The pachytene checkpoint prevents accumulation and phosphorylation of the meiosis-specific transcription factor Ndt80

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In budding yeast, many mutants defective in meiotic recombination and chromosome synapsis undergo checkpoint-mediated arrest at the pachytene stage of meiotic prophase. We recovered the *NDT80* gene in a screen for genes whose overexpression bypasses the pachytene checkpoint. Ndt80 is a meiosis-specific transcription factor that promotes expression of genes required for exit from pachytene and entry into meiosis I. Herein, we show that the Ndt80 protein accumulates and is extensively phosphorylated during meiosis in wild type but not in cells arrested at the pachytene checkpoint. Our results indicate that inhibition of Ndt80 activity is one mechanism used to achieve pachytene arrest.

Checkpoints ensure the correct order of events within the mitotic cell cycle by preventing the initiation of late events until earlier events have been executed successfully (1). Checkpoints also operate in meiosis. In both yeast and mammals, a checkpoint prevents cells from exiting the pachytene stage of meiotic prophase until meiotic recombination and synaptonemal complex formation have been completed (2). Because recombination and synapsis are necessary for proper chromosome segregation at the first meiotic division, this "pachytene checkpoint" ensures the production of viable meiotic products.

zip1, *dmc1*, and *hop2* are three budding yeast mutants that undergo checkpoint-mediated arrest at the pachytene stage. The *ZIP1* gene encodes a structural component of the synaptonemal complex (3–7), which holds homologous chromosomes in close apposition along their lengths at the pachytene stage. *zip1* mutants arrest or delay in meiosis with unsynapsed chromosomes and unresolved double Holliday junctions (3–6, 8). The *DMC1* gene encodes a homolog of the bacterial RecA strand exchange enzyme (9); in *dmc1* strains, synapsis is delayed and cells arrest or delay in meiosis with unrepaired double-strand breaks (9, 10). The *hop2* mutant undergoes extensive SC formation between nonhomologous chromosomes and fails to complete meiotic recombination (11).

Mutations in a number of genes inactivate the pachytene checkpoint by interfering with the production or transmission of the signal(s) indicating a defect in recombination and synapsis. These include the Rad24, Rad17, and Mec1 proteins, which are also involved in the DNA damage checkpoint that arrests vegetative cells in response to unrepaired double-strand breaks (12). In addition, the pachytene checkpoint requires the chromatin silencing factor Sir2 and the meiosis-specific checkpoint protein Pch2, which colocalizes with Sir2 in the nucleolus (13). Pachytene arrest also requires the meiosis-specific Red1 and Mek1 proteins (14). Red1 is a component of the cores of meiotic chromosomes (15); Mek1 is a protein kinase that associates with and phosphorylates Red1 (16, 17). Reversal of Mek1-mediated phosphorylation (presumably of Red1) is required for exit from the pachytene stage (18).

Recently, the Swe1 protein kinase has been shown to be one of the downstream targets of the pachytene checkpoint in budding yeast (19). Swe1 phosphorylates and thereby inactivates the major cyclin-dependent kinase, Cdc28 (20), whose activity is required for the exit from pachytene (14, 21). Deletion of the *SWE1* gene allows meiotic mutants that normally arrest at the pachytene checkpoint to complete meiosis; a *cdc28* mutation that renders Cdc28 nonphosphorylatable by Swe1 has a similar effect (19). In response to defects in recombination and synapsis, the Swe1 protein increases in abundance, becomes hyperphosphorylated, and is presumably activated (19).

Although a *swe1* mutation restores sporulation to mutants that arrest at pachytene (e.g., *hop2*), sporulation in the corresponding double mutants (e.g., *hop2 swe1*) is delayed compared with that in wild type, suggesting that Swe1 is not the exclusive target of the pachytene checkpoint (19). Furthermore, there is evidence indicating that the Ndt80 protein is also regulated at the pachytene checkpoint (22, 23). Ndt80 is a meiosis-specific transcription factor (22) whose activity is absolutely required for exit from the pachytene stage (24). Ndt80 activates transcription of the middle class of meiotic genes (23, 25), including Clb1, the major cyclin required for meiosis I (26, 27). In *dmc1* cells arrested at the pachytene checkpoint, transcription of *CLB1* and other genes regulated by Ndt80 is inhibited (23, 25). However, if the *dmc1*-induced arrest is inactivated by a checkpoint mutation such as *rad17* (12), transcription of Ndt80-dependent genes is restored (23, 25). Thus, Ndt80 activity seems to be regulated negatively at the pachytene checkpoint. Consistent with this hypothesis, the delay in sporulation observed in *hop2 swe1* strains can be eliminated by introduction of a multicopy plasmid carrying the *CLB1* gene (19). These results indicate that arrest at pachytene is caused by inhibition of both Cdc28 kinase activity and Ndt80-promoted transcription.

