Role for the Silencing Protein Dot1 in Meiotic Checkpoint Control

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During the meiotic cell cycle, a surveillance mechanism called the "pachytene checkpoint" ensures proper chromosome segregation by preventing meiotic progression when recombination and chromosome synapsis are defective. The silencing protein Dot1 (also known as Pch1) is required for checkpoint-mediated pachytene arrest of the *zip1* and *dmc1* mutants of *Saccharomyces cerevisiae*. In the absence of *DOT1*, the *zip1* and *dmc1* mutants inappropriately progress through meiosis, generating inviable meiotic products. Other components of the pachytene checkpoint include the nucleolar protein Pch2 and the heterochromatin component Sir2. In *dot1*, disruption of the checkpoint correlates with the loss of concentration of Pch2 and Sir2 in the nucleolus. In addition to its checkpoint function, Dot1 blocks the repair of meiotic double-strand breaks by a Rad54-dependent pathway of recombination between sister chromatids. In vegetative cells, mutation of *DOT1* results in delocalization of Sir3 from telomeres, accounting for the impaired telomeric silencing in *dot1*.

INTRODUCTION

During eukaryotic cell division, faithful transmission of genetic information is ensured by the operation of cell-cycle checkpoints. These surveillance mechanisms arrest or delay cell cycle progression in response to defects in cellular processes, thereby preventing the initiation of late events until earlier events have been successfully completed (Hartwell and Weinert, 1989). In the mitotic cell cycle, several checkpoint controls have been characterized. For example, the "DNA damage checkpoint" responds to genome injuries by arresting the cell cycle at G1/S or G2/M, or slowing progression through S phase, thus allowing time to repair the damage and preventing the replication or segregation of damaged chromosomes (reviewed by Weinert, 1998; Lowndes and Murguia, 2000). In *Saccharomyces cerevisiae*, several components of the DNA damage checkpoint have been identified. Rad9 and the Rad24 group of proteins (Rad24, Rad17, Mec3, and Ddc1) are thought to be involved in sensing damage and/or generating a signal in response to damage (reviewed by Wein-

ert, 1998; Lowndes and Murguia, 2000). This signal is transduced through a cascade of protein kinases, including Mec1, Rad53, and Chk1, that act on downstream effectors, ultimately triggering cell cycle arrest (reviewed by Weinert, 1998; Lowndes and Murguia, 2000). A number of checkpoint proteins initially characterized in budding and fission yeasts have counterparts in mammals, demonstrating the conservation of these surveillance mechanisms (Weinert, 1998).

Meiosis is a specialized form of cell division that generates haploid gametes from diploid parental cells in sexually reproducing organisms. Proper distribution of chromosomes to the haploid progeny depends on a coordinated series of interactions between homologous chromosomes (homologs) that occur during meiotic prophase, including reciprocal genetic exchange and the intimate association of homologs in the context of the synaptonemal complex (SC) (reviewed by Roeder, 1997). As in the mitotic cell cycle, checkpoints operate in meiosis to ensure the accurate transmission of genetic information. The same checkpoint controls that arrest progression of the mitotic cell cycle in response to DNA damage, blocks in replication, or defects in spindle integrity also function in meiosis (reviewed by Bailis and Roeder, 2000a). In addition, meiotic cells possess a surveillance mechanism called the "pachytene checkpoint" that monitors events specific to meiosis, such as recombination and chromosome synapsis (i.e., SC formation), that are critical for proper meiotic chromosome segregation (reviewed by Roeder, 1997). In mutants of budding yeast, mouse, and *Caenorhabditis elegans* that are defective at intermediate steps in recombination and/or synapsis, the pachytene checkpoint

triggers arrest at midmeiotic prophase (reviewed by Bailis and Roeder, 2000a). In the case of mouse and worm germ cells, arrest is followed by apoptotic death.

In yeast, meiotic recombination occurs concurrently with chromosome synapsis and is required for SC formation (reviewed by Roeder, 1997). Recombination is initiated by double-strand breaks (DSBs) that occur before synapsis. The breaks are rapidly processed to expose single-stranded tails that then invade homologous sequences usually in a nonsister chromatid. Strand invasion is followed by repair synthesis and branch migration to form double Holliday junctions, around the time of SC formation. Mature recombinants are produced near the end of pachytene as the SC disassembles. During meiotic recombination, a number of intermediates are formed in which DNA molecules are not intact or are interlocked; thus, any attempt to segregate chromosomes before completion of recombination would be deleterious. The pachytene checkpoint prevents meiotic nuclear division in the presence of recombination intermediates (Bailis and Roeder, 2000a).

In *S. cerevisiae*, *dmc1* and *zip1* are two well-characterized examples of mutants that undergo checkpoint-mediated arrest at pachytene. Dmc1 is a meiosis-specific homolog of the bacterial RecA strand-exchange enzyme (Bishop *et al.*, 1992). In the *dmc1* mutant, synapsis is delayed and DSBs remain unrepaired (Bishop *et al.*, 1992; Rockmill *et al.*, 1995). Zip1 is a major structural component of the central region of the SC (Sym *et al.*, 1993; Sym and Roeder, 1995; Tung and Roeder, 1998; Dong and Roeder, 2000). The *zip1* mutant arrests in meiosis with unsynapsed chromosomes and unresolved Holliday junctions (Sym *et al.*, 1993; Storlazzi *et al.*, 1996).

Several components of the pachytene checkpoint have been identified in *S. cerevisiae*. During meiotic recombination, DSBs and regions of single-stranded DNA occur; checkpoint proteins that respond to these types of lesions in vegetative cells also monitor recombination in meiosis. Thus, a subset of DNA damage checkpoint proteins, including Rad17, Rad24, Mec3, Ddc1, and Mec1, function in the pachytene checkpoint (Lydall *et al.*, 1996; San-Segundo and Roeder, 1999; Hudson, San-Segundo, and Roeder, unpublished data). The pachytene checkpoint also requires the SC-associated proteins Red1 and Mek1 (Xu *et al.*, 1997; Bailis and Roeder, 2000b; Bailis *et al.*, 2000). Checkpoint-dependent cell-cycle arrest at pachytene is achieved by accumulation of hyperphosphorylated Swe1, which presumably inactivates Cdc28 (Leu and Roeder, 1999), and by inhibiting the activity of the Ndt80 transcription factor, which is required for transcription of the *CLB1* gene encoding the major cyclin active at meiosis I (MI) (Chu and Herskowitz, 1998; Hepworth *et al.*, 1998).

A genetic screen for pachytene checkpoint mutants uncovered a meiosis-specific protein, Pch2, required for *zip1* meiotic arrest (San-Segundo and Roeder, 1999). Pch2 localizes to the ribosomal DNA region (rDNA) and this nucleolar localization depends on the silencing factor Sir2, which is also necessary for pachytene checkpoint function. Silencing is a position-dependent, gene-independent form of repressed chromatin structure that affects large chromosomal domains. In yeast, three regions are subjected to silencing: the telomeres, the *HML/HMR* silent mating-type loci, and the rDNA array. Whereas telomeric and *HML/HMR* silencing involves the silent information regulators Sir2, Sir3, and

Sir4, only the Sir2 protein is required for rDNA silencing (reviewed by Lustig, 1998).

Presented here is the characterization of another protein, Dot1, identified in our screen for components of the pachytene checkpoint (San-Segundo and Roeder, 1999). The *DOT1* gene was independently isolated in a screen for high-copy disruptors of telomeric silencing and shown also to affect *HML/HMR* and rDNA silencing (Singer *et al.*, 1998), further implicating chromatin silencing in the pachytene checkpoint. In the absence of Dot1, the *zip1* and *dmc1* mutants fail to arrest; they proceed through meiosis and sporulation to produce inviable spores. In addition to its checkpoint function, Dot1 inhibits the operation of a Rad54-dependent intersister recombination pathway that repairs DSBs in the absence of Dmc1. The nucleolar Pch2 and Sir2 proteins, as well as the telomeric Sir3 protein, are mislocalized in the absence of Dot1.

