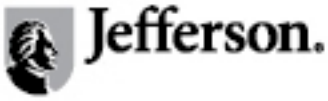

6-15-2014

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Mol. Cell. Biol. **2014, 34(12):2249. DOI: 10.1128/MCB.01413-13.**
Published Ahead of Print 7 April 2014.

MCB
Journals.ASM.org

Multisite Phosphorylation of the Sum1 Transcriptional Repressor by S-Phase Kinases Controls Exit from Meiotic Prophase in Yeast

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Activation of the meiotic transcription factor Ndt80 is a key regulatory transition in the life cycle of *Saccharomyces cerevisiae* because it triggers exit from pachytene and entry into meiosis. The *NDT80* promoter is held inactive by a complex containing the DNA-binding protein Sum1 and the histone deacetylase Hst1. Meiosis-specific phosphorylation of Sum1 by the protein kinases Cdk1, Ime2, and Cdc7 is required for *NDT80* expression. Here, we show that the S-phase-promoting cyclin Clb5 activates Cdk1 to phosphorylate most, and perhaps all, of the 11 minimal cyclin-dependent kinase (CDK) phospho-consensus sites (S/T-P) in Sum1. Nine of these sites can individually promote modest levels of meiosis, yet these sites function in a quasiadditive manner to promote substantial levels of meiosis. Two Cdk1 sites and an Ime2 site individually promote high levels of meiosis, likely by preparing Sum1 for phosphorylation by Cdc7. Chromatin immunoprecipitation reveals that the phosphorylation sites are required for removal of Sum1 from the *NDT80* promoter. We also find that Sum1, but not its partner protein Hst1, is required to repress *NDT80* transcription. Thus, while the phosphorylation of Sum1 may lead to dissociation from DNA by influencing Hst1, it is the presence of Sum1 on DNA that determines whether *NDT80* will be expressed.

Key regulatory proteins in eukaryotic cells are often phosphorylated on multiple residues. Multisite phosphorylation can produce graded changes in protein activity (1) and switch-like transitions (2), and it can also impart distinct conformations to proteins that specify alternative outcomes (3). Despite the critical role of multisite phosphorylation in shaping cellular behavior, there are only a few examples of hyperphosphorylated proteins for which the regulatory consequences of all, or even most, of the phosphomodifications have been established.

Exit from meiotic prophase is a significant point of regulation in meiotic development. In the yeast *Saccharomyces cerevisiae*, this transition is controlled by a transcriptional switch involving a repressor (Sum1) and an activator (Ndt80) that bind sites in middle meiotic promoters termed middle sporulation elements (MSEs) (4). The transition from the Sum1-bound state (when middle genes are silent) to the Ndt80-bound state (when middle genes are expressed) is a point when preceding events in the meiotic program are monitored and when "readiness" for nuclear segregation is assessed. This transition occurs at approximately the same time as commitment, the point after which the inducing signal (starvation) is no longer required for completion of the meiotic program (5). Ndt80 can competitively displace Sum1 from MSE DNA *in vitro*, demonstrating that an MSE can be bound by either Sum1 or Ndt80 but not both proteins (6). Another property that contributes to the switch-like properties of this transition is the positive autoregulatory loop wherein Ndt80 activates its own promoter (7).

The *NDT80* positive autoregulatory loop is induced only during meiotic prophase due to the combination of MSE and URS1 elements in the *NDT80* promoter (8, 9). URS1s are occupied by the Ume6 DNA-binding protein/Rpd3-Sin3 histone deacetylase complex during vegetative growth. Upon starvation of diploids, Ume6 is degraded (10) and URS1 elements acquire the ability to activate transcription via the Ime1 transcription factor (the master regulator of meiotic induction that is produced upon starvation of **a**/α cells) (11, 12). However, Ime1 cannot activate *NDT80* transcription due to the Sum1/MSE complex. Sum1 interacts with the NAD-dependent histone deacetylase Hst1 via the bridging protein Rfm1 (9, 13). The MSE/Sum1/Rfm1/Hst1 complex at the *NDT80* promoter therefore functions as a "meiotic gatekeeper" in cells that have converted URS1s to activating elements (i.e., meiotically induced cells that are expressing early meiosis-specific genes).

