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B-type cyclins *CLB5* and *CLB6* control the initiation of recombination and synaptonemal complex formation in yeast meiosis

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Background: The life cycle of most eukaryotic organisms includes a meiotic phase, in which diploid parental cells produce haploid gametes. During meiosis a single round of DNA replication is followed by two rounds of chromosome segregation. In the first, or reductional, division (meiosis I), which is unique to meiotic cells, homologous chromosomes segregate from one another, whereas in the second, or equational, division (Meiosis II) sister centromeres disjoin. Meiotic DNA replication precedes the initiation of recombination by programmed Spo11-dependent DNA double-strand breaks. Recent reports that meiosis-specific cohesion is established during meiotic S phase and that the length of S phase is modified by recombination factors (Spo11 and Rec8) raise the possibility that replication plays a fundamental role in the recombination process.

Results: To address how replication influences the initiation of recombination, we have used mutations in the B-type cyclin genes *CLB5* and *CLB6*, which specifically prevent premeiotic replication in the yeast *Saccharomyces cerevisiae*. We find that *clb5* and *clb5 clb6* but not *clb6* mutants are defective in DSB induction and prior associated changes in chromatin accessibility, heteroallelic recombination, and SC formation. The severity of these phenotypes in each mutant reflects the extent of replication impairment.

Conclusions: This assemblage of phenotypes reveals roles for *CLB5* and *CLB6* not only in DNA replication but also in other key events of meiotic prophase. Links between the function of *CLB5* and *CLB6* in activating meiotic DNA replication and their effects on subsequent events are discussed.

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Background

The highly conserved process of meiosis consists of a single round of replication followed by two successive rounds of chromosomal segregation without an intervening S-phase (meiosis I and II). This scenario results in a halving of the chromosomal complement and ensures that each gamete receives one and only one copy of each chromosome. A central aspect of meiosis is a high level of recombination, which by creating physical linkages between paired homologs has a critical role in ensuring their proper segregation at the first meiotic division. Most if not all meiotic recombination is initiated by DNA double-strand breaks (DSBs), which form throughout the genome early in meiotic prophase [1] and are repaired by interaction between homologous chromosomes. In many organisms meiotic DSB repair is associated with the formation of the synaptonemal complex (SC), a proteinaceous structure that functions in the maturation of recombinational intermediates and is essential for proper disjunction of homologs at anaphase I [2–4]. The SC is composed of two lateral elements, each of which connects a pair of replicated sister chromatids, and a central element that joins the two. Prior to their incorporation into the SC, lateral elements are referred to as axial elements.

The temporal order of the major molecular events of prophase I has been described in detail for the rapidly sporulating *Saccharomyces cerevisiae* strain SK1 [5]. One of the earliest detectable landmarks is replication, which takes place after transfer of cells to sporulation medium and which precedes DSB formation by 1.5–2 hr, as assayed by both fluorescence-activated cell sorting (FACS) [5] and two-dimensional gel analysis [6]. Around the time of replication, alterations of chromosome structure at future DSB sites also occur [7]. Several studies have shown that all natural and artificially created DSB sites examined so far are located in regions of chromatin that are hypersensitive to DNaseI and micrococcal nuclease (MNase) in mitotic as well as in meiotic cells [8, 9]. Moreover, hypersen-

sitivity to nuclease increases during meiotic prophase, before DSBs appear [9]. This change likely reflects the establishment of a local chromatin configuration favorable for DSB induction [1, 10].

It has been shown that the same origins of replication are used in both meiotic and mitotic cells [11], and it is thought that the same replication machinery is responsible for bulk DNA synthesis in both meiotic and mitotic cells. However, there are important features that distinguish meiotic from mitotic replication. Meiotic S phase is strikingly longer than mitotic S phase in all organisms examined to date [13]. In *S. cerevisiae*, meiotic replication requires about 60 min, in contrast to 17 min for mitotic DNA synthesis [14]. In *Schizosaccharomyces pombe*, recent results suggest that the mechanisms that control the initiation of replication and the coupling of replication to the meiotic cell cycle are partially distinct [12]. Additionally, concommitant with meiotic replication is the establishment of sister chromatid cohesion mediated by such meiosis-specific proteins as Rec8, which binds the length of chromosomes [15]. These observations have prompted the idea that phenomena associated with meiotic replication help to establish a framework favorable for recombination later on [14]. More specifically, they have also led us to ask whether meiotic replication is indeed an absolute requirement for the initiation of recombination.

Previous studies in *S. cerevisiae* have shown that blocking meiotic replication also prevents recombination [16–18], but these observations have been difficult to interpret. This is because the inhibition of recombination could reflect either a direct coupling of replication to recombination or, instead, the existence of checkpoint systems that sense defects in replication and then indirectly prevent recombination by blocking cell cycle progression. Recent evidence favoring a direct link between replication and the initiation of recombination is provided by the finding that DSBs do not form in cells lacking the *MEC1* replication-dependent checkpoint when these cells are treated with the replication inhibitor hydroxyurea [6]. This study also revealed a temporal relationship between the initiation of replication and DSB formation [6].

In the present study, we have taken advantage of mutations in the B-type cyclin genes *CLB5* and *CLB6* to determine how the absence of replication influences the initiation of recombination and associated meiotic events. In mitotic cells, Clb5 and Clb6 are the principal activators of the S phase function of the Cdc28 cyclin-dependent kinase; their absence confers only a delay in the initiation of replication [19–21], which is eventually assured by functionally related B-type cyclins encoded by *CLB1–CLB4* [22]. In contrast, Clb5 and Clb6 are essential for the initiation of meiotic replication [23, 24]. In wild-type (WT) cells of the SK1 background, chromosomal duplication, as assessed by FACS analysis, is completed within 4 hr after the induction of sporulation, but in the *clb5 clb6* double mutant, meiotic replication is totally undetectable [23, 24]. In the *clb5* single mutant, meiotic replication is delayed, prolonged, and incomplete. Cells lacking *CLB6* are phenotypically WT, but the complete absence of replication in the *clb5 clb6* double mutant reveals a role for Clb6 activity. Despite these defects, *clb5* and *clb5 clb6* cells progress into meiotic prophase and undergo one or both meiotic M phases [23–25], in part because the *MEC1* checkpoint is not activated [23]. Here, we report that *clb5* and *clb5 clb6* but not *clb6* mutants are defective in DSB induction as well as prior chromatin changes, heteroallelic recombination, and SC formation. Altogether, our results are consistent with the idea that replication is required for DSB formation, but the connection between these two fundamental processes remains unknown. We propose hypotheses to explain the links between the function of *CLB5* and *CLB6* in activating meiotic DNA replication and in controlling the initiation of recombination.

