologous centromeric interactions mediated by heterochromatin and SC-directed centromere pairing are illustrated.

Figure 6

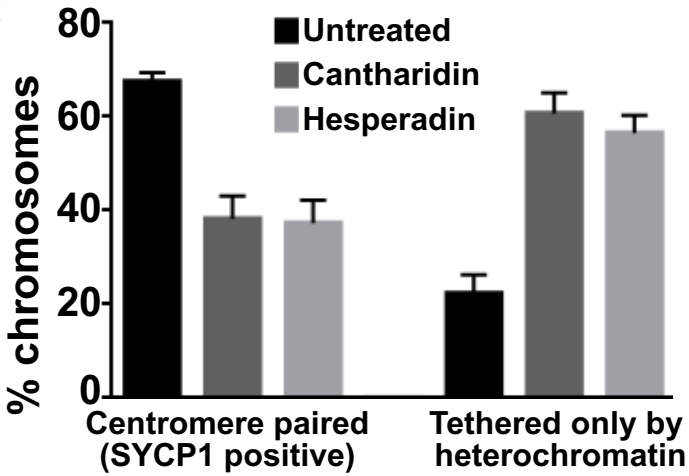
A

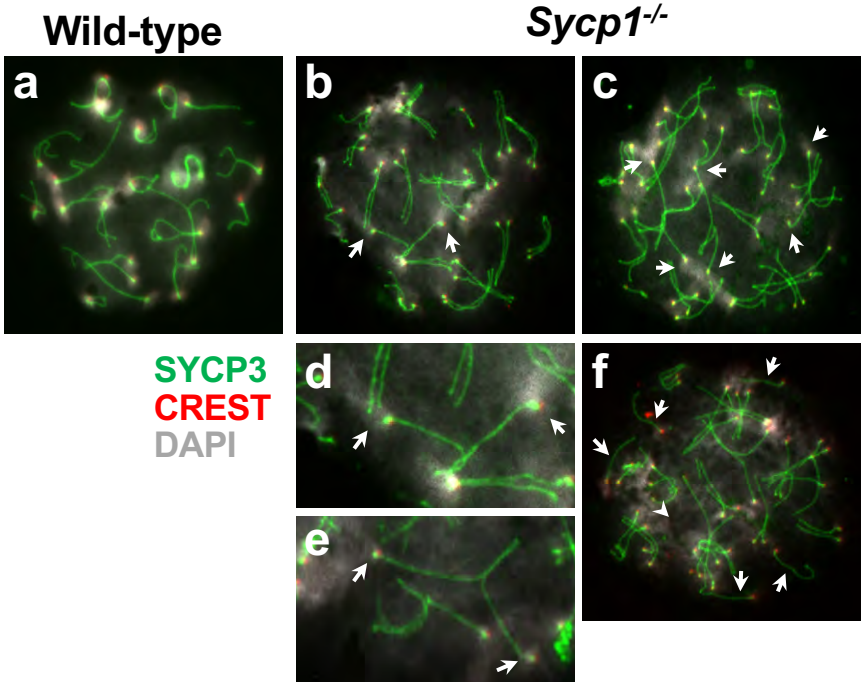


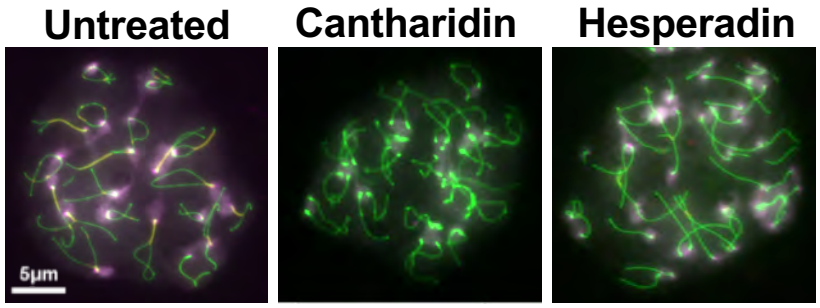
B

Experimental condition	% chromosomes with paired centromeres	% chromosomes with unpaired centromeres	% centromeres SYCP1 positives
No treatment	67.4±10.7%	26.2±11.3%	67.6±10.8%
Cantharidin (10μM)	38.0±4.9% p<0.002	60.7±4.5% p<0.002	38.2±4.9% p<0.002
Hesperadin (10μM)	37.3±4.9% p<0.002	56.2±3.8% p<0.002	37.3±4.9% p<0.002

