# Meiotic Centromere Coupling and Pairing Function by Two Separate Mechanisms in *Saccharomyces cerevisiae*

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**ABSTRACT** In meiosis I, chromosomes become paired with their homologous partners and then are pulled toward opposite poles of the spindle. In the budding yeast, *Saccharomyces cerevisiae*, in early meiotic prophase, centromeres are observed to associate in pairs in a homology-independent manner; a process called centromere coupling. Later, as homologous chromosomes align, their centromeres associate in a process called centromere pairing. The synaptonemal complex protein Zip1 is necessary for both types of centromere association. We aimed to test the role of centromere coupling in modulating recombination at centromeres, and to test whether the two types of centromere associations depend upon the same sets of genes. The *zip1-S75E* mutation, which blocks centromere coupling but no other known functions of Zip1, was used to show that in the absence of centromere coupling, centromere-proximal recombination was unchanged. Further, this mutation did not diminish centromere pairing, demonstrating that these two processes have different genetic requirements. In addition, we tested other synaptonemal complex components, Ecm11 and Zip4, for their contributions to centromere pairing. *ECM11* was dispensable for centromere pairing and segregation of achiasmate partner chromosomes; while *ZIP4* was not required for centromere pairing during pachytene, but was required for proper segregation of achiasmate chromosomes. These findings help differentiate the two mechanisms that allow centromeres to interact in meiotic prophase, and illustrate that centromere pairing, which was previously shown to be necessary to ensure disjunction of achiasmate chromosomes, is not sufficient for ensuring their disjunction.

**KEYWORDS** Zip1; Zip4; centromere pairing; chromosome segregation; meiosis; synaptonemal complex

ACCURATE chromosome segregation in meiosis is important for preservation of the genome of an organism through multiple generations. In meiosis I, the cell is presented with a unique challenge in which homologous chromosomes must segregate from one another. This is followed by a mitosis-like segregation of the sister chromatids in meiosis II. In *Saccharomyces cerevisiae*, chromosomes interact with other chromosomes in meiosis I in four defined ways that will be introduced here: crossing over, centromere coupling, synaptonemal complex (SC) formation, and centromere pairing (reviewed in Kurdzo and Dawson 2015).


doi: 10.1534/genetics.116.190264
Manuscript received April 8, 2016; accepted for publication November 25, 2016; published Early Online December 2, 2016.
Supplemental material is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.116.190264/-/DC1.
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During meiotic prophase, homologous chromosome partners go through a series of events that culminate in the formation of crossovers between the homologous partners. In early prophase, double-strand breaks (DSBs) in the DNA are created by the endonuclease Spo11 (Keeney *et al.* 1997), homologous partners align, and a proteinaceous structure called the SC assembles along the axes of the homologs (reviewed in Kurdzo and Dawson 2015). The SC is comprised of two axial elements that run along the axes of each homolog and a central region that joins the axial elements together along their length. Repair of the DSBs by homologous recombination is critical for the formation of crossovers that, together with sister chromatid cohesion, serves to tether homologous partners together as they go through the process of attaching to the meiotic spindle (Keeney *et al.* 1997; Celerin *et al.* 2000).

Coincident with the formation of DSBs, centromeres undergo a period of pairwise associations that are homology independent and are referred to as centromere coupling

(Tsubouchi and Roeder 2005; Obeso and Dawson 2010). Similar coupling or clustering of centromeres, or regions of pericentric heterochromatin, have been observed in a number of organisms, including onion (Church and Moens 1976), wheat (Bennett 1979; Martínez-Pérez *et al.* 1999), rice (Prieto *et al.* 2004), fission yeast (Ding *et al.* 2004; Tsubouchi and Roeder 2005; Obeso and Dawson 2010), maize (Zhang *et al.* 2013), and mouse (Scherthan *et al.* 1996; Takada *et al.* 2011). The reason behind this centromere coupling or clustering remains unclear, but recent studies in yeast suggest the chromosomes show a length-dependent preference for partner choice during centromere coupling, which may improve the efficiency of homologous pairing later in meiosis (Lefrancois *et al.* 2016).

As the chromosomes begin to synapse with their homologs, the centromeres seem to transition individually from nonhomologous coupling to pairing with their homologous centromere, as there is never a time in wild-type (WT) cells when all the centromeres are fully dispersed between the coupling and pairing stages (Obeso and Dawson 2010). When the homologous partners are fully synapsed (pachytene stage), remaining pairs of natural or artificial chromosomes that have failed to recombine (achiasmate partners) can be seen to pair at their centromeres (called centromere pairing) (Kemp *et al.* 2004; Gladstone *et al.* 2009; Newnham *et al.* 2010).

As the cells transition out of pachytene, the SC largely disassembles to reveal a small stretch of Zip1 left behind at the centromeres; suggesting that, like achiasmate partners, the chiasmate homologous chromosomes are also joined at their centromeres in pachytene. This type of pairing has been observed in yeast (Kemp *et al.* 2004; Gladstone *et al.* 2009; Newnham *et al.* 2010), but similar centromere-centromere interactions in late prophase (after SC disassembly) have also been observed in *Drosophila* (Dernburg *et al.* 1996; Takeo *et al.* 2011), fission yeast (Davis and Smith 2003; Ding *et al.* 2004), and mouse spermatocytes (Bisig *et al.* 2012; Qiao *et al.* 2012). Centromere pairing has been proposed to serve as an alternative means to tether partners that have failed to become joined by chiasmata (Dawson *et al.* 1986), and has been shown in genetic experiments to promote the biorientation of homologous chiasmate partners on the spindle (Gladstone *et al.* 2009).

The genes that are necessary for centromere coupling and pairing remain largely undefined. When centromere coupling was first discovered, it became clear that the protein Zip1 was necessary to tack the two centromeres together (Tsubouchi and Roeder 2005). Zip1 is a component of the transverse filament of the central region of the SC in budding yeast (Sym *et al.* 1993; reviewed in Kurdzo and Dawson 2015). Cohesin was found to be necessary for coupling as well, but this might be because faulty cohesin leads to a lack of correct Zip1 localization (Chuong and Dawson 2010). Zip1 is a member of the ZMM group of proteins (Zip1, Zip2, Zip3, Zip4, Mer3, Msh4, Msh5, and Spo16), also known as the synapsis initiation complex, that are required for SC assembly (Chua and Roeder 1998; Agarwal and Roeder 2000; Novak *et al.* 2001; Borner *et al.* 2004; Fung *et al.* 2004; Jessop *et al.* 2006; Shinohara *et al.* 2008). *ZIP2* and *ZIP3* are dispensable for centromere coupling, as is *RED1*, which encodes an axial element protein (Chuong and Dawson 2010). *ECM11* and *GMC2*, which encode SC central element proteins in budding yeast, were also found to be unnecessary for coupling to occur (Humphryes *et al.* 2013). DSBs and the signaling mechanisms they trigger are not necessary for centromere coupling, because coupling occurs in mutants lacking *SPO11* (Tsubouchi and Roeder 2005; Obeso and Dawson 2010). Exactly what is required for coupling besides *ZIP1* and *REC8* remains to be elucidated.

It is not clear at the mechanistic level how centromere coupling and pairing differ from one another besides their timeline in prophase. It has been hypothesized that coupling and pairing could result from the same mechanism, as they both require *ZIP1*, but this has not been formally tested. The centromere pairing (but not centromere coupling) has a partial dependence on *ZIP2*, *ZIP3*, or *ZIP4*, which could suggest a mechanistic difference (Gladstone *et al.* 2009; Newnham *et al.* 2010). Alternatively, these proteins may affect centromere pairing indirectly by affecting the availability of Zip1 for centromere pairing in late prophase, but not affect centromere coupling before SC assembly has begun.

Also unclear is the role, if any, that centromere coupling provides to the cell. It has been proposed that coupling nonhomologous centromeres together could provide a means to sequester homologous centromeres away from each other, preventing the formation of crossovers near the centromeres (Obeso and Dawson 2010). By this model, nonhomologous coupling would force centromere-proximal meiotic DSBs to be repaired from sister chromatids. Yeast and many other organisms exhibit repression of crossing over around centromeres and such crossovers have been shown to predispose chromosomes to higher rates of meiotic chromosome segregation errors (Koehler *et al.* 1996; Lamb *et al.* 1997; Rockmill *et al.* 2006; reviewed in Hassold and Hunt 2001).

The experiments here take advantage of a phosphomimetic mutation of *ZIP1*, *zip1-S75E*, that has been identified as a separation-of-function allele (Falk *et al.* 2010). Serine 75 was identified as a target of the kinase, Mec1 (ATR), in response to DSBs, and its phosphorylation promotes additional phosphorylation of Zip1, presumably by other kinases. Cells expressing the *zip1-S75E* allele appeared WT for *ZIP1*-related functions, such as recombination and SC assembly, but exhibited a severe defect in centromere coupling (Falk *et al.* 2010). Here, we take advantage of the *zip1-S75E* allele and mutations in other genes related to SC function to explore the role of centromere coupling and the requirements for centromere coupling and centromere pairing. The results demonstrate that centromere coupling does not measurably influence centromere-proximal recombination, and that centromere coupling and centromere pairing operate by nonidentical molecular mechanisms that both require the protein Zip1.

## Materials and Methods

### Yeast strains and culture conditions

Genotypes of the strains used in this study are listed in Supplemental Material, Table S1. Strains are isogenic derivatives of a rapidly sporulating diploid strain that is formed by mating haploids from lines called X and Y, which are primarily of S288C and W303 ancestry. These strains were derived in the Rochelle Easton Esposito laboratory (Dresser *et al.* 1994). We used standard yeast media and culture techniques (Burke *et al.* 2000). To induce meiosis, cells were grown in YPAcetate to 3–4 $\times$ $10^7$ cells/ml, then shifted to 1% potassium acetate at $10^8$ cells/ml.

