Meiotic Cohesin Promotes Pairing of Nonhomologous Centromeres in Early Meiotic Prophase

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A period of pairing between nonhomologous centromeres occurs early in meiosis in a diverse collection of organisms. This early, homology-independent, centromere pairing, referred to as centromere coupling in budding yeast, gives way to an alignment of homologous centromeres as homologues synapse later in meiotic prophase. The regulation of centromere coupling and its underlying mechanism have not been elucidated. In budding yeast, the protein Zip1p is a major component of the central element of the synaptonemal complex in pachytene of meiosis, and earlier, is essential for centromere coupling. The experiments reported here demonstrate that centromere coupling is mechanistically distinct from synaptonemal complex assembly. Zip2p, Zip3p, and Red1p are all required for the assembly of Zip1 into the synaptonemal complex but are dispensable for centromere coupling. However, the meiotic cohesin Rec8p is required for centromere coupling. Loading of meiotic cohesins to centromeres and cohesin-associated regions is required for the association of Zip1 with these sites, and the association of Zip1 with the centromeres then promotes coupling. These findings reveal a mechanism that promotes associations between centromeres before the assembly of the synaptonemal complex, and they demonstrate that chromosomes are preloaded with Zip1p in a manner that may promote synapsis.

INTRODUCTION

More than 30 y ago, the centromeres in onion meiocytes were shown to organize in nonhomologous pairs or clusters before the alignment of homologous chromosomes (Church and Moens, 1976). Similar observations have since been made in several other organisms (reviewed in Stewart and Dawson, 2008). Little is known about the mechanism by which this early meiotic centromere pairing occurs. Recently, it was discovered that in a *spo11Δ* mutant of budding yeast, which is blocked for the creation of meiotic DNA double-strand breaks (DSBs) and the subsequent synapsis of homologous chromosomes, centromeres arrange themselves in pairs during meiosis (Tsubouchi and Roeder, 2005). This pairing, referred to in budding yeast as centromere coupling (Tsubouchi and Roeder, 2005), is homology independent and depends upon Zip1p, a component of the central element of the synaptonemal complex (SC) (Sym *et al.*, 1993). How Zip1p promotes early meiotic homology-independent centromere pairing (centromere coupling) is not known, but its role of in SC assembly is better characterized. In pachytene, Zip1p bridges the axial elements that run along each homologue (Sym *et al.*, 1993; Sym and Roeder, 1995; Dong and Roeder, 2000). In many organisms, the initiation of recombination between the homologous chromosomes is a prerequisite to normal SC assembly (Loidl *et al.*, 1994; Weiner and Kleckner, 1994; Bhalla and Dernburg, 2008)

reviewed in (Bhalla and Dernburg, 2008). A number of proteins collectively referred to as the ZMM (*ZIP1, ZIP2 ZIP3 ZIP4 MER3 MSH4 MSH5*) or synaptic initiation complex proteins groups, have been identified that are required for efficient SC assembly (reviewed in Lynn *et al.*, 2007). Mer3p, Msh4p, and Msh5p are involved in the creation of early recombination intermediates (Ross-Macdonald and Roeder, 1994; Hollingsworth *et al.*, 1995; Nakagawa and Ogawa, 1999), and Zip2p and Zip3p localize at sites of newly created DSBs (Chua and Roeder, 1998; Agarwal and Roeder, 2000; Novak *et al.*, 2001), where they are thought to promote the polymerization of Zip1p and the zippering of axial elements.

Zip1p does not require DSBs to associate with the chromosomes. In *spo11Δ* mutants, Zip1p associates with chromosomes as punctate foci, but it assembles continuous SC-like structures inefficiently (Bhuiyan and Schmekel, 2004; Henderson and Keeney, 2004). Many of the punctate Zip1 foci seen in *spo11Δ* mutants were reported to colocalize with centromeres (Tsubouchi and Roeder, 2005), and recent studies suggest that synapsis of homologous chromosomes may often initiate from paired homologous centromeres (Tsubouchi *et al.*, 2008).

The mechanism by which nonhomologous centromeres are paired is not known. To address these issues, we have performed experiments to investigate whether proteins that contribute to the assembly of Zip1p into the synaptonemal complex are also required for centromere coupling, and the loading of Zip1p onto chromosomes, at centromeres and at other sites in early meiosis. The results demonstrate that centromere coupling and the initial loading of Zip1p onto meiotic chromosomes are mechanistically distinct from synaptonemal complex assembly and lend new insight into the ways in which chromosomes are prepared for later events in meiosis.

MATERIALS AND METHODS

Yeast Strains and Culture Conditions

All diploid strains used were obtained by matings of TSP50 and TSP52, or their isogenic derivatives. We used standard yeast media and culture methods (Burke *et al.*, 2000). To induce meiosis, cells were grown in YP-acetate to 3–4 × 10^7 cells per ml, and then shifted to 1% potassium acetate at 10^8 cells/ml.

Strain Construction

Genetic methods were performed according to standard protocols (Burke *et al.*, 2000). Polymerase chain reaction (PCR)-based methods were used to create complete deletions of open reading frames and epitope-tags (Longtine *et al.*, 1998). Some deletions were created by using PCR to amplify deletion-KANMX insertions from the yeast gene deletion collection (Invitrogen, Carlsbad, CA), and these products were then used for transformations. Diagnostic PCRs were performed to confirm each gene modification. The *spo11Δ::hisG-URA3-hisG* (Kateneva *et al.*, 2005) and the *REC8-GFP* (Kateneva *et al.*, 2005) strains were described previously. The *rec8::P_{REC8}-SCC1-3HA* strain was constructed as described previously (Toth *et al.*, 2000) by using C4056 (a gift from Kim Nasmyth, University of Oxford). The plasmid pJN2 (256 lacO arrays targeting to chromosomal coordinates 153583–154854) was integrated at *CEN1* and the correct integration was confirmed genetically. The P_{CYC1}-lacI-green fluorescent protein (GFP) cassette was inserted as part of pAFS1520 (a gift from Aaron Straight, Stanford University School of Medicine). Strain genotypes are listed in Table 1.

Meiotic Chromosome Spread Preparation

Meiotic nuclear spreads were prepared according to Dresser and Giroux (1988), with the following modifications. Cells were spheroplasted using 20 mg/ml Zymolyase (AMSBIO, Abingdon, United Kingdom) 100T for ~30 min. Spheroplasts were briefly suspended in minimal essential medium [100 mM 2-(*N*-morpholino)ethanesulfonic acid, 10 mM EDTA, and 500 μM $MgCl_2$] containing 1 mM phenylmethylsulfonyl fluoride, fixed with 4% paraformaldehyde plus 0.1% Tween 20, and spread onto poly-L-lysine–coated slides (Fisherbrand Superfrost Plus; Thermo Fisher Scientific, Waltham, MA). Slides were blocked with 4% nonfat dry milk in phosphate-buffered saline for at least 30 min and incubated overnight at 4°C with primary antibodies. Primary antibodies were mouse anti-Zip1p (see Figure 1) (a gift from Rebecca Maxfield, The Jackson Laboratory), goat anti-Zip1 (sc-48716; Santa Cruz Biotechnology, Santa Cruz, CA) (see Figures 4 and 6), rabbit anti-MYC (A190-105A; Bethyl Laboratories, Montgomery, TX), mouse anti-MYC (gift from S. Rankin, Oklahoma Medical Research Foundation), chicken anti-GFP (AB16901; Millipore Bioscience Research Reagents, Temecula, CA), and rabbit anti-GFP (A11122; Invitrogen). Secondary antibodies were Alexa Fluor 488-conjugated goat anti-chicken immunoglobulin (Ig)G, Alexa Fluor 546-conjugated goat anti-mouse IgG, Alexa Fluor 647-conjugated goat anti-rabbit IgG, Alexa Fluor 568-conjugated anti-mouse, and Alexa Fluor 568-conjugated donkey anti-goat (all from Invitrogen). Secondary antibody incubations were for two hours at room temperature.