MATERIALS AND METHODS

Strains and Plasmids

Yeast strain genotypes are listed in Table 1. *DOT1* was cloned as an ~6.5-kb *Eco*RI-*Bgl*II fragment from *S. cerevisiae* λ clone 5513 (ATCC 70580) into the *Eco*RI-*Bam*HI sites of Bluescript SK$^+$ to generate pSS24. *DOT1* was disrupted by transformation with pSS30 (*dot1::URA3*) or pSS44 (*dot1::TRP1*). To generate the disruption plasmids, an ~2.8-kb fragment containing *DOT1* was amplified by polymerase chain reaction (PCR) by using oligonucleotides ORF26#1 (5′-GGG<u>GGATCC</u>AGGAACACTGAAGAACGGG-3′) and ORF26#2 (5′-GGG<u>GGGCCC</u>AGGTACCTGGTCCACGGCGC-3′) as primers and DNA from λ clone 5513 as template. *Bam*HI and *Apa*I sites added in the primers are underlined. The PCR fragment was cut with *Bam*HI and *Apa*I and cloned into the same sites of Bluescript SK$^+$ to generate pSS25. pSS30 was constructed by replacing a *Hin*dIII fragment of pSS25 containing most of the *DOT1* coding region (from nucleotides − 414 to +1373) with a *Hin*dIII fragment (from pR1333) containing *URA3*. pSS44 was constructed by replacing the *Bst*BI-*Nhe*I fragment of pSS25 containing most of the *DOT1* coding region (from nucleotides −109 to +1278) with a *Cla*I-*Spe*I fragment (from pSS43) containing *TRP1*. pSS43 was made by cloning an ~0.85-kb *Eco*RI-*Bgl*II fragment containing *TRP1* (but not *ARS1*) from YRp7 (Struhl *et al.*, 1979) between the *Eco*RI and *Bam*HI of Bluescript SK$^+$. Both pSS30 and pSS44 were cut with *Bam*HI and *Kpn*I before transformation into yeast. Although *dot1* mutants have been shown to be defective in transcriptional silencing of a reporter gene inserted at the *HM* loci (Singer *et al.*, 1998), we have not detected a mating defect in *dot1* haploids; therefore, *dot1* homozygous diploids were made directly by mating the haploid parents. A *rad54::LEU2* disruption plasmid (pSM31) was provided by D. Schild (Lawrence Berkeley Laboratories, Berkeley). Plasmids used for other gene disruptions have been described previously: *zip1::LEU2* (Sym *et al.*, 1993), *zip1::LYS2* (Sym and Roeder, 1994), *zip1::URA3* (Sym and Roeder, 1995), *dmc1::ARG4* (Bishop *et al.*, 1992), and *rad24::TRP1* (Lydall and Weinert, 1997). Strains containing *SIR3-HA* integrated at the *ura3-1* locus were provided by E. Hong and B. Rockmill (Yale University). Plasmids pSS63 and pSS64 containing *DOT1-HA* and *DOT1-GFP*, respectively, in the high-copy vector pRS424 (Christianson *et al.*, 1992) were constructed by cloning in frame a *Not*I-*Not*I fragment carrying three copies of the hemagglutinin (HA) epitope or green fluorescent protein (GFP)-coding sequences (from plasmids p264 and p266, respectively; provided by B. Santos; Yale University) into a *Not*I site generated by PCR after the second codon of *DOT1*. Additional details of plasmid and strain constructions are available upon request. Media, sporulation conditions, and determination of the frequency of sporulation and meiotic nuclear division have been described (Sym *et al.*, 1993; Sym and Roeder, 1994; San-Segundo and Roeder, 1999).

Table 1. Yeast strains

Strain	Genotype
BR2495	*MAT**a*** *leu2-27 his4-280 trp1-1 arg4-8 thr1-1 ura3-1 ade2-1 cyh10*
	MATα leu2-3,112 his4-260 trp1-289 ARG4 thr1-4 ura3-1 ade2-1 CYH10
MY63	BR2495 but homozygous *zip1*::*LEU2*
DP138	BR2495 but homozygous *dot1*::*URA3*
DP139	BR2495 but homozygous *zip1*::*LEU2 dot1*::*URA3*
MY152	BR2495 but homozygous *zip1*::*URA3*
DP381	BR2495 but homozygous *zip1*::*URA3 dot1*::*TRP1*
DP382	BR2495 but homozygous *zip1*::*URA3 dot1*::*TRP1 rad54*::*LEU2*
DP201	BR2495 but homozygous *zip1*::*LEU2 PCH2-HA*
DP285	BR2495 but homozygous *zip1*::*LEU2 dot1*::*TRP1 PCH2-HA*
BR1919-2N	*MAT**a*** *leu2-3,112 his4-260 trp1-289 ARG4 thr1-4 ura3-1 ade2-1 CYH10*
	MATα leu2-3,112 his4-260 trp1-289 ARG4 thr1-4 ura3-1 ade2-1 CYH10
DP365	BR1919-2N but homozygous *sir3*::*TRP1 SIR3-HA*::*URA3*
DP366	BR1919-2N but homozygous *sir3*::*TRP1 SIR3-HA*::*URA3 dot1*::*TRP1*
DP185	*MAT**a*** *CENIII* *leu2-hisG* *HIS4* *lys2 ho*::*LYS2 trp1-H3 ura3*
	MATα CENIII::*TRP1 leu2-hisG his4-B-LEU2 lys2 ho*::*lys2 trp1-H3 ura3*
DP184	DP185 but homozygous *dot1*::*URA3*
DP246	DP185 but *zip1*::*LEU2*
	zip1::*LYS2*
DP371	DP185 but *zip1*::*LEU2* and homozygous *dot1*::*URA3*
	zip1::*LYS2*
DP338	*MAT**a*** *leu2 lys2 ho*::*LYS2 trp1 ura3 arg4*
	MATα leu2 lys2 ho::*LYS2 trp1 ura3 arg4*
DP339	DP338 but homozygous *dmc1*::*ARG4*
DP341	DP338 but homozygous *dmc1*::*ARG4 dot1*::*TRP1*
DP343	DP338 but homozygous *dmc1*::*ARG4 dot1*::*TRP1 rad54*::*LEU2*
DP352	DP338 but homozygous *dmc1*::*ARG4 rad24*::*TRP1*
DP342	DP338 but homozygous *dmc1*::*ARG4 rad54*::*LEU2*

Strains DP185, DP184, DP246, DP371, DP338, DP339, DP341, DP343, DP352 and DP342 are isogenic with SK1.

Cytology

Chromosome spreads and immunostaining of Pch2-HA, Sir2, Zip1, and Red1 were performed as described previously (San-Segundo and Roeder, 1999). To detect Sir3-HA and Dot1-HA, mouse monoclonal anti-HA antibody (HA.11; Babco, Richmond, CA) was used at 1:100 and 1:150 dilution, respectively. Rat antitubulin antibody (YOL 1/34; Sera-Lab, Crawley Down, Sussex, United Kingdom) was used at 1:100 dilution. Rabbit anti-Rad51 antibody (a gift of D. Bishop, University of Chicago) was used at 1:400 dilution. Anti-Nsr1 antibodies were described previously (San-Segundo and Roeder, 1999). To observe the Dot1-GFP fluorescent signal combined with 4′-6-diamidino-2-phenylindole (DAPI) staining in whole cells, 0.1 ml of 37% formaldehyde was added to aliquots (0.9 ml) of vegetative or sporulating cultures. After fixation for 15–20 min (mitotic cells) or 45–60 min (meiotic cells) at 30°C with rotation, cells were washed twice with phosphate-buffered saline (PBS) and incubated for 15 min in PBS containing 0.1% Triton X-100 and DAPI (2 μg/ml). Cells were washed twice with PBS, resuspended in a small volume of PBS, and observed at the fluorescence microscope (Nikon Eclipse E800, Plan Apo 100×/1.4 oil objective) equipped with an FITC HYQ filter (Chroma Technology Corporation, Brattleboro, VT) to visualize the GFP signal. Images were captured with a Sensys charge-coupled device camera (Photometrics, Tucson, AZ).

Dityrosine Fluorescence Assay

To examine dityrosine fluorescence as an indicator of sporulation, patches of cells were grown on YPAD plates and replica-plated to sporulation plates overlaid with nitrocellulose filters (Protran BA85; Schleicher & Schuell, Keene, NH). After 1–3 d incubation at 30°C

(depending on strain background), dityrosine fluorescence was visualized by illuminating the plates from the top (without the lid) with 302-nm UV light. Photographs were taken using 667 Polaroid film and a Wratten gelatin filter (No. 47B; Kodak, Rochester, NY).

RESULTS

Identification of DOT1

To identify genes involved in the pachytene checkpoint in *S. cerevisiae*, a screen for mutations that alleviate the meiotic arrest of the *zip1* mutant was performed; mutations were introduced by transformation with a transposon-mutagenized yeast genomic library (San-Segundo and Roeder, 1999). In addition to insertions in three other genes (*RAD24*, *DDC1*, and *PCH2*), two transposon insertions were found in the open reading frame YDR440w, which we initially called *PCH1* (for pachytene checkpoint). During the course of this study, the same gene was isolated in a screen for high-copy disruptors of telomeric silencing and named *DOT1* (Singer *et al.*, 1998). Therefore, the name *DOT1* is used throughout this article.