While Ndt80 can competitively displace Sum1 from DNA, Sum1 is removed from the *NDT80* promoter in the absence of the Ndt80 protein during meiotic prophase (14). The *NDT80*-independent removal of Sum1 from DNA is promoted by a CDK, Cdk1 (also known as Cdc28), and the meiosis-specific CDK-like kinase Ime2, which downregulate Sum1 in meiotic cells (15). The Cdc7/Dbf4 S-phase regulatory kinase (referred to as Cdc7 below) controls multiple steps in meiotic cells that are required for meiosis I (MI) (16–18). Similar to Cdk1 and Ime2, Cdc7 phosphorylates Sum1 in meiotic prophase and downregulates Sum1 (19, 20).

Sum1 contains a single Ime2 phospho-consensus site (residue T306) and 11 minimal Cdk1 phospho-consensus sites (S/T-P) that are located throughout the 1,062-residue Sum1 protein (15). A mutant in which all 11 Cdk1 phosphoacceptor consensus sites in Sum1 are rendered nonphosphorylatable (*sum1-c*) completes meiosis and forms spores. Similarly, a mutant that renders T306 nonphosphorylatable (*sum1-i*) completes meiosis and forms spores. In contrast, a mutant containing the Cdk1 consensus substitutions and the Ime2 phosphoacceptor substitution (*sum1-ci*) arrests in late prophase with an *ndt80*Δ-like phenotype and undetectable levels of *NDT80* mRNA. The *sum1-ci* arrest phenotype

Received 24 October 2013 Returned for modification 20 November 2013
Accepted 1 April 2014
Published ahead of print 7 April 2014
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doi:10.1128/MCB.01413-13

can be bypassed by ectopic *NDT80* expression, by mutation of an MSE in the *NDT80* promoter, or by deletion of either *RFM1* or *HST1*. These data indicate that Ime2 and Cdk1 can promote pachytene exit by phosphorylating Sum1 and that these phosphomodifications lead to the downregulation of repression at the gatekeeper MSE. It has been speculated that the phosphorylation of Sum1 causes downregulation of Hst1 (15, 19). How changes in localized Hst1 activity influence *NDT80* transcription is unknown. Irrespectively, once derepression of *NDT80* takes place, the active Ndt80 that is produced is capable of competitively displacing Sum1. These interactions lead to induction of the *NDT80* positive autoregulatory loop, increased levels of active Ndt80, and exit from pachytene.

In this study, we show that the S-phase-promoting cyclin Clb5 activates Cdk1 to phosphorylate most and perhaps all of the 11 S/T-P motifs in Sum1. Our findings demonstrate that while no single Cdk1 site in Sum1 is required for meiosis, most and perhaps all of these phosphoregulatory sites collectively control meiotic progression. Clb5/Cdk1 phosphoacceptor sites with the greatest regulatory influence prepare adjacent residues for phosphorylation by Cdc7. We also show that Sum1 can repress *NDT80* transcription independently of Hst1. These findings show that Sum1 functions as an integrator of signals from the three key S-phase-promoting kinases to control *NDT80* promoter activity and exit from pachytene.

MATERIALS AND METHODS

Yeast growth and sporulation. The SK1 genetic background was used for all the experiments described in this study except for the Northern blotting experiments, which were performed using the W303 background (Table 1). Cells were propagated on yeast extract-peptone-dextrose (YEPD) or yeast extract-peptone-acetate (YEPA) (each supplemented with adenine at 40 mg/liter) at 30°C. For sporulation, cells were grown in liquid YEPA to a density of no more than 10^7 cells/ml, harvested by centrifugation, washed once in SPO (2% acetate plus 10 mg/liter adenine, 4.8 mg/liter uracil, 28.8 mg/liter L-leucine, 7.2 mg/liter lysine, 9.6 mg/liter L-tryptophan, 4.8 mg/liter L-histidine), resuspended in SPO at 4×10^7 cells/ml, and incubated at 30°C for the indicated times.