We recovered the *NDT80* gene in a screen for genes whose overexpression can bypass the pachytene checkpoint. Our results demonstrate that the Ndt80 protein increases in abundance and becomes extensively phosphorylated in wild-type cells but not in cells arrested at the pachytene checkpoint. We propose that Ndt80 must be phosphorylated to be activated, and inhibition of this phosphorylation is one mechanism used to achieve checkpoint-induced arrest at the pachytene stage.

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Materials and Methods

Yeast Strains and Genetic Methods. Yeast manipulations were performed, and media were prepared as described (6). Cells

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Abbreviations: kb, kilobase; CIP, calf intestinal phosphatase; HA, hemagglutinin.

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were grown and induced for meiosis at 30°C unless otherwise indicated. Most experiments were carried out in the diploid strain BR2495 (6). A derivative of BR2495 carrying the *cdc28–63* mutation was obtained from Xu *et al.* (14). The wild-type and *zip1* SK1 strains used are identical to MY261 and MY262 (4), respectively, except that *CEN3* is not marked with *URA3*.

The multicopy library described by Engebrecht *et al.* (28) was screened for suppressors of the *zip1* sporulation defect by monitoring expression of a *ysw1*::*lacZ* fusion gene. *YSW1* is a late meiotic gene that is not expressed in cells arrested at the pachytene checkpoint. *ysw1*::*lacZ* was introduced into *zip1* BR2495 by transformation with pTP36. Transformants carrying suppressors turn blue when sporulated cells are exposed to 5-bromo-4-chloro-3-indolyl β-D-galactoside.

Plasmids. pTP36, containing *ysw1*::*lacZ*, was derived from yeast strain M77 as described (29). The plasmid from M77 was cut with *Sal*I and *Sma*I, and the fragment containing *ysw1*::*lacZ* was inserted between the *Sal*I and *Nae*I sites of pRS306 (30). pTP36 was targeted for integration at *YSW*I by cutting with *Bgl*II.

Plasmids for disruption of *DMC1* (9), *PCH2* (13), *HOP2* (11), and *ZIP1* (3–5) have been described. The *mek1*::*URA3* plasmid pTP187 was constructed by replacing the *Sac*I–*Hpa*I fragment containing most of the *MEK1* coding region (−77 to +1,134) in pB124 (31) with a 1.4-kilobase (kb) *Sac*I–*Hpa*I fragment containing the *URA3* gene. pTP187 was targeted for substitutive transformation by cutting with *Eco*RI and *Rsr*II.

One of the plasmids isolated in the screen for *zip1* suppressors, p6B6, carries a 7.1-kb insert containing *NDT80*. *ndt80*::*URA3* and *ndt80*::*LEU2* disruption plasmids were constructed as follows. First, a 2.9-kb *Xba*I–*Kpn*I fragment containing *NDT80* from p6B6 was subcloned between the *Xba*I and *Kpn*I sites of pBluescript SK(+) to create pTP76. Second, pTP77 was generated by deleting a 0.7-kb *Bam*HI fragment from pTP76 to remove an *Eco*RI site downstream of *NDT80*. Third, the 1.1-kb *Bgl*II–*Eco*RI fragment of *NDT80* in pTP77 was replaced with a 1.5-kb *Bam*HI–*Eco*RI fragment containing *URA3* from YEp24 (32) to create pTP78. Next, the 0.9-kb *Eco*RV fragment of *URA3* in pTP78 was replaced with a 2.1-kb *Pvu*II–*Hpa*I fragment containing *LEU2* from YEp351 (33) to create pTP89, containing *ndt80*::*LEU2*. pTP78 and pTP89 were targeted for substitution by cutting with both *Xba*I and *Bam*HI.

For *NDT80* overexpression, a 2.9-kb *Xba*I–*Bam*HI fragment containing *NDT80* from p6B6 was inserted at the *Xba*I–*Bam*HI sites in YEp351 (33) to create pTP73. A 2.9-kb *Sac*I–*Sal*I fragment containing *NDT80* from pTP73 was inserted at the *Sac*I–*Sal*I sites of YEp352 (33) to make pTP94. To construct *NDT80-HA*, a *Not*I site was created at the end of *NDT80* on pTP73 by PCR mutagenesis, and two copies of a sequence encoding three copies of the hemagglutinin (HA) epitope (34) were inserted at this site. A 3.1-kb *Sac*I–*Sal*I fragment containing the *NDT80-HA* allele was subcloned into the *Sac*I–*Sal*I sites of YEp352 (33) and pRS316 (30) to generate pTP118 and pTP115, respectively. A 1.4-kb *Hin*dIII–*Sca*I fragment including the epitope-coding sequences and the flanking regions of *NDT80-HA* was inserted at the *Hin*dIII–*Sma*I sites of the integrating vector, pRS306 (30), to create pTP121. pTP121 was used to replace the chromosomal copy of *NDT80* with *NDT80-HA* by two-step transplacement, targeting for integration by cutting with *Bgl*II.