DOT1 encodes a basic (predicted pI 9.7) 582-amino-acid protein with no other homologs in the yeast genome. The N-terminal half of Dot1 is unique and has a high content of lysine residues (Figure 1A). This lysine-rich domain contains at least two consensus monopartite nuclear localization signals (Chelsky *et al.*, 1989; Figure 1A). The C terminus of Dot1
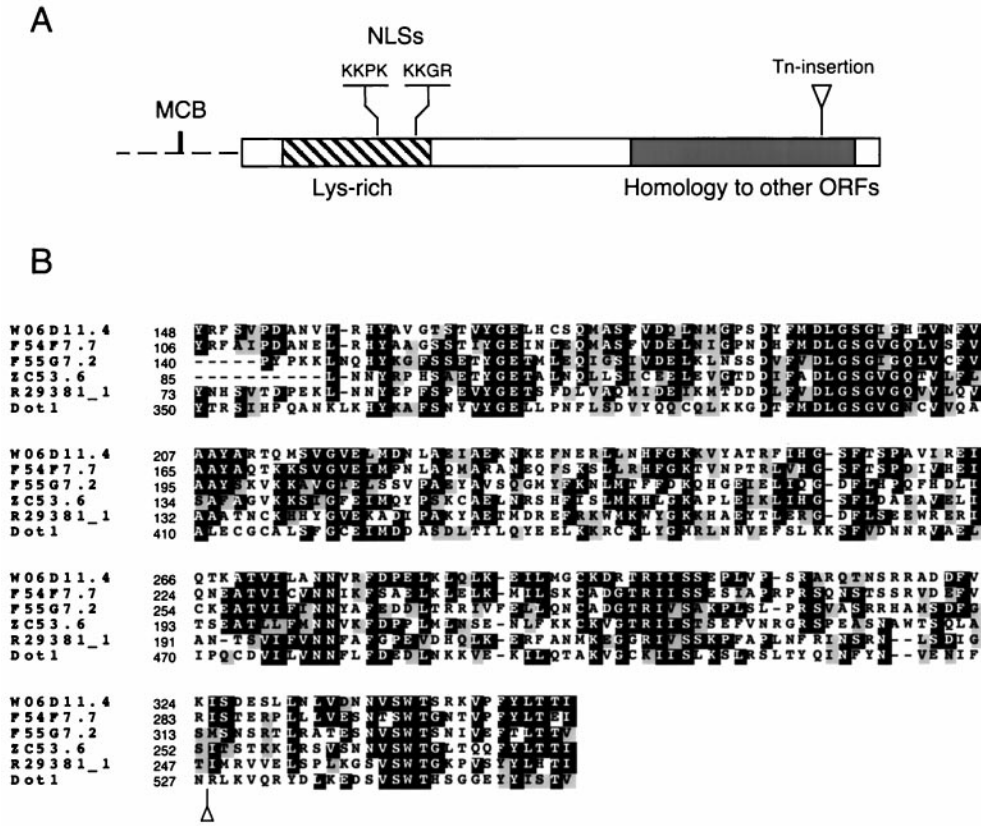
Figure 1. DOT1 sequence features. (A) Diagram of the Dot1 protein (rectangle) and *DOT1* promoter (discontinuous line). Indicated are a lysine-rich domain (striped box; 21.5% of residues between amino acids 35 and 169 are lysines), two putative NLSs starting at positions 126 and 162, and the region of homology to human and *C. elegans* ORFs (gray area). The sequence ACGCGTCA at positions −172 to −165 in the *DOT1* promoter corresponds to a consensus MCB element (MluI cell cycle box site is underlined). (B) Alignment of the regions of homology from *S. cerevisiae* Dot1, human ORF R29381_1 and *C. elegans* ORFs W06D11.4, F54F7.7, F55G7.2, and ZC53.6. Identities are highlighted in black and conservative substitutions in gray. Numbers at the left represent amino acid positions. Alignment and shading were performed with ClustalW 1.7 and Boxshade 3.21, respectively, at the BCM Search Launcher (http://dot.imgen.bcm.tmc.edu:9331/). In A and B, the position of the C-terminal transposon insertion recovered from the mutant screen is marked with a triangle. (C) Percentage identity and similarity of Dot1 to the human and nematode homologs over the indicated length. The significance of the homology is indicated by the BLAST E-values.

displays significant homology over a region of ~200 amino acids with putative proteins identified in systematic sequencing of the human and *C. elegans* genomes (Figure 1). One of the mutant alleles of *DOT1* recovered from the screen encodes a truncated protein lacking only the last 55 amino acids, thus removing the last conserved block (Figure 1, A and B). This allele displays a phenotype indistinguishable from that of the *dot1* null mutant (see below; our unpublished results).

Examination of the *DOT1* promoter revealed the presence of a "*Mlu*I cell cycle box" (Figure 1A). This element is the binding site for the MBF transcription factor, which activates transcription of genes at the G1/S transition in the mitotic cell cycle (Johnston and Lowndes, 1992; Koch and Nasmyth,

1994). Indeed, genome-wide analysis of gene expression during the mitotic cell cycle has shown that transcription of *DOT1* is cell-cycle regulated with the peak of expression at G1 (Spellman *et al.*, 1998). In contrast, *DOT1* transcription is not regulated during the meiotic cell cycle (Chu *et al.*, 1998).

Dot1 Is Required for Meiotic Prophase Arrest or Delay in the zip1 Mutant

To characterize the role of Dot1 in meiosis, null mutants were constructed and analyzed in diploid strains. The *dot1* single mutant displays the wild-type level of sporulation (Figure 2A) and high levels of spore viability and crossing over (Table 2). Thus, Dot1 is not normally required for

Figure 2. Dot1 is required for checkpoint-induced meiotic arrest (or delay) of *zip1* and *dmc1* mutants. (A) Mutation of *DOT1* alleviates the meiotic arrest of *zip1* in BR2495 isogenic strains. Time course of sporulation of strains BR2495 (wild type), MY63 (*zip1*), DP138 (*dot1*), and DP139 (*zip1 dot1*). (B) Mutation of *DOT1* bypasses the meiotic delay of *zip1* in the SK1 strain background. Dityrosine fluorescence, an indicator of sporulation, was examined after 1 and 2 d on sporulation plates. Strains are DP185 (wild type), DP246 (*zip1*), and DP371 (*zip1 dot1*). (C) Distribution of tetrad types. The percentages of tetrads with 4, 3, 2, 1 and 0 viable spores (4-sv, 3-sv, 2-sv, 1-sv, and 0-sv, respectively) are represented. Tetrads (389 and 513) were dissected from DP246 (*zip1*) and DP371 (*zip1 dot1*), respectively. (D) Bypass of *dmc1* arrest by *dot1* and effects of the *rad54* mutation. Dityrosine fluorescence was examined after 2 d on sporulation plates. (%) MI + MII represents the fraction of cells that have undergone at least one meiotic nuclear division (i.e., containing two or more DAPI-stained bodies) after 24 h in liquid sporulation medium. (%) Spo. represents the fraction of cells that formed mature or immature asci. The asterisks indicate that most asci are immature. At least 300 cells were scored for each strain. Strains are DP338 (wild type), DP339 (*dmc1*), DP341 (*dmc1 dot1*), DP343 (*dmc1 dot1 rad54*), DP352 (*dmc1 rad24*), and DP342 (*dmc1 rad54*).
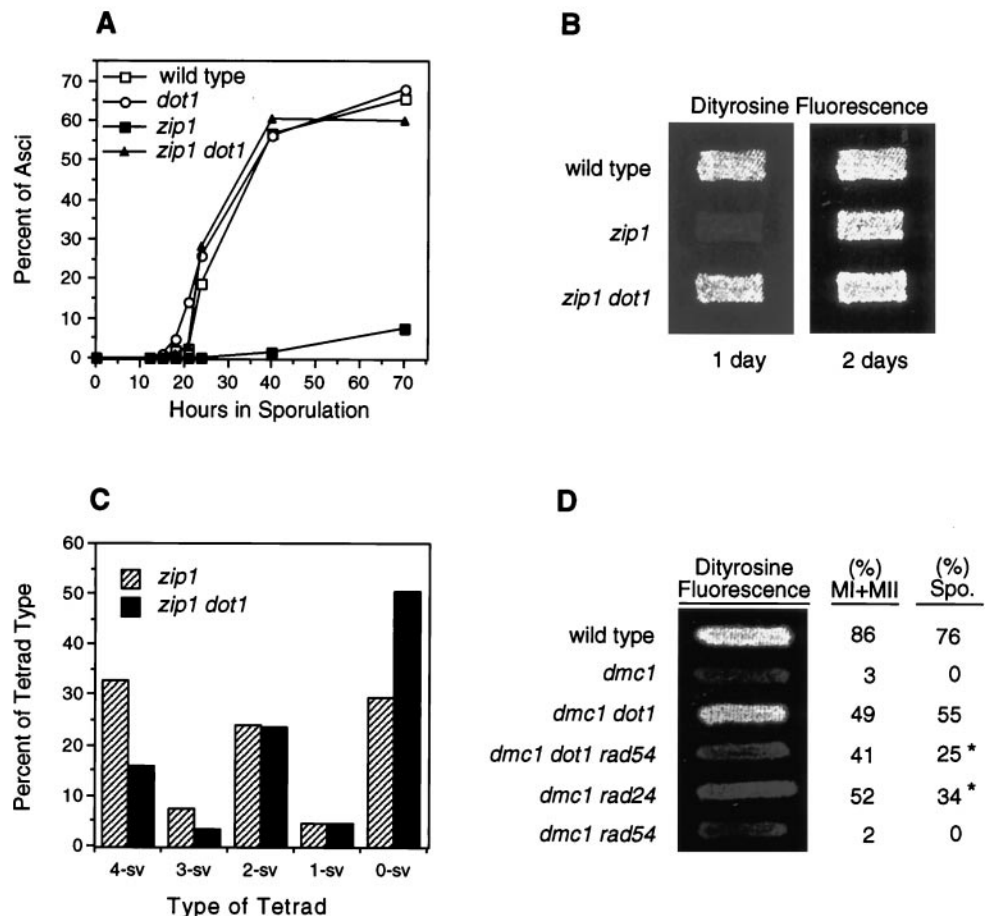
meiotic progression, recombination, chromosome segregation and spore formation.

The sporulation phenotype of the *zip1* mutant depends on strain background, though chromosomes fail to synapse in all *zip1* strains tested (Sym *et al.*, 1993; Sym and Roeder, 1994). In the BR2495 strain background, *zip1* arrests at pachytene, and no mature recombinants are produced (Sym *et al.*, 1993; Roeder, 1997). Deletion of *DOT1* completely alleviates the *zip1* meiotic arrest; the kinetics and frequency of sporulation in *zip1 dot1* are similar to wild type (Table 3 and Figure 2A). However, spore viability is reduced to ~37% (Table 3), suggesting that some or all of the defects

Table 2. Spore viability and crossing over in SK1 strains

Genotype	Spore viability (%)	Crossing over (cM)	Tetrads dissected	Strains
Wild type	98.0	50.8	215	DP185
dot1	93.5	45.0	128	DP184
zip1	52.3	15.2	389	DP246
zip1 dot1	31.5	13.6	513	DP371

Crossover data are the sum of the map distances in the *MAT-CENIII* and *CENIII-HIS4* intervals on chromosome III. Calculations are based on the following numbers of 4-spore-viable tetrads: 203 for wild type, 112 for *dot1*, 129 for *zip1* and 84 for *zip1 dot1*.