Construction of yeast strains and plasmids. The mutant form of Sum1 that cannot be phosphorylated by Ime2 due to the T306A substitution (*sum1-i*), the mutant that cannot be phosphorylated by Cdk1 due to substitution of the 11 S/T-P Cdk1 phospho-consensus sites in Sum1 to A-P (*sum1-c*), and the combination mutant containing both the Ime2 and Cdk1 site substitutions (*sum1-ci*) were derived from the integrating *URA3*-based plasmids pMES42 (*sum1-i*), pMES77 (*sum1-c*), and pMES71 (*sum1-ci*) as previously described (Tables 1 and 2) (15). In all of these plasmids, the translational initiator ATG had been changed to GCG (referred to as *sum1-nostart* below). Digestion of these plasmids with HindIII linearizes the plasmid in the *SUM1* coding sequence, and homologous recombination of the linearized plasmids in yeast generates a *sum1-mutant*::*URA3*::*sum1-nostart* duplication allele in which only the mutant *sum1* mRNA is translated due to the absence of an in-frame initiator ATG in the *nostart* allele.

The *sum1-ci-A379S* (pMES81) and *sum1-ci-A512S* (pMES82) reversion alleles were generated using the QuikChange site-directed mutagenesis system (Stratagene, La Jolla, CA) to change codon 379 or 512, respectively, in the pMES71 (*sum1-ci*) plasmid from GCT to TCT. The *sum1-S379A* (pDRC6) and *sum1-S512A* (pDRC7) alleles were generated using the QuikChange system to change codon 379 and codon 512 in the pMES39 (*sum1-wt*) plasmid from TCT to GCT. The *sum1-306A-379A-512A* allele was generated by "stitching" overlapping PCR fragments prepared from *sum1-T306A* plasmid (pMES42) and *sum1-S512A* plasmid (pDRC7) templates using oligonucleotides that span the overlap region that contained codon 379 TCT-to-GCT changes. The stitched PCR product was subsequently digested with HindIII and BglII restriction endonucleases, and this fragment was used to replace the same fragment in pMES42 to generate pDRC14. A similar PCR stitching and cloning strategy was used to generate *sum1-c,S305A* (pDRC16), *sum1-ci,A379S,S378A* (pDRC18), and *sum1-ci,A512S,S511A* (pDRC17) using *sum1-c* (pMES77), *sum1-ci-A379S* (pMES81), and *sum1-ci-A512S* (pMES82) as the templates for the PCRs, respectively. The plasmids used as PCR templates in these constructions were also used as the plasmids to receive the mutated HindIII/BglII fragments after digestion with the same enzymes. The presence of the mutations in all plasmids generated in this study was confirmed by sequencing. All plasmid inserts in this study that were derived from PCR were sequenced in their entirety. A cassette containing eight histidine codons and the hemagglutinin (HA) epitope (HH) was added to the end of the *SUM1* gene as previously described (21).

Mapping potential phosphoacceptor sites by yeast-mediated recombination. The series 1 recombination-based strategy to identify regulatory phosphosites in Sum1 started with a haploid strain containing *sum1-ci*::*URA3*::*sum1-nostart* (MSY331) (see Fig. 2A). *sum1-ci/SUM1* chimeras were generated by plating 3×10^6 cells grown in liquid dropout culture on YEPD plates. Cells were grown overnight into a lawn that was replica plated onto 5-fluoroorotic acid (5-FOA) plates and incubated for 6 days at 30°C, after which the FOA-resistant colonies were analyzed by DNA sequence analyses of *SUM1* PCR fragments to identify a representative selection of chimeras. Subsequently, chimeras were mated with MSY341 and diploids were selected on SD-URA/Geneticin (G418) sulfate (KAN) plates in which monosodium glutamate was used as a nitrogen source instead of ammonium sulfate (ammonium sulfate interferes with Geneticin uptake). The series 2 chimeric analyses started with a *SUM1*::*URA3*::*sum1-ci-nostart* haploid that was generated by integrating the *SUM1-nostart* plasmid pMES39 as described above into a *sum1-ci* strain that was recovered in the series 1 experiments (VYY1009) (see Fig. 2B). The series 3 chimeric analyses started with a *sum1-i*::*URA3*::*sum-ci-nostart* haploid that was generated by integrating the *sum1-i-nostart* plasmid pMES42 as described above into a *sum1-ci* strain (see Fig. 2C).