Cytology. Meiotic chromosome spreads were prepared, and immunofluorescence procedures were performed as described (6), except that both FBS (GIBCO/BRL) and BSA were used for blocking slides. Rabbit polyclonal anti-HA antibody (Babco, Richmond, CA) was used at 1:50 dilution for the double localization of tubulin and Ndt80-HA. Mouse monoclonal anti-HA antibody (Babco, HA11) was used at 1:200 dilution for the double localization of Zip1 and Ndt80-HA. Rabbit anti-Zip1 antibody (3) was used at 1:100 dilution. Monoclonal anti-tubulin antibody YOL1/34 (Sera-Lab, Crawley Down, Sussex, U.K.) was used at 1:20 dilution. Goat anti-mouse IgG conjugated to Cy3 or FITC and goat anti-rabbit IgG conjugated to Cy3 or FITC (Jackson ImmunoResearch) were used as secondary antibodies at 1:200 dilution. Images were captured and processed as described (6).

Protein Extraction and Western Blot Analysis. To prepare protein extracts for Western blot analysis, 10 ml of meiotic cells were collected from sporulating cultures. For time course analysis, cells were collected at the time points indicated. For total protein extracts, pelleted cells were washed and then lysed with glass beads in 300 μl of lysis buffer (1 mM DTT/0.1% Nonidet P-40/250 mM NaCl/5 mM EDTA/50 mM Tris, pH 7.5) containing protease inhibitors (4 mM PMSF/24 μg/ml pepstatin/24 μg/ml leupeptin/14 μg/ml antipain/3 μg/ml aprotinin). Cells were broken by vortexing nine times for 45 s each in the presence of glass beads at 4°C. Extracts were fractionated by electrophoresis on SDS/8% polyacrylamide gels. After electrophoresis, proteins were blotted onto Immobilon-P (Millipore) and probed with monoclonal anti-HA antibody (Babco, HA11) at a 1:1,000 dilution. Primary antibody was detected by using anti-mouse alkaline phosphatase-conjugated antibody (Jackson ImmunoResearch) at a 1:2,000 dilution followed by CDP-Star (Roche Molecular Biochemicals).

Immunoprecipitation and Calf Intestinal Phosphatase (CIP) Treatment. For immunoprecipitation of Ndt80-HA and treatment with CIP, 12 ml of cells was collected, washed, and then resuspended in 200 μl of RIPA buffer (50 mM Tris·HCl, pH 7.5/1% Nonidet P-40/0.1% SDS/0.5% sodium deoxycholate) to which 1 mM DTT and protease inhibitors (as described above) had been added. Samples were vortexed nine times for 30 s in the presence of glass beads at 4°C. Lysates were centrifuged at 14,000 rpm for 2 min in an Eppendorf 5415C centrifuge to remove cell debris. RIPA buffer (400 μl) containing protease inhibitor was added to the cleared lysate, and anti-HA antibody (Babco, HA11) was then added at a dilution of 1:120.

Immunoprecipitations were carried out at 4°C with shaking. After 1.5 h, 65 μl of protein A-Sepharose (Pierce) was added, and incubation was continued for another 1.5 h. Immune complexes were collected by centrifugation, washed twice with RIPA buffer plus protease inhibitors, and then washed twice in CIP buffer (20 mM $MgCl_2$/40 mM KCl/50 mM Tris, pH 8.0). Immune complexes were resuspended in 300 μl of CIP buffer. Aliquots of the immunoprecipitate (100 μl) were exposed to 100 units of CIP (New England Biolabs) at 37°C for 10 min in the presence or absence of 5 mM β-glycerolphosphate, a CIP inhibitor. Samples were analyzed by immunoblotting as described above.

Results

Overproduction of Ndt80 Suppresses the *zip1* Sporulation Defect. In a BR2495 strain background, the *zip1* null mutant undergoes checkpoint-induced arrest at pachytene (6). In an SK1 strain background, the *zip1* null mutant sporulates; however, the efficiency of sporulation is reduced, and sporulation is delayed compared with that in wild type (4, 8). In a screen for multicopy suppressors that allow a *zip1* BR2495 strain to sporulate, the *NDT80* gene was recovered. The presence of a multicopy plasmid carrying *NDT80* improves sporulation in *zip1* BR2495, although sporulation is reduced and delayed compared with that in wild type (Table 1 and Fig. 1 *A* and *B*). Overproduction of Ndt80 in a *zip1* SK1 strain causes nuclear division and spore formation to occur earlier than in the *zip1* mutant alone

Table 1. Sporulation, spore viability, and crossing over in cells overproducing Ndt80

	BR2495 background		SK1 background			
					Crossing over, centimorgans	
Strains	Sporulation efficiency, %	Spore viability, %	Sporulation efficiency, %	Spore viability, %	*MAT/CEN3*	*CEN3/HIS4*
Wild type + vector	76.0	97.9	96.0	98.9	11.6	31.9
Wild type + Ndt80-OP	54.6	95.8	80.4	96.5	12.4	32.7
zip1 + vector	0		65.5	60.0	5.7	3.7
zip1 + Ndt80-OP	18.0	44.8	54.0	48.8	8.8	4.3

Sporulation efficiencies represent the averages of four independent cultures, with 100 cells counted for each culture. For BR2495 spore viability data, 48 tetrads were dissected from each strain. For spore viability and crossover frequencies in wild-type SK1, 190 tetrads were dissected for each strain. For *zip1* SK1 strains, at least 450 tetrads were dissected. OP, overproduced.