Results

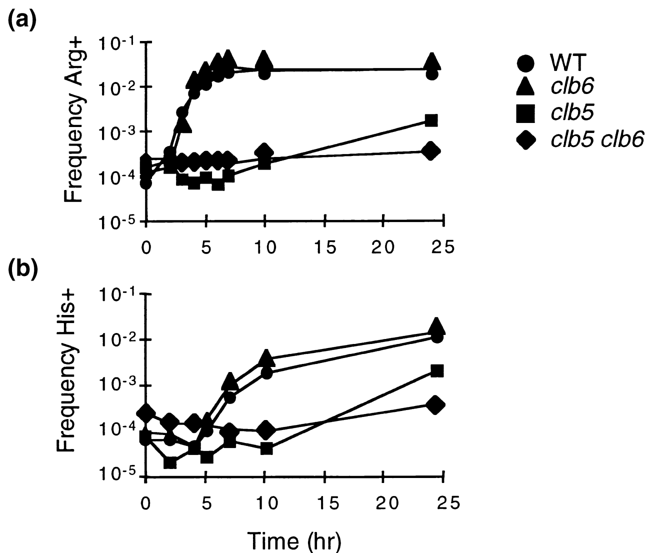
Meiotic recombination is impaired in *clb5* and *clb5 clb6* but not *clb6* diploids

We analyzed heteroallelic recombination at the *ARG4* and *HIS4* loci in diploid WT, *clb5*, *clb6*, and *clb5 clb6* strains. Because spore formation is defective in both *clb5* and *clb5 clb6* homozygous diploids [23–25], we measured recombination by the return-to-growth assay, which allows for the recovery of diploid cells that have initiated meiotic recombination by DSBs but that are unable to repair them at later stages [26]. Both WT and *clb6* strains exhibit frequencies of 2.5×10^{-2} Arg+ and 1.2×10^{-2} His+ prototrophs after 24 hr in sporulation medium, which are approximately 200- and 160-fold increases over their respective mitotic frequencies (Figure 1). In contrast, the frequency of Arg+ and His+ prototrophs rises only 10- to 30-fold in a *clb5* strain, and this increase is delayed (after 10 hr) relative to the time at which prototrophs appear in WT and *clb6* strains. The *clb5 clb6* double mutant does not show any increase in the frequency of recombination at either locus.

Meiotic recombination genes are expressed in *clb5 clb6* mutants

We first considered whether the recombination and sporulation defects of *clb* mutants could result from a failure to express key genes required for various aspects of meiotic recombination. Many meiosis-specific genes whose products are required at early, intermediate, and late stages of sporulation are induced normally in *clb5 clb6* diploids [23, 24], but genes relevant to recombination were not included in these studies. By northern analysis we observed that transcripts required for the initiation of recombination (*SPO11*, *REC102*, *REC104*, *REC114*, *MEI4*, *MEK1*, *HOP1*, *RED1*, reviewed in [2, 4]), meiotic sister

Figure 1



Meiotic recombination is impaired in *clb5* and *clb5 clb6* mutants. Heteroallelic recombination was assayed at the *ARG4* and *HIS4* loci in WT (circles), *clb6* (triangles), *clb5* (squares), and *clb5 clb6* (diamonds) diploids. **(a)** Aliquots of cells heterozygous for the *arg4-nsp* and *arg4-bgl* alleles and **(b)** heterozygous for the *his4-B* and *his4-X* alleles were removed from the sporulation medium at various times and were plated on medium lacking arginine or histidine to select for Arg+ or His+ prototrophs. Relevant genotypes of the strains used in this experiment were (a) ORD5740 (*Mata/α arg4-nsp/arg4-bgl*), ORD5737 (*Mata/α clb5::KanMX/" arg4-nsp/arg4-bgl)* ORD5733(*Mata/α clb6::TRP1/" arg4-nsp/arg4-bgl)*, and ORD5732 (*Mata/α clb5::KanMX/" clb6::TRP1/" arg4-nsp/arg4-bgl*), and below, ORD6518 (*Mata/α his4-B/his4-X*), ORD6513 (*Mata/α clb5::KanMX/" his4-B/his4-X*), ORD6516 (*Mata/α clb6::TRP1/" his4-B/his4-X*), and ORD6509 (*Mata/α clb5::KanMX/" clb6::TRP1/" his4-B/his4-X*).

chromatid cohesion and axial-element formation (*REC8* [15]), strand exchange between homologous chromosomes (*DMC1* [2, 4]), and SC formation (*ZIP1* [2, 4]), which are all induced in WT cells [27], are also meiotically expressed in *clb5 clb6* mutants (Figure 2a,b and data not shown). In all cases, with the exception of a 3- to 4-fold reduction in the level of *SPO11* transcript, which encodes the catalytic subunit of the DSB transesterase [28, 29], these genes are expressed at levels comparable to (greater than 75% of) WT levels.

DSB formation is defective in *clb5* and *clb5 clb6* diploids

To determine whether the impairment of recombination in *clb5* and *clb5 clb6* diploids is due to a defect in the initiation of recombination (which might result from the observed reduction in *SPO11* transcript levels) or in the processing of the recombination intermediates, we next examined meiotic DSB formation. In both WT and *clb6* strains, break fragments at the YCR047c/YCR048w [30] and *CYS3* [31] recombinational hot spots (located on chromosomes III and I, respectively) are clearly detected after 3 hr in sporulation medium (Figure 3). In contrast, their appearance is delayed by up to 2 hr and their total frequency is dramatically reduced (at least 10-fold) in the *clb5* diploid. DSBs are undetectable at either locus in the *clb5 clb6* double mutant. These results demonstrate that Clb5 activity has a major influence on the initiation of meiotic recombination, and the more extreme phenotype of the *clb5 clb6* double mutant suggests that Clb6 can partially substitute for Clb5. Significantly, the extent of DSB formation in each strain mirrors both the partial and total defects in meiotic DNA replication [23, 24], and this suggests that these two processes are under coordinate regulation.