C







SYCP3/SYCP1/CREST/DAPI

Figure 6. The SC is required for establishment but not maintenance of homologous heterochromatin-mediated centromere connections. A. Quantitation of *Sycp1*$^{-/-}$ diplotene spermatocytes; a high number of homologous centromeres engage in heterologous heterochromatin associations. Note in contrast to wild-type spermatocytes, S*ycp1*$^{-/-}$ cells had a high number of unpaired homologs due to loss of homologous pericentromeric interactions. Examples of diplotene spermatocyte chromosome spreads from wild-type (panel a) and S*ycp1*$^{-/-}$ (mid-diplotene, panel b-e and late-diplotene, panel f) littermates are presented. Arrows indicate the centromeric ends of zygotene chromosomes engaged in heterologous heterochromatin interactions. In panel f, arrows mark unpaired chromosomes. Scale bar = 5 μm for all images except the magnified images of individual chromosomes. **B**. Small molecule inhibitor treatment of diplotene spermatocytes resulted in loss of SYCP1 from centromeres and centromere pairing disruption. **C**. After cantharidin and hesperadin treatment pairs of homologous chromosomes from cultured wild-type diplotene spermatocytes remained tethered only by centromeric heterochromatin. The pictures show examples of spermatocytes treated with small molecule inhibitors to prematurely remove the centromeric SC. An untreated cell is shown as a control. Scale bar = 5 μm.

Supplementary Data

Heterochromatin and the Synaptonemal Complex Maintain Homologous Centromere Interactions to Promote Meiotic Chromosome Segregation in Mouse Spermatocytes

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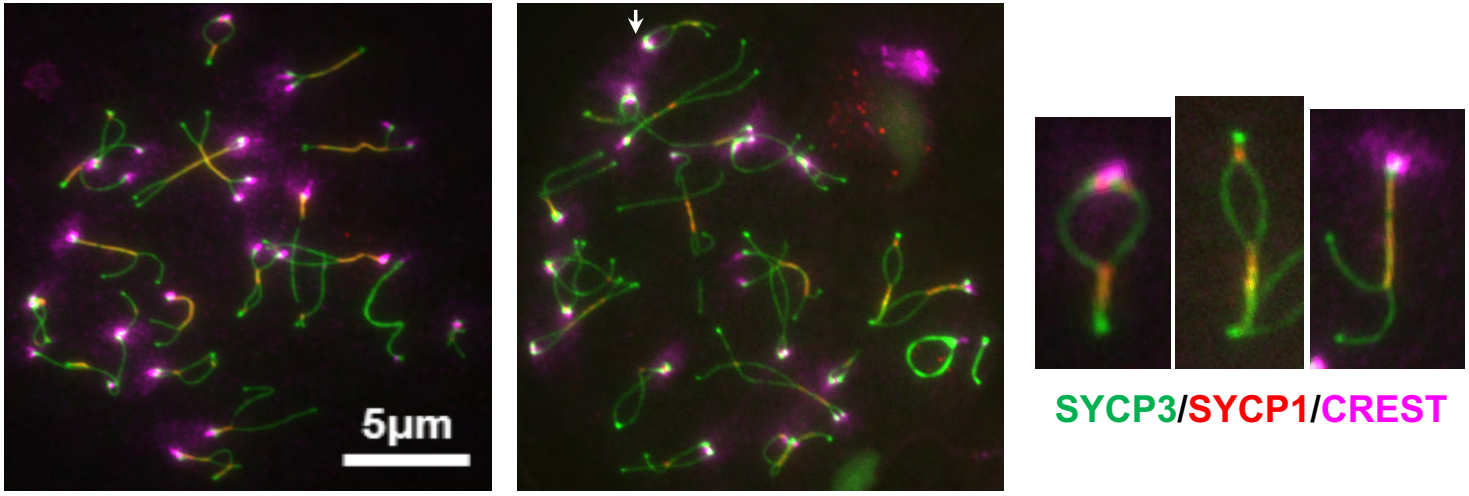
Table S1

	Cell stage	% cells with no het. Connection	% cells with het. connection	% cells with Conjoined FISH signal
Chromosome 8	Spg. B, n=350	49	11	40
	S-phase, n=80	81	8.1	8.8
	Leptotene, n=35	78	13	10
	Zygotene, n=60	58	17	25
	Pachytene, n=190	0	0	100
Chromosome 15	Spg. B, n=287	58	3	42
	S-phase, n=59	72	7	18
	Leptotene, n=30	70	8	31
	Zygotene, n=66	51	10	30
	Pachytene, n=127	0	0	100
Chromosome 19	Spg. B, n=1037	85	4	9
	S-phase, n=34	84	0	18
	Leptotene, n=32	98	0	3
	Zygotene, n=53	60	2	30
	Pachytene, n=205	0	0	100

Figure S1. Pattern of homologous chromosome desynapsis in diplotene mouse spermatocytes. A. Example of diplotene spermatocyte chromosome spreads showing chromosomal regions undergoing synaptonemal complex removal. Magnified chromosomes are examples of chromosomes showing different desynapsed chromosome areas. Scale bar = 5 μm for all images except for magnified images of individual chromosomes. **B**. Quantitation of diplotene chromosomes with different desynapsed areas.