### Strain construction

Polymerase chain reaction (PCR)-based methods were used to create complete deletions of open reading frames and epitope-tagged versions of genes (Longtine *et al.* 1998; Janke *et al.* 2004). Some deletions were created by using PCR to amplify gene deletion-*kanMX4* insertions from the gene-deletion collection (Invitrogen, Carlsbad, CA), and these products were then used for transformations. The $P_{CYC1}$-*lacI-GFP-LYS2* cassette was inserted in the *LYS2* locus as part of pLL1. The $P_{DMC1}$-*lacI-GFP-LYS2* cassette was inserted in the *LYS2* locus as part of pMDE798, a gift from Mike Dresser. The plasmid ptetR-tdTomato-LEU2 (a gift from Zachariae laboratory), containing the *tdTomato* gene fused to the 3′ end of the *tetR* coding sequence under the control of the *URA3* promoter and with *ADH1* transcriptional terminator at the 3′ end of the fusion gene, was inserted at *URA3*. The plasmid pD212 targeted a cassette (~10 kb) of 256 *tet* operon operator (tetO) repeats to *CEN3*, coordinates 113,101–113,583. The plasmid pD214 targeted 256 *lac* operon operator (lacO) repeats to *CEN3*, coordinates 113,101–113,583 (Straight *et al.* 1996; Michaelis *et al.* 1997). The plasmid pMNS25 targeted 256 lacO repeats to *CEN4*, coordinates 447,580–448,580; the plasmid pELK20 targeted 256 tetO repeats to *CEN4*, coordinates 447,580–448,580. Correct integration was confirmed genetically.

A *zip1-S75E* point mutant was built by a modified gene two-step replacement method. Briefly, WT *ZIP1* was amplified using high-fidelity fusion PCR with primer sets containing the GAA codon instead of AGT at *ZIP1* position 222–225 of the *ZIP1* ORF, creating a *ZIP1* DNA coding for glutamic acid instead of a serine at position 75. This fragment was cloned in the PCR-Blunt II TOPO vector (Invitrogen). Yeast cells, in which the *ZIP1* gene was previously replaced by *URA3*, were transformed with the restriction-enzyme digested plasmid DNA and colonies selected on plates containing 5-fluoro-orotic acid (Boeke *et al.* 1984). The sequence of the *zip1-S75E* gene was determined and no additional mutations were present. A hygromycin resistance gene (*hphMX4*) marker was then added downstream of *zip1-S75E* in the 3′ untranslated region (between 2721 and 2820 bp downstream of the *ZIP1* start codon) using PCR-based standard techniques (Janke *et al.* 2004). The hygromycin resistance gene position was confirmed by PCR.

### Microscopy

Images were collected using a Carl Zeiss (Thornwood, NY) AxioImager microscope with band-pass emission filters, a Roper HQ2 charge coupled device, and AxioVision software or, where noted, a Deltavision OMX-SR structured illumination imaging station.

### Meiotic chromosome spreads

Two chromosome-spreading methods were used. For the experiments in Figure 1, Figure 2, and Figure 3, chromosome spreads were prepared from cells harvested 5 hr (Figure 1) or 7 hr (Figure 2 and Figure 3) after induction of sporulation at 30°, and meiotic nuclear spreads were prepared according to Dresser and Giroux (1988) with minor modifications. Cells were spheroplasted using 20 mg/ml zymolyase-100T for ~30 min. Spheroplasts were briefly suspended in MEM [100 mM 2-(*N*-morpholino)ethanesulfonic acid, 10 mM EDTA, 500 μM $MgCl_2$] containing 1 mM phenylmethane sulfonyl fluoride, fixed with 4% paraformaldehyde plus 0.1% Tween 20, and spread onto poly-L-lysine-coated slides (Fisherbrand Superfrost Plus). Slides were blocked with 4% nonfat dry milk in phosphate buffered saline (PBS) for at least 30 min, and incubated overnight at 4° with primary antibodies. Primary antibodies were mouse anti-Zip1 (used at 1:1000 dilution; gift from S. Rankin), rabbit anti-Zip1 (used at 1:1000 dilution, y-300 SC-33733; Santa Cruz), rabbit anti-MYC (1:400, A190–105A; Bethyl Laboratories), mouse anti-MYC (used at 1:1000 dilution; gift from S. Rankin), chicken anti-GFP (used at 1:500 dilution, AB16901; Millipore, Bedford, MA), rabbit anti-DsRed (used at 1:1000–1:2000 dilution, 632496; Clontech), and rabbit anti-RFP (1:500, 600-401-379; Thermo Scientific). Secondary antibodies were Alexa Fluor 488-conjugated goat anti-chicken IgG (used at 1:1200 dilution), Alexa Fluor 568-conjugated goat anti-mouse IgG (used at 1:1000 dilution), Alexa Fluor 647 conjugated goat anti-rabbit IgG (used at 1:1200 dilution), and Alexa Fluor 568-conjugated goat anti-rabbit IgG (used at 1:1000 dilution).

For the chromosome spreads for Figure 4, Figure 5, and Figure S1, cells were harvested 5 hr after induction of sporulation at 30° (Figure 4) or 13 hr after induction of sporulation at 23° (Figure 5). Chromosome spreads for Figure 4, Figure 5, and Figure S1 were prepared as described by Grubb *et al.* (2015) with the following modifications. After chromosome spreads were created and dried overnight, the slides were rinsed gently with 0.4% Photoflo. The slide was then incubated with PBS/4% milk at room temperature for 30 min in a wet chamber. Milk was drained off of the slide, and primary antibody diluted in PBS/4% milk was incubated on the slide overnight at 4°. A control slide with PBS/4% milk was used for each experiment. The following day, the slides were washed in PBS, and incubated with secondary antibody diluted in PBS/4% milk for 2 hr in a wet chamber at room temperature. The slides were gently washed in PBS. Then, 1 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI) was added

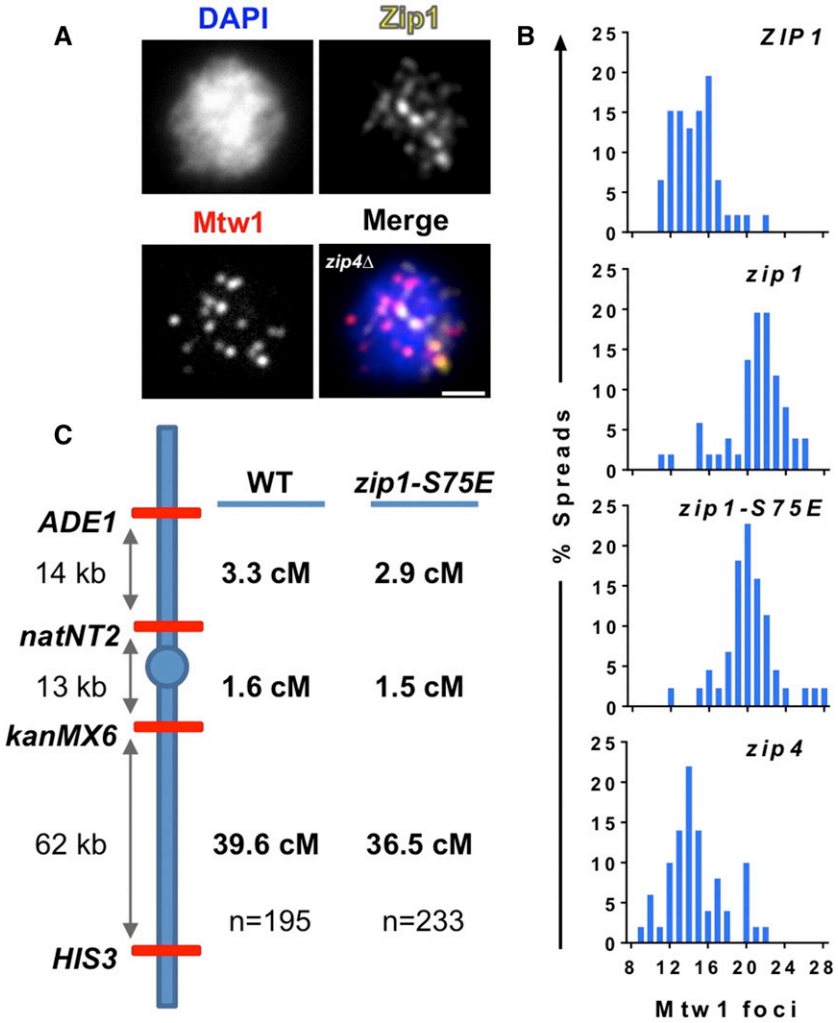


**Figure 1** Centromere coupling does not repress centromere-proximal crossing over. (A and B) The *zip1-S75E* mutation diminishes centromere coupling. (A) Centromere coupling was monitored by creating chromosome spreads that were assayed for the number of Mtw1 foci (the representative spread that is shown is from the *zip4*Δ *spo11*Δ mutant strain; DEK330). Scale bar equals 2 μm. (B) Histograms show the number of Mtw1 foci for WT (*ZIP1/zip1*Δ *spo11*Δ/*spo11*Δ; DDO133), *zip1*Δ/*zip1*Δ *spo11*Δ/*spo11*Δ (DDO134), *zip1-S75E*//*zip1*Δ *spo11*Δ/*spo11*Δ (DDO132), and *zip4*Δ/*zip4*Δ *spo11*Δ/*spo11*Δ (DEK330). Averages, 95% C.I., and the number of cells scored for each strain are as follows: *ZIP1*: 14.6, 13.9–15.3, 46; *zip1*Δ: 20.8, 19.9–21.7, 51; *zip1-S75E*: 20.2, 19.4–21.1, 44; *zip4*Δ: 14.8, 13.9–15.7, 50. The difference in coupling between the *ZIP1/zip1* strain and the *zip1-S75E* and *zip4* strains was significant, as was the difference between the *zip4* strain and the *zip1-S75E* and *zip1* strains (Kruskal–Wallis; $P < 0.0001$; Dunn's *post hoc* test for these comparisons $P < 0.0001$); the difference between *ZIP1/zip1* and *zip4* was not significant, nor was the difference between *zip1-S75E* and *zip1*. (C) Mapping crossing over in *zip1-S75E* strains. Diploid strains were constructed to allow the assessment of crossing over on chromosome *I* in WT (*ZIP1/zip1*Δ; DDO140 and DDO145) and *zip1-S75E* (*zip1-S75E1/zip1*Δ; DDO143) strains. *n*, the number of four spore viable tetrads that were analyzed.

to each slide and allowed to incubate at room temperature for 10 min. Slides were then washed gently in PBS and 0.4% Photoflo, then allowed to dry completely before a coverslip was mounted. Antibodies used for this spread protocol match those of the spread protocol used for Figure 1 and Figure 3.