(Table 1 and Fig. 1 *C* and *D*). Thus, in both strain backgrounds, Ndt80 overproduction partially suppresses the *zip1* defect in sporulation.

In principle, Ndt80 overproduction could allow *zip1* to sporulate either by suppressing the *zip1* defects in meiotic chromosome metabolism or by overriding the checkpoint that causes *zip1* cells to arrest. To examine the effect of Ndt80 overproduction on chromosome synapsis, meiotic chromosomes from a *zip1* BR2495 strain were surface spread, stained with silver nitrate, and viewed in the electron microscope. No difference in chromosome morphology was observed between the *zip1* mutant containing the multicopy *NDT80* plasmid and *zip1* cells carrying a control plasmid. In both strains, the axial cores of meiotic chromosomes become fully developed, and homologs pair with each other; however, chromosomes do not become intimately synapsed (data not shown). In *zip1* SK1 strains, spore viability is reduced to about 50% of that in wild type, and meiotic crossing over is reduced 2- to 3-fold (Table 1; refs. 4 and 8). Overproduction of Ndt80 does not correct the defect in crossing over, nor does it improve spore viability (Table 1). Thus, overproduction of Ndt80 bypasses the pachytene checkpoint without suppressing the *zip1* defects in recombination, synapsis, and chromosome segregation.

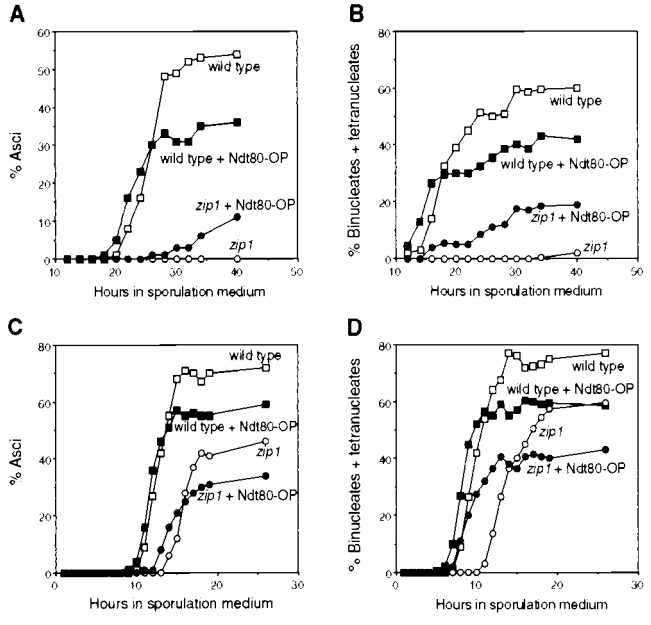

Fig. 1. Kinetics of meiosis in cells overproducing Ndt80. Spore formation and nuclear division in wild-type and *zip1* strains overexpressing *NDT80* were examined throughout meiosis. (*A* and *B*) Strains used are BR2495 derivatives: wild type + YEp351, wild type + pTP73, *zip1*::*URA3* + YEp351, and *zip1*::*URA3* + pTP73. (*C* and *D*) Strains used are SK1 derivatives: wild type + YEp352, wild type + pTP94, *zip1* + YEp352, and *zip1* + pTP94. These experiments were repeated at least three times, with qualitatively similar results. OP, overproduced.

Overproduction of Ndt80 also improves sporulation in *dmc1* strains (3% in *dmc1* BR2495 vs. 15% in *dmc1* + Ndt80-overproduced BR2495), indicating that this effect is not specific to the *zip1* mutant. However, Ndt80 overproduction does not restore sporulation in the *hop2* mutant (data not shown). The inability of Ndt80 overproduction to bypass *hop2* is consistent with previous results suggesting that the meiotic defect in *hop2* is more severe than in *zip1* and *dmc1* (11).

Although Ndt80 overproduction improves sporulation in *zip1* and *dmc1* BR2495 strains, it reduces the overall efficiency of sporulation in wild-type BR2495 and in both wild-type and mutant SK1 strains (Table 1 and Fig. 1 *A* and *C*). This effect occurs before meiosis I, because the frequency of cells entering nuclear division is correspondingly decreased (Fig. 1 *B* and *D*). However, those cells that do sporulate undergo meiotic nuclear division earlier than wild-type cells carrying a control plasmid. One explanation for these opposing effects is that different intracellular concentrations of Ndt80 protein (caused by variation in plasmid copy number) have different effects on sporulation. Up to a certain point, excess Ndt80 protein may speed up the meiotic process; however, an even higher level of Ndt80 may be inhibitory to meiotic progression (both in wild-type and mutant cells). This inhibitory effect cannot be detected in *zip1* (or *dmc1*) BR2495 cells, which undergo little or no sporulation in the absence of Ndt80 overproduction.