Centromere Coupling Assays

Except where stated centromere coupling assays were performed with chromosome spreads prepared, as described above, from cells harvested at 5.5 h after the induction of meiosis (transfer to 1% potassium acetate). Indirect immunofluorescence microscopy was used to determine the number of kinetochore foci in each spread. Statistical comparisons of the average number of kinetochore foci were performed with unpaired *t* tests.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed according to Meluh and Koshland (1997), with minor modifications. Approximately 2 × 10^8 cells were used per ChIP experiment (mock, immunoprecipitation [IP], and input). Chromatin was formaldehyde-cross-linked for 30 min at room temperature and sonicated to average fragment sizes of 500–700 base pairs. Antibodies used for ChIP was rabbit polyclonal anti-Zip1p (sc-33733; Santa Cruz Biotechnology) and rabbit polyclonal anti-GFP (A11122; Invitrogen). Protein G-Sepharose beads were from Invitrogen. After reversal of cross-linking, overnight at 65°C, DNA was purified using QIAquick PCR purification kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. PCR was used to amplify selected chromosomal regions. Primers were chosen to amplify ~300-base pairs fragments except for the primer-walk evaluation of *CEN1* in which the primers were spaced ~400–450 base pairs apart. Primer sequences and coordinates are listed in Supplemental Tables 2–5. The number of PCR cycles to be used for each primer was determined empirically so as not to reach saturation. Input DNA was diluted 120 times except for *ARE1*, *ARG4*, and *CARC3* for which it was diluted 600 times. PCRs were performed with Hot-Start Taq DNA Polymerase (Denville Scientific, Metuchen, NJ). Twenty-five to 30-μl PCR reactions were run on a 1.2% agarose gels. Images were obtained with an Image Station 4000R system (Eastman Kodak, Rochester, NY). Band intensities were measured using Molecular Imaging Software, version 4 (Eastman Kodak).

Each chromatin immunoprecipitation experiment was done with samples from two independent immunoprecipitations. Similar results were obtained in the repetitions of each experiment, and a representative example is shown for each. Error bars represent SE of the mean for multiple PCR reactions from one chromatin immunoprecipitation sample. Except where stated ChIP assays were performed with cells harvested at 5.5 h after the induction of meiosis (transfer to 1% potassium acetate).

SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting

For protein extraction, cells were harvested and suspended in 16.6% cold trichloroacetic acid and were lysed with glass beads. Total cellular protein was precipitated and dissolved in SDS-PAGE sample buffer. SDS-PAGE and Western blotting onto polyvinylidene difluoride membrane were performed using XCell SureLock electrophoresis system and XCell II Blot Module (Invitrogen), according to the manufacturer's instructions.
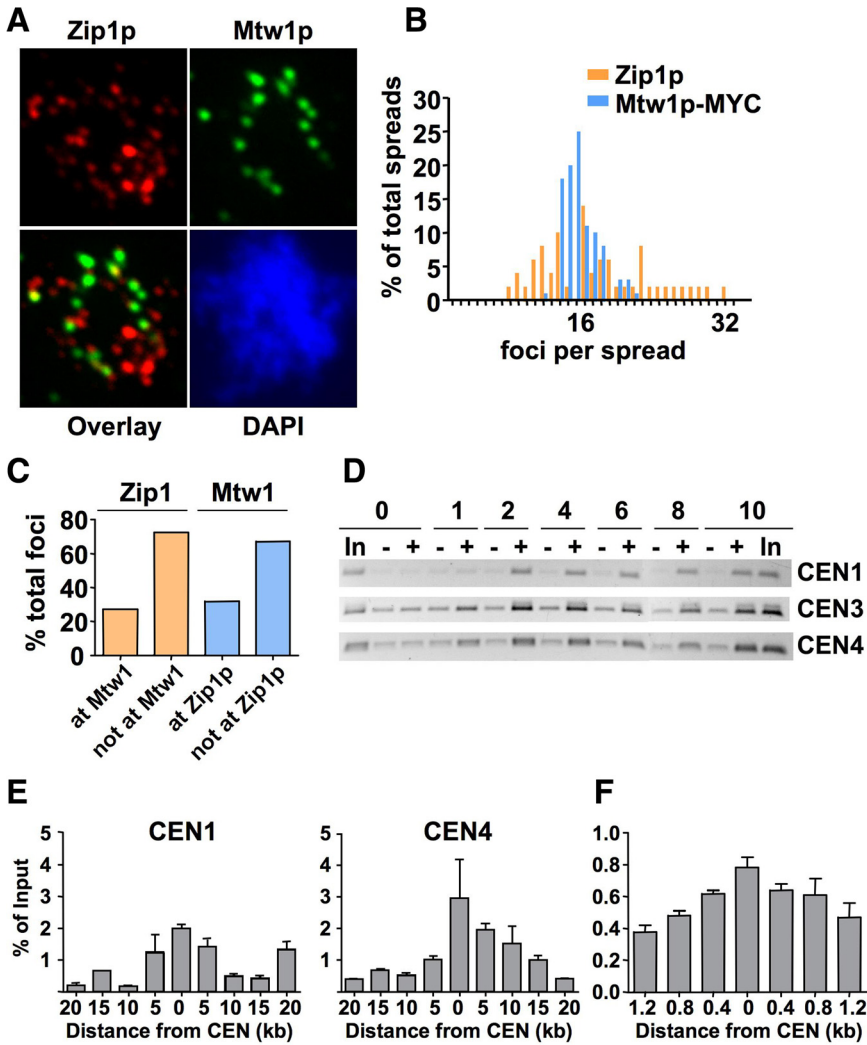
RESULTS

Zip1p Associates with Centromeric Chromatin Early in Meiosis

In budding yeast, centromeres have been shown to pair in a homology-independent manner in pachytene, when homologous partners are fully synapsed (Kemp *et al.*, 2004), and also at earlier stages of meiosis (Tsubouchi and Roeder, 2005) before the assembly of the synaptonemal complex. This early centromere pairing that occurs sometime before pachytene has been termed "centromere coupling" (Tsubouchi and Roeder, 2005), and we will use this term here. Zip1 was been shown previously to be required for centromere coupling and to localize to paired nonhomologous centromeres (Tsubouchi and Roeder, 2005). We examined Zip1p localization in *spo11Δ* mutants in early meiotic chromosome spreads (5.5 h after induction of meiosis) in our laboratory strain background. We detected very little colocalization of Zip1p foci with kinetochores (Mtw1-13XMYC) (Figure 1, A–C), contrary to the previous report (Tsubouchi and Roeder, 2005). Although the previous analysis of centromere coupling reported ≈16 Zip1 foci per chromosome spread in prophase *spo11Δ* cells, localized to the ≈16 kinetochore foci (Tsubouchi and Roeder, 2005), we observed considerable variation in the total number of Zip1p foci per spread, consistent with previous studies (Bhuiyan and Schmekel, 2004; Henderson and Keeney, 2004), ranging from seven to 30 three foci per nuclear spread (Figure 1B). The number of Mtw1p (kinetochore) foci in these chromosome spreads was tightly centered around 16 per cell (Figure 2B), the number expected if the centromeres of the 32 chromosomes were organized as pairs. When colocalization of the Zip1p foci with kinetochores was measured in spreads that had 16 Mtw1p foci, only ~25% of the Zip1p foci colocalized with the Mtw1p foci (Figure 1C). Similar results were obtained when chromosome spreads were prepared from cells harvested at multiple early meiotic time points (data not shown). In contrast, in isogenic *SPO11* diploids, and diploids of other strain backgrounds, Zip1p exhibits clear colocalization with centromeres after exit from pachytene when the synaptonemal complex disassembles but centromeric Zip1 is preserved (Gladstone *et al.*, 2009; Newnham *et al.*, 2010). The lack of colocalization of Zip1 with coupled centromeres in early meiotic prophase of *spo11Δ* mutants in our immunofluorescence experiments does not mean that Zip1p is not localized to the centromeres in these cells at the time of centromere coupling. Instead, it is possible that a minor population of Zip1p, too small to detect in our assays, is localized to centromeres, but this level of may be sufficient for centromere coupling.