### *Coupling assay*

Centromere coupling in Figure 1 was monitored as described previously (Obeso and Dawson 2010). Mtw1 (an inner kinetochore protein) foci were quantified in spreads that were ≥5.4 μm in diameter to ensure centromeres were spread enough to assay. Centromere coupling would theoretically yield 16 kinetochore (Mtw1) foci, while absence of coupling would yield 32 kinetochore foci. All strains were *spo11Δ/spo11Δ* to block progression beyond the coupling stage (Falk *et al.* 2010; Obeso and Dawson 2010).

### *Genetic mapping*

Diploid strains heterozygous for several markers located at different positions on chromosome *I* were created by PCR-based methods. Briefly, haploids; in which *ADE1* has been replaced by *LYS2* at position 169,375 of chromosome *I* (Chuong and Dawson 2010), *kanMX4* has been inserted at position 143,400 (5 kb to the left of *CEN1*), the *natNT2* gene has been inserted at position 156,285 (adjacent to *SWD1*, 8 kb to the right of *CEN1*), and *HIS5* has been added at position 80,587 (after the *MTW1* gene); were mated with strains WT for *ADE1*, with the *TRP1* gene instead of *HIS5* added after the *MTW1* gene and without the *kanMX4* and *natNT2* insertions. Tetrads were dissected and the spore phenotypes were tested by replica plating onto selective media. The percentage of crossing over between two markers was determined by scoring the percentage of tetratypes (T) and nonparental ditypes (NPD) among all four spore viable tetrads (total). The distance in centimorgans was estimated as $100 \times (0.5T + 3NPD)/total$ (see Perkins 1949 and Amberg *et al.* 2005 for descriptions of mapping functions).

### *Power analysis of genetic mapping experiment*

With a lack of significance between incidents of recombination in WT and *zip1-S75E* cells within intervals that included a 13-kb region which included *CEN1* and 14 kb directly adjacent, we wanted to test our power. Using G*Power, we performed both *post hoc* and *a priori* tests to judge current power

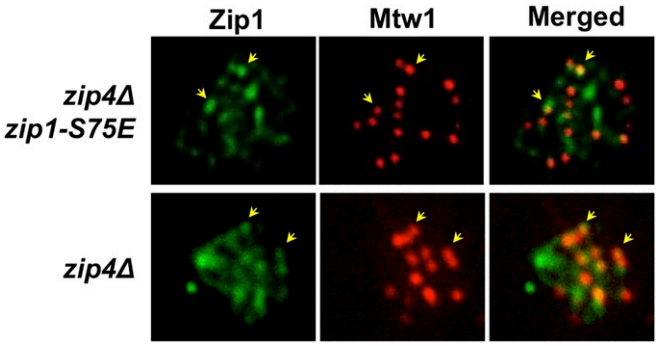


**Figure 2** Zip1-S75E localizes to centromeres. Colocalization of Zip1 and Mtw1 staining on *zip4*Δ (DDO172) and *zip4*Δ *zip1-S75E* double mutants (DDO182). Cells were induced to sporulate in liquid medium, and samples were harvested 7 hr later for the preparation of chromosome spreads. Yellow arrowheads indicate examples of colocalization of Zip1 and Mtw1 immunofluorescence. The level of Mtw1/Zip1 colocalization in both *zip4* (32 spreads, 438 Mtw1 foci) and *zip4 zip1-S75E* (24 spreads, 356 Mtw1 foci) strains was quantified and compared to the Mtw1/Zip1 overlap that was seen when the foci were randomized (see *Materials and Methods* for details).

and the necessary number of tetrads to dissect to achieve significance. The outcomes of these tests can be found in Table S2.

### *Pachytene pairing assays*

Centromere pairing in pachytene for Figure 3, Figure 5, and Figure S1 was assessed using published methods (Gladstone *et al.* 2009) in which lacO and tetO arrays were either inserted adjacent to the centromeres of two chromosomes or two plasmids. These cells expressed a *GFP-lacI* gene fusion under the control of a *DMC1* meiotic promoter, and a *tetR-tdTomato* gene fusion under the control of the *URA3* promoter. This produced fluorescent foci at the operator arrays. Chromosome spreads were prepared and indirect immunofluorescence was used to identify the hybrid proteins and Zip1 localization. Only cells that exhibited "ropey" DAPI staining were scored in this assay, and they were disqualified for assessment if there was more than one GFP focus or more than one tdTomato focus. In these cells, the distance between the center of the green focus and the center of the red focus was measured using AxioVision software. Foci within 0.6 μm were scored as paired, and those separated by 0.6 μm or a larger distance were scored as unpaired.

To monitor pairing of kinetochores (Mtw1-13×Myc), chromosome spreads were prepared as in Figure 3 (see above). Spreads were stained for Mtw1-13×MYC (mouse anti-MYC, Developmental Studies Hybridoma Bank, 1:200) and Red1 (guinea pig anti-Red1, from Marta Kasperzyk, 1:1000) using methods described above. Secondary antibodies were Alexa Fluor 568 goat anti-mouse (Invitrogen, 1:1000) and Alexa Fluor 488 donkey anti-guinea pig (Jackson ImmunoResearch, 1:800). *Z*-series images were collected with a Deltavision OMX in the OMRF Imaging Core. Images were then deconvolved and reconstructed using softWoRx. Presented images (Figure 4) are a quick projection generated using softWoRx. Spreads with continuous Red1 staining were identified and these spreads were scored for whether the Mtw1-13×MYC foci were singlets or doublets by evaluating the projected images and by scrolling through the deconvolved *Z*-series. Foci were scored as doublets if they could be resolved as two separated foci that were associated with the same pair of Red1 axes.

### *Mtw1 colocalization with Zip1-S75E*

To determine whether Mtw1 foci showed a significant overlap with Zip1-S75E foci, images were cropped to contain the DAPI-staining region. ImageJ software was used to define the perimeters of each Zip1 focus. Mtw1 foci that overlapped edges of the Zip1 foci were scored as colocalized. The Zip1 images were then flipped 180° and Mtw1 colocalization was again scored. A statistical analysis was then performed to determine whether actual colocalization was significantly greater than the randomized (flipped) control. A paired *t*-test was used to compare the number of Mtw1 foci that colocalized within the actual *vs.* flipped Zip1 foci.

### *Meiosis I nondisjunction assay*

Nondisjunction frequencies of CEN plasmids were determined in a manner similar to how homeologous chromosomes were assayed in a previously published work (Gladstone *et al.* 2009). Diploid cells were induced to enter meiosis at 23° (because *zip1*, *zip4*, and *ecm11* mutants in this strain background arrest in pachytene at 30°) and cells were harvested at 24 hr (when many anaphase I cells are present). Harvested cells were either assayed fresh, or were frozen in 15% glycerol and 1% potassium acetate until the time at which they were assayed. Preparation for assaying the cells included staining the cells with DAPI and then mounting the cells on agarose pads for viewing (Kim *et al.* 2013). Anaphase I cells were identified by the presence of two DAPI masses on either side of elongated cells, indicating that the chromosomes had segregated. To avoid scoring cells with duplicated or lost CEN plasmids, only cells with one GFP focus and one tdTomato focus were assayed. A *Z*-series of each cell was collected to assess whether CEN plasmids had disjoined or nondisjoined.

The representative images in Figure 6B were edited using AxioVision software, using a constrained iterative deconvolution algorithm and a wavelet-based extended focus algorithm to collapse the *Z*-stacks into a single two-dimensional image.

### *Statistics*

GraphPad Prism software was used for all statistical calculations. Continuous data were tested for normality and compared using the Kruskal–Wallis test. Dunn's *post hoc* test was done for direct comparisons between genotypes. Experiments that required 180° rotation of a fluorescent channel to test overlap employed student's paired *t*-test (Wilcoxon signed-rank test). Categorical data were compared using Fisher's exact test and Bonferroni's correction was used to adjust the *P*-value for multiple comparisons where we have