Figure S1

A



B

No. of Partially Synapsed Bivalents, N (%)		Centromeric	Interstitial	Telomeric	Separated centromeres
Early diplotene	301(24)	55(15)	36(29)	116(26)	94(29)
Middle diplotene	634(50)	209(57)	71(58)	227(51)	127(39)
Late diplotene	326(26)	105(29)	16(13)	104(23)	101(31)
Total	1,261	369(29)	123(10)	447(35)	322(26)

Figure S2. Example of spermatocytes at different stages of prophase I showing immunolocalization of H3K9me3 and H3K9me2. SYCP3 antibodies were used to mark chromosome cores. Scale bar = 5 μm for all images.

Figure S2

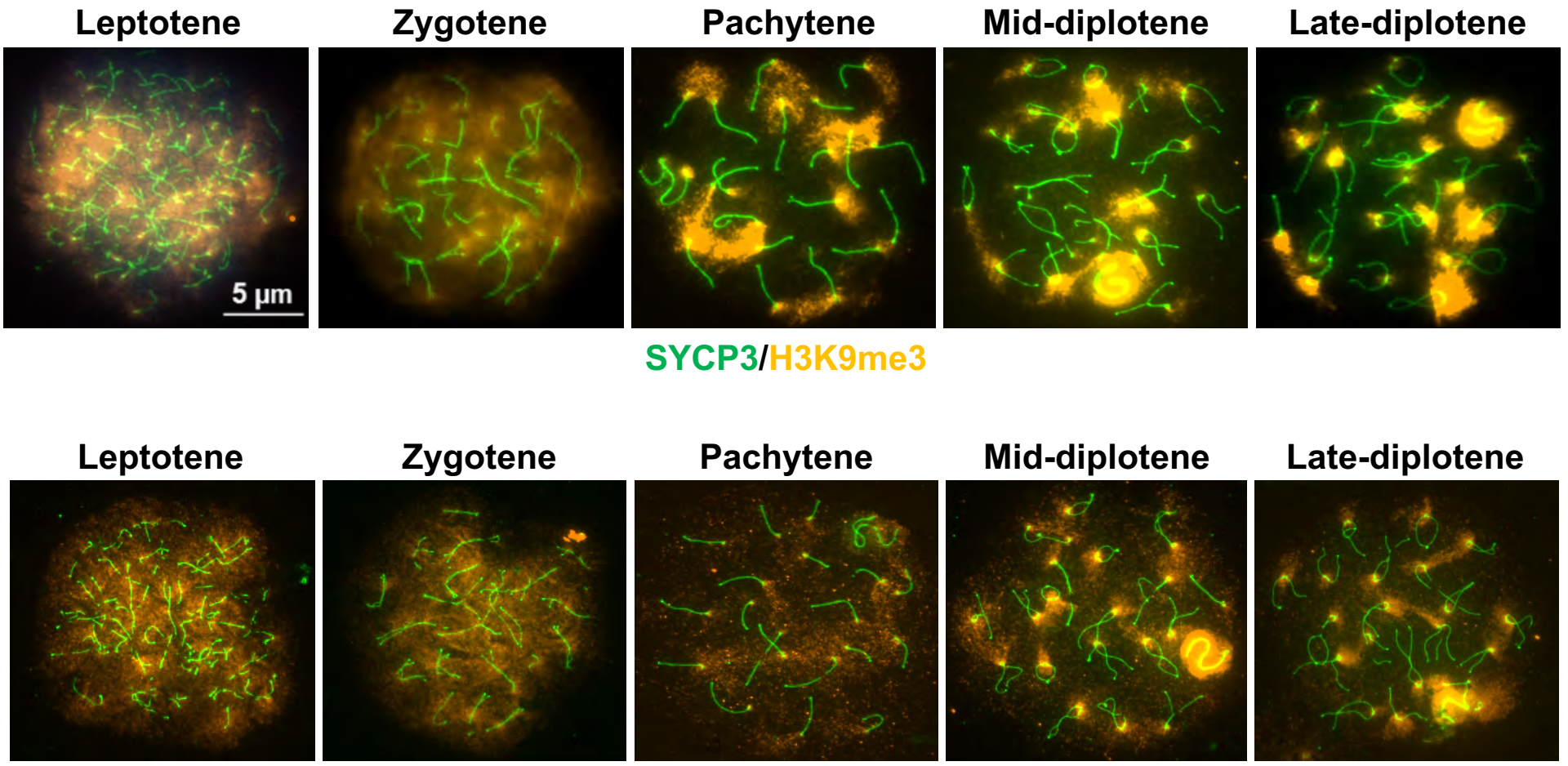

Leptotene
Zygotene
Pachytene
Mid-diplotene
Late-diplotene
5 µm
SYCP3/H3K9me3
Leptotene
Zygotene
Pachytene
Mid-diplotene
Late-diplotene
SYCP3/H3K9me2

Table S1. Numbers of cells showing heterochromatin connections. Quantitation of pre-meiotic cells and spermatocytes at different stages of prophase I with and without heterochromatin connections between homologous centromere pairs.