Despite the lack of clear colocalization of Zip1 to coupled centromeres in early meiotic cells by immunofluorescence, we found, as was described previously (Tsubouchi and Roeder, 2005), that Zip1 is essential for normal levels of centromere

Table 1. Yeast strains used in this study

| Name	Genotype
TSP50	MATα, ura3-13, trp1-63, his3-1, leu2, met13-c, tyr1-2, lys2-2, cyh2-1
TSP52	MAT**a**, ura3-13, trp1-63, his3-1, leu2, met13-d, tyr-1, lys2-1, can1
ABY130-16c	MATα, ura3-13, trp1-63, his3-1, leu2, met13-d, tyr1, lys2-1, can1, spo11::HisG-URA3 MTW1-13Myc::TRP1
ABY128-3a	MAT**a**, ura3-1, trp1-63, his3-1, tyr1-2, ade2, lys2-2, cyh2-1, spo11::HisG-URA3-HisG, MTW1-13xMYC-His3MX
ABY130-1a	MATα, ura3-13, trp1-63, his3-1, met13-d, tyr1, lys2-1, can1, zip1::KanMX6, spo11::HisG::URA3 MTW1-13Myc::TRP1
ABY128-2b	MAT**a**, ura3-1, trp1-63, his3-1, leu2, met13-c, tyr1-2, ade2, lys2-2, cyh2-1, zip1::KanMX6, spo11::HisG-URA3-HisG MTW1-13Myc-His3MX6
ABY160	MAT**a**, ura3-13, trp1-63, his3-1, ade2, lys2-2, cyh2-1, spo11::HisG-URA3-HisG red1::KanMX MTW1-13Myc-His3MX6
ABY165-6a	MATα trp1-63, his3-1, ade2, lys2-2, cyh2-1, spo11::HisG-URA3-HisG rec8::TRP1 MTW1-13myc-His3MX his3-Δ1 ura3-13:: [P_{CYC1}-LacI-GFP-URA3]
ABY162-27b	MAT**a** ura3-13, trp1-63, his3-1, leu2, met13-d, tyr1-1, lys2-1 spo11::HisG-URA3-HisG rec8::TRP1 MTW1-13myc-TRP1 CEN 4:: [pMNS25-lacO-LEU2]
ABY158	MATα, ura3-13, trp1-63, his3-1, leu2, can1, spo11::HisG::URA3 MTW1-13Myc::TRP1 red1::KanMX
ABY180	MATα, his3-1, ura3-13::pAFS152[URA3 P_{CYC1}-GFP-lacI], spo11::HisG-URA3-HisG, rec8::TRP1 P_{REC8}::P_{REC8}-SCC1-3HA::LEU2, MTW1-13xmyc-His3MX
DHC57.2a-5d	MATα, his3-1, ura3-52, spo11::HisG-URA3-HisG, rec8::TRP1 P_{REC8}::P_{REC8}-SCC1-3HA::LEU2, CEN1::pJN2[lacO256 LEU2], MTW1-13xmyc-His3MX, SPC42-GFP-TRP1
ABY182-12a	MAT**a**, ura3-13, trp1-63, his3-1, leu2, met13-d, tyr1-1, lys2-1, CEN1::pJN2[lacO256 LEU2], spo11::HisG-URA3-HisG, rec8::TRP1 P_{REC8}:: P_{REC8}-SCC1-3HA::LEU2, MTW1-13myc-TRP1
ABY190-14d	MATα, his3-1, met13-c, ade2, spo11::HisG-URA3-HisG rec8::TRP1 P_{REC8}::P_{REC8}-SCC1-3HA::LEU2 MTW1-13xmyc-His3MX
ABY195	X114: MAT**a**, ura3-13, trp1-63, his3-1, +, leu2, met13-d, lys2-1, zip1::KanMX6, CEN1::pJN2[lacO256 LEU2], spo11::HisG-URA3-HisG rec8::TRP1 P_{REC8}::P_{REC8}-SCC1-3HA::LEU2 MTW1-13xMyc::TRP1
ABY252-2b	MATα, leu2, lys2-2, met13-c, tyr1-2, REC8-GFP::URA3, trp1-Δ63, cyh2, his3-Δ1, MTW1-13xMYC-His3MX
ABY251-1a	MAT**a**, tyr1-1, met13-d, can1, lys2-1, REC8-GFP:URA3, trp1-63, leu2, MTW1-13xMYC-TRP1, his3-Δ1, ura3-13
TSP64.1	MATα, ura3-13, trp1-63, his3-1, leu2, met13-d, tyr1-1(-2?), lys2-1, can1, spo11::HisG-URA3 MTW1-13Myc::TRP1 zip2::NAT
TSP63.3	MAT**a**, ura3-1, trp1-63, leu2, his3-1, tyr1-2, ade2, lys2-2, cyh2-1, spo11::HisG-URA3-HisG MTW1-13Myc-His3MX6 zip2::NAT
TSP78.1	MATα, ura3-13, trp1-63, his3-1, leu2, met13-d, tyr1-1(-2?), lys2-1, can1, spo11::HisG-URA3 MTW1-13Myc::TRP1 zip3::KanMX
ABY136	MATα, ura3-13, trp1-63, his3-1, leu2, met13-d, tyr1, lys2-1, can1, spo11::HisG-URA3 mam1::NAT, MTW1-13Myc::TRP1
ABY128-3a	MAT**a**, ura3-1, trp1-63, his3-1, tyr1-2, ade2, lys2-2, cyh2-1, spo11::HisG-URA3-HisG, mam1::NAT, MTW1-13xMYC-His3MX
TSP79.3	MAT**a**, ura3-1, trp1-63, leu2, his3-1, tyr1-2, ade2, lys2-2, cyh2-1, spo11::HisG-URA3-HisG MTW1-13Myc-His3MX6 zip3::KanMX
ABY363-12a	MATα, leu2, lys2-2, met13-c, tyr1-2, ura3-1, trp1-Δ63, cyh2-1, spo11::HisG-URA3-HisG, zip1::KANMX, REC8-GFP::URA3, ade2-?, MTW1-13xMYC-His3MX
ABY375	MATα, ura3-13, trp1-Δ63, leu2, tyr1-1, lys2-1, met13-d, can1-R, spo11::HisG-URA3-HisG, zip1::KANMX, MTW1-13 × MYC-TRP1, Rec8-GFP::URA3
ABY202-3d	MATα, leu2, lys2-2, met13-c, tyr1-2, ura3-1, trp1-Δ63, cyh2-1, spo11::HisG-URA3-HisG, REC8-GFP::URA3, ade2
ABY197-6b	MAT**a**, ura3-13, trp1-Δ63, leu2, tyr1-1, lys2-1, met13-d, can1-R, spo11::HisG-URA3-HisG, Rec8-GFP::URA3
ABY178	MATα trp1-63, his3-1, leu2, met13-c, tyr1-2, lys2-2, cyh2-1 spo11::HisG-URA3-HisG rec8::TRP1 pREC8::pREC8-SCC1-3HA::LEU2 MTW1-13myc-His3MX ura3-13:: [P_{CYC1}-LacI-GFP-URA3]
ABY312-10c	MATα ura3-13, trp1-63, his3-1, leu2, met13-d, tyr-1, lys2-1, can1 spo11::kanMX MTW1-13myc-TRP1
ABY312-16b	MAT**a** spo11::kanMX rec8::TRP1 MTW1-13myc-TRP1 ura3-13, trp1-63, his3-1, leu2, met13-d, tyr-1, lys2-1, can1
DDO54.3-15b	MAT**a**, leu2, lys2-2, tyr1-2, met13-c, ura3::[pAFS152:URA3 PCYC-GFP-lacI], trp1-Δ63, cyh2-1, his3-Δ1, CEN1::pJN2[lacO256 LEU2], MTW1-13xMYC-HIS3
ABY194-Nd	MATα, ura3-13, trp1-63, his3-1, HIS7, leu2, met13-d, ADE2, ADE5, lys2-1, ade1::LYS2, ura3::pAFS152[URA3 PCYC-GFP-lacI], CEN1::pJN2[lacO256 LEU2], MTW1-13xMYC-TRP1, MTW1-13xMyc::TRP1 ade1::LYS2
DAB195-2b	MATα lys2 leu2 his3 ura3 trp1 spo11::NAT MTW1-13Myc::His3MX6
DAB195-17c	MAT**a** lys2 leu2 his3 ura3 trp1 spo11::NAT MTW1-13Myc::His3MX6
TAB196	MATα lys2 leu2 his3 ura3 spo11::NAT Δzip1::KanMX MTW1-13Myc::His3MX6
TAB198	MAT**a** lys2 leu2 his3 ura3 spo11::NAT Δzip1::KanMX MTW1-13Myc::His3MX6
ABY133	(ABY128-3a × 130-16c) 2n
ABY144	(ABY136 × ABY134) 2n
ABY271	(ABY130-1a × ABY128-2b) 2n
ABY168	(ABY158 × 160) 2n
DD701	(TSP63.3 × TSP64.1) 2n
DSP2	(TSP78.1 × TSP79.3) 2n
ABY174	(ABY162-27b × 165-6a) 2n
ABY184	(ABY180 × ABY182-12a) 2n
ABY198	(ABY195 × ABY190-14d) 2n
ABY372	(ABY252-2b × ABY251-1a) 2n
ABY418	(ABY375 × ABY363-12a) 2n
ABY428	(ABY197-6b × ABY202-3d) 2n
ABY326	(ABY128-3a × ABY312-10c) 2n
ABY327	(ABY178 × ABY312-16b) 2n
DAB199	(DAB195-2b × DAB195-17c) 2n
DAB205	(TAB196 × TAB198) 2n
DHC58	(ABY182-12a × DHC57.2a-5d) 2n