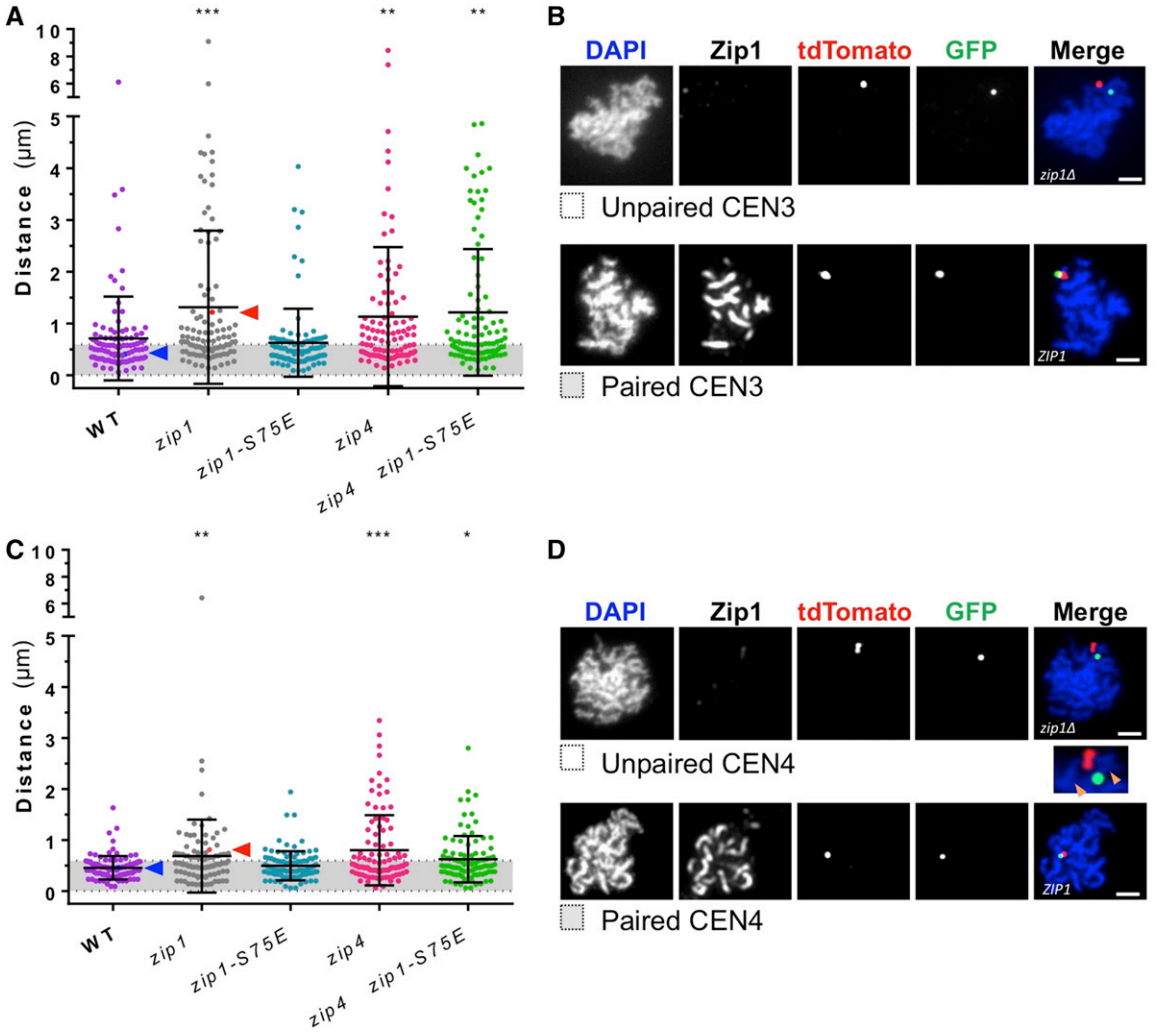


**Figure 3** The *zip1-S75E* mutation does not affect pairing of homologous pericentric regions at pachytene. (A and B) *zip4*Δ affects pairing between pericentromeric regions of chromosome *III*. Cells were induced to sporulate in liquid medium, and samples were taken at time of 5 hr for chromosome spreads. tdTomato and GFP foci, which localized to *tet* and *lac* operator repeats situated near the centromere of chromosome *III* (*CEN3*), were visualized using indirect immunofluorescence and the distance between foci was measured. (A) The measured distance between *CEN3* foci. CEN pairing, as defined as <0.6-μm distance (gray shading). The observed pairing values for the strains tested were: 61% WT (median = 0.51 μm; DEK350, DEK360), 41% *zip1*Δ (median = 0.71 μm; DEK337, DEK361), 68% *zip1-S75E* (median = 0.46 μm; DEK357, DEK362), 46% *zip4*Δ (median = 0.70 μm; DEK338, DEK363), and 44% *zip4*Δ *zip1-S75E* double mutant (median = 0.63 μm; DEK339, DEK364); *n* = 100 spreads per genotype consisting of two separate experiments. The data points indicated by the red and blue arrowheads correspond to chromosome spreads shown in (B). See Figure S2 for a graphical representation of the centromere pairing data shown in (A). (B) Representative spreads with unpaired [*zip1*Δ, DEK361; 1.22 μm distance, red arrowhead and data point in (A)] and paired [WT, DEK360; 0.41 μm distance, blue arrowhead and data point in (A)] tdTomato and GFP foci are shown. Bar, 2 μm. (C) Centromere pairing (*CEN4*) was assessed by localization of tetR-tdTomato and GFP-lacI foci localized to *tet* and *lac* operator repeats respectively inserted adjacent to *CEN4*. CEN pairing, as defined as <0.6-μm distance (gray shading), was exhibited between 83% WT (median = 0.43 μm; DEK359, DEK365), 55% *zip1*Δ (median = 0.55 μm; DEK258, DEK370), 75% *zip1-S75E* (median = 0.41 μm; DEK353), 59% *zip4*Δ (median = 0.49 μm; DEK351, DEK368), and 63% *zip4 zip1-S75E* double mutant (median = 0.50 μm; DEK352, DEK369); *n* = 100 spreads per genotype consisting of two separate experiments. (D) Representative spreads used for the quantifications in (C) with unpaired [*zip1*Δ, DEK370; 0.81 μm distance, red arrowhead and data point in (C)] and paired [WT, DEK365; 0.45 μm distance, blue arrowhead and data point in (C)]. Orange arrowheads in inset indicate axial associations of chromosome *IV* pair that display no pairing. Bar, 2 μm. The errors bars in (A) and (C) represent mean and SD. Statistical comparisons were performed with Kruskal–Wallis; (A) $P < 0.0001$, (C) $P = 0.0002$; multiple comparisons to WT done by Dunn's *post hoc* test $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

noted. Raw *P*-values can be found in Table S3, Table S4, and Table S5.

### Data availability

Strains and plasmids are available upon request. Table S1 includes names and genotypes of each strain used in this work.

## Results

### Centromere coupling does not repress centromere-proximal crossing over

The characterization of the *zip1-S75E* separation-of-function allele (Falk *et al.* 2010) has made it possible to test the hypothesis that the coupling of centromeres with nonhomologous

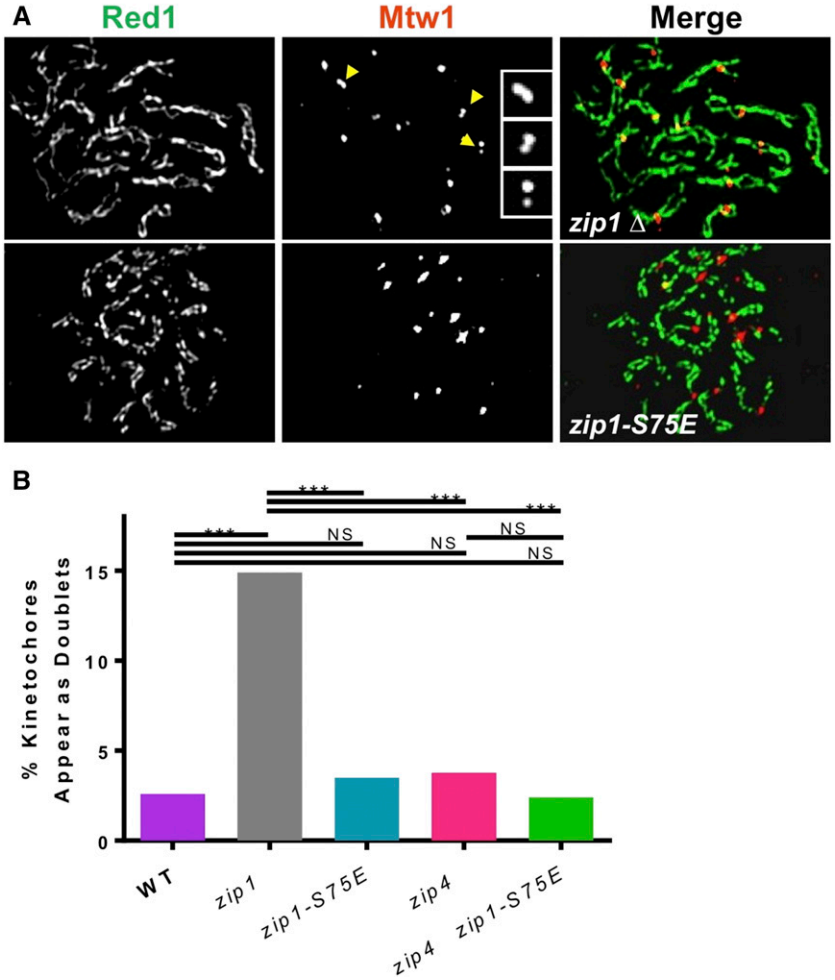


**Figure 4** Zip1 promotes pairing of chromosomal centromeres. The pairing of centromeres of homologous chromosomes was monitored on chromosome spreads from pachytene cells. Chromosome axes were stained with anti-Red1 antibodies (green) and kinetochores were stained with anti-MYC antibodies (red). (A) Examples of spreads from *zip1*Δ and *zip1-S75E* strains are shown. Yellow arrowheads indicate examples of separated Mtw1 foci associated with parallel Red1 axes that were scored as "doublets." The indicated doublets are shown in magnified images in the insets. (B) Histogram of the percentage of Mtw1 foci scored as doublets in five different strains. *n*, total Mtw1 foci scored. Strains: WT (*ZIP1*), DHC349, *n* = 114; *zip1*Δ, DHC350, *n* = 179; *zip1-S75E*, DHC351, *n* = 159; *zip4*Δ, DHC352, *n* = 177; *zip4*Δ *zip1-S75E*, DHC353, *n* = 122. ***$P \leq 0.001$. Raw *P*-values can be found in Table S3.