To test the possibility that the overall level of *SPO11* RNA might be below some threshold value required for DSB activity in *clb5* and *clb5 clb6* mutants, we placed the *SPO11* coding region under the control of the strong and early meiosis-specific *HOP1* promoter [32], which is comparably active in both WT and *clb5 clb6* strains (Figure 2a). This construct (pKSY1, see Materials and methods) expresses *SPO11* RNA at levels greater than that expressed by the endogenous *SPO11* gene in a *clb5 clb6* strain (Figure 2c) and, furthermore, it does so at times when DSBs normally form in WT cells. However, pKSY1 does not restore DSB formation to this same *clb5 clb6* strain (data not shown), and this finding suggests that a simple reduction in *SPO11* transcript levels does not alone account for the DSB defect of *clb5 clb6* diploids.

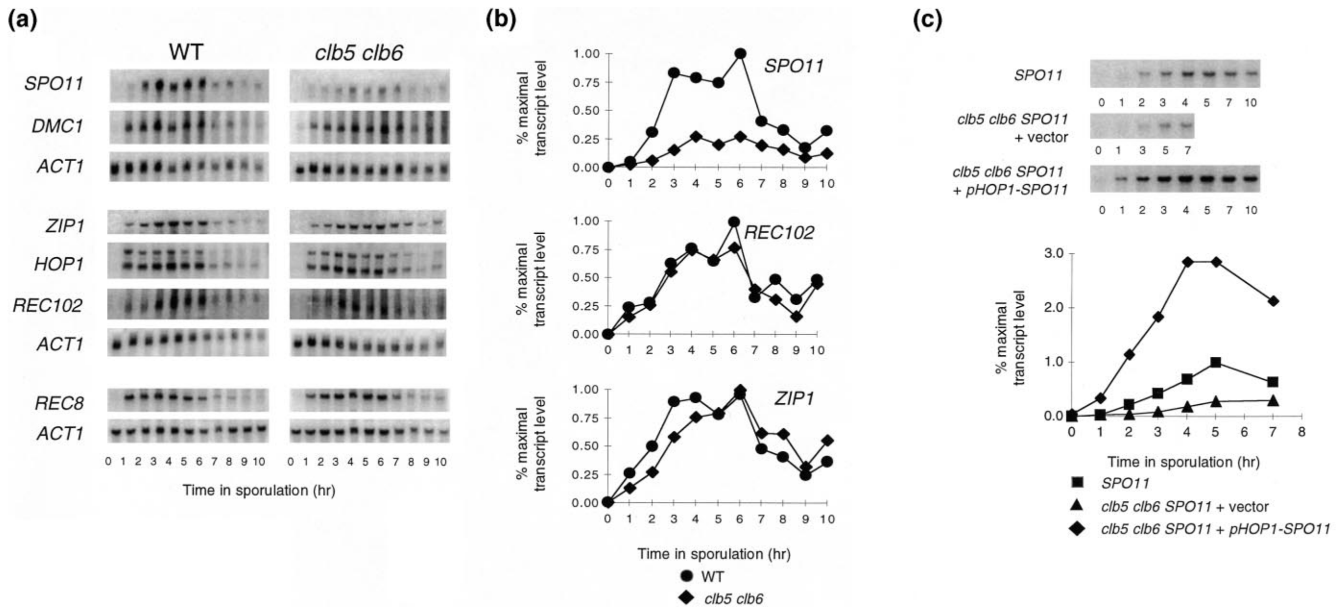
The meiotic increase of chromatin accessibility in DSB regions does not take place in *clb5* and *clb5 clb6* diploids

To determine whether Clb5 and Clb6 functions are required for the meiotic increase in hypersensitivity to nucleases observed at future DSB sites, we examined MNase hypersensitivity at the *CYS3* locus in WT and *clb* mutant strains. In all cases, we found a similar distribution of MNase hypersensitive sites, which overlap the DSB sites *CYS3*-I and *CYS3*-II (Figure 4). Like WT cells, *clb6* cells exhibit a meiosis-specific increase in MNase sensitivity after 4 hr in sporulation medium (Figure 4), a time by which WT SK1 diploids have completed replication and have undergone DSB formation (Figure 3). In contrast, no detectable increase in sensitivity was observed in *clb5* and *clb5 clb6* diploids during meiosis, and this indicates that the chromatin changes at DSB sites are dependent on these B cyclin functions.

Chromosomal morphogenesis and SC formation

Other chromosomal phenomena relevant to recombination that take place in early meiotic prophase, even before DSB formation, include the establishment of sister chromatid cohesion [15] and the preparation for interhomolog interactions [14] that culminate in full-length SC [3, 33].