Figure 1. Zip1p localization with centromeres in *spo11∆* mutants. (A) Indirect immunofluorescence was used to evaluate Zip1p and Mtw1p localization on meiotic chromosome spreads. (B) Zip1p and Mtw1-Myc13p foci per chromosome spread (n = 100 chromosome spreads). (C) Colocalization of Zip1p and Mtw1-Myc13p foci. Zip1p foci were counted from spreads (n = 25) that had 16 Mtw1-Myc13p foci. Overlapping foci were scored as colocalized. Adjacent foci were not scored as colocalized. (D) Chromatin immunoprecipitation was used to evaluate the association of Zip1p with *CEN1*, *CEN3*, and *CEN4* in cells harvested from a meiotic time course. (In, total sheared chromatin; −, mock-precipitated chromatin; +, anti-Zip1p precipitated chromatin). (E) Quantification of Zip1p association across *CEN1* and *CEN4*. (F) Mapping of Zip1p binding across *CEN1* by primer walk. Strain: *spo11∆*, ABY133.

coupling both in our strain background and in strains in which the phenomenon was initially described (Supplemental Figure S1; see below). Because Zip1p is necessary for centromere coupling, we examined by ChIP whether Zip1p is associated with centromeric chromatin at early meiotic time points. The experiments were done in a *spo11∆* strain because cells exhibiting centromere coupling are easily detected in this background (Tsubouchi and Roeder, 2005). Meiotic cells were harvested at timed intervals and ChIP (Meluh and Koshland, 1997) was performed using antibodies against Zip1p. PCR reactions with primers corresponding to three different centromeres (*CEN1*, *CEN3*, and *CEN4*) were used to assay the immunoprecipitate for the presence of centromeric DNA. Before the induction of meiosis (T = 0), there was no detectable amplification of centromeric DNA from the immunoprecipitated chromatin. One hour after the induction of meiosis, the centromeric DNA signal was slightly elevated in the ChIP samples but not in the controls lacking antibody (Figure 1D). The ChIP signals for all three centromeres increased by 2 h of meiotic induction and persisted throughout the course of the experiment. The early association of Zip1p with centromeric chromatin, and its persistence, is consistent with a role for Zip1p at centromeres promoting centromere coupling, and the persistence of centromere coupling in the *spo11∆* mutant.

Zip1p Binding Is Most Prominent Close to the Core Centromere

To test whether Zip1p is localized to core centromeres, or more generally, in centromere proximal regions, we used ChIP to map Zip1p association across a 20-kb centromere region of two different chromosomes (I and IV) (Figure 1E). Samples were taken after the induction of meiosis (T = 5.5 h) in a *spo11∆* strain. For both chromosomes, Zip1p binding was most prominent around the centromere and gradually declined on both sides (Figure 1E). In this and other experiments, *CEN4* always gave higher signals than the other centromeres, perhaps suggesting that more Zip1p localizes to some centromeres than others (Figure 1A).

To define more precisely Zip1p binding around the centromere, we performed a higher resolution mapping across *CEN1* with a primer-walk. Primers were chosen to amplify 400–450 base pairs segments with each segment overlapping its neighboring segments by 20 base pairs. Because the sonicated chromatin fragments were up to 700 bp, this yielded a resolution of ~300 base pairs on either side of the amplified product. Again, Zip1p binding was most prominent around the core centromere (Figure 1F), consistent with a mechanism of Zip1 loading in which Zip1, or a protein
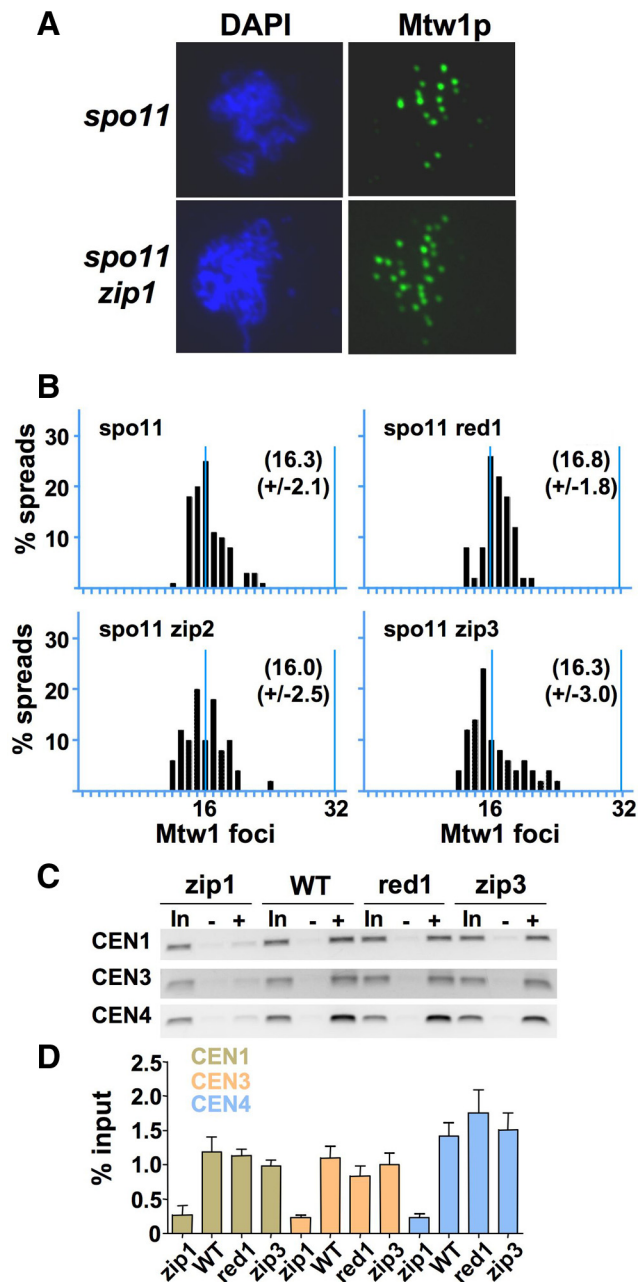
Figure 2. Centromere coupling does not require Red1p, Zip2p, or Zip3p. Centromere pairing and Zip1p localization to centromeres was evaluated in strains deleted for genes that are required to promote assembly of Zip1p into synaptonemal complexes. Strains: *spo11*Δ, ABY133; *spo11*Δ *red1*Δ, ABY168; *spo11*Δ *zip2*Δ; *spo11*Δ *zip3*Δ. (A) Indirect immunofluorescence was used to evaluate the numbers of Mtw1-13XMYCp foci. Chromosome spreads were stained with 4,6-diamidino-2-phenylindole (DAPI), and antibodies against Zip1p and MYC. Zip1p-positive nuclei (except for the *zip1*Δ strain) with condensed chromosomes were chosen for the analysis. Representative examples of pairing (16 foci) and incomplete pairing (26 foci) are shown. (B) Quantification of Mtw1p foci. The vertical lines at 16 and 32 correspond to complete pairing and the absence of pairing (n ≥ 50 for all experiments). The average and SD is indicated for each distribution. The average number of Mtw1 in the *spo11 red1*, *spo11 zip2*, and *spo11 zip3* strains was not significantly different from the *spo11* strains (unpaired *t* test, p > 0.2 in each case). (C) Association of Zip1p with centromere regions in *red1*Δ and *zip3*Δ mutants was evaluated by ChIP as described in Figure 1D. All strains carry the *spo11*Δ mutation. (D) Quantification of Zip1p association with centromeres in *spo11*Δ (WT), *spo11*Δ *red1*Δ, and *spo11*Δ *zip3*Δ strains was performed using ChIP.

with which it associates, binds first at the kinetochore then spreads outward along the arms.

RED1, ZIP2, and ZIP3 Are Not Essential for Centromere Coupling and RED1 and ZIP3 Are Not Required for Localization of Zip1p to Centromeres

Because Zip1p is a component of the SC, we tested whether other proteins needed for SC assembly are required for centromere coupling. Red1p is a component of the lateral element of the SC, and SCs do not form in *red1* deletion mutants (Rockmill and Roeder, 1990; Smith and Roeder, 1997). Normal SCs also do not form in *zip2* and *zip3* deletion mutants (Chua and Roeder, 1998; Agarwal and Roeder, 2000; Borner *et al.*, 2004). However, Zip3p localizes at centromeres of *spo11*Δ mutant cells that are engaged in centromere coupling and Zip2p localizes with centromere-associated Zip1p foci in wild-type cells (Tsubouchi *et al.*, 2008). Centromere pairing was assayed by monitoring Mtw1p foci in meiotic chromosomes spreads. In *spo11*Δ spreads, meiotic nuclei had an average of 16.3 ± 2.1 Mtw1p foci, implying that the centromeres of the 32 chromosomes were organized as pairs (Figure 2, A and B) as described previously (Tsubouchi and Roeder, 2005). Although deletion of *ZIP1* disrupts this pairing deletion of *RED1*, *ZIP2*, or *ZIP3* does not (Figure 2B); in all three mutants, the average number of Mtw1p foci was also ≈16 and not significantly different from the *spo11* strain (16.8 ± 1.8, 16 ± 2.5, and 16.3 ± 3, respectively). Thus, although these proteins are essential for assembly of Zip1p into SCs, none are essential for centromere coupling. Therefore, centromere coupling is not dependent on the pathway required for normal SC formation.