Table 3. Sporulation and spore viability in BR2495 strains

Genotype	Sporulation efficiency[a] (%)	Spore viability[b] (%)	Strains
Wild type[c]	51	95.3	BR2495
zip1	3	—	MY152
zip1 dot1	57	36.9	DP381[d]
zip1 dot1 rad54	54	13.7	DP382[e]

[a] Sporulation efficiency was determined by counting the fraction of cells forming asci after 3 days on sporulation plates; 400 cells were scored for each strain.

[b] Spore viability was assessed by tetrad dissection.

[c] Viability data of wild type was presented by San-Segundo and Roeder (1999), and is based on the dissection of 256 tetrads.

[d] Eighty-eight tetrads were dissected for DP381.

[e] Eighty-seven tetrads were dissected for DP382.

conferred by *zip1* persist in the double mutant. In fact, chromosomes do not synapse in *zip1 dot1* (see DISCUSSION).

In the SK1 strain background, the *zip1* mutant sporulates, but MI is delayed and Holliday junctions persist longer than in wild type (Sym and Roeder, 1994; Storlazzi *et al.*, 1996); however, recombination intermediates are eventually resolved, and ~50% of the spores produced are viable (Table 2; Sym and Roeder, 1994). *DOT1* is required for the meiotic delay of *zip1* in the SK1 strain background; *zip1 dot1* sporulates with wild-type kinetics (Figure 2B; our unpublished results). SK1 strains were used to compare recombination and chromosome segregation in the *zip1* and *zip1 dot1* mutants. The reduction in spore viability in *zip1* (Table 2; Sym and Roeder, 1994) is mostly due to pairs of homologous chromosomes segregating to the same pole at MI because of a failure to cross over (Sym and Roeder, 1994). Relief of the meiotic delay of *zip1* by a *dot1* mutation leads to a further decrease in spore viability from ~52 to ~31% (Table 2). However, crossing over in *zip1 dot1* is not significantly different from *zip1*, at least for the chromosome III intervals examined (Table 2). Homolog nondisjunction at meiosis I in *zip1* is manifested by a nonrandom pattern of spore death (Sym and Roeder, 1994), with 0-, 2-, and 4-spore-viable tetrads predominating (Figure 2C). The same pattern of spore death is exhibited by *zip1 dot1* (Figure 2C), but the proportion of 0-spore-viable tetrads is increased at the expense of tetrads with four viable spores (Figure 2C). Thus, in the absence of *DOT1*, *zip1* fails to arrest or delay in meiosis, resulting in chromosome missegregation and the formation of aneuploid meiotic products.

Dot1 Prevents Cell Cycle Progression and Rad54-dependent Intersister Recombination in the dmc1 Mutant

In SK1 strains, the *dmc1* mutant arrests in meiosis with unrepaired DSBs. To determine whether Dot1 is required for this checkpoint-dependent arrest, *dmc1 dot1* double mutants were examined. Deletion of *DOT1* relieves the meiotic arrest of *dmc1*. The *dmc1 dot1* double mutant undergoes high levels of meiotic nuclear division and sporulation (Figure 2D); however, spore viability is very low (~5% for *dmc1 dot1* vs. >95% for wild type; 110 and 227 tetrads dissected, respectively).

Strikingly, a high proportion of the *dmc1 dot1* meiotic products are enclosed in asci-containing mature spores similar to those of wild type, as evidenced by microscopic examination (our unpublished results) and formation of dityrosine (Figure 2D), a fluorescent component of spore walls (Briza *et al.*, 1990). In contrast, the *dmc1 rad24* double mutant proceeds through the meiotic divisions with unrepaired DSBs, resulting in the formation of nuclei with fragmented DNA and few asci with mature spores (Lydall *et al.*, 1996), manifested by the almost complete absence of dityrosine fluorescence (Figure 2D). The fact that *dmc1 dot1* forms morphologically normal spores suggests that a significant fraction of meiotic DSBs in *dmc1* are repaired in the absence of Dot1.

To examine the presence of recombination intermediates relative to meiotic progression, the cytological assay described by Lydall *et al.* (1996) was used. This method is based on the use of anti-Rad51 antibodies to detect recombination intermediates (presumably unrepaired DSBs) in combination with tubulin staining to identify cells that have initiated MI. In wild type, all Rad51 foci have disappeared when the MI spindle is formed (Lydall *et al.*, 1996; Figure 3A). In the arrested *dmc1* mutant, a single tubulin-stained focus characteristic of duplicated but unseparated spindle pole bodies is present, and numerous Rad51 foci persist (Table 4 and Figure 3B). Nuclei in the *dmc1 dot1* mutant fall into three classes (Table 4). Some nuclei (38%) are similar to those of wild type (i.e., MI spindle and no Rad51 foci), indicating that all DSBs have been repaired (Figure 3C). In other nuclei (56%), a few Rad51 foci persist concomitantly with the elongated spindle (Figure 3D). Occasionally (6%), MI nuclei containing 20–35 Rad51 foci are observed (Table 4; our unpublished results). Thus, whereas ~63 Rad51 foci accumulate in the *dmc1* single mutant, only approximately four foci (on average) persist in *dmc1 dot1* nuclei undergoing MI (Table 4). These results indicate that most DSBs are repaired when *dmc1* arrest is bypassed by mutation of *DOT1*; in ~40% of cells, all DSBs are repaired. Because most *dmc1 dot1* spores are dead, this repair presumably occurs using sister chromatids as donors instead of homologs (interhomolog recombination would ensure proper disjunction at meiosis I).

Several studies indicate that meiotic DSBs in the *dmc1* mutant can be repaired by a Rad54-dependent pathway of recombination between sister chromatids, if *RED1* is mutated or cells are returned to growth medium (Schwacha and Kleckner, 1997; Xu *et al.*, 1997; Arbel *et al.*, 1999; Bishop *et al.*, 1999). To test whether this alternative pathway is active in *dmc1 dot1*, the effects of a *rad54* mutation were examined. The *dmc1 dot1 rad54* triple mutant proceeds through the meiotic nuclear divisions (Figure 2D); however, the triple mutant is unable to make mature spores (Figure 2D; our unpublished results). Furthermore, numerous Rad51 foci are detected in *dmc1 dot1 rad54* cells that have exited prophase and elongated the MI spindle (Table 4 and Figure 3E). In this respect, *dmc1 dot1 rad54* is similar to *dmc1 rad24*, which enters MI with unrepaired DSBs (Table 4 and Figure 3F; Lydall *et al.*, 1996). Thus, the Rad54-dependent pathway is active in *dmc1 dot1*, but blocking repair of DSBs by a *rad54* mutation does not prevent meiotic progression.

Repair of DSBs in zip1 dot1 Is Largely Independent of Rad54

In contrast to *dmc1*, which accumulates DSBs, the *zip1* mutant accumulates primarily unresolved Holliday junctions; a small fraction (~10%) of DSBs also remain unrepaired (Storlazzi *et al.*, 1996). Although the spore viability of the *zip1 dot1* double mutant is significantly lower than that of wild type, the moderate fraction of viable spores produced (Tables 2 and 3) indicates that DSBs have been repaired. To test whether the repair of DSBs in *zip1 dot1* depends on Rad54, a *zip1 dot1 rad54* strain was analyzed. The triple mutant sporulates with the same efficiency as wild type or *zip1 dot1* (Table 3) and generates mature spores (our unpublished results), suggesting that most (if not all) DSBs are repaired even in the absence of Rad54.

To confirm this conclusion, Rad51 and tubulin staining was carried out. In the arrested *zip1* mutant, no spindle is formed and ~25 Rad51 foci are present (Table 4 and Figure

DAPI   Rad51 + Tubulin
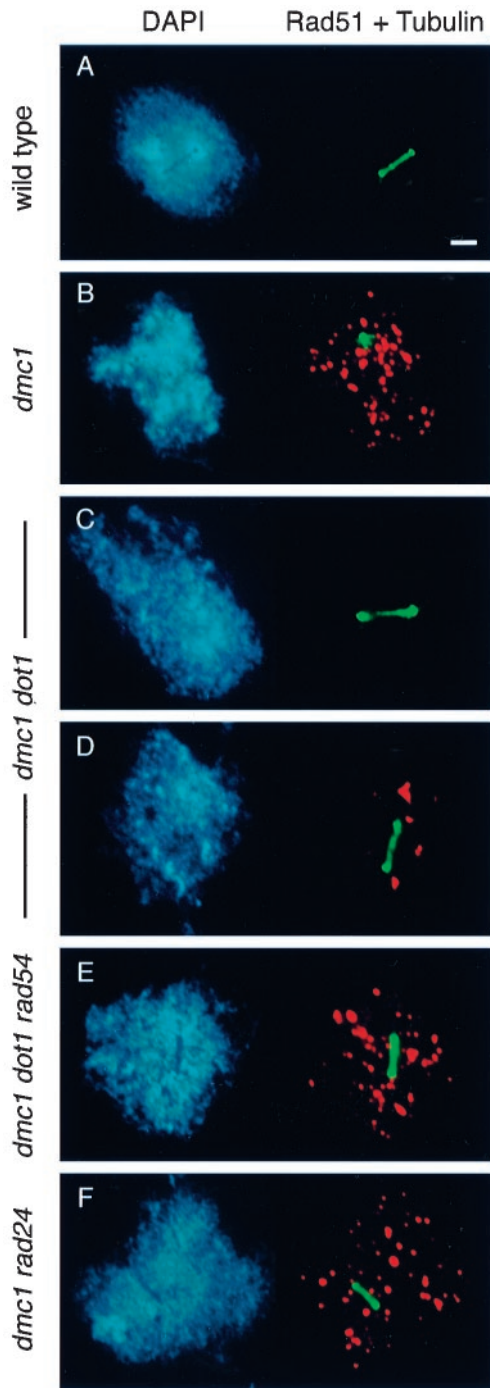
Figure 3. Cytological analysis of unrepaired DSBs and spindle elongation. Spread meiotic nuclei from strains DP338 (wild type; A), DP339 (*dmc1*; B), DP341 (*dmc1 dot1*; C and D), DP343 (*dmc1 dot1 rad54*; E), and DP352 (*dmc1 rad24*; F), stained with DAPI (left) and anti-Rad51 (red) and anti-tubulin (green) antibodies (right). Spreads were prepared after 6 h in sporulation medium. Bar, 2 μm.