Ndt80 Localizes to Chromosomes at Meiosis I and Meiosis II. To immunolocalize the Ndt80 protein, six copies of the HA epitope were fused to the carboxyl terminus of Ndt80, and anti-HA antibodies were used to localize the Ndt80-HA fusion protein. The *NDT80-HA* fusion gene fully complements an *NDT80* deletion (data not shown), indicating that the tagged protein is functional. Meiotic chromosomes were surface spread and stained with anti-HA and anti-tubulin or anti-Zip1 antibodies. Tubulin staining was used to identify cells undergoing the meiosis I and meiosis II divisions, and Zip1 staining was used as a marker for the pachytene stage.

Immunofluorescence analysis revealed that the Ndt80-HA fusion protein localizes to numerous foci on chromosomes during both meiotic divisions (Fig. 2 *B* and *E*). This staining pattern is specific for Ndt80-HA, because no staining is observed in spread nuclei from control cells lacking the HA-tagged version of Ndt80 (data not shown). Ndt80 staining is not detected on chromosomes at the pachytene stage (Fig. 2*H*) or on chro-

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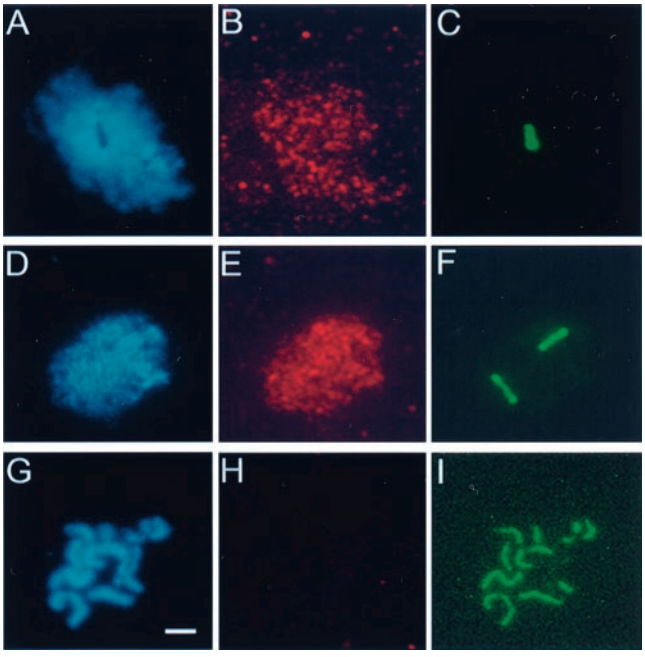

Fig. 2. Localization of Ndt80 to meiotic chromosomes. Spread nuclei from strains carrying *NDT80-HA* were stained with a DNA-binding dye (*A*, *D*, and *G*), anti-HA antibody (*B*, *E*, and *H*), and anti-tubulin antibody (*C* and *F*) or anti-Zip1 antibody (*I*). (*A–C*) Meiosis I nucleus. (*D–F*) Meiosis II nucleus. (*G–I*) Pachytene nucleus. The strain used in *A–F* is *ndt80*::*LEU2* BR2495 carrying *NDT80-HA* on a single-copy plasmid (pTP115). The strain used in *G–I* is BR2495 homozygous for *NDT80-HA*. (Bar = 2 μm.)