Figure 2



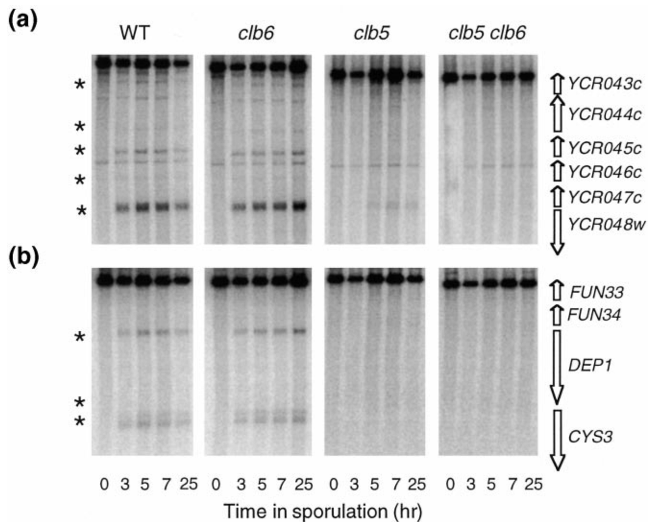
Expression of meiosis-specific genes during sporulation of *WT*, *clb6*, *clb5*, and *clb5 clb6* cells. **(a)** Northern blots of total RNA prepared from WT (ORD5740) and *clb5 clb6* (ORD5732) cells at hourly intervals after transfer to sporulation medium were hybridized with the indicated probes (*SPO11*, *DMC1*; *HOP1*, *ZIP1*, *REC102*; and *REC8*). The rehybridization of each blot with an *ACT1* probe provided a basis of comparison. **(b)** Graphs quantifying the level of selected transcripts (*SPO11*, *ZIP1*, and *REC102*) with respect to the level of *ACT1* RNA at each time point over the course of meiosis for WT (circles) and *clb5 clb6* (diamonds) strains. The maximal ratio was normalized to unity in each case. **(c)** A 3-fold overexpression of *SPO11* is not sufficient to restore DSB formation to a *clb5 clb6* diploid. Shown are northern analyses of *SPO11* RNA over the course of sporulation in a WT strain, ORD5748 ("*SPO11*"); a *clb5 clb6* strain carrying a plasmid without *SPO11* sequences, ORT3372 ("*clb5 clb6 SPO11* + vector"); and a *clb5 clb6* strain carrying pKSY1, a plasmid bearing *SPO11* under the control of the *HOP1* promoter, ORT3373 ("*clb5 clb6 SPO11* + *pHOP1-SPO11*"). Quantification of the level of *SPO11* transcript with respect to the level of *ACT1* RNA (not shown) is provided by the graph, in which the maximal ratio of *SPO11* to *ACT1* transcript in the WT strain ORD5748 is normalized to unity. The presence of pKSY1 increases the level of *SPO11* transcript in *clb5 clb6* cells almost 10-fold (ORT3373, diamonds) relative to *clb5 clb6* cells containing only the vector (ORT3372, triangles), and it increases this level about 3-fold over that of the transcript seen in WT cells (ORD5748, squares). Despite the overexpression of *SPO11*, even at early times at which DSBs form in ORT5748, DSBs cannot be detected in ORT3373 (not shown). The relevant genotypes for the strains in this experiment are as follows. ORD5748, *Mata/α CLB5/" CLB6/" SPO11/" rad50S::URA3/"*; ORT 3372, *Mata/α clb5::URA3/" clb6::TRP1/" SPO11/" rad50S::URA3/" pICM34*; and ORT3373, *Mata/α clb5::URA3/" clb6::TRP1/" SPO11/" rad50S::URA3/" pKSY1.*

Sister chromatid cohesion, chromosome condensation, and homologous synapsis all require the meiosis-specific cohesin Rec8, which is expressed around the time of replication [15]. Rec8 remains associated with sister chromatids throughout SC development, and it is displaced from chromosome arms only at anaphase I and from centromeres at anaphase II [15].

To determine whether SC formation depends on Clb5 and Clb6 functions, we performed a cytological analysis of *clb* mutants. We first used a fully functional HA3-tagged version of Rec8 [15] to monitor the development of cohesion axes. Rec8 is present in >90% of nuclei after 3 hr in sporulation medium in all strains tested (WT, *clb6*, *clb5*, and *clb5 clb6*). In WT and *clb6* nuclei Rec8 forms linear elements, which soon coalesce and coincide with the SC (Figure $5a_{i,ii}$). This process is reduced and delayed in the *clb5* mutant, but a small fraction of cells eventually does succeed in forming extensive SC (Figure $5a_{iii,iv}$,d_{iv}). Unreplicated chromosomes in the double mutant can undergo considerable condensation and sometimes develop thread-like Rec8 cores (Figure $5a_{v}$). This observation indicates that Rec8 may recognize and bind its normal binding sites, even on unreplicated chromosomes. Similarly, the mitotic counterpart of Rec8, Scc1, is known to bind chromatin in the absence of replication [34]. Interestingly, we also found that in *clb5* cells Rec8 persists on chromosomes and that those few cells that undergo anaphase I do so with Rec8 still bound to chromosome arms (F. Klein and A. Penkner, personal communication).

We next monitored the progression of synapsis by immunostaining with an antibody to Zip1, a component of the central element of the SC [35]. In WT and *clb6* cells Zip1 is uniformly distributed along the core of pachytene chromosomes except in the asynaptic rDNA region (Fig-