4A). In *zip1 dot1* nuclei that have entered MI, all Rad51 foci have disappeared (Table 4 and Figure 4B). In the *zip1 dot1 rad54* triple mutant, two kinds of MI nuclei are found: ~43% are like *zip1 dot1* (i.e., no Rad51 foci coexist with the spindle; Table 4; our unpublished results), whereas ~56% nuclei still contain a few Rad51 in conjunction with a spindle (Table 4 and Figure 4, C and D). Consistent with the small number of unrepaired DSBs present in *zip1 dot1 rad54* (Table 4), the triple mutant exhibits a further decrease in spore viability compared with *zip1 dot1* (Table 3) and loss of the distinctive 4-, 2-, and 0-spore-viable pattern of spore death (Figure 4E). Thus, unlike the situation in *dmc1 dot1*, only a minor fraction of the DSBs accumulated by *zip1* requires Rad54 for repair when meiotic arrest is relieved by a *dot1* mutation.

Dot1 Is a Nuclear Protein That Associates with Chromosomes

To study the cellular localization of Dot1, the protein was tagged at the N terminus with GFP or with three copies of the HA epitope. Both fusion proteins are fully functional (our unpublished results).

In mitotic diploid cells containing a high-copy plasmid carrying *DOT1-GFP*, the Dot1-GFP signal is detected in the nucleus, but is often enriched toward one side of the nucleus in a region that stains faintly with the DNA-binding dye DAPI (Figure 5A) and corresponds to the nucleolus (Pintard *et al.*, 2000). Dot1-GFP is found in cells at different stages of the mitotic cell cycle. The same pattern of nuclear localization with nucleolar accumulation is present in vegetative cells expressing Dot1-GFP from a low-copy plasmid, suggesting that this localization is not an artifact of overexpression (our unpublished results). In cells at different stages of meiotic progression, Dot1-GFP is also found in the nucleus, but accumulation in the nucleolus is not evident until meiosis has been completed and spores are being formed (Figure 5B). In meiotic cells, visualization of Dot1-GFP depends on the use of a high-copy plasmid.

To determine whether Dot1 is free in the nucleoplasm or associated with chromosomes, nuclei from vegetative and meiotic cells carrying *DOT1-HA* on a high-copy plasmid were surface spread and analyzed by indirect immunofluorescence by using anti-HA antibodies. In spread mitotic nuclei, two patterns of Dot1-HA localization are found. In ~29% of nuclei, Dot1 is homogeneously distributed throughout chromatin (Figure 5C, bottom). In the remaining nuclei, Dot1 is clearly enriched in the region of the nucleus corresponding to the nucleolus (Figure 5C, top). Nucleolar localization of Dot1 was confirmed by double staining for Dot1-HA and the nucleolar protein Nsr1 (our unpublished results). In spread meiotic nuclei, Dot1 is uniformly dispersed throughout the whole DAPI-stained area during all stages of meiotic prophase (Figure 5D; our unpublished results). These results indicate that Dot1 binds directly or indirectly to chromosomes. However, only a small fraction (~5%) of spread nuclei (especially from meiotic cells) displays Dot1 staining, suggesting that the interaction of Dot1 with chromosomes is weak and/or sensitive to the spreading procedure.

Dot1 Is Required for Localization of Sir2 and Pch2

The checkpoint defect of *zip1 dot1* (Tables 2 and 3) is similar to that of *zip1 pch2* and *zip1 sir2* (San-Segundo and Roeder,

Table 4. Quantitation of Rad51 foci associated with MI spindle

Genotype	Nuclei (%)	Rad51 foci (No.)	MI spindle	Nuclei scored	Strain
dmc1	100	62.7 ± 7.0	−	20	DP339
dmc1 dot1[a]	38	0	+		DP341
	56	4.0 ± 3.0	+	50	
	6	25.6 ± 6.0	+		
Average[a]		3.8 ± 6.4			
dmc1 dot1 rad54	100	56.7 ± 10.5	+	22	DP343
dmc1 rad24	100	58.0 ± 12.9	+	20	DP352
zip1	100	25.1 ± 4.3	−	15	MY152
zip1 dot1	100	0	+	20	DP381
zip1 dot1 rad54[b]	43	0	+	30	DP382
	56	2.3 ± 1.6	+		
Average[b]		1.3 ± 1.6			

[a] MI Nuclei of *dmc1 dot1* are grouped into three classes. The overall number of Rad51 foci per nucleus is also represented (average).
[b] MI Nuclei of *zip1 dot1 rad54* are grouped into two classes. The overall number of Rad51 foci per nucleus is also represented (average).

1999; our unpublished results). Furthermore, like Sir2, Dot1 is involved in chromatin silencing (Singer *et al.*, 1998). These observations suggest that Pch2, Sir2, and Dot1 act at the same step in the pachytene checkpoint pathway and raise the possibility that proper localization of Pch2 and/or Sir2 requires Dot1.

To address this question, localization of Pch2 was examined in spread meiotic chromosomes of the *zip1 dot1* mutant. In *zip1*, the Pch2 protein is detected only in the rDNA region (Figure 6A, top; San-Segundo and Roeder, 1999). In contrast, in the *zip1 dot1* double mutant, Pch2 is fairly evenly distributed throughout chromatin (Figure 6A, bottom). Because Pch2 localization depends on Sir2, the possibility that Sir2 is also mislocalized in the absence of Dot1 was explored. In wild-type meiotic chromosomes, Sir2 accumulates in the nucleolus and is also present in foci mostly located at telomeric positions (Figure 6B, top; San-Segundo and Roeder, 1999). In contrast, in the *dot1* mutant, a significant fraction of the Sir2 protein is dispersed to other chromosomal locations (Figure 6B, bottom). The localization pattern of Dot1 in mitotic cells is unaffected by a *sir2* mutation (our unpublished results). The localization dependence implies the following order of action for these proteins: Dot1 → Sir2 → Pch2.

Sir3 Is Mislocalized in the dot1 Mutant

In addition to the meiotic checkpoint function described here, Dot1 has been shown to be important for telomeric silencing in vegetative cells (Singer *et al.*, 1998). The observation that Sir2 localization is altered in the *dot1* mutant raised the possibility that the defect in telomeric silencing is caused by the failure to recruit and/or stabilize the Sir protein complex at telomeres. To explore this hypothesis, the localization of Sir3 was studied in spread mitotic nuclei of wild type and *dot1*, using a functional HA-tagged version of Sir3. In wild type, a few very strong Sir3 foci are present that likely represent clusters of telomeres (Gotta *et al.*, 1996; Figure 6C, top). Also, some Sir3-HA is detected in the rDNA region (the possibility of low-level binding of Sir3 to the rDNA has been previously reported; Gotta *et al.*, 1997).

However, in the *dot1* mutant, Sir3 is dispersed all over chromatin (Figure 6C, bottom). A few stronger foci can be observed that perhaps correspond to telomeric clusters, but they clearly contain less Sir3 protein than those in wild type. Sir3 is also mislocalized in meiotic chromosomes from the *dot1* mutant (our unpublished results). Thus, localization of Sir3 (and Sir2) is altered in the absence of the Dot1 protein, presumably accounting for the defect in telomeric silencing in the *dot1* mutant.

DISCUSSION

Dot1 Is Required for the Pachytene Checkpoint and for Preventing Repair of DSBs by Intersister Recombination

The results presented here indicate that the product of the *DOT1* gene of *S. cerevisiae* is necessary for function of the pachytene checkpoint. The *dot1* single mutant shows no apparent defect in spore viability, recombination, or sporulation, arguing that *DOT1* is not required during an unperturbed meiosis. In contrast, *DOT1* is essential to trigger meiotic prophase arrest (or delay) when the SC fails to assemble due to lack of Zip1. The defects in chromosome synapsis (see Red1 staining in Figure 6A) and interhomolog exchange characteristic of *zip1* are still manifest, or even enhanced, in the *zip1 dot1* double mutant. Thus, relief of the meiotic block by the *dot1* mutation is not due to suppression of the defects that trigger checkpoint-induced arrest, but rather is due to disruption of the checkpoint per se.