partners blocks recombination between homologous centromere regions. We first confirmed that the *zip1-S75E* allele has a coupling defect in our strain background like that previously shown by Falk *et al.* (2010). To ensure we were examining centromere coupling between nonhomologous chromosomes, and not centromere pairing that occurs later in prophase between centromeres of homologous chromosomes, we deleted *SPO11* (Keeney *et al.* 1997) which prevents homologous centromere pairing (Falk *et al.* 2010; Obeso and Dawson 2010). We assayed centromere coupling by quantifying the number of Mtw1 (an inner kinetochore protein) foci using indirect immunofluorescence of a MYC-tagged version of Mtw1 5 hr after the induction of meiosis by transfer to a sporulation medium (Figure 1A). In this assay, each of the 32 chromosomes is comprised of two sister chromatids with a shared kinetochore, which appears as a single focus. If the centromeres of the 32 chromosomes form 16 couples, they appear as ~16 foci (some foci may overlap or be undetectable) when visualized using conventional wide-field microscopy (Obeso and Dawson 2010; Tsubouchi and Roeder 2005). Our WT chromosome spreads, on average, showed ~16 kinetochore foci (average 14.6 foci)—indicative of complete coupling, as do *zip4* mutants (Figure 1B). At this stage of meiosis, Zip1 appears as dispersed foci of varying intensities (Figure 1A). As shown previously, many of these colocalize with or are adjacent to kinetochore foci, consistent with the requirement for Zip1 for efficient centromere coupling (Tsubouchi and Roeder 2005; Chuong and Dawson 2010; Falk *et al.* 2010; Obeso and Dawson 2010). The number of foci is significantly increased in *zip1*Δ mutants (average 20.8 foci). Note that in these assays the maximum unpaired number of kinetochore foci (32) is not typically observed (Tsubouchi and Roeder 2005; Chuong and Dawson 2010; Falk *et al.* 2010; Obeso and Dawson 2010). This may be because of some residual pairing capability in the *zip1* mutants, or because some kinetochores are too faint to detect, or are obscured, or overlap one another in the chromosome spreads. There was no distinguishable difference between coupling in the *zip1-S75E* strain (average 20.2 foci) and *zip1*Δ mutants (Figure 1, legend). Thus, in our strain background, the *zip1-S75E* allele behaves as described previously (Falk *et al.* 2010) and appears as defective in coupling as a *zip1* deletion mutation.

To test whether loss of coupling affected centromere-proximal crossing over, we integrated markers that would allow us to monitor crossing over in a region including the

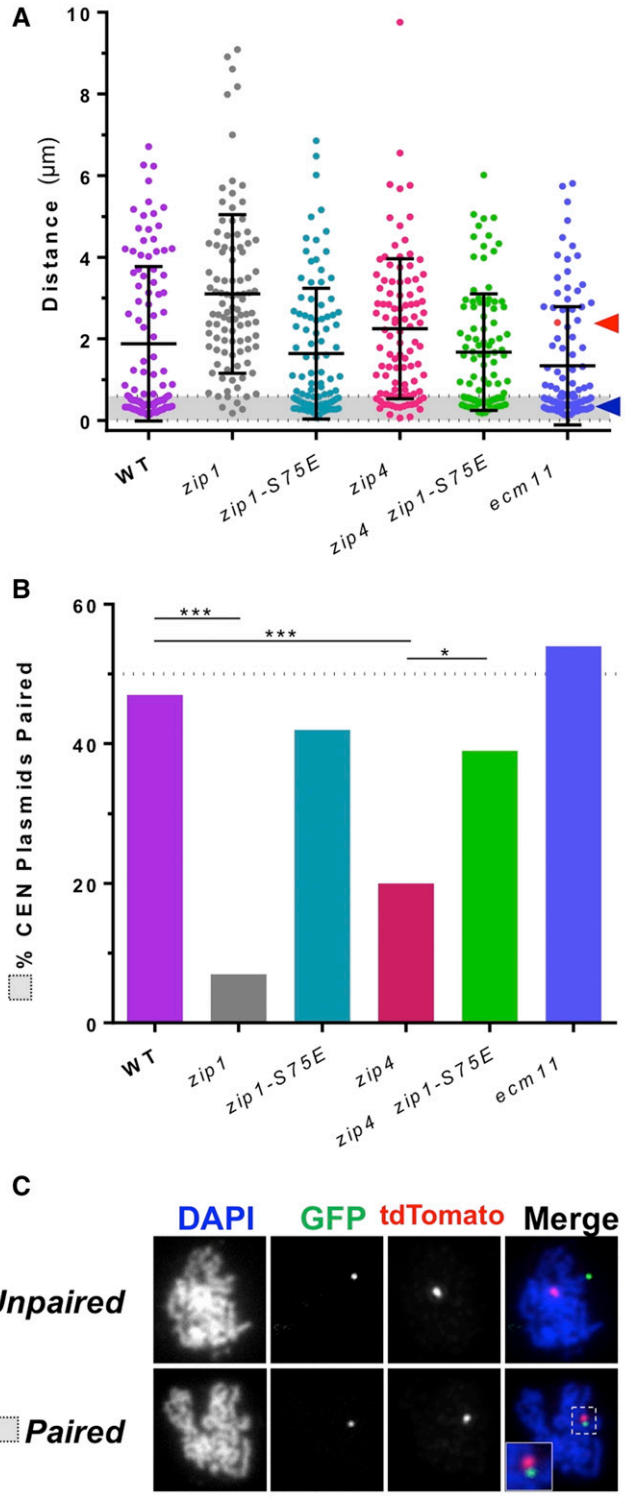


**Figure 5** Zip1-S75E promotes centromere pairing of CEN plasmids. (A) Centromere pairing of CEN plasmids was assessed by monitoring the pairing of tetR-tdTomato and lacI-GFP foci localized to *tet* and *lac* operator repeats respectively inserted into a plasmid that contains 5.1 kb of *CEN3* sequence. Chromosome spreads were prepared from samples taken at time 13 hr after transfers of cultures to sporulation medium and sporulated at 23°. tdTomato and GFP foci were visualized using indirect immunofluorescence and pairing, as defined as <0.6-μm distance (gray shading), and was assessed to occur between 47% WT (median = 0.65 μm, DEK303, DEK323), 7% *zip1*Δ (median = 2.7 μm, DEK320, DEK325), 42% *zip1-S75E* (median = 0.87 μm, DEK267, DEK324), 20% *zip4*Δ (median = 2.1 μm, DEK304, DEK326), 39% *zip4*Δ *zip1-S75E* double mutant (median = 1.2 μm, DEK335), and 54% *ecm11*Δ (median = 0.57 μm, DEK321, DEK327). Sample size $n$ = 100 cells from at least two experiments. Statistical comparisons were performed with Kruskal–Wallis, $P < 0.0001$; multiple comparisons to WT done by Dunn's *post hoc* test ****$P \leq 0.0001$. (B) Histogram of percentage of pairing between CEN plasmids, from gray shading in (A). Statistical comparisons were performed with Fisher's exact test to compare all genotypes to WT, and *zip4*Δ to both *zip1*Δ and *zip4*Δ *zip1-S75E* double mutant. Bonferroni's correction was used to adjust for the number of comparisons: *$P \leq 0.05$, ***$P \leq 0.001$. Raw $P$-values can be found in Table S4. (C) Representative spreads with unpaired [2.4 μm distance; red arrowhead and data point in (A); DEK321] and paired [0.38 μm distance; navy blue arrowhead in (A); DEK321] tdTomato and GFP foci are shown.

centromere as well as flanking intervals on the arms of chromosome *I* (Figure 1C). The genetic distance measured between the markers straddling the centromere in WT cells was 1.6 cM. The similarly sized adjacent arm interval was measured as 3.3 cM, consistent with repression of crossing over between homologous partners near the centromeres. The map distances exhibited by the *zip1-S75E* mutant were indistinguishable and not significantly different from those in the WT strain (Figure 1C and Figure S1). To assess whether we had examined an adequate number of tetrads to make conclusions based on our data, we performed a power analysis (Table S2), which assured us that our sample size was large enough to conclude there was no significant difference between centromere-proximal crossovers in WT and *zip1-S75E* cells. We conclude that absence of centromere coupling does not affect the level of crossover repression that occurs at the centromere, corroborating similar work recently published by Marston and colleagues (Vincenten *et al.* 2015).

### *The zip1-S75E allele is not defective in associations of homologous peri-centromeric regions during pachytene*

Neither coupling nor pairing is dependent on the homology of the underlying DNA sequence near the centromere, but centromere pairing most often occurs between centromeres of homologous chromosomes because the homologous chromosomes are paired with one another during pachytene. The exception to this occurs when two nonhomologous chromosomes are left without homologous partners during pachytene. The centromere regions of these partners pair with one another, even if they are not homologous, and they segregate away from each other in anaphase I (Kemp *et al.* 2004; Gladstone *et al.* 2009; Newnham *et al.* 2010). The fact that both coupling and pairing of centromeres are Zip1 dependent and homology independent raises the question of whether they operate using the same mechanism, but at different times in the meiotic program. To address this question, we tested whether the *zip1-S75E* mutation ablates centromere-pairing interactions just as it does centromere coupling.