Figure 3

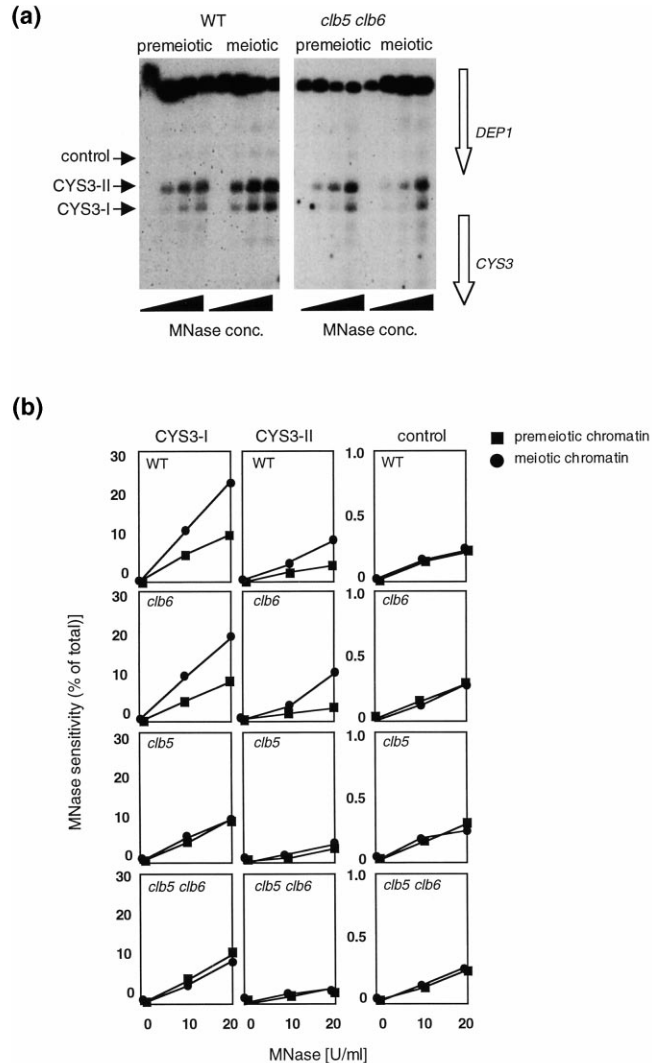


(a) DSB formation at the *YCR047c/YCR048w* hotspot and **(b)** at the *CYS3* locus occurs normally in WT (ORD1181) and *clb6* (ORD5717) diploids but is defective in *clb5* (ORD5715) and *clb5 clb6* strains (ORD5701). DNA was extracted from cells taken from sporulation medium at the indicated times, was digested with *Ase*I, and was probed with (a) sequences specific to YCR048w or (b) with a probe representing the full length *CYS3* ORF. The DSB sites in the promoter regions adjacent to the ORFs in these fragments give rise to the bands designated by asterisks. The diagrams at right show the positions of open reading frames in the (a) 9.6 kb and (b) 4.7 kb parental *Ase*I fragments. All strains are homozygous for the *rad50S-KI81::URA3* allele and accumulate unprocessed DSBs [42]. The relevant genotypes for the strains in this experiment are as follows: ORD1181, *Mata/α rad50S::URA3/"* ORD5717, *Mata/α clb6::TRP1/" rad50S::URA3/"*; ORD5715, *Mata/α clb5::KanMX/" rad50S::URA3/"*; ORD5701, *Mata/α clb5::KanMX/" clb6::TRP1/" rad50S::URA3/"*.

ure $5a_{i,ii}$); in these strains SC formation peaks at 5 hr (Figure $5d_{iii}$). In *clb5* cells chromosomal synapsis, like replication, is delayed and, except in a small subset of cells, incomplete (Figure $5a_{iii,iv}$). A maximum of 12% of *clb5* cells exhibit near-complete or complete SC by 8 hr, whereas 40% of WT cells form extensive SC by 5 hr (Figure $5d_{iii}$). In addition, aggregates of Zip1 (termed polycomplex) are generally present in *clb5* and *clb5 clb6* cells, which is similar to what is observed for many meiotic mutants that cannot form SC [35]. The double mutant is incapable of assembling even partial SC. Instead, the frequency of *clb5 clb6* nuclei with Zip1 foci, which resemble early WT nuclei, remains high (Figure $5a_v$,$d_{i–iii}$).

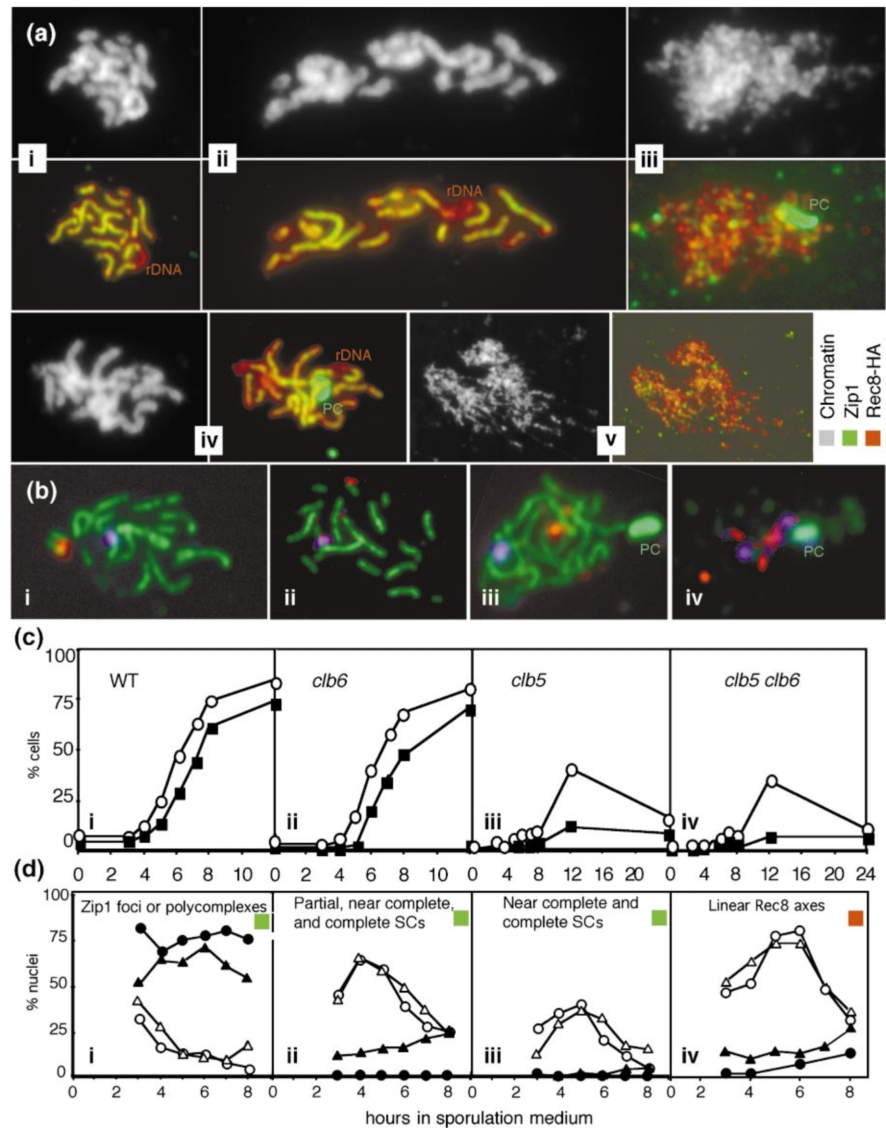
In those few *clb5* nuclei in which extensive synapsis takes place, we could not distinguish 16 isolated Zip1-staining axes corresponding to the expected 16 bivalents that develop in WT and *clb6* nuclei (Figure $5a_{i,ii}b_{i,ii}$). Instead, we noted branched structures or networks (Figure $5a_{iv}$,b_{iii}). These apparently interconnected chromosomes could result from strand switching during nonhomologous synap-