The inappropriate meiotic progression of *zip1 dot1* results in chromosome missegregation and the formation of aneuploid spores. The pattern of spore death in *zip1 dot1* resembles that of the *zip1* single mutant, suggesting that the cause of spore death in both cases is homolog nondisjunction at MI (Sym and Roeder, 1994). The fraction of viable spores produced by *zip1 dot1* (~35%) is higher than expected if chromosomes segregate before recombination intermediates are resolved (segregation before completion of recombination would result in chromosome breakage and extensive spore
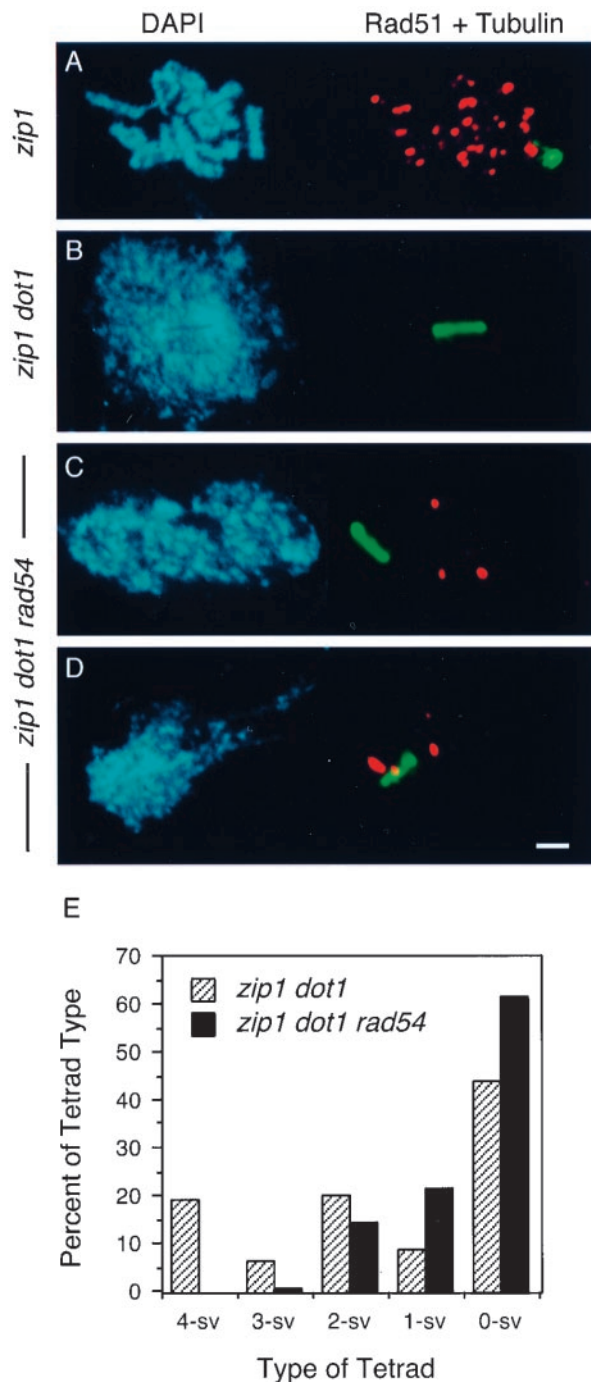
Figure 4. Repair of DSBs in *zip1 dot1* is largely independent of Rad54. (A–D) Cytological analysis of ongoing recombination and entry into MI, assessed by the presence of Rad51 foci and spindle elongation, respectively. Spread meiotic nuclei from strains MY152 (*zip1*; A), DP381 (*zip1 dot1*; B), and DP382 (*zip1 dot1 rad54*; C and D), stained with DAPI (left) and anti-Rad51 (red) and anti-tubulin (green) antibodies (right). Spread nuclei were prepared after 16 h in sporulation medium. Bar, 2 μm. (E) Distribution of tetrad types. The percentages of tetrads with 4, 3, 2, 1, and 0 viable spores (4-sv, 3-sv, 2-sv, 1-sv, and 0-sv, respectively) are represented. Tetrads (88 and 87) were dissected from strains DP381 (*zip1 dot1*) and DP382 (*zip1 dot1 rad54*), respectively.

death). Thus, most or all Holliday junctions and DSBs that accumulate in *zip1* must become resolved/repaired as the arrest is bypassed by a *dot1* mutation. A similar situation (~40% spore viability) is found when *zip1* meiotic arrest is relieved by mutation of *SWE1*, the major downstream target of the pachytene checkpoint (Leu and Roeder, 1999). Swe1 inhibits cyclin-dependent kinase activity by phosphorylating tyrosine 19 of Cdc28 (Booher et al., 1993). It is unlikely that Swe1 function directly affects the recombination machinery; however, recombination is completed in *zip1 swe1* (Leu and Roeder, 1999) as in *zip1 dot1*. These observations raise the possibility that the accumulation of recombination intermediates in *zip1* is the consequence, rather than the cause, of the meiotic arrest. The defect in SC assembly may be the primary cause of checkpoint-induced arrest in *zip1* strains.

Checkpoint-dependent arrest in the *dmc1* mutant also requires *DOT1*. Physical assays of recombination have demonstrated that the *dmc1* mutant accumulates processed DSBs (Bishop et al., 1992). Cytologically, the presence of recombination intermediates is manifested by the persistence of multiple Rad51-containing complexes on chromosomes. Although most spores produced by *dmc1 dot1* are dead, immunostaining with anti-Rad51 antibodies revealed that a significant fraction of *dmc1 dot1* cells that progress into MI have repaired the DSBs (i.e., Rad51 foci have disappeared). The high levels of spore inviability of *dmc1 dot1* can be explained if the resolution of recombination does not involve homologous chromosomes and, therefore, does not ensure proper segregation.

It has been recently shown that *dmc1* cells can use a Rad54-dependent pathway for repairing DSBs by using sister chromatids instead of homologs as templates for repair (Arbel et al., 1999; Bishop et al., 1999). Indeed, Rad51 foci persist in the *dmc1 dot1 rad54* triple mutant, indicating that repair of DSBs in *dmc1 dot1* requires *RAD54*. However, despite the presence of unresolved recombination intermediates, the triple mutant proceeds through meiosis. The spores produced are immature and probably contain fragmented chromosomes, similar to the situation in *dmc1 rad24*. The *dmc1 rad54* double mutant does arrest (Figure 2D), demonstrating that the failure of *dmc1 dot1 rad54* to arrest is due to the lack of Dot1 and not the lack of Rad54 function. These data argue that Dot1 performs two distinct functions. Dot1 executes a genuine checkpoint function, preventing meiotic cell cycle progression when recombination is incomplete. In addition, Dot1 blocks a Rad54-dependent pathway that repairs DSBs by intersister recombination when Dmc1 is absent.