If Zip1p physically promotes centromere coupling through its localization to centromeres, then Zip1p should associate with centromere regions in cells with mutations that disrupt SC formation but not centromere coupling. This was tested using ChIP assays in *red1*Δ and *zip3*Δ mutants by using antibodies against Zip1p. The association of Zip1p with centromeres, as evidenced from the amount of PCR amplification of centromere DNA, was indistinguishable in the *spo11*Δ, *spo11*Δ *red1*Δ, and *spo11*Δ *zip3*Δ strains (Figure 2, C and D). Thus, Red1p and Zip3p are not essential for Zip1p binding to the centromeres. This is consistent with the centromere coupling results described above.

REC8 Is Needed for Centromere Coupling

Rec8p is a meiosis-specific component of the cohesin complex that maintains sister chromatid cohesion during meiosis I and II (Klein *et al.*, 1999). Because of Rec8p's specialized role in meiotic kinetochore function (Watanabe and Nurse, 1999; Buonomo *et al.*, 2000; Kitajima *et al.*, 2003) and because SC formation is defective and Zip1p does not show normal localization in a *rec8*Δ mutant (Klein *et al.*, 1999), we tested whether Rec8p is needed for centromere coupling. Premature separation of sister chromatids in the *rec8*Δ strain (Klein *et al.*, 1999) background would lead to extra Mtw1 foci and complicate the interpretation of the assay. To prevent this, we expressed the mitotic cohesin *MCD1/SCC1* from the *REC8* promoter as described previously (Toth *et al.*, 2000). The *spo11*Δ *rec8*Δ::P_{REC8}-*SCC1* strain do not form normal SCs but maintain efficient sister chromatid cohesion during meiotic prophase (Toth *et al.*, 2000). We confirmed that in our strain background sister centromeres remain together in meiosis I of *spo11*Δ *rec8*Δ::P_{REC8}-*SCC1* cells (Figure 3A). This strain therefore allowed us to test Rec8p's role in centromere pairing in the *spo11*Δ mutant.

A *zip1* deletion strain was constructed to allow the evaluation of the requirement for Zip1p for centromere coupling
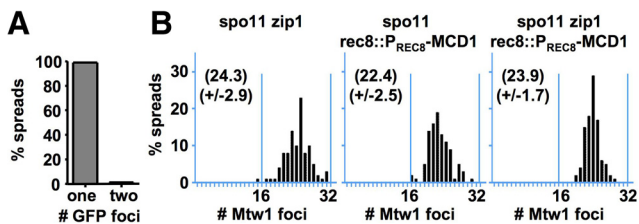
Figure 3. REC8 is required for centromere coupling. (A) Sister chromatid cohesion is maintained during meiotic prophase in a *spo11Δ rec8Δ P_REC8-SCC1* strain. lacO arrays were inserted ~2 kb from *CEN1*, in one of the copies of chromosome *I*. Chromosome spreads were prepared 5.5 h after induction of meiosis and stained with anti-GFP antibody and the DNA-dye 4,6-diamidino-2-phenylindole (DAPI). Chromosome spreads with a single undivided nucleus, and condensed chromosomes were regarded as prophase cells and selected for analysis. A single GFP dot implies normal cohesion; two GFP dots implies loss of cohesion (n = 96). (B) Quantification of Mtw1p foci in *rec8* mutants. Chromosome spreads were used to assay centromere pairing as described in Figure 2 (n = 100 for each strain). The average and SD is indicated for each distribution. Strains: *spo11Δ zip1Δ*, ABY271; *spo11Δ rec8::P_REC8-SCC1*, ABY184; *spo11Δ rec8::PREC8-SCC1 zip1Δ*, ABY198. All three strains exhibited significantly higher numbers of Mtw1 foci than the *spo11* control (see Figure 2) (unpaired *t* test, p < 0.0001 in each case).

in our laboratory strain background. In a previous report, the *zip1Δ* mutation led to a nearly complete loss of centromere pairing, yielding 32 kinetochore foci, one for each unpaired kinetochore (Tsubouchi and Roeder, 2005). We observed ≈24 kinetochore foci (24.3 ± 2.9) in *spo11Δ zip1Δ* strains. This is fewer than reported previously, but a significant reduction in centromere coupling compared with the *spo11Δ* strain (16.3 ± 2.1). The failure to observe 32 kinetochore foci suggests that factors independent of Zip1p also might promote centromere coupling, or alternatively that our chromosome spreading and quantification methods differ from those described previously (Tsubouchi and Roeder, 2005). We tested the effect of a *ZIP1* deletion in three different strains (our laboratory strain, SK1, and BR1919—the strain in which the *zip1Δ* centromere pairing defect was originally described; Tsubouchi and Roeder, 2005). In all three strains the *ZIP1* deletion significantly increased the number of kinetochore foci above that seen in the *spo11Δ* strain, but none resulted in a complete loss of centromere coupling (Figure 3 and Supplemental Figure S1).

Deletion of *REC8* resulted in loss of centromere coupling like that resulting from deletion of ZIP1. The *spo11Δ rec8Δ::P_REC8-SCC1* strain had an average of 22.4 ± 2.5 Mtw1p foci, significantly, more than that observed in the *spo11* strain (unpaired *t* test, p < 0.0001) (Figure 3B).

The fact that the *spo11Δ zip1Δ* strain and the *spo11Δ rec8::P_REC8-SCC1* strain had similar centromere coupling defects indicates that Zip1p and Rec8p might function in the same pathway to promote centromere coupling. To test this, we examined the number of Mtw1p foci in a strain deleted for both *ZIP1* and *REC8*. The *spo11Δ zip1Δ rec8Δ::P_REC8-SCC1* strain had a similar number of Mtw1p foci (23.9 ± 1.7) as was found in the single mutants (Figure 3B). This is consistent with the model that these proteins promote centromere coupling through a common mechanism.

Rec8p Localizes to Kinetochores Early in Meiosis

Rec8p assembles onto chromosomes in meiotic S phase and localizes first to kinetochores before it is found along the chromosome cores (Klein *et al.*, 1999). Zip1p begins to as-