Figure 4



The meiotic chromatin transition at DSB sites at the *CYS3* locus is abolished in *clb5* and *clb5 clb6* mutants. Mutant effects are observed only at the *CYS3* DSB sites and not at a nearby control site and are independent of the MNase concentration. **(a)** Chromatin was isolated from WT (ORD5740), *clb5* (ORD5737), *clb6* (ORD5733), and *clb5 clb6* (ORD5732) cells in presporulation medium ("premeiotic") or from cells after 4 hr in sporulation medium ("meiotic"). Chromatin samples were treated with 0, 10, 20, or 30 U/ml of MNase, as represented by the solid triangles, and they were analyzed by an indirect end-labeling method as described [9, 10]. The diagram at right shows the positions of open reading frames in the 1.6 kb parental *Pst*I fragment. Arrows indicate MNase-hypersensitive sites ("*CYS3*-I" and "*CYS3*-II") and an MNase-sensitive site used as an internal standard ("control"). The CYS3-I and CYS3-II sites here are coincident with the DSB sites marked by asterisks in the lower panels of Figure 3. **(b)** Quantification of the MNase hypersensitivity of the two *CYS3* DSB sites ("site I" and "site II") and of a nearby mitotically hypersensitive site at which DSBs do not form ("control"). The extent of MNase hypersensitivity is expressed as the percentage of radioactivity for the given band relative to total lane radioactivity and is plotted as a function of MNase concentration. Closed squares represent premeiotic chromatin; closed circles represent meiotic chromatin.

Figure 5



CLB5 and *CLB6* are required for progression in meiosis and synaptonemal complex formation. **(a)** SC formation is similar in (i) WT and (ii) *clb6* cells, (iii, iv) proceeds to various degrees in *clb5* mutants, and (v) is abolished in *clb5 clb6* mutants. Staining with DAPI (gray) allowed the visualization of chromatin; Rec8::HA (red) and Zip1 (green) proteins were detected with specific antibodies. (iii, iv) Two different *clb5* nuclei illustrate the variable extent of synapsis seen in this mutant; the *clb5 clb6* double mutant can form numerous linear Rec8 elements but does not undergo synapsis (v). Zip1 polycomplex (PC) can be seen in (iii, iv) *clb5* and (not shown) *clb5 clb6* nuclei. Rec8 forms cohesion axes in the ribosomal DNA (rDNA) region of chromosome XII, but this region does not undergo synapsis, as the absence of Zip1 shows. **(b)** Consecutive immunostaining with an antibody to Zip1 (green) and FISH analysis of spreads of (i) WT, (ii) *clb6,* (iii) *clb5*, and (iv) *clb5 clb6* nuclei, with probes that identify specific loci on Chromosome VI (red) and Chromosome XI (blue). Zip1 polycomplex (PC) can be seen in (iii) *clb5* and (iv) *clb5 clb6* nuclei. Nuclei from (iii) *clb5* cells often have "sticky," apparently interconnected, SCs, in contrast to the 16 typically distinct elements seen in (i) WT or (ii) *clb6* nuclei. In extensively synapsed nuclei, whether from (i–iii) WT*, clb6*, or *clb5* cells, homologous signals are always paired. Homologous FISH signals in (iv) *clb5 clb6* cells are never paired and often appear less compact than those in WT*, clb6*, or *clb5* cells. **(c)** Progression of (i) WT, (ii) *clb6,* (iii) *clb5* and (iv) *clb5 clb6* strains through meiosis I and meiosis II shows that cells lacking *CLB5* exhibit a cell cycle delay and a reduction in the number of nuclei that carry out the two divisions. Cells that have undergone the first meiotic division contain two or four nuclei (circles); cells that have undergone both divisions contain four nuclei (solid squares). Nuclei were visualized by DAPI. n = 100 per time point. **(d)** Graphical representation of axis formation in WT (circles), *clb6* (triangles), *clb5* (solid triangles), and *clb5 clb6* cells (solid circles). The panels illustrate for each strain the percentage of nuclei that exhibit (i) Zip1 foci or polycomplex only, (ii) partial to complete SC, (iii) near-complete to complete SC, (iv) and Rec8 axes. Green or red squares indicate that data were obtained by visualization of Zip1 or Rec8::HA, respectively. n = 100 per time point. Strains used for all experiments were ORD5789 ("WT," *Mata/α REC8::HA3::URA3/"*); ORD5787 ("clb6" *Mata/α clb6::TRP1/" REC8::HA3::URA3/"*); ORD5785 ("clb5," *Mata/α clb5::KanMX4/" REC8::HA3::URA3/"*); and ORD5783 ("clb5 clb6," *Mata/α clb5::KanMX4/" clb6::TRP1/" REC8::HA3::URA3/"*).

sis [36], or they could be due to some feature of *clb5* chromosomes that causes them to be "sticky." To distinguish between these two possibilities, we labeled synapsed axes with Zip1 antibody, identified 14 nuclei exhibiting extensive synapsis, and for these nuclei performed FISH analysis by using DNA probes that recognize two different loci. In all 14 cases, the two signals marking each locus were fused, and this result indicates that synapsis in the *clb5* mutant is always homologous (Figure $5b_{iii}$). As expected, signals on fully synapsed WT and *clb6* chromosomes also colocalize (Figure $5b_{i,ii}$), but each signal is distinct in the *clb5 clb6* double mutant, which never undergoes synapsis (Figure $5b_{iv}$).