Unlike *dmc1*, the formation and disappearance of meiotic DSBs in the *zip1* mutant is essentially normal, except that a small fraction of DSBs (~10%) remains unrepaired (Storlazzi et al., 1996; Xu et al., 1997). In agreement with these findings, ~25 foci of Rad51 persist in nuclei of the arrested *zip1* mutant. When *zip1* arrest is relieved by the *dot1* mutation, Rad51 foci disappear, consistent with the notion that recombination intermediates are resolved (see above). However, for the most part, this repair does not require Rad54, because only a very small number of Rad51 foci persist when *zip1 dot1 rad54* cells enter MI. Assuming that the Rad54-dependent pathway is mostly involved in intersister recombination (Arbel et al., 1999), this result suggests that most DSBs
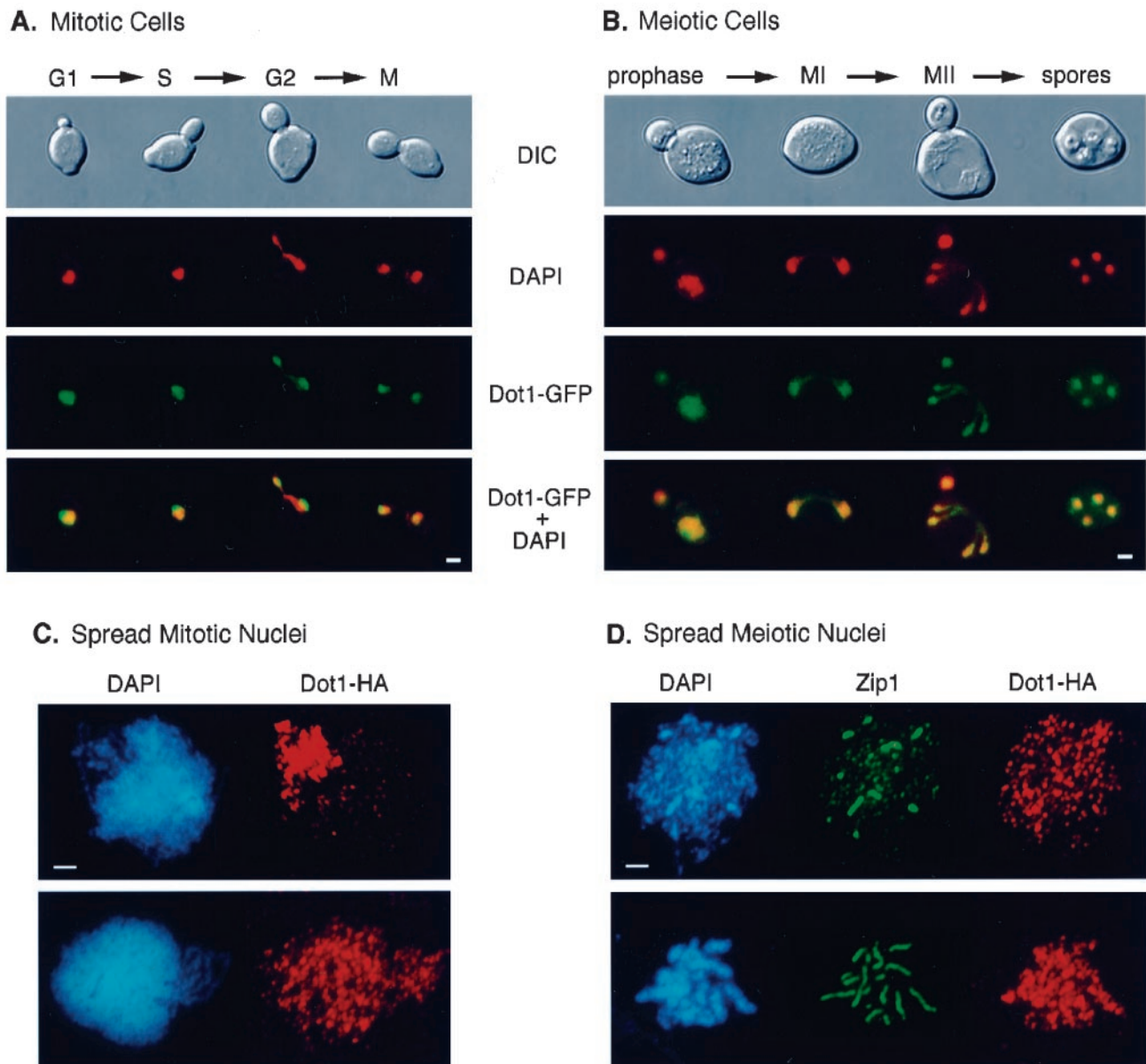
Figure 5. Nuclear localization of Dot1. (A and B) Cells from strain DP138 (*dot1*) transformed with pSS64 (2 μ *DOT1-GFP*) growing vegetatively (A) or at different times in sporulation medium (B) were fixed, stained with DAPI, and observed at the fluorescence microscope. The merged images of DAPI (red) and Dot1-GFP (green) are shown in the bottom panels. Overlap appears yellow. The corresponding differential interference contrast images are also presented (top). The approximate cell-cycle stage was estimated based on bud size and nuclear morphology and position. In B, only the large mother cells have entered meiosis; the small buds should not be considered. (C) Spread nuclei from strain DP138 (*dot1*) transformed with pSS63 (2 μ *DOT1-HA*) growing vegetatively, stained with DAPI (blue) and anti-HA antibodies (red). (D) Spread leptotene/zygotene (top row) and pachytene (bottom row) nuclei from strain DP138 (*dot1*) containing pSS63 (2 μ *DOT1-HA*) stained with DAPI (blue) and anti-Zip1 (green) and anti-HA (red) antibodies. Bar, 2 μm.

accumulated in *zip1* are already "committed" to undergo repair by using a nonsister chromatid. In contrast, in the *dmc1* mutant, the preference for interhomolog recombination is lost (Schwacha and Kleckner, 1997), and DSBs can be repaired by sister-sister recombination when the block to the

pathway is relieved by mutation of *DOT1* (this work) or *RED1* (Xu *et al.*, 1997; Bishop *et al.*, 1999). The small number of Rad54-dependent (likely intersister) recombination events that occur in *zip1 dot1* may account for the further reduction in spore viability compared with *zip1*.
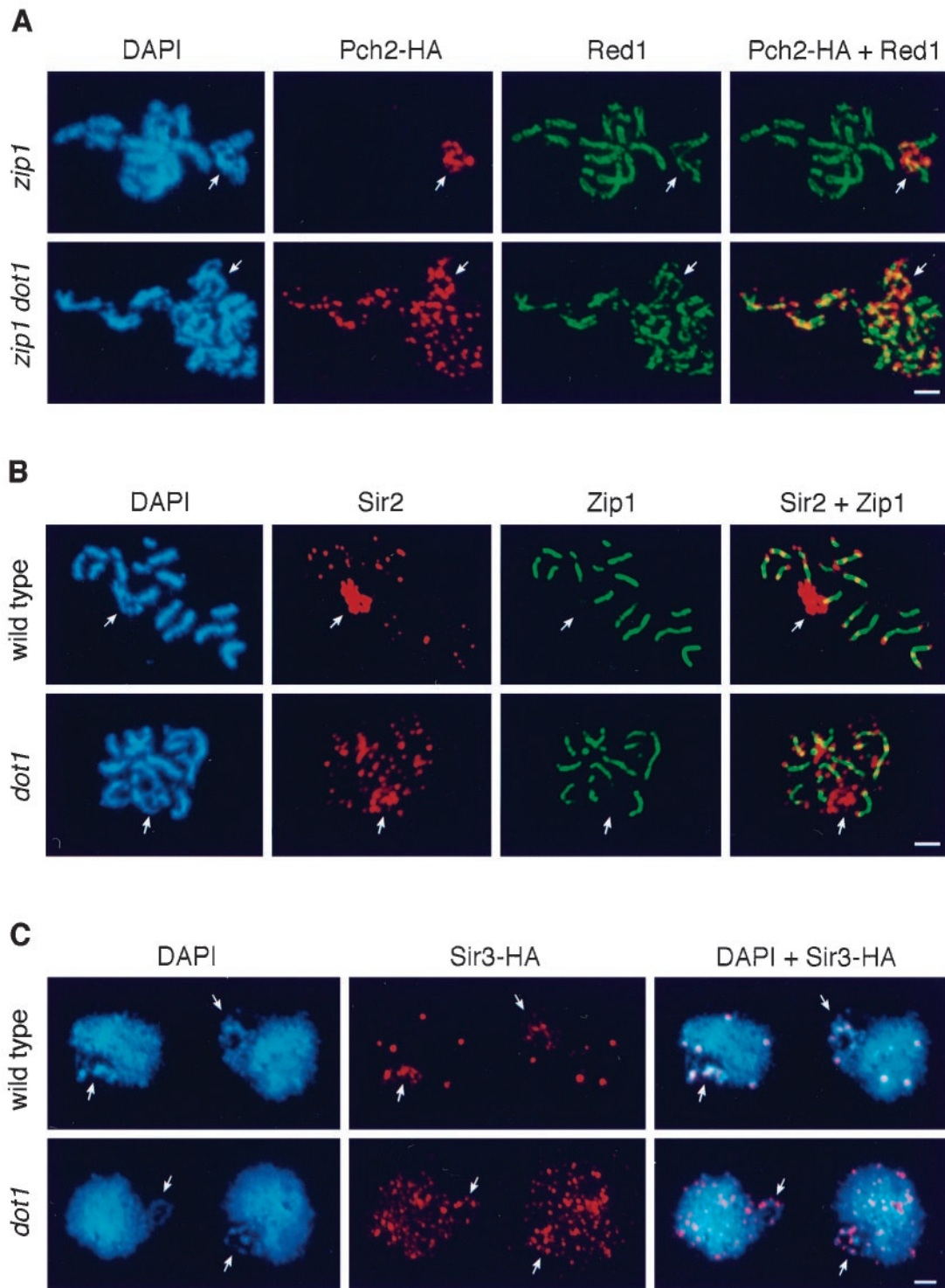
Figure 6. Pch2, Sir2, and Sir3 are mislocalized in the absence of *DOT1*. (A) Spread meiotic nuclei from DP201 (*zip1 PCH2-HA*, top) and DP285 (*zip1 dot1 PCH2-HA*, bottom) stained with DAPI (blue) and anti-HA (red) and anti-Red1 (green) antibodies. (B) Spread pachytene nuclei from BR2495 (wild type, top) and DP138 (*dot1*, bottom) stained with DAPI (blue) and anti-Sir2 (red) and anti-Zip1 (green) antibodies. (C) Spread mitotic nuclei from DP365 (*SIR3-HA*, top) and DP366 (*dot1 SIR3-HA*, bottom) stained with DAPI (blue) and anti-HA (red) antibodies. In all panels, arrows point to the nucleolar region. Bar, 2 μm.

Comparison of Dot1 Function with That of Other Pachytene Checkpoint Proteins

The meiosis-specific proteins Red1 and Mek1 are also involved in the pachytene checkpoint (Xu *et al.*, 1997; Bailis and Roeder, 2000b). Red1 is a component of the lateral elements of the SC (Smith and Roeder, 1997), and Mek1 is a kinase that phosphorylates Red1 (Bailis and Roeder, 1998; de los Santos and Hollingsworth, 1999). Mutation of *DOT1* or *RED1* has similar effects when combined with *dmc1*: both bypass meiotic arrest, and both channel the repair of DSBs into a Rad54-dependent pathway (this work; Xu *et al.*, 1997; Bishop *et al.*, 1999). It has been proposed that Red1 (and Mek1) promote a meiosis-specific chromosomal context in which interhomolog recombination is monitored (Xu *et al.*, 1997; Bailis *et al.*, 2000). Recent findings indicate that Mek1-dependent phosphorylation of Red1, triggered by the presence of recombination intermediates, activates the pachytene checkpoint (Bailis and Roeder, 2000b). Dot1 appears to be a chromatin-associated protein that might also contribute to chromosomal context. However, Dot1 is not meiosis specific; in addition, the phenotypes of *dot1* and *red1* single mutants are clearly different, suggesting that the proteins perform different functions. Whereas the *red1* mutant is severely defective in chromosome synapsis, recombination, chromosome segregation, and the production of viable spores (Rockmill and Roeder, 1988, 1990), *dot1* is not defective in these processes.