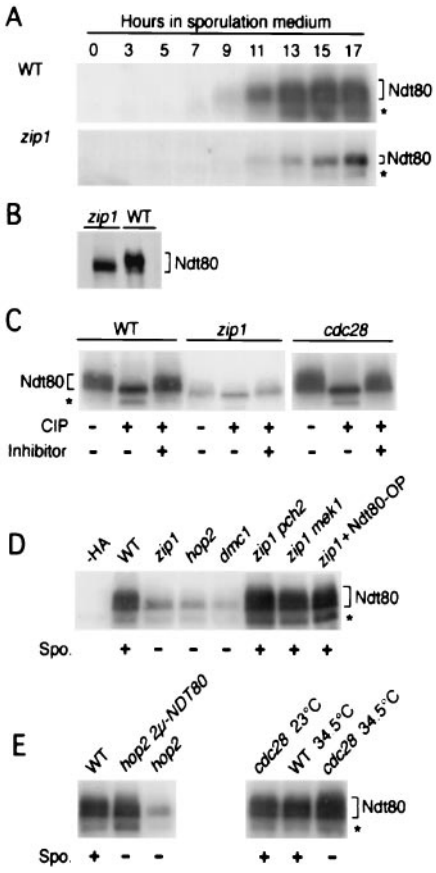

Fig. 3. Phosphorylation of Ndt80 is prevented by the pachytene checkpoint. (*A*) Production of Ndt80 in wild-type (WT) and *zip1*::*LEU2* cells was monitored throughout meiosis by Western blot analysis with anti-HA antibodies. (*B*) Western blot analysis in which the amount of *zip1* extract loaded was five times that of wild type; extracts were prepared after 15.5 h of sporulation. (*C*) Ndt80-HA from wild-type, *zip1*::*LEU2*, and *cdc28–63* cells was immunoprecipitated, treated with CIP, and analyzed by immunoblotting. *cdc28–63* cells were grown at 23°C, incubated in sporulation medium at 23° for 3 h, and then shifted to 34.5°C. (*D*) Immunoblot analysis of Ndt80-HA in wild type, *zip1*::*LEU2*, *hop2*::*URA3*, *dmc1*::*LEU2*, *zip1*::*LEU2 pch2*::*URA3*, *zip1*::*LEU2 mek1*::*URA3*, and *zip1*::*LEU2* containing a multicopy plasmid carrying *NDT80-HA* (pTP118). (*E*) Immunoblot analysis of Ndt80-HA in wild type, *hop2*::*LEU2* carrying the *NDT80-HA* multicopy plasmid (pTP118), *hop2*::*URA3*, and *cdc28–63*. For *cdc28* and the wild-type control, cells were grown at 23°C, incubated in sporulation medium for 3 h, and then either shifted to 34.5°C or maintained at 23°C, as indicated. The + and − signs below each lane in *D* and *E* indicate the ability of the strain to sporulate (Spo.). Equal amounts of extract (≈30 μg) were loaded per lane, except in *B*. Unless otherwise noted, cells were harvested after 17 h in sporulation medium. All strains are isogenic with BR2495 and homozygous for *NDT80-HA*. The band indicated by an asterisk is presumed to be a degradation product of Ndt80.

mosomes from *zip1* cells arrested at the pachytene checkpoint (data not shown). In rare cases, weak foci of both Ndt80 and Zip1 staining are observed in the same spread nucleus (data not shown). Thus, Ndt80 appears to localize to chromosomes after Zip1 dissociates from chromosomes, and this localization persists throughout both meiotic divisions.

Phosphorylation of Ndt80 Is Inhibited at the Pachytene Checkpoint. To investigate the regulation of Ndt80 during meiosis, Ndt80 was analyzed by Western blotting in wild-type and *zip1* cells. The amount of Ndt80 protein present in wild type is much greater than in *zip1* (Fig. 3*A*). Furthermore, in wild type, but not in the *zip1* mutant, the mobility of the Ndt80 protein on an SDS/polyacrylamide gel changes as meiosis progresses such that the protein migrates more slowly at later time points (Fig. 3*A*). The difference in Ndt80 mobility between wild type and *zip1* is apparent even when the amount of extract from the mutant is increased to compensate for the decrease in Ndt80 abundance (Fig. 3*B*).

To determine whether the Ndt80 gel mobility shift results from phosphorylation, Ndt80 was immunoprecipitated from lysates of wild-type and *zip1* cells and then treated with phosphatase. In extracts from wild type, the slower-migrating forms of Ndt80 are converted to a faster-migrating form (Fig. 3*C*). This conversion does not occur in the presence of a phosphatase inhibitor, β-glycerolphosphate (Fig. 3*C*), indicating that Ndt80 is phosphorylated in wild type. In contrast, Ndt80 undergoes very little modification in *zip1* cells (Fig. 3 *A* and *C*). The *dmc1* and *hop2* mutants are similar to *zip1*, with respect to the abundance and mobility of the Ndt80 protein (Fig. 3*D*). Thus, Ndt80 is extensively phosphorylated in wild-type cells but undergoes only a very low level of modification in mutants that arrest at the pachytene checkpoint.

Mutations that allow *zip1* to sporulate by inactivating the checkpoint include *pch2* (13) and *mek1* (14). Consistent with the restoration of sporulation, the *pch2* and *mek1* mutations restore phosphorylation and accumulation of Ndt80 in the *zip1* mutant (Fig. 3*D*). Similarly, overproduction of Ndt80 in *zip1* strains allows Ndt80 to be phosphorylated to the same extent as in wild type (Fig. 3*D*). These results suggest that Ndt80 must be phosphorylated to be activated, and this phosphorylation is prevented when the pachytene checkpoint is triggered.

An alternative interpretation is that Ndt80 phosphorylation is the consequence, rather than the cause, of meiotic cell cycle progression. The failure to accumulate and modify Ndt80 in checkpoint-arrested mutants may be an indirect consequence of arrest at a stage in the cell cycle when Ndt80 is not normally

stabilized or phosphorylated. To address this possibility, Ndt80 from *hop2* cells overproducing Ndt80 was examined by Western blot analysis. In this case, Ndt80 is modified (Fig. 3*E*), despite the fact that cells remain arrested at the pachytene stage. Thus, exit from pachytene is not required for Ndt80 modification.