For scoring meiosis in the *SUM1/sum1-ci* chimeras, the set of chimeric mutants and the *sum1-ci* and wild-type controls were grown overnight to a density of 0.8 to 1.4 cells in YEPA, and cells were washed and transferred to SPO medium, incubated at 30°C for 24 h, and then scored for meiosis as described above. The chimeric series containing most of the mutants was analyzed at least 3 times, with similar results being obtained each time. The data shown in Fig. 2 represent a single analysis for each chimeric series that was carried out in parallel and represent at least 200 cells counted for each strain.

Phosphotransferase assays. Peptides were synthesized by Genescript and contain an N-terminal biotin-Ahx (flexible motif) modification attached to the following amino acid sequences from Sum1 (p denotes phosphorylation on the next residue): NGKERPSTANSSSI (299–312 peptide); NGKERPSpTANSSSI (phospho-299–312-peptide); KFHQIPSS PSNPV (372–384 peptide); KFHQIPSpSPSNPV (phospho-372–384-peptide). All peptides were dissolved in dimethyl sulfoxide (DMSO) to generate 20 mM stock solutions.

Protein kinase reactions were carried out in 100 mM HEPES-KOH at pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 100 μM ATP supplemented with 10 μCi of 3,000 Ci/mmol [γ-^{32}P]ATP, and 20 ng of yeast Cdc7/Dbf4 (DDK) purified from baculovirus as described previously (22). Reaction mixtures were incubated at 30°C for 10 min, and reactions were terminated by addition of 10 μl of 7.5 M guanidine-HCl. Fifteen microliters of the terminated-reaction mixtures was spotted onto SAM^2 biotin capture membranes (Promega), incubated for 1 min, and washed 4 times in 2 M NaCl and 4 times in 2 M NaCl plus 1% H_3PO_4 for 3 min for each wash and 2 times in water for 1 min for each wash. Membranes were dried, and the radioactivity was quantitated using a scintillation counter.

Antibodies and immunoblot analyses. For the *sum1-HH* and *sum1-ci-HH* time course, 4.4×10^8 cells were collected at the indicated times

TABLE 1 Yeast strains[a]