The fact that a maximum of 12% of *clb5* nuclei eventually complete synapsis raised the possibility that some of them might successfully complete both meiotic divisions. Analysis of nuclear segregation by DAPI staining indicated that progression through meiosis I and II is similar in WT and *clb6* cells but is strongly inhibited in cells lacking Clb5 (Figure $5c_{i–iii}$). In *clb5* strains few cells complete both meiotic divisions by 12 hr, and the percentage of cells

containing two nuclei decreases at 24 hr relative to 12 hr because *clb5* nuclei degenerate, which gives rise to anucleate cells. We find that up to 3% of *clb5* cells produce four-spored asci, but among 20 tetrads analyzed only two contained viable spores. This result suggests that a subpopulation of *clb5* cells (about 0.3%) eventually completes DNA replication and undergoes both divisions to produce viable spores. A greater percentage of *clb5* cells (about 10%) forms extensive SC (Figure $5a_{iv}$,b_{iii}) and may complete both divisions (Figure $5c_{iii}$) but fails to make viable products.

These results indicate that cells lacking Clb5 have profound defects in chromosome synapsis and that these defects are more severe in the *clb5 clb6* double mutant than in the *clb5* single mutant. However, chromosome condensation and even Rec8 axis formation can be observed on unreplicated (*clb5 clb6*) or partially replicated (*clb5*) chromosomes (Figure $5_{aiii–v}$,d_{iii}). Our results show that cells lacking Clb5 also exhibit a significant cell cycle block, in that the percentage of cells that execute one or both meiotic divisions is greatly reduced.

Discussion

The data reported here demonstrate that *clb5* and *clb5 clb6* mutants, which are severely impaired in their ability to carry out meiotic replication [23, 24], are also defective with respect to their ability to undergo meiosis-specific changes in chromatin accessibility at DSB sites, to initiate recombination by DSBs, and to form SC. Furthermore, the extent of impairment differs among the *clb* mutants; *clb5 clb6* double mutants are completely defective in all of these processes, *clb5* mutants exhibit partial and variable defects, and *clb6* mutants undergo a WT meiosis. These results indicate that the B-type cyclin Clb5 but not Clb6 is indispensable for meiotic recombination.

Roles for *CLB5* and *CLB6* in meiosis

We can consider two major classes of hypothesis to account for the recombinational defects of *clb5 clb6* and *clb5* mutants. First, in addition to their role in promoting replication, Clb5 and Clb6 may act independently at one or more points in meiosis and thereby control progression through the successive phases of chromosomal development. In an extreme version of this hypothesis, chromatin reorganization, DSB formation, SC formation, and chromosomal segregation would be independently impaired in the absence of Clb5 and Clb6 activities. Second, all of these defects may result in some way solely from the impairment of replication, which as a consequence prevents the initiation of recombination. Based on our data, we cannot rigorously exclude either of these interpretations, in part because the specific targets of the activated Clb5-Clb6/Cdc28 complex that are relevant to the initiation of replication, and to other meiosis-specific processes, remain to be identified in both mitotic and meiotic cells. Nonetheless, current evidence indicates that the primary role of Clb5 and Clb6 early in meiosis is to promote replication as it does in mitotic cells [19–21, 37]. Furthermore, consistent with previous observations that several genes expressed at early, intermediate, and late stages of sporulation are induced normally in *clb5 clb6* diploids [23, 24], we have found no evidence that the absence of Clb5 and Clb6 adversely affects the expression of genes relevant to meiotic recombination. *CLB5* and *CLB6* likely have later functions in meiosis besides their early role in promoting S-phase, as both genes are transcriptionally induced shortly before the first meiotic division, well after the completion of replication [25]. We conclude from these observations that the global program of meiotic gene expression is not severely impaired in these B-type cyclin mutants, but the possibility of discrete effects on gene regulation cannot be fully excluded and warrants further study.

Linkage of replication to DSB formation

The alternative hypothesis — that replication is essential for DSB induction and associated chromosomal development — is an attractive explanation for the meiotic defects of *clb* mutants. The failure of *clb* mutants to form DSBs can in turn fully account for later defects in SC assembly. In *S. cerevisiae* temporal, cytological, and molecular analyses clearly demonstrate that SC formation occurs well after DSB formation and, without exception, mutants that do not make DSBs (although are competent for replication) also fail to make SC [3, 33]. Therefore, additional roles in promoting SC formation need not be invoked for Clb5 and Clb6.

We can envisage two different, but not necessarily incompatible, hypotheses to explain how DSB formation could be coupled to replication. One possibility is that an as-yet-unidentified checkpoint mechanism might act to prevent DSB formation until replication has taken place, as defined by the *clb5 clb6* meiotic defect [23, 24]. Since *CLB5* and *CLB6* function in the transition from preinitiation to active replication complexes [19–21, 37], a checkpoint that links DSB formation to replication could be sensitive to some aspect of the preinitiation complex assembled at replication origins [23]. The replication-dependent *MEC1* checkpoint likely acts downstream of this point by blocking cell cycle progression due to replication fork stalling or to the presence of DNA damage [6, 23], but it is unclear whether *MEC1* also monitors the initiation of replication and, indeed, whether it could be a component of a surveillance mechanism linking replication and DSB formation. The function of such a mechanism in WT cells would be to coordinate the initiation of replication and initiation of recombination to ensure that DSBs do not occur on unreplicated chromosomes. There is ample precedent for checkpoints that link later meiotic events. For example, the pachytene checkpoint, which is sensitive to the pres-

ence of recombinational intermediates or improperly synapsed chromosomes, prevents anaphase I from taking place while recombination is underway [38]. The partial cell cycle block exhibited by cells lacking Clb5 ([23, 24] and present results) may reflect the action of one or more checkpoint mechanisms that sense either defects in DSB formation or the presence of rare recombination intermediates and that act to restrain anaphase until chromosomes are correctly aligned on the spindle.