Ndt80 Is Not a Substrate of Cdc28. As noted above, Cdc28 is phosphorylated and consequently inactivated at the pachytene checkpoint (19). In principle, Ndt80 might lie downstream of Cdc28 in the checkpoint pathway, and the failure of Ndt80 phosphorylation in checkpoint-arrested cells might be caused by the absence of active Cdc28. To investigate this possibility, Ndt80 modification was examined in a temperature-sensitive *cdc28* mutant (*cdc28–63*) incubated in sporulation medium at the nonpermissive temperature. The *cdc28–63* mutant arrests at pachytene, not because the checkpoint is triggered, but as a direct consequence of the absence of Cdc28 kinase activity. Ndt80 is modified in the *cdc28–63* mutant sporulated at the nonpermissive temperature (Fig. 3 *C* and *E*), demonstrating that Cdc28 function is not required for phosphorylation of Ndt80. Thus, Ndt80 is not downstream of Cdc28 in the pachytene checkpoint pathway.

The behavior of the *cdc28–63* mutant provides additional evidence that the failure of Ndt80 to be phosphorylated in meiotic mutants is not an indirect consequence of cell cycle arrest. Like the *zip1*, *dmc1*, and *hop2* mutants, *cdc28–63* arrests at pachytene, but Ndt80 is nevertheless modified.

Discussion

Ndt80-dependent genes are not transcribed in cells arrested at the pachytene checkpoint, leading to the hypothesis that Ndt80 is a downstream target of the checkpoint pathway (23, 25). Our identification of the *NDT80* gene as a multicopy suppressor of the *zip1* sporulation defect provides additional support for this hypothesis. Furthermore, our studies provide insight into the molecular mechanisms involved in Ndt80 regulation. We have demonstrated that the Ndt80 protein is both less abundant and less extensively phosphorylated in cells arrested at the pachytene checkpoint.

Although significant amounts of Ndt80 mRNA (22, 23) and protein (Fig. 3) are present in cells arrested at pachytene, there is no detectable expression of Ndt80 target genes (22, 23), suggesting that Ndt80 is inactive as a transcription factor. The difference in Ndt80 phosphorylation state between wild-type and checkpoint-arrested cells provides a simple explanation for the inability of Ndt80 protein to function at the pachytene checkpoint; presumably Ndt80 needs to be phosphorylated to be active. An increase in Ndt80 activity resulting from phosphorylation can also account for the increase in Ndt80 abundance in wild-type cells compared with cells arrested at pachytene. Studies of *NDT80* transcription (22, 23) have shown that a limited amount of *NDT80* transcription occurs in advance of expression of the middle meiotic genes and is independent of Ndt80 function; however, induction of *NDT80* transcription at later stages requires an active Ndt80 protein. Thus, we propose that the critical step in regulating Ndt80 activity is phosphorylation of the protein. This phosphorylation activates the protein to serve as a transcription factor, promoting its own expression as well as the transcription of target genes (such as *CLB1*). The increased amount of Ndt80 protein, resulting from Ndt80-mediated transcription of *NDT80*, further enhances the transcription of target genes.

A formal possibility that cannot be excluded is that the increased transcription of Ndt80 target genes, in wild-type versus checkpoint-arrested cells, is caused entirely by increased abundance of the Ndt80 protein. However, this hypothesis does not explain the failure of transcription of Ndt80 target genes in cells arrested at the pachytene checkpoint, nor does it account for the change in Ndt80 electrophoretic mobility between wild-type and checkpoint-arrested cells. It is possible that phosphorylation stabilizes the Ndt80 protein without affecting its enzymatic activity.