semble onto chromosomes in late leptotene/zygotene (Borner *et al.*, 2004). Because the above-mentioned results implicate both proteins in centromere coupling we evaluated the relative timing of the association of these proteins with chromosomes by immunofluorescence microscopy. Cells enter meiosis with their centromeres clustered, the result of being pulled to the spindle pole body in the previous mitosis (Hayashi *et al.*, 1998; Jin *et al.*, 1998). The early meiotic centromere clusters are preserved in our chromosome spreads (Figure 4A). At early meiotic time points, Rec8-GFP (Kateneva *et al.*, 2005) was often observed as a single focus (Figure 4, A and B) or small collection of foci that colocalize with clustered centromeres (Figure 4A) consistent with the initial description of Rec8 loading in early meiosis (Klein *et al.*, 1999). In *spo11* mutants, cells in which the Rec8p was detected only at the centromeres, had either no detectable Zip1p staining or a few foci of Zip1p, most of which are outside of the centromere cluster (Figure 4 C, top two rows). At later time points, including 5.5 h after meiotic induction—the time point at which the centromere coupling and ChIP assays are performed—Rec8 is found through out the chromatin mass (Figure 4, B and C, bottom row) and Zip1p is found as numerous foci. Thus, consistent with previous evaluations of the timing of Rec8p and Zip1p localization to chromosomes, Rec8p localizes to centromere regions before the time at which Zip1p is associated with chromosomes, and before Zip1 was detected at centromeres by ChIP assays (Figure 1D).
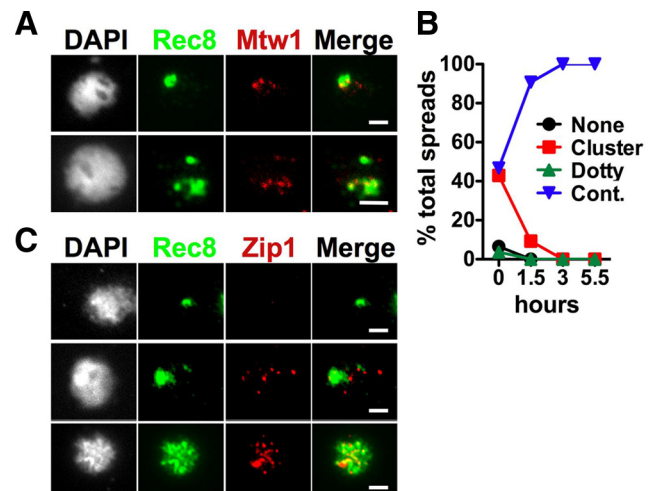
Figure 4. Rec8 localizes to centromere regions before most Zip1 association with chromosomes. Indirect immunofluorescence was used to evaluate the relative timing of Rec8p and Zip1p association with meiotic chromosomes. After induction of meiosis, cells were removed from cultures at timed intervals, chromosome spreads were prepared, and indirect fluorescence microscopy was used to evaluate localization of Rec8p with respect to kinetochores (A) or relative localization of Rec8p and Zip1p (B and C). (A) In early time points, Rec8p was often found as a single focus (top row). This focus always overlapped with the cluster of kinetochores (Mtw1p) characteristic of early meiotic cells (Hayashi *et al.*, 1998; Jin *et al.*, 1998). As centromeres disperse, the Rec8p exhibits dotty foci that overlap the Mtw1p foci (bottom row). Strain: ABY372 (*REC8-GFP MTW1-13XMYC*). (B) Time course showing Rec8p localization patterns. Rec8p staining moves from a single cluster to continuous staining as meiosis proceeds. n > 50 for each time point. Strain: ABY428 (*spo11 REC8-GFP*). (C) Representative Zip1p and Rec8p staining patterns observed in cells from the time course in B. Chromosome spreads in which Rec8p appears as a cluster, dots, or as continuous staining on chromatin are shown. Bars, 2 μm.

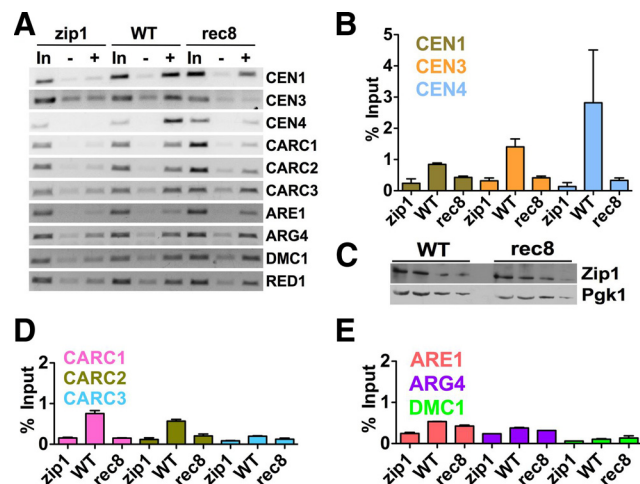### Deletion of REC8 Reduces Zip1p's Binding at Core Centromeres

The early loading of Rec8p at centromeres and the centromere coupling phenotypes of zip1Δ and rec8Δ single and double mutants, suggested that Rec8p might be needed for Zip1p's association with centromeres. We used ChIP assays to compare the association of Zip1p with centromere regions in meiotic extracts from spo11Δ and spo11Δ rec8Δ:: P_{REC8}-SCC1 strains (Figure 5, A and B). The association of Zip1p with centromeric DNA was greatly diminished (but not lost altogether) in the spo11Δ rec8Δ::P_{REC8}-SCC1 strain (Figure 5, A and B). Western blots revealed there were no distinguishable differences in Zip1p levels between the two strains (Figure 5C); thus, the reduced levels of centromere DNA in the Zip1p immunoprecipitate from the spo11Δ rec8Δ::P_{REC8}-SCC1 strain implies that in this strain Zip1p has a reduced ability to associate with the centromeres.

Zip1p Associates with Cohesin-rich Regions in Meiosis in a Rec8p-dependent Manner

Because Zip1p's centromeric association depended upon the meiotic cohesin Rec8p, we tested Zip1p binding at other cohesin-rich and cohesin-poor loci. Mitotic and meiotic cohesins do not bind uniformly all along the chromosomes but are organized into cohesin-rich and cohesin-poor regions (Laloraya et al., 2000; Glynn et al., 2004). In both meiotic and mitotic cells, centromeres are specifically cohesin dense, and cohesin binds over a 20- to 50-kb range across the centromeres (Blat and Kleckner, 1999; Glynn et al., 2004; Weber et al., 2004). We tested Zip1p binding at CARC1, CARC2, and CARC3 on chromosome III (Laloraya et al., 2000) (Figure 5, A and D). Zip1p was enriched at all three cohesin-associated regions (CARs), but less so than at centromeres. As with the centromeres, levels of Zip1p association with the CARs var-

ied from one to another and deletion of REC8 reduced the ChIP signals to a similar low level. The level of Zip1p association with CARs and centromeres in the REC8 deletion strain (spo11Δ rec8Δ::P_{REC8}-SCC1) is higher than that seen for some other loci even when REC8 is present (see below), suggesting that Zip1p may have a weak ability to associate with these CARs and centromere regions independent of Rec8p.

We also examined Zip1p binding at cohesin-poor regions. Meiotic cohesin tends to be excluded from regions surrounding meiotic DSBs and from regions that are transcriptionally active in early meiosis (Glynn et al., 2004). We evaluated Zip1 association at two DSB hot spots, located near the promoter regions of ARE1 (Goldway et al., 1993) and ARG4 (Sun et al., 1989), and two genes that are specifically expressed in meiosis, RED1 and DMC1 (Chu et al., 1998). The DMC1 locus binds mitotic cohesins but association with cohesins is diminished in meiotic cells (Glynn et al., 2004). The DMC1 locus and the ARG4 and ARE1 DSB hot spots showed levels of PCR amplification that were consistently above background but much lower than the signals at centromeres or CARs (Figure 5, A and E). Unlike the centromere regions and CARs, deletion of REC8 had no detectable influence on Zip1p association with these sites. No PCR amplification above background was detected at the RED1 locus (Figure 5A). Together, these data show that in the spo11Δ mutant meiosis, Zip1p is enriched at known cohesin-rich regions, including centromeres, and these associations are largely Rec8p dependent. Zip1p associates at much lower levels, or with reduced affinity, at regions that are known to have sparse cohesin binding. Although our semi-quantitative PCR may not be able to detect reduction in Zip1 association with these already low binding sites, the large dependence of Zip1p on Rec8p to bind to cohesin-rich regions lead us to suggest that Zip1p associations with the cohesin-poor regions are largely Rec8p independent.
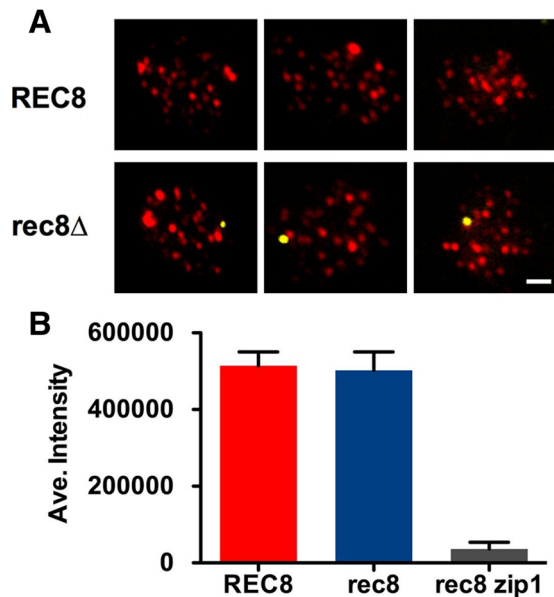
Zip1 Associates with Chromosomes Independently of Rec8

The finding that Zip1 association with centromeres and CARs is dependent on Rec8 raises the question of whether the Zip1 foci observed on chromosome spreads are dependent on Rec8 for their association with the chromosomes. Chromosome-associated Zip1 was evaluated in spo11 strains with or without REC8. To allow a direct comparison Zip1 loading in isogenic rec8Δ and REC8 strains, the cells from both strains were mixed together, induced to enter meiosis, cells were harvested, chromosome spreads were prepared, and the level of Zip1p association with the spreads evaluated. The total fluorescence corresponding to Zip1 was determined in individual chromosome spreads by using indirect immunofluorescence microscopy. The rec8 strain carried a SPC42-DSRed gene that produced fluorescently tagged spindle pole bodies, making it possible to determine which chromosome spreads were from which strain background. In this way, it was possible to quantify Zip1 staining intensities in spreads from the two strains that were acquired in a single image (Figure 6A). The overall level of Zip1 associated with the chromatin in the two strains was indistinguishable (Figure 6, A and B). This result is similar to a previous observation made in SPO11 strains by Brar et al. (2008) and demonstrates that a significant population of Zip1p associates with chromosomes in a Rec8p-independent process.