Strain	Genotype	Reference or source
LNY150	*MAT***a**/*MATα ura3/ura3 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2/lys2 his4-N/his4-G ho::LYS2/ho::LYS2*	51
SSY102	*MAT***a**/*MATα ndt80Δ::LEU2/ndt80Δ::LEU2 SUM1-HH::URA3/SUM1-HH::URA3*	This study
SSY103	*MAT***a**/*MATα ndt80Δ::LEU2/ndt80Δ::LEU2 sum1-ci-HH::URA3/sum1-ci-HH::URA3*	This study
DCY65	*MAT***a**/*MATα ndt80Δ::LEU2/ndt80Δ::LEU2 sum1-c-HH::URA3/sum1-c-HH::URA3*	This study
DCY66	*MAT***a**/*MATα ndt80Δ::LEU2/ndt80Δ::LEU2 sum1-c-S305A-HH::URA3/sum1-c-S305A-HH::URA3*	This study
DCY75	*MAT***a**/*MATα ndt80Δ::LEU2/ndt80Δ::LEU2 dmc1Δ::LEU2/dmc1Δ::LEU2 SUM1-HH::URA3/SUM1-HH::URA3*	This study
MSY331	*MATα sum1-ci::URA3::sum1-nostart SMK1-HA::KAN*	15
VYY1024	*MATα SUM1::URA3::sum1-ci-nostart SMK1-HA::KAN*	This study
MSY341	*MAT***a** *sum1-ci::URA3::sum1-nostart*	15
DCY47	*MAT***a**/*MATα sum1-T306A-S379A-S512A(3A)::URA3::sum1-nostart/sum1-T306A-S379A-S512A(3A)::URA3::sum1-nostart*	This study
MSY392	*MAT***a**/*MATα sum1-ci::URA3::sum1-nostart/sum1-ci::URA3::sum1-nostart*	15
MSY348	*MAT***a**/*MATα sum1-c::URA3::sum1-nostart/sum1-c::URA3::sum1-nostart*	15
ASY19	*MAT***a**/*MATα sum1-ci-A379S::URA3::sum1-nostart/sum1-ci-A379S::URA3::sum1-nostart*	This study
VYY29	*MAT***a**/*MATα sum1-ci-A512S::URA3::sum1-nostart/sum1-ci-A512S::URA3::sum1-nostart*	This study
MSY294	*MAT***a**/*MATα ndt80Δ::LEU2/ndt80Δ::LEU2 SMK1-HA::KAN/SMK1-HA::KAN*	This study
NAY228	*MAT***a**/*MATα ndt80Δ::LEU2/ndt80Δ::LEU2 sum1-T306A(i)::URA3::sum1-nostart/sum1-T306A(i)::URA3::sum1-nostart SMK1-HA::KAN/SMK1-HA::KAN*	14
SSY109	*MAT***a**/*MATα ndt80Δ::LEU2/ndt80Δ::LEU2 sum1-S379A::URA3::sum1-nostart/sum1-S379A::URA3::sum1-nostart SMK1-HA::KAN/SMK1-HA::KAN*	This study
SSY110	*MAT***a**/*MATα ndt80Δ::LEU2/ndt80Δ::LEU2 sum1-S512A::URA3::sum1-nostart/sum1-S512A::URA3::sum1-nostart SMK1-HA::KAN/SMK1-HA::KAN*	This study
GOY29	*MAT***a**/*MATα SSP2-myc::KAN/SSP2-myc::KAN SMK1-HA::HIS3/SMK1-HA::HIS3*	This study
DCY99	*MAT***a**/*MATα SMK1-HA::KAN/SMK1-HA::KAN clb5Δ::KANMX4/clb5Δ::KANMX4 clb6Δ::TRP1/clb6Δ::TRP1*	This study
DCY104	*MAT***a**/*MATα SMK1-HA::KAN/SMK1-HA::KAN clb5Δ::KANMX4/clb5Δ::KANMX4*	This study
MSY352	*MAT***a**/*MATα SMK1-HA::KAN/SMK1-HA::KAN sum1-T306A(i)::URA3::sum1-nostart/sum1-T306A(i)::URA3::sum1-nostart*	15
DCY100	*MAT***a**/*MATα SMK1-HA::KAN/SMK1-HA::KAN clb5Δ::KANMX4/clb5Δ::KANMX4 clb6Δ::TRP1/clb6Δ::TRP1 sum1-T306A(i)::URA3::sum1-nostart/sum1-T306A(i)::URA3::sum1-nostart*	This study
DCY101	*MAT***a**/*MATα SMK1-HA::KAN/SMK1-HA::KAN clb5Δ::KANMX4/clb5Δ::KANMX4 sum1-T306A(i)::URA3::sum1-nostart/sum1-T306A(i)::URA3::sum1-nostart*	This study