As a first test of whether the Zip1-S75E protein might promote centromere pairing, we determined whether it

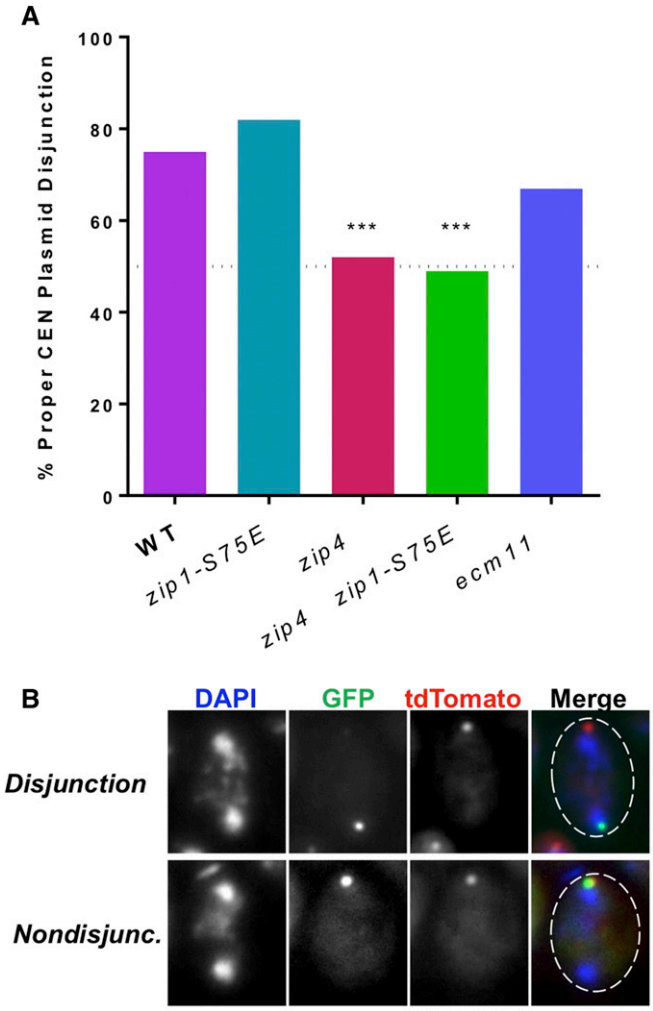


**Figure 6** CEN plasmids disjunction requires *ZIP1* and *ZIP4*. (A) Segregation of CEN plasmids in anaphase I was assessed by monitoring the tetR-tdTomato and lacI-GFP foci localized to *tet* and *lac* operator repeats on the plasmids. Cells from meiotic cultures (23°, time = 26 hr post meiotic induction) were stained with DAPI and scored for segregation of CEN plasmid. (A) shows the percentage of spreads with correctly segregated CEN plasmids. Disjunction was assessed to occur in 75% WT (DEK303, DEK323), 82% *zip1-S75E* (DEK267, 324), 52% *zip4*Δ (DEK304, DEK326), 49% *zip1-S75E zip4*Δ (DEK335), and 67% *ecm11*Δ (DEK321, DEK327). Sample size represents $n$ = 150 cells, split between three experiments. Statistical comparisons were performed with Fisher's exact test to compare all genotypes to WT. Bonferroni's correction was used to adjust for the number of comparisons; ***$P \leq 0.001$. Raw $P$-values can be found in Table S5. (B) Representative binucleate cells with disjoined and nondisjoined CEN plasmids. Both sample photos are from WT cells (DEK323).

can localize to centromeres as the WT protein does (Figure 2). This was done in *zip4*-strain backgrounds, as these do not assemble a continuous SC; leaving discernable Zip1 foci of varying intensities, some of which colocalize with kinetochores (Tsubouchi *et al.* 2008). In this experiment, Zip1 and Zip1-S75E showed similar levels of localization with Mtw1 (51 and 59%, respectively), which were significantly greater than randomized controls ($P < 0.0001$).

Next we monitored the proximity of centromere regions using fluorescence microscopy in *zip1-S75E* mutants. We inserted linearized plasmids containing (~10 kb) arrays of the bacterial *lac* operator or *tet* operator sequences ~1 kb from the centromeres of homologous chromosomes, and expressed GFP-lacI or tetR-tdTomato to tag the centromere regions of the homologous partners (Straight *et al.* 1996; Michaelis *et al.* 1997). The centromeres of both a small (*CEN3*) and a large (*CEN4*) chromosome were tagged in this manner. Diploids with the tagged centromeres were induced to enter meiosis (sporulation), and at a time point (5 hr) corresponding to pachytene, cells were harvested and chromosome spreads were prepared (Grubb *et al.* 2015) and analyzed by indirect fluorescence microscopy. To assay centromere pairing in these cells, the distances between the centers of the fluorescent GFP and tdTomato foci were measured in pachytene cells, which were identified by the dense ropey appearance of the chromosomes when stained with DAPI (Figure 3A, representative photos in Figure 3B). For *CEN3*, WT cells showed a median separation of the dots of 0.51 μm, with ~61% of the dots separated by 0.6 μm or less, which we defined as "paired" for the purposes of this assay. As a negative control, we used a *zip1*Δ strain, which exhibited a significantly elevated median of 0.71 μm and a drop to 41% of measurements scored as paired (Figure 3, A and B). The *zip1-S75E* mutant was indistinguishable from the WT strain (median 0.46 μm, 68% of centromeres paired). The higher level of centromere associations in the WT and *zip1-S75E* cells compared to the *zip1*Δ cells could be due to the formation of the SC in the pericentric region, keeping centromeres near one another; or due to centromere pairing, independent of SC formation. To test this, we assayed the effect of the *zip1-S75E* mutation in a *zip4*Δ background (Tsubouchi *et al.* 2008). The *zip4* mutant resulted in a significant increase in median focus-to-focus distance compared to WT cells (median 0.70 μm, 46% paired), suggesting that at least a portion of the associations were due to the SC. The pairing in the *zip4*Δ *zip1-S75E* double mutant cells (median 0.63 μm, 44% paired) was indistinguishable from the *zip4*Δ mutants (Figure 3, A and B). Both mutants exhibited a level of pairing similar to that seen in *zip1* null mutants. But the fact that pairing in the *zip4*Δ background [which eliminates the SC but not pairing (Tsubouchi *et al.* 2008)] is similar to the pairing exhibited in the *zip1*Δ mutation, which eliminates both the SC and pairing (Gladstone *et al.* 2009; Newnham *et al.* 2010), suggests that associations of peri-centromeric dots in WT and *zip1-S75E* spreads is mainly attributable to the SC, not centromere pairing.

A similar approach was taken to examine pairing of the pericentric region of the much larger chromosome *IV* and similar results were obtained. That is, *zip1-S75E* mutants were not significantly different from WT, while *zip1*Δ (median 0.55 μm, 55% paired), *zip4*Δ (median 0.49 μm, 59% paired), and *zip4*Δ *zip1-S75E* double mutants (median 0.50 μm, 63% paired) all exhibited significantly larger distances between the GFP and tdTomato foci than were seen in WT spreads (Figure 3C, representative photos in Figure 3D).

At both the chromosomal *CEN3* and *CEN4* regions, the association of GFP and tdTomato arrays that persists in the *zip4*Δ and *zip1*Δ strains (~40–50%, Figure S2) is presumably promoted by the tethering of the homologous pericentric regions by adjacent crossovers, since in the absence of both *ZIP1* and crossing over, homologous centromeres do not associate (Obeso and Dawson 2010). The persistent association of the pericentric regions even in *zip1*Δ mutants could be compromising our ability to monitor centromere pairing. In fact, our observation that *zip1*Δ and *zip4*Δ mutations similarly reduce peri-centromeric associations of 10-kb arrays inserted adjacent to the centromere is in contrast to the findings found by Tsubouchi *et al.* (2008), wherein a *zip4*Δ mutant exhibited paired kinetochores late in prophase, as assayed by Ctf19 immunofluorescence, while this pairing vanished in *zip1*Δ strains.

### *The zip1-S75E allele promotes pairing of homologous centromeres during pachytene*

To directly test whether the Zip1-S75E protein can promote pairing of centromere regions, we used an assay similar to that described by Tsubouchi *et al.* (2008), except that structured illumination microscopy was used to evaluate the association of centromeres in the chromosome spreads rather than conventional indirect fluorescence microscopy. Chromosome spreads from five different isogenic strains were stained with antibodies against Red1, to reveal chromosome axes, and Mtw1-13×MYC, to reveal kinetochores. Spreads with paired/aligned axial elements were scored for whether the Mtw1 foci for axis pairs appeared as a single focus (paired) or as a doublet (unpaired) (examples in Figure 4A). High levels of doublet kinetochore foci could be seen in the *zip1*Δ strain (Figure 4, A and B) as described previously (Tsubouchi *et al.* 2008), indicating a loss of centromere pairing. In contrast, *ZIP1*, *zip1-S75E*, and *zip4*Δ mutants were indistinguishable, with high levels of pairing (Figure 4B). Even in *zip4*Δ *zip1-S75E* double mutants the kinetochores remain paired, demonstrating that even in the absence of an SC, the Zip1-S75E protein can promote centromere pairing.

### *Achiasmate centromere pairing in pachytene requires ZIP1 but not ZIP4 or ECM11*

To evaluate *zip1-S75E* phenotypes in a system in which the association of partner centromeres would not be affected by adjacent crossovers, we used circular mini-chromosomes [or centromere (CEN) plasmids] that are small enough that they rarely recombine with one another. These plasmids contain a 5-kb block of chromosome *III* that contains the centromere (*CEN3*); selectable yeast genes *TRP1* and *LEU2* or *URA3*, so that their segregation could be followed using genetic assays; and *lac*O and *tet* O arrays, so that they could be followed by fluorescence microscopy in cells expressing GFP-lacI and tetR-tdTomato fusion proteins, respectively (Straight *et al.* 1996; Michaelis *et al.* 1997).

To probe centromere pairing (Figure 5), cells were induced to enter meiosis [at 23° to circumvent the pachytene arrest that occurs when SC proteins are disrupted (Borner *et al.* 2004)]. Cells were harvested coincident with entry into pachytene, and chromosome spreads were prepared. The purpose of the low sporulation temperature was to allow for these cells to later be assayed for their ability to segregate chromosomes in anaphase I (below). We identified pachytene cells by the condensed ropey appearance of chromosomes after staining with DAPI. Only cells with a single GFP focus and a single tdTomato focus (one copy of each plasmid) were evaluated in this assay. The distance between the two foci was then measured and plotted to show the distribution of these distances (Figure 5A). WT cells showed a median of 0.65 μm between foci. For the CEN plasmids, the separation of dots that did not pair (>0.6 μm) is much larger than was seen with the chromosomal *CEN3* and *CEN4* dots (compare Figure 5A and Figure 3, A and C). This illustrates how the CEN plasmids, unrestrained by crossovers, can truly disassociate when not paired with one another. It is notable that the chromosomal *CEN4* focus-to-focus distances were markedly less than those observed for chromosomal *CEN3* (Figure 3C, representative images in Figure 3D). The reason behind this is unclear, but one possible explanation is that chromosome *IV*, being the larger chromosome, might be more compacted than chromosome *III* as appears to be the case in mitotic cells (Neurohr *et al.* 2011).