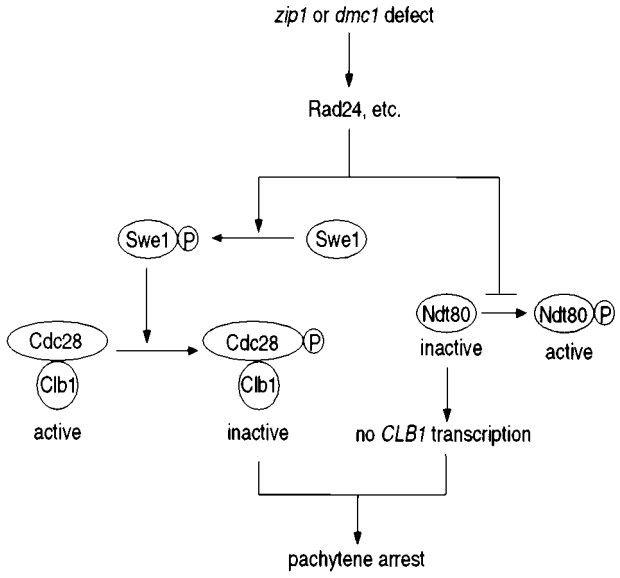

Fig. 4. A model for the pachytene checkpoint. Defects in recombination and synapsis in the *zip1* and *dmc1* mutants are sensed and transmitted by several proteins including Mek1, Pch2, Red1, Rad24, Rad17, and Mec1. The activated checkpoint prevents phosphorylation of Ndt80 and thereby inhibits transcription of *CLB1* and other genes required for the exit from pachytene. Rather than affecting kinase activity directly, the checkpoint may increase the abundance or activity of protein that inhibits Ndt80 phosphorylation, perhaps by direct physical interaction. In parallel with the inhibition of Ndt80 phosphorylation, the checkpoint increases Swe1 activity, which phosphorylates and inhibits Cdc28.

In principle, the pachytene checkpoint could regulate Ndt80 activity by limiting the abundance or activity of the kinase that phosphorylates Ndt80. However, the efficient phosphorylation of Ndt80 observed on overproduction of Ndt80 suggests that the relevant kinase is not a limiting factor. Another possibility is that the pachytene checkpoint increases the abundance or activity of an inhibitor of Ndt80 phosphorylation. This inhibitor might bind to Ndt80 and thereby prevent its access to the kinase. Excess Ndt80 protein resulting from overproduction would titrate out the inhibitor, thus allowing the bulk of the Ndt80 protein to be phosphorylated.

In *hop2* cells overexpressing *NDT80*, the Ndt80 protein accumulates and becomes phosphorylated (as in wild type), but the checkpoint is not bypassed. This result is consistent with previous observations indicating that checkpoint-mediated arrest in the *hop2* mutant is tighter than in *zip1* and *dmc1* strains. Whereas the extent of arrest in *zip1* and *dmc1* strains varies with yeast strain background (3, 4, 8–10), the *hop2* mutant displays an absolute failure of sporulation in all strains tested (11). In addition, a *pch2* mutation does not allow *hop2* to sporulate, through it does restore sporulation in *zip1* and *dmc1* strains (13). The behavior of *hop2* strains overproducing Ndt80 indicates that accumulation and phosphorylation of Ndt80 are not sufficient to inactivate the pachytene checkpoint, although they are necessary for full checkpoint bypass. Perhaps Cdc28 is more tightly regulated by Swe1 in *hop2* than in *zip1* and *dmc1*, such that an excess of Clb1 protein has less impact on the overall level of Cdc28/Clb1 activity.

Ndt80 localizes to chromosomes later than Zip1, and localization of the two proteins seems to be largely mutually exclusive.

The latter observation is unexpected, because Ndt80 function is required for the exit from pachytene and the dissociation of Zip1 from chromosomes (24). One interpretation of these results is that Ndt80 does localize to chromosomes during pachytene, but the amount of protein is too low to be detected by immunofluorescence. At later times, as Ndt80 increases in abundance and activity, Ndt80 localization becomes more pronounced.

DNA microarray analysis has been used to identify the targets of the Ndt80 transcription factor (25). More than 200 genes are induced when Ndt80 is ectopically expressed during vegetative growth. In meiotic cells, about 150 genes display a pattern of expression consistent with being targets of Ndt80. In the upstream region of each Ndt80 target gene is one or a few copies of the middle sporulation element that serves as a binding site for Ndt80 (22, 25). The number of Ndt80-staining foci observed by immunofluorescence is roughly consistent with the number of genes activated by Ndt80. However, one or a few molecules of the Ndt80 protein are not expected to be sufficient to generate a detectable signal by immunofluorescence, raising the intriguing possibility that Ndt80 plays another role in meiosis, in addition to its role as a transcriptional activator.

Previous studies have demonstrated that Swe1, and hence Cdc28, is a major downstream target of the pachytene checkpoint pathway (19). The results presented herein demonstrate that Ndt80 is also regulated at the pachytene checkpoint. Two observations suggest that Swe1 and Ndt80 are the targets of two parallel branches of the checkpoint pathway (Fig. 4). First, Cdc28 activity is not required for phosphorylation of Ndt80. Second, deletion of *SWE1* and overproduction of Clb1 have additive or synergistic effects on sporulation in mutants that arrest at the pachytene checkpoint (19). Thus, the checkpoint apparently prevents cell cycle progression by limiting the activity or abundance of both components of the Cdc28/Clb complex.

Sporulation is delayed in *hop2 swe1* strains, and this delay is bypassed by introduction of a multicopy plasmid carrying *CLB1* (19). The delay in sporulation in *hop2 swe1* is correlated with a delay in *CLB1* mRNA accumulation (19) and a corresponding delay in phosphorylation of Ndt80 (data not shown). These observations suggest that there may be some crosstalk between the two branches of the pathway, such that accumulation of active Cdc28 somehow relieves the inhibition of Ndt80 phosphorylation.

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