An alternative hypothesis that would explain the link between replication and the initiation of recombination is that replication may be essential to create the proper substrate for later DSB formation. One characteristic of this substrate is represented by the increase in nuclease sensitivity at DSB sites. It is well established that there is a correlation between the meiotic increase of chromatin accessibility and DSB formation. Analyses of recombinational hotspots in strains with alterations of *cis*-acting sequences or with mutations in *trans*-acting factors, either transcription factors that bind DSB regions or proteins required for DSB formation, indicate that the frequencies of DSB formation and recombination generally reflect the extent of chromatin accessibility [7]. Although the molecular nature of nuclease hypersensitivity at DSB sites is not yet elucidated [10], one idea is that increases in sensitivity reflect programmed changes in chromatin structure in early meiosis that may be necessary to provide a proper substrate for the Spo11 nuclease. Alternatively, these modifications could be a consequence of the assembly of a preinitiating recombination complex at the future DSB site, which would be reminiscent of the molecular strategy used in the initiation of replication [39]. While the precise temporal relationship between the initiation or completion of replication and the establishment of an appropriate meiotic chromatin structure has not been determined, the failure of *clb5 clb6* mutants to undergo an increase in meiotic accessibility strongly supports the idea that meiotic replication precedes and is required for the development of chromatin at future DSB regions. In addition, the requirement for replication in creating a proper DSB substrate is compatible with both regional and global control over DSB formation. Strong evidence for the importance of local control is provided by studies showing that the interval between replication and DSB formation in a given region is held constant — at approximately 1 1/2 hr — regardless of whether replication occurs early or late at that site [6]. Our results cannot exclude additional influences over DSB formation by Clb activity on a more cell-wide basis, such as would be expected for a checkpoint system.

The elaboration of the proper local substrate for DNA cleavage likely also includes many chromosomal morphogenetic events that must occur between replication and DSB formation. Such events probably include the development of sister chromatid cohesion [15] and the establishment of chromosomal pairing [40]. These activities may also account for the extended length of meiotic, as opposed to mitotic, S phase [14]. Intriguingly, the Spo11 protein specifically regulates the length of S phase, and this indicates that it may coordinate replication with the initiation of recombination [14]. In conclusion, our results demonstrate that the initiation of recombination and SC formation require B-type cyclin functions, and we favor the interpretation that this requirement may stem from the role of Clb5 and Clb6 in promoting meiotic replication.

Materials and methods

Strains, media, and growth conditions

Media and general methods for culturing and sporulating yeast were as described [5, 41] except that the sporulation medium for cytological experiments was 2% potassium acetate. All strains are SK1 derivatives. All strains used in this report were constructed from progenitor strains analyzed for replication defects and kindly supplied by D. Stuart [23]. We independently verified the absence of meiotic replication in ORD5732 (*clb5 clb6*) by FACS analysis. Derivatives were created by appropriate genetic crosses using strains bearing the *clb5::KanMX* or *clb5::URA3* and the *clb6::TRP1* alleles, which are null [23]. The *rad50S::URA3* [42], *REC8::HA::URA3* [15], and *spo11::URA3* (F. K.) alleles were introduced by crosses. ORD1181 has been described [41]. The relevant genotypes for each strain used are given in the figure legends; details of strain construction are available upon request. The *SPO11* overproducing plasmid pKSY1 and the control vector pICM34 (which lacks *SPO11* sequences) are derivatives of the high–copy number vector pRS324, which contains the 2 μm origin of replication. pICM34 contains the KanMX selectable marker (introduced by C. Mézard) and a 204 bp fragment of the *HOP1* promoter (coordinates −207 to −4, where +1 designates the first nucleotide of the *HOP1* open reading frame, amplified by B. de Massy from pNH33-2, provided by Nancy Hollingsworth). We fused the complete *SPO11* open reading frame to the *HOP1* promoter of pICM34 to create pKSY1. Construction details and sequences of pKSY1 and pICM34 are also available upon request. Plasmids were introduced into yeast strains by transformation with the lithium acetate procedure [43]. We confirmed that pKSY1 also complements the spore inviablity of *spo11*Δ cells.

Recombination and DSB assays

To assess recombinational proficiency by the return-to-growth protocol, we recovered cells from sporulation medium at various times and plated appropriate dilutions on −Arg and −His dropout and on YPD plates to determine the percentage of Arg+ or His+ prototrophs. DSBs at the *YCR047c/YCR048w* hotspot of Chromosome III [30] were detected with a radiolabeled *YCR48w* probe [41] hybridized to Southern blots of *Ase*1-digested genomic DNA prepared from sporulating cells, as described [31]. We rehybridized the same blots with a radiolabeled, amplified *CYS3* probe (GENEPAIRS, see below) to visualize DSBs at the *CYS3* locus [31]. Bands representing DSBs were visualized by the exposure of blots to Phosphorimager screens with the use of ImageQuant software (Molecular Dynamics).

Northern analysis

RNA was prepared from frozen cell pellets with the Fast-RNA RED kit (Bio 101), was subjected to electrophoresis on formaldehyde gels according to standard protocols [44], and was transferred to Gene Screen membranes (NEN Life Science Products). All probes were prepared by amplification of GENEPAIRS PCR products with universal primers (Research Genetics) except for the *ACT1* probe, which was isolated as a *Xba*1-*Hin*DIII fragment from pSK-actin. Hybridization of radiolabeled fragments to membranes in a 50% formamide solution and stripping for successive hybridizations were as recommended by the manufacturer. Transcripts were visualized by exposure of blots to Phos-

phorimager screens and were quantified with the ImageQuant volume analysis feature and the local median background.

Analysis of chromatin hypersensitivity

Chromatin was isolated from premeiotic cells and from cells incubated for 4 hr in sporulation medium and digested with micrococcal nuclease (MNase), as described [9]. After MNase treatment, DNA was digested with *Pst*I and was analyzed by southern hybridization, for which the probe was the [^{32}S]*Pst*I-*Sal* I fragment from pCYS3. Fragments representing hypersensitive sites were visualized with a Fuji-BAS2000 image analyzer and were quantified as previously described [9].

Cytology

Nuclear staining with DAPI (0.5 μg/ml), chromosome spreading, immunocytology and FISH were performed as described [45]. We used mouse anti-Zip1 or rabbit anti-Zip1 antibody to demonstrate synapsis and mouse monoclonal 12CA5 antibody to decorate Rec8::HA3. The FISH probe labeled with Cy3 is a 13.8 kb fragment from the right arm of chromosome VI (cosmid 70,468) and the probe marked with Cy5 consists of two overlapping fragments covering the centromere of chromosome XI, with a cumulative size of 47.5 kb (cosmids 041 and 021) [46].

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