DCY102	*MAT***a**/*MATα SMK1-HA::KAN/SMK1-HA::KAN clb6Δ::TRP1/clb6Δ::TRP1 sum1-T306A(i)::URA3::sum1-nostart/sum1-T306A(i)::URA3::sum1-nostart*	This study
DCY50	*MAT***a**/*MATα sum1-c-S305A::URA3::sum1-nostart/sum1-c-S305A::URA3::sum1-nostart*	This study
DCY58	*MAT***a**/*MATα sum1-ci-A379S-S378A::URA3::sum1-nostart/sum1-ci-A379S-S378A::URA3::sum1-nostart*	This study
DCY56	*MAT***a**/*MATα sum1-ci-A512S-S511A::URA3::sum1-nostart/sum1-ci-A512S-S511A::URA3::sum1-nostart*	This study
DCY72	*MAT***a**/*MATα cdc7-as3-myc9/cdc7-as3-myc9 SMK1-HA::KAN/SMK1-HA::KAN TRP1/TRP1*	This study
DCY73	*MAT***a**/*MATα ndt80Δ::LEU2/ndt80Δ::LEU2 cdc7-as3-myc9/cdc7-as3-myc9 SMK1-HA::KAN/SMK1-HA::KAN TRP1/TRP1*	This study
NH452F	*MAT***a**/*MATα cdc7-as3-myc9/cdc7-as3-myc9 ura3/ura3 leu2/leu2 TRP1/TRP1 ARG4/arg4-NSP his4-x/HIS4 ho::LYS2/ho::LYS2 lys2/lys2*	19
DCY51	*MAT***a**/*MATα cdc7-as3-myc9/cdc7-as3-myc9 sum1-ci::URA3::sum1-nostart/sum1-ci::URA3::sum1-nostart TRP1/TRP1*	This study
DCY53	*MAT***a**/*MATα cdc7-as3-myc9/cdc7-as3-myc9 sum1-c::URA3::sum1-nostart/sum1-c::URA3::sum1-nostart TRP1/TRP1*	This study
DCY52	*MAT***a**/*MATα cdc7-as3-myc9/cdc7-as3-myc9 sum1-ci-A379S::URA3::sum1-nostart/sum1-ci-A379S::URA3::sum1-nostart TRP1/TRP1*	This study
DCY54	*MAT***a**/*MATα cdc7-as3-myc9/cdc7-as3-myc9 sum1-ci-A512S::URA3::sum1-nostart/sum1-ci-A512S::URA3::sum1-nostart TRP1/TRP1*	This study
DCY55	*MAT***a**/*MATα cdc7-as3-myc9/cdc7-as3-myc9 sum1-T306A-S379A-S512A(3A)::URA3::sum1-nostart/sum1-T306A-S379A-S512A(3A)::URA3::sum1-nostart TRP1/TRP1*	This study
MSY252	*MAT***a**/*MATα sum1-T306A(i)::URA3::sum1-nostart/sum1-T306A(i)::URA3::sum1-nostart*	15
DCY90	*MAT***a**/*MATα cdc7-as3-myc9/cdc7-as3-myc9 sum1-T306A(i)::URA3::sum1-nostart/sum1-T306A(i)::URA3::sum1-nostart SMK1-HA::KAN/SMK1-HA::KAN TRP1/TRP1*	This study
KMSY1016	*MAT***a** *ura3 leu2::hisG trp1::hisG lys2 his4-N* or *his4-G ho::LYS2 SUM1-HH::URA3*	This study
KMSY1048	*MAT***a** *ura3 leu2::hisG trp1::hisG lys2 his4-N* or *his4-G ho::LYS2 SUM1-K359R-HH::URA3*	This study
W303a	*MAT***a** *can1-100 ssd1-d ade2-1 leu2-3,112 his3-11,15 ura3 trp1-1 lys2 ho::LYS2*	52
SSY1001	*MAT***a** *ume6Δ::HIS3*	This study
SSY1002	*MATα sum1Δ::KANMX4*	This study
SSY1005	*MAT***a** *hst1Δ::URA3*	This study
SSY1006	*MATα hst1-N291A*	This study
SSY1009	*MAT***a** *ume6Δ::HIS3 hst1Δ::URA3*	This study
SSY1010	*MATα ume6Δ::HIS3 hst1-N291A*	This study
US	*MAT***a** *ume6Δ::HIS3 sum1Δ::KANMX4*	9

[a] Most strains in this table are in the SK1 genetic background. The SK1 strains contain the genetic markers indicated for LNY150 except for *his4*, which could be either *his4-N* or *his4-G* in each case (not tested). The exceptions are the last 8