In this experiment, the frequency of Zip1 foci that colocalized with Mtw1 in the REC8 and rec8Δ strains also was evaluated (as described in Figure 1A). The levels of colocalization of Zip1p and Mtw1p foci in REC8 and rec8Δ strains

Figure 5. REC8 is required for Zip1p localization to centromeres in early meiosis. (A) ChIP was used to evaluate the requirement for Rec8p in Zip1p association with centromeres, CARs, and other loci. Primer sequences are in Supplemental Table 2. Strains: (WT) spo11Δ, ABY326; (zip1) spo11Δ zip1Δ, ABY271; (rec8) spo11Δ rec8Δ::P_{REC8}-SCC1, ABY327. (B) Quantification of Zip1p association with centromere regions. Values shown in D, F, and G are averages, with SEM, of three PCR reactions from two independent ChIP experiments. (C) Total cellular proteins were extracted from equivalent numbers of spo11Δ and spo11Δ rec8Δ cells and twofold serial dilutions were subjected to Western blot analysis using antibodies against Zip1p or Pgk1p. (D) Quantification of Zip1p association with CARs. (E) Quantification of Zip1p association with Rec8-poor loci.

Figure 6. Rec8 is not required for Zip1 association with chromosomes. Indirect immunofluorescence was used to evaluate association of Zip1 with chromosomes in *spo11Δ* mutant strains. Strains: (REC8) *spo11 REC8 ZIP1*, ABY133; (rec8) *spo11Δ rec8Δ::P_REC8-SCC1*, *ZIP1*, *SPC42-DSRed*, DHC58; (zip1) *spo11Δ*, *REC8*, *zip1Δ*, ABY198. For the experiment presented here, Zip1p-positive chromosome spreads (>70% of the spreads) from the 3-h time point were used. Total fluorescence of each spread was quantified using AxioVision software (Carl Zeiss, Jena, Germany). (A) Representative images showing Zip1p association (Red) with chromosome spreads from *REC8* and *rec8Δ* strains. The SPC42 localization can be seen as a yellow focus in *rec8Δ* spreads. *rec8Δ zip1Δ* strains gave no detectable foci (data not shown). (B) Average fluorescence (arbitrary units) corresponding to Zip1p on chromosome spreads prepared from *REC8*, *rec8Δ*, and *rec8Δ zip1Δ* cells. Error bars represent SE of the mean. Bar, 2 μm.

were statistically indistinguishable (*REC8*, n = 21, 23.2% colocalization; *rec8* n = 23, 21.7% colocalization; unpaired *t* test, p = 0.97). Thus, the Zip1p/Mtw1p colocalizations observed in the immunofluorescence assays (Figure 1A) do not represent the (Rec8p-dependent) Zip1p association with centromere regions that is detected ChIP assays.

The Association of Rec8 with Centromere Regions Is Zip1p Independent

Because deletion of *REC8* reduced or abolished Zip1p's centromere binding in ChIP assays, we tested whether deletion of *ZIP1* would interfere with Rec8p's centromere binding. We used a strain expressing a Rec8-GFP protein for this experiment. Sheared chromatin was immunoprecipitated from *spo11Δ REC8-GFP* and *spo11Δ zip1Δ REC8-GFP* strains after 5.5 h of induction of meiosis, using an antibody against GFP. A *spo11Δ* strain in which Rec8p was not tagged with GFP was used as a negative control for the immunoprecipitations. As expected from prior studies (Glynn *et al.*, 2004), centromere DNA was enriched in the Rec8-GFP immunoprecipitates (Figure 7, A and B). There were no distinguishable differences in the amounts of PCR-amplified centromere DNA between the strains with and without *ZIP1* (Figure 7, A and B). This demonstrated that Zip1p is not essential for Rec8p's association with centromeric regions and suggested that *REC8* lies upstream of Zip1p in the establishment of centromere coupling machinery.
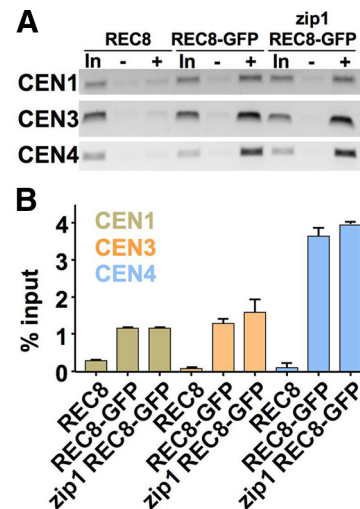

Figure 7. Rec8p association with centromeres is independent of Zip1p. (A) ChIP was used to evaluate the association of Rec8-GFP with *CEN1*, *CEN3*, and *CEN4* in *ZIP1* and *zip1Δ* strains by using methods described in Figure 1. (B) Quantification of ChIP reactions (F). Strains: (REC8) *spo11Δ REC8*, ABY133; (REC8-GFP) *spo11Δ REC8-GFP*, ABY428; (zip1p REC8-GFP) *spo11Δ zip1Δ REC8-GFP*, ABY418. Immunoprecipitations were performed using an antibody against GFP.

DISCUSSION

The pairing of nonhomologous centromeres in early stages of meiosis has been reported in evolutionarily distant organisms, but the regulation of this pairing has not been revealed. Centromere coupling requires Zip1p, and we have found that the localization of Zip1p to centromeres in early prophase requires the meiotic cohesin protein Rec8p. Rec8p is loaded onto centromere regions in premeiotic S phase (Klein *et al.*, 1999). This loading of Rec8 precedes centromere coupling and the association of Zip1 with the chromosomes, which occurs in meiotic prophase. The loading of Rec8 to centromeres is independent of Zip1 (Figures 7 and Figure 8ii). Zip1 can associate with chromosomes independently of Rec8 but does not associate with centromere regions in a way that yields elevated signals in ChIP assays. Together, these data are consistent with a model in which Rec8p directs the organization of Zip1p at centromeres early in meiosis, which then is the trigger for centromere pairing (Figure 8iii). As in previous studies (Tsubouchi and Roeder, 2005), our analysis of the localization of Zip1p foci with nonhomologously paired centromeres shows clear evidence of both centromere/Zip1p colocalization and examples of noncolocalization. However, the Zip1p foci detected in our studies were of variable intensities, and we saw considerably more examples of Zip1p foci that were not localized to centromeres than was reported previously (Tsubouchi and Roeder, 2005). Our observation of centromeres engaged in centromere coupling but with no corresponding Zip1p foci suggests that only modest levels of Zip1p are necessary to promote pairing. Furthermore, Zip1p was also found to localize in a Rec8p-dependent manner at CARs. The Zip1p at CAR sites also could be engaged in pairing interactions, but if so, they cannot compete efficiently for centromeres as pairing partners, or we would not so consistently observe 16 centromere foci in cells engaged in centromere coupling. Fluorescence microscopy experiments showed that, in early meiosis, association of Zip1 with chromosomes was indistinguishable in *rec8Δ* and wild-type strains, suggesting that

Figure 8. Stages of centromere organization in early meiosis. Chromosomes are depicted as being condensed at all stages to simplify the illustration. Ovals on each chromosome represent the core centromere and flanking pericentric regions. (i) Chromosomes are replicated in premeiotic S phase. (ii) In premeiotic S phase and early prophase, Rec8p (blue) is preferentially localized around centromeres. (iii) Zip1p (green) becomes associated with centromeres and CARs. Not shown is Zip1 that is localized as Rec8-independent foci on chromosomes. Localization of Zip1 at centromeres allows initiation of centromere coupling. (iv) Loading of Zip1p at homologous centromeres and CARs, before SC formations may promote alignment of homologous partners, and the propagation (arrowheads) of SC from both centromeres or sites of recombination (yellow).
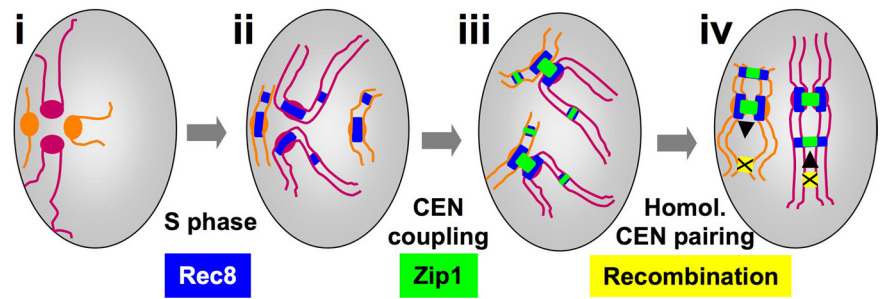
Rec8, whereas essential for organization of Zip1p at centromeres (this work) or into synaptonemal complex (Klein *et al.*, 1999; Brar *et al.*, 2008), is not required for loading of Zip1p onto chromosomes.