In the WT control, 47% of cells showed pairing (focus-to-focus distances <0.6 μm; Figure 5, A and B, representative images in Figure 5C). In contrast, only 7 of the 100 assayed *zip1*Δ spreads showed a focus-to-focus distance of <0.6 μm (Figure 5B). The *zip1-S75E* mutant exhibited levels of pairing (42%, Figure 5B) that were not significantly different from WT cells.

Previous studies of the role of *ZIP4* in mediating centromere associations have yielded different results depending on the assay used. *ZIP4* (like *ECM11*, *GMC2*, and *REC8*) is not required for centromere coupling (Figure 1, A and B). When pairing of homologous centromeres was examined using native chromosomes it was found that in the absence of *ZIP1*, centromeres were often separated, while in *zip4* mutants they were paired even though the flanking arm regions were not synapsed (Tsubouchi *et al.* 2008). However, in *zip4* mutants, centromere pairing of nonexchange chromosome partners was significantly diminished (Newnham *et al.* 2010). We found that in the *zip4*Δ background, CEN plasmids were unable to pair at WT levels in pachytene but did exhibit a low level of centromere pairing, much as was seen by Newnham *et al.* (2010). Though this amount of pairing was not significantly more than in *zip1*Δ mutants, given the numbers of spreads examined, it is striking that the number of *zip4*Δ cells that exhibited centromere pairing between CEN plasmids was >20% (Figure 5B). One possible explanation for these results is that centromere pairing is possible, but weakened, in *zip4* mutants. In this scenario, for chromosomes tacked together along their length by crossovers (as examined by Tsubouchi *et al.* 2008 and this work, Figure 4), the weakened centromere-pairing mechanism may still be

sufficient to hold centromeres together. In contrast, with nonexchange chromosomes or plasmids in which centromere pairing is the only mechanism holding the partners together during the spreading process, the weakened centromere pairing may fail to hold the partners together in every chromosome spread. In both assay systems, elimination of Zip1, which is required for the pairing mechanism, leads to complete loss of pairing. When the *zip4*Δ *zip1-S75E* double mutant was assayed, we were surprised to see a rescue phenotype; indeed, the *zip4*Δ *zip1-S75E* double mutant cells had no significant decrease in pairing compared to WT cells, but a significant gain of centromere pairing compared to *zip4*-Δ alone (Figure 5B). This indicated to us that the *zip1-S75E* mutant version of Zip1 promotes more stable centromere associations in late prophase than its WT counterpart—completely contradictory to its role in centromere coupling in early prophase.

A newly described protein of the central element of the SC, Ecm11, has also been discovered to be important for linear loading of Zip1 along the chromosome arms (Zavec *et al.* 2008; Humphryes *et al.* 2013). Though found to be dispensable for centromere coupling (Humphryes *et al.* 2013), it has not been examined closely in its role in centromere pairing or segregation of achiasmate chromosomes. We examined the effects of deletion of *ECM11* on CEN plasmid centromere pairing because we were curious whether it was necessary for late centromere pairing. The *ecm11*Δ strain exhibited no defect in centromere pairing between CEN plasmids (Figure 5B, and representative images in Figure 5C). So despite the importance of *ECM11* in stabilizing a continuous SC, it is not necessary at the centromere to mediate coupling or pairing.

From these data, we conclude that some but not all SC proteins play a role in centromere pairing. While Zip1 appears to play one of the most important roles, the importance of other components that affect a mature SC assembly cannot be discounted.

### *CEN pairing is not sufficient to ensure disjunction at anaphase*

Prior work has shown that Zip1-dependent centromere pairing of nonexchange partner chromosomes in prophase is correlated with their proper disjunction in anaphase (Gladstone *et al.* 2009; Newnham *et al.* 2010). There are several steps in meiotic progression (Meyer *et al.* 2013) that separate centromere pairing in pachytene from homolog disjunction in anaphase, and it is not clear how pairing in pachytene translates into better segregation fidelity at anaphase. We asked whether the connections we observed between CEN plasmids in pachytene resulted in proper disjunction of these chromosomes in anaphase I.

We induced meiosis in the cells described in Figure 5, but collected cells at a time when anaphase cells were most prevalent in WT strains. These cells were collected and stained with DAPI and those with two DAPI masses (indicative of the segregated homologs on either side of the cell at anaphase) were scored for either disjunction or nondisjunction of the fluorescently labeled CEN plasmids (Figure 6A, with representative photos in Figure 6B). In the WT control strain, CEN plasmids segregated properly 75% of the time—this is about the segregation fidelity that would be expected if the plasmids that had paired in pachytene (47%, Figure 5) segregated properly, and the remaining 53% of cells segregated randomly—disjoining correctly by chance half of the time. This amount of pairing and disjunction is similar to levels observed previously with achiasmate pairing partners (Guacci and Kaback 1991; Gladstone *et al.* 2009; Newnham *et al.* 2010). In a *zip1*Δ control strain, very few cells completed meiosis I, despite the lower sporulation temperature so these cells were not scored for plasmid segregation. Yet historically, in a different strain background (S288C), nonexchange chromosomes showed elevated levels of nondisjunction when *ZIP1* was deleted (Gladstone *et al.* 2009; Newnham *et al.* 2010). By comparison, the *zip1-S75E* mutant exhibited WT levels of segregation; 82% of CEN plasmids segregated properly (Figure 6A).

In contrast, *zip4*Δ (similar to previous reports, Newnham *et al.* 2010) and the *zip4*Δ *zip1-S75E* double mutant both exhibited random segregation of the CEN plasmids at meiosis I (Figure 6A). The fact that the *zip4*Δ mutant showed a modest level of pairing and the *zip4*Δ *zip1-S75E* double mutant displayed WT levels of pairing during pachytene, yet segregated their plasmids randomly, demonstrates that the pairing that occurs in pachytene may be necessary but not sufficient to ensure disjunction at anaphase I.

Previous experiments have shown that mutants (*zip1*, *zip2*, *zip3*, and *zip4*) that fail to efficiently assemble the SC are defective in pairing and segregation of nonexchange chromosome partners (Gladstone *et al.* 2009; Newnham *et al.* 2010). Here we tested whether a fully WT SC structure is necessary. We found that an *ecm11*Δ mutant showed proper disjunction near WT levels (67%, Figure 6A). Therefore, not all SC structural-component proteins are required to faithfully segregate the nonexchange CEN plasmids in meiosis.

## Discussion

This study has provided evidence that centromere coupling and pairing are controlled by independent mechanisms that are both dependent on *ZIP1*, and that *ZIP4* is critical for proper pairing and disjunction of achiasmate partner chromosomes in meiosis I. The major conclusions from this work are (1) that there is no measureable defect in crossover repression near centromeres in the absence of centromere coupling, (2) that there is a difference between the mechanisms that allow for early centromere coupling and late centromere pairing, and (3) that centromere associations that occur in pachytene do not necessarily lead to correct segregation in anaphase.

### *zip1-S75E mutants are incapable of coupling but still protect the centromere from crossovers in meiosis*

Crossovers that occur close to the centromere are repressed in many organisms including budding yeast, flies, and humans. This is likely due to the fact that these crossovers lead to

error-prone disjunction during anaphase I (Sears *et al.* 1995; Koehler *et al.* 1996; Lamb *et al.* 1996; Ross *et al.* 1996; reviewed in Hassold and Hunt 2001). We had originally hypothesized that centromere coupling might protect homologous centromeres from forming centromere-proximal crossovers (Obeso and Dawson 2010); however, we found no conclusive evidence that such protection is provided to the cells by coupling, as a coupling-deficient *zip1-S75E* mutant shows WT levels of crossovers both at the centromere and along the chromosome arms. This confirms what Vincenten *et al.* (2015) have recently published when they examined crossovers in a *zip1-S75E* mutant in SK1 yeast cells. Their studies confirm that repression of crossing over near centromeres requires *ZIP1* (Chen *et al.* 2008), but that loss of coupling, by using the *zip1-S75E* allele, yielded no significant change in crossover repression (Vincenten *et al.* 2015).

### *zip1-S75E is capable of pairing centromeres in late prophase*

The question remained whether early centromere coupling and late centromere pairing could be controlled by similar mechanisms. If coupling and pairing occur by exactly the same mechanism we reasoned that *zip1-S75E* mutants, defective for centromere coupling, would also struggle to pair centromeres in late prophase, which would hinder proper disjunction of achiasmate chromosomes. To the contrary, we found that the *zip1-S75E* mutant showed full centromere pairing between CEN plasmids, an otherwise achiasmate chromosome system. This leads us to conclude that centromere coupling and pairing are mediated by two different mechanisms. This conclusion is supported by the observation that *ZIP2*, *ZIP3*, and *ZIP4* are required for efficient centromere pairing, but it could be argued that the role of these proteins in centromere pairing is indirect—that is, supporting centromere associations by modulating the availability of Zip1 for the process.

### *ZIP4 is required for disjunction-promoting pairing of CEN plasmids*

In *zip4* mutants, CEN plasmids exhibit modest levels of pairing at pachytene but segregate randomly. This result is unique since other SC regulatory components examined have either been required for both pairing and disjunction (as is the case for *ZIP1*, *ZIP2*, and *ZIP3*; Gladstone *et al.* 2009; Newnham *et al.* 2010) or for neither (*ECM11*, this article). In budding yeast, Zip4 plays an important role in linear SC assembly and therefore crossover frequency, but how it contributes specifically is not fully understood (Tsubouchi *et al.* 2006). In *atzip4 Arabidopsis* mutant lines, there is abrupt separation of homolog pairing partners after diplotene despite normal synapsis (Kuromori *et al.* 2008), but this is likely due at least in part to the loss of the normal number of crossovers. Similarly, in *ZIP4H*-deficient mice (also known as *TEX11*), synapsis levels are normal but crossover number and timing are altered, leading to increased arrest and apoptosis (Adelman and Petrini 2008).