Another set of proteins required for checkpoint-dependent arrest of *zip1* and *dmc1* are the DNA damage checkpoint proteins Rad24, Rad17, Mec1, and Ddc1 (Lydall *et al.*, 1996; San-Segundo and Roeder, 1999). It is generally believed that these proteins are involved in the detection of broken DNA both in mitotic and meiotic cells (Lydall and Weinert, 1995; Lydall *et al.*, 1996; Longhese *et al.*, 1998; Weinert, 1998). The distribution of Dot1 throughout chromatin is consistent with such a role. However, the meiotic phenotypes of *dot1* and DNA damage checkpoint mutants differ in several aspects. Whereas *DOT1* is not required during an unperturbed meiosis, the *rad24*, *rad17*, and *mec1-1* single mutants show decreased crossing over, increased ectopic recombination, increased unequal sister-chromatid exchange, defective chromosome synapsis, and reduced spore viability (Lydall and Weinert, 1995; Lydall *et al.*, 1996; Grushcow *et al.*, 1999; Thompson and Stahl, 1999). Both *DOT1* and *RAD24* are required for *dmc1* arrest, but the outcome of bypassing arrest is different. The *dmc1 dot1* mutant completes meiosis with most DSBs repaired via a Rad54-dependent pathway generating mature, though largely inviable, spores. In contrast, the Rad54 pathway appears to remain inactive in *dmc1 rad24*, resulting in progression through meiosis with unrepaired DSBs and the formation of immature spores.

Recent evidence indicates that the ultimate consequence of activation of the pachytene checkpoint is inhibition of cyclin-dependent kinase activity by Swe1-mediated phosphorylation of Cdc28 and limiting Ndt80-dependent transcription of *CLB1* (Chu and Herskowitz, 1998; Hepworth *et al.*, 1998; Leu and Roeder, 1999). A *zip1 swe1* mutant sporulates to wild-type levels but after a significant delay; this delay is overcome by simultaneous overexpression of *CLB1* (Leu and Roeder, 1999). Because *zip1 dot1* sporulates with wild-type efficiency and kinetics, this implies that Dot1 function lies upstream of the bifurcation of the checkpoint pathway into the two regulatory branches of cyclin-dependent kinase activity.

Localization of Pch2, Sir2, and Sir3 Depends on Dot1

The predominantly nucleolar proteins Pch2 and Sir2 are also required for the pachytene checkpoint (San-Segundo and Roeder, 1999), revealing an unexpected link between chromatin silencing and control of meiotic progression. Like Dot1, Pch2 and Sir2 are dispensable during a normal meiosis, but they are essential to prevent progression of meiosis in the absence of Zip1. Several lines of evidence suggest that these three proteins act at the same step in the pachytene checkpoint pathway. First, the meiotic phenotypes of *zip1 dot1* are similar to those of *zip1 pch2* or *zip1 sir2*; all three double mutants sporulate with wild-type kinetics, resulting in similar levels and patterns of spore viability (San-Segundo and Roeder, 1999; this work; our unpublished results). Second, like Sir2, Dot1 is involved in chromatin silencing (Singer *et al.*, 1998). Third, proper localization of Pch2 and Sir2 depends on Dot1. Singer *et al.* (1998) have shown that both Dot1 overproduction and *DOT1* deletion disrupt telomeric silencing, suggesting that Dot1 is part of a protein complex in which the proper balance of components is critical for silencing. Indeed, the absence of Dot1 results in mislocalization of the Sir3 protein, which is an essential component of a multiprotein complex required for telomeric silencing (Grunstein, 1998; Lustig, 1998). Furthermore, overexpression of *DOT1* in *zip1* cells results in partial bypass of the meiotic arrest (our unpublished results). These considerations lead to the speculation that Dot1 is a critical component of protein complexes assembled in heterochromatic regions (nucleolus and/or telomeres) with functions in checkpoint and/or silencing processes. In vegetative cells, Dot1 accumulates in the nucleolus. In meiotic nuclei, Dot1 is also present, but not enriched, in the rDNA region. However, the localization data for Dot1 in spread nuclei should be considered with caution because they are based on overproduction of the protein. The fact that Dot1 does not seem to be localized exclusively in heterochromatic regions is not incompatible with a role in silencing; other essential components of silenced chromatin, such as histones H3 and H4, and Rap1, are also present in both silenced and nonsilenced regions (Shore, 1994; Grunstein, 1998).

Considerations about Heterochromatin, Checkpoints, and DNA Damage Responses

The silencing factor Sir2 is required for meiotic arrest of the *zip1* mutant, demonstrating a connection between chromatin silencing and the pachytene checkpoint. The checkpoint defect of *sir2* is presumably due to the failure to localize the meiosis-specific Pch2 protein to the nucleolus (San-Segundo and Roeder, 1999). These findings are extended by the results presented here demonstrating a role for another silencing protein, Dot1, in meiotic checkpoint control. In the absence of Dot1, the nucleolar confinement of Sir2 and Pch2 is lost, and the pachytene checkpoint is inactive. The involvement of nucleolar proteins in checkpoint mechanisms is not an exclusive feature of meiotic cells. The human Rad17 protein (homolog of the *S. cerevisiae* Rad24 checkpoint pro-

tein) localizes to the nucleolus in mitotic cells and is redistributed throughout the nucleus upon UV irradiation (Chang *et al.*, 1999). In budding yeast, the cellular localization of most DNA damage checkpoint proteins, including Rad24, remains to be established.

The precise role of the nucleolus in the pachytene checkpoint is not yet understood. However, recent studies have demonstrated a role for the nucleolus in another cell-cycle regulatory process, namely the exit from mitosis (reviewed by Garcia and Pillus, 1999). The Cdc14 phosphatase is sequestered in the nucleolus during most of the mitotic cell cycle by association with the Net1/Cfi1 protein, but Cdc14 is released from the nucleolus in late anaphase, dispersing throughout the cell and triggering mitotic exit (Shou *et al.*, 1999; Visintin *et al.*, 1999). Importantly, localization of Sir2 to the rDNA also requires Net1, and the *net1* mutant is defective in rDNA silencing (Straight *et al.*, 1999). It will be of interest to investigate whether Net1, and perhaps Cdc14, are implicated in the pachytene checkpoint.

Several observations also point to a link between telomeric heterochromatin and cellular responses to DSBs/DNA damage in both mitotic and meiotic cells. The Pch2 protein is normally found in the nucleolus, but it localizes to telomeres under some circumstances (*SIR4* overexpression or rDNA deletion). Under these conditions, Pch2 still provides at least partial checkpoint function (San-Segundo and Roeder, 1999), arguing that some feature of heterochromatin is important for the pachytene checkpoint. A physical interaction between Mec3 (a DNA damage checkpoint protein) and Set1 (a protein required for telomeric silencing) has been reported (Corda *et al.*, 1999). In addition, analysis of telomeric silencing, telomere length and DNA repair revealed genetic interactions between *mec3* and *set1* mutants (Corda *et al.*, 1999). In fission yeast, mutations in the *rad26⁺*, *rad1⁺*, *rad17⁺*, and *rad3⁺* checkpoint genes (the last three genes are homologs of *S. cerevisiae RAD17*, *RAD24*, and *MEC1*, respectively), result in telomere shortening (Dahlen *et al.*, 1998) and, in the case of the *rad3* mutant, reduced telomeric silencing (Matsuura *et al.*, 1999). A mutation in *MEC1* also leads to shorter telomeres (Ritchie *et al.*, 1999) and impaired telomeric silencing (Craven and Petes, 2000). The Sir proteins have not been implicated in the DNA damage checkpoint, but they do facilitate (directly or indirectly) the repair of DSBs (reviewed by Haber, 1999). The Sir proteins, as well as the Ku complex, are redistributed from telomeres to the sites of DSBs. This relocalization requires the function of DNA damage checkpoint proteins and results in decreased telomeric silencing (Martin *et al.*, 1999; McAnish *et al.*, 1999; Mills *et al.*, 1999). The Sir2-related Hst3 and Hst4 proteins of *S. cerevisiae* and Hst4 of *Schizosaccharomyces pombe* also contribute to radiation resistance (Brachmann *et al.*, 1995; Freeman-Cook *et al.*, 1999). In consonance with all these observations, the *dot1* mutant, which shows reduced telomeric silencing and altered telomere length (Singer *et al.*, 1998), also displays defects in the localization of Sir proteins and in the meiotic checkpoint that responds to unrepaired DSBs. Moreover, mutation of *DOT1* increases the DNA damage sensitivity of the *rad24* checkpoint mutant (San-Segundo and Roeder, unpublished observations), suggesting that Dot1 also participates in the DNA damage response in vegetative cells.

The connection between telomere function and cell-cycle checkpoints is not restricted to yeast. Human cells deficient in the ATM checkpoint protein undergo telomere shortening (Metcalfe *et al.*, 1996). Mutation of the *mrt-2* gene of *C. elegans* (homolog of *RAD17* in *S. cerevisiae*) results in defects in telomere maintenance and defective responses to the accumulation of meiotic recombination intermediates and DNA damage (Ahmed and Hodgkin, 2000; Gartner *et al.*, 2000). It is tempting to speculate that the metazoan proteins that share homology with Dot1 (Figure 1) have roles in heterochromatin assembly, telomere metabolism, meiotic checkpoint control, and/or cellular responses to damaged DNA.

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