In budding yeast, Rec8p has been shown to have functions in meiotic interhomologue recombination, maintenance of chiasmata before anaphase I, and maintenance of a physical link between sister kinetochores until anaphase II (Klein *et al.*, 1999; Buonomo *et al.*, 2000; Brar *et al.*, 2008). Rec8p is also implicated in sister kinetochore mono-orientation during meiosis I in some organisms (Kitajima *et al.*, 2003; Yokobayashi *et al.*, 2003; Chelysheva *et al.*, 2005).

We have uncovered a new role of Rec8—that of promoting Zip1-dependent centromere coupling. How does Rec8p promote centromere coupling? Three results suggest that Rec8p and Zip1p act in the same process to promote centromere pairing. First, ChIP experiments demonstrated that Rec8p promotes the association of Zip1p with centromere regions. Zip1p binding at the centromeres is reduced or lost in the absence of Rec8p. Second, the fact that the numbers of unpaired kinetochores in *zip1Δ* and *rec8Δ* single mutants are similar and do not increase in the double mutant is consistent with this conclusion. Third, Rec8p most probably acts upstream in this process; Rec8p is observed on the chromosomes earlier in meiosis that Zip1, and whereas deletion of *REC8* reduced Zip1p association with centromeres, deletion of Zip1p had no effect on Rec8p association.

This function of Rec8p in centromere coupling is independent of sister chromatid cohesion; whereas sister chromatid cohesion is normal in *spo11Δ rec8Δ P_REC8-SCC1* cells in meiotic prophase (Toth *et al.*, 2000; Brar *et al.*, 2008; also see Figure 3A), both centromere pairing and Zip1p association at centromeric regions are disrupted. We therefore conclude that Rec8p-mediated, Zip1-dependent centromere pairing is a cohesion-independent phenomenon. The demonstrations that 1) Zip1p assembly into SC can occur in the absence of sister chromatids, and hence of sister chromatid cohesion (Pukkila and Skrzynia, 1995; Brar *et al.*, 2008), and 2) Rec8p expressed only after completion of premeiotic DNA replication, and hence probably noncohesive (Watanabe *et al.*, 2001), can support assembly into SC, are consistent with this (Toth *et al.*, 2000; Brar *et al.*, 2008).

Our ChIP experiments demonstrate that Rec8p also promotes the association of Zip1p at other CARs and in the absence of Rec8p, Zip1p association with CARs was significantly reduced. ChIP experiments yielded low, Rec8p-independent signals for cohesin-poor loci. Whether this reflects a weak association of Zip1p with these regions or experimental background is not clear. But because we did not detect any Zip1p association at the *RED1* locus but did reproduc-

ibly detect Zip1 association with other cohesin-poor loci, it is possible that the weak signals seen in the other loci reflect a genuine association with Zip1p.

REC8 is also required for meiotic chromosome axis (axial element) formation; in the absence of Rec8p, one of the structural components of the axial elements, Red1p (Smith and Roeder, 1997), is improperly localized on chromosomes (Klein *et al.*, 1999). Red1p appears only as punctate foci in *REC8* deletion mutants. Does centromere coupling require an intact chromosomal axis? Although we do not know whether an intact axis (Red1p ribbons) forms in *spo11Δ rec8Δ P_REC8-SCC1* cells, the fact that centromere coupling is intact in a *spo11Δ red1Δ* mutant (16 kinetochore foci) and Zip1p associates with centromere regions to a similar extent as it does in a *spo11Δ* mutant, makes it unlikely that an intact axial element is required for centromere coupling. These data also exclude the possibility that centromere coupling requires SC-like structures, because continuous SCs do not form in *red1Δ* mutants (Smith and Roeder, 1997). The requirement for SC-like structures in centromere coupling is also ruled out by both the occurrence of centromere coupling in *zip2Δ* and *zip3Δ* mutants and the apparently wild-type levels of Zip1p association with centromeric regions in the *zip3Δ* mutant. Zip1p association with noncentromeric cohesin-rich chromosomal sites (CARC1 and CARC2) also did not depend on the presence of Red1p and Zip3p (data not shown). Zip1p binding at centromeres and other cohesin-rich regions in a *spo11Δ* mutant, therefore, depends largely on Rec8p but does not need synaptonemal complex-related structures.

Does centromere coupling depend upon the factors that allow the centromeres of sister chromatids to orient toward the same pole (mono-orientation) at meiosis I? In budding yeast, mono-orientation depends upon the monopolin complex (Toth *et al.*, 2000; Rabitsch *et al.*, 2003; Petronczki *et al.*, 2006) but does not require Rec8p. Mam1 seems to load onto chromosomes in pachytene, after centromere coupling (Clyne *et al.*, 2003; Lee and Amon, 2003). Furthermore, Mam1p loading onto chromosomes, unlike centromere coupling, is independent of Rec8p, because Scc1p, when expressed in place of Rec8p can support Mam-dependent mono-orientation (Toth *et al.*, 2000; Rabitsch *et al.*, 2003; Petronczki *et al.*, 2006). Consistent with these data, a *spo11Δ mam1Δ* strain had ≈16 kinetochore foci at a stage when a *spo11Δ zip1Δ* strain had ≈24 foci (Supplemental Figure S2). Thus, centromere coupling seems independent of sister kinetochore mono-orientation or a functional monopolin complex.

How does Rec8p promote Zip1p association at centromeres and other cohesin-rich regions? One possibility could

be recruitment, by direct contact, of Zip1p by Rec8p. Alternatively, it is possible that Rec8p promotes Zip1p association indirectly by recruiting factors needed for Zip1p loading or altering chromosomal topology in a way that exposes Zip1p binding sites. Clearly, further experiments are needed to determine the manner in which Rec8p-promotes Zip1p association with the chromosomes in early meiosis.

An unresolved question is how centromeres disengage from their nonhomologous partners and become aligned with their homologous partners. In *zip4* mutants, which have no SC, homologous centromeres are paired and this pairing is dependent on Zip1p (Tsubouchi *et al.*, 2008). This is consistent with the model that Zip1p acts in an SC-independent manner to pair homologous centromeres later in prophase, just as it promotes pairing of nonhomologous centromeres earlier in prophase, but unanswered is the question of how the transition is made from one partner to another. When the transition is made from nonhomologous to homologous centromere alignment, is the pairing apparatus transferred with the centromeres? It seems likely that a significant portion of the Zip1p remains with the centromeres. In our experiments (data not shown), and those performed by others (Tsubouchi and Roeder, 2005; Tsubouchi *et al.*, 2008), recently paired homologous centromeres are often associated with bright Zip1p foci. The alignment of homologous centromeres, preloaded with a significant accumulation of Zip1p might promote the synaptic process in multiple ways. First, the alignment of homologous centromeres that are preloaded with synaptic components may provide an initiation point for efficient synapsis, outward from the centromere, as suggested previously (Tsubouchi and Roeder, 2005; Tsubouchi *et al.*, 2008; Figure 8iv, orange chromosomes, black arrowhead). Second, if homologous centromeres, preloaded with pairing factors become firmly linked, this connection would keep homologous axes in proximity in the face of the rapid chromosome movements that are ongoing when chromosomes are initiating synapsis (Conrad *et al.*, 2008; Koszul *et al.*, 2008). The linking of the homologous axes by centromere pairing might also stabilize alignments in a way that makes SC propagation from nearby recombination sites toward the centromere more favorable than from other recombination initiation sites in regions where the axes are not so well tethered. This would result in the formation of SC patches near centromeres in the early stages of the synapsis process, like those that have been reported previously (Tsubouchi *et al.*, 2008). Not to be overlooked in either model is the potential contribution of Zip1p loaded at CAR sites along chromosomes arms. In wild-type cells, these sites might act as reservoirs of Zip1p for SC assembly, or as a series of contact points that could bring homologues into register (Figure 8iv, maroon chromosome), and tack them together in a manner that promotes SC assembly.

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