Centromere pairing in *zip4* mutant strains is not sufficient to promote subsequent disjunction at anaphase. This suggests that Zip4 might aid in the stability of pairing interactions beyond pachytene, or Zip4 could promote an environment in which a different, more stable, connection can be formed—and it is this connection that could promote proper disjunction to occur. The fact that the majority of Zip1 (and SYCP1 in mouse spermatocytes) has vanished from centromeres before segregation occurs suggests the second model may be correct: pachytene pairing may allow for the formation of another connection that is critical for disjunction. A possible example of this is seen in *Drosophila* females, where centromeres associate in prophase (Dernburg *et al.* 1996; Takeo *et al.* 2011), but later, in metaphase, elastic connections composed of peri-centromeric heterochromatic DNA have been detected between achiasmate partner chromosomes. These connections have been suggested to promote disjunction of the chromosomes (Hughes *et al.* 2009). Perhaps this form of connection is created in the pachytene environment of centromere pairing, but not in *zip4* mutants.

### *zip1-S75E stabilizes centromere pairing in late prophase*

We found that the *zip1-S75E* allele rescues the defect in pairing seen in *zip4*Δ mutants during pachytene. How could a version of Zip1 that cannot promote centromere coupling in early prophase provide a stabilizing force in centromere associations late in prophase? The Zip1-S75E protein mimics a version of Zip1 that not only has a phosphorylation at residue 75, but multiple phosphorylated amino acids along the protein (Falk *et al.* 2010). The initial phosphorylation is completed by Mec1 (ATR), a DSB-activated checkpoint sensor kinase, and serves as a regulatory step to connect meiotic timing with DSB formation (Falk *et al.* 2010). It is not clear why the Zip1-S75E protein can provide more centromere pairing in the *zip4* background than the WT Zip1 protein. Perhaps the Zip1-S75E version of the protein promotes a more stable SC structure than the nonphosphorylated version of Zip1 and it is this sort of structure that promotes centromere pairing. Consistent with this, the Zip1-S75E protein is competent to form an SC that is functional and indistinguishable from a WT SC, where tested. In contrast, centromere coupling relies on a mechanism that is independent of other tested ZMM proteins and is only known, thus far, to require Zip1 and Rec8 at the centromere (Tsubouchi and Roeder 2005; Chuong and Dawson 2010).

### *Ecm11 is unnecessary for centromere-centromere associations in late prophase*

Ecm11, being a newly identified central element protein important for stabilizing Zip1 on the chromosomes (Zavec *et al.* 2008; Humphryes *et al.* 2013), was an attractive candidate to test for its effects on centromere pairing. In our hands, however, centromere pairing showed no dependence on *ECM11*. When anaphase cells were examined, it was also found that *ECM11* was dispensable for proper segregation of CEN plasmids; indicating to us that though the Ecm11 protein is critical for full synapsis of natural chromosomes, it is not necessary to the mechanism of pairing and segregation of achiasmate chromosomes.

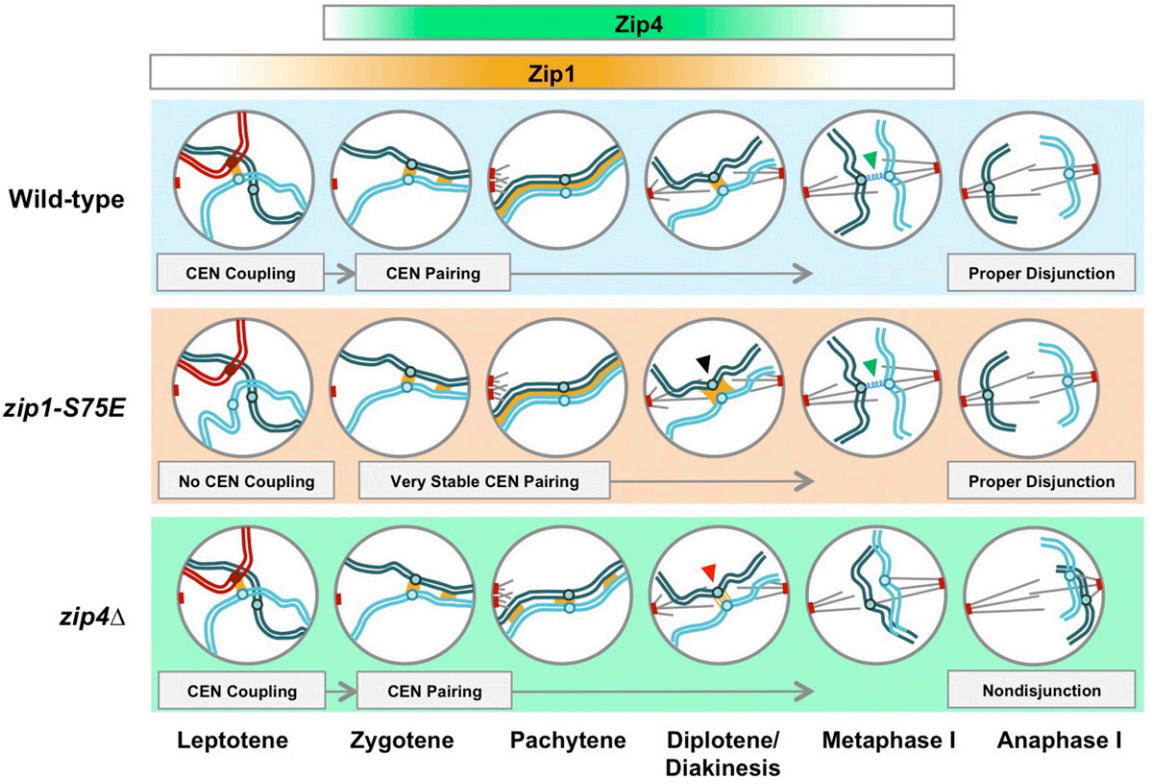


**Figure 7** A model for how Zip1 and Zip4 may contribute to centromere coupling and pairing. A progression of one pair of achiasmate homologous chromosomes proceeding from early prophase through anaphase I. Gradients of Zip1 and Zip4 at the top indicate their contributions to centromere interactions and synaptonemal complex formation during this time. In WT cells, nonhomologous centromere coupling resolves into centromere pairing and SC formation. After SC disassembly, centromere pairing can be seen more clearly until Zip1 unloads and yields a proposed flexible tether that leads to proper disjunction of chromosomes during anaphase I. *zip1-S75E* cells display defective centromere coupling, but very stable centromere pairing (black arrow) that helps contribute to proper disjunction of achiasmate partners, except in the absence of Zip4. *zip4*Δ mutants exhibit WT levels of coupling, defective SC formation, and weak centromere pairing during pachytene (red arrow) that leads to a lack of a stable but flexible interaction thereafter (green arrows in metaphase I). This ultimately leads achiasmate chromosomes to behave independently of one another in anaphase I, contributing to random segregation of pairing partners.

### *A model for centromere-centromere interactions in prophase in budding yeast*

The data presented here provide sufficient evidence to conclude that centromere coupling and centromere pairing operate via separate mechanisms. We propose a model (Figure 7) in which Zip1 and Zip4 play a role in pairing centromeres and segregating achiasmate chromosomes. In this model, centromeres that normally couple in WT and *zip4*Δ cells are unable to couple in *zip1-S75E* cells. Once DSBs have been created, Zip4 becomes active in helping Zip1 form linear stretches of SC between homologous partners (Tsubouchi *et al.* 2006). It is notable here that Zip4 is unable to load onto chromosomes in the absence of DSBs, as seen in a *spo11* mutant (Tsubouchi *et al.* 2006). In the absence of Zip4, Zip1 can still be found in short stretches along the chromosomes and at the centromere, but its ability to form linear stretches along the lengths of chromosomes is impeded (Tsubouchi *et al.* 2006).

How Zip4 helps Zip1 load at a mechanistic level is still not well understood. After pachytene and the disassembly of the majority of the SC, a small stretch of Zip1 stays behind to mediate centromere pairing. Given the ability of Zip4 to promote SC assembly, it may be that there is less Zip1 at the centromeres in *zip4* mutants, and this might explain why pairing is somewhat deficient between achiasmate partner chromosomes in the *zip4*Δ single mutant. It could also be true that the SC that forms in *a zip4*Δ mutant is less stable and more apt to disassemble prematurely, as supported by observations of premature bivalent separation made in *Arabidopsis* and mouse spermatocyte *ZIP4* mutants (Adelman and Petrini 2008; Kuromori *et al.* 2008).

The results here could have implications for human health. Intriguingly, mutations in *TEX11* in humans, the homolog of *ZIP4*, lead to infertility and nonobstructive azoospermia (Yang *et al.* 2015). Though this is likely due to a pachytene arrest from defects in chiasma or SC formation, it remains clear that understanding *ZIP4* better might allow us to understand mutations that are relevant to fertility issues in humans. Additionally, measurements of recombination and segregation patterns in humans suggest that the frequency of achiasmate chromosome *21*'s is larger than the frequency of conceptuses that are aneuploid for this chromosome. This

has led to the suggestion that humans may have mechanisms beyond chiasma formation for ensuring the proper segregation of homologous chromosomes (Oliver *et al.* 2008; Cheng *et al.* 2009; Fledel-Alon *et al.* 2009). Indeed, a recent study has uncovered previously unappreciated segregation mechanisms in human oogenesis that appear to reduce the risks of aneuploidy from achiasmate partners (Ottolini *et al.* 2015). The centromere-pairing assisted segregation described in this work might provide clues to understanding the fates of achiasmate chromosomes in humans.

## Acknowledgments

We thank Michael Dresser for plasmid pMDE798; Wolfgang Zachariae for plasmid ptetR-tdTomato-LEU2; and members of the Dawson laboratory, past and present, for contributions of plasmids and yeast strains that were used in these studies. Members of the Program in Cell Cycle and Cancer Biology contributed to the intellectual development of the project. Structure illumination microscopy was performed in the Oklahoma Medical Research Foundation Imaging Core with their expert consultation and technical assistance. The project was supported by National Institutes of Health grant R01 GM-087377 to D.S.D.

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*Communicating editor: N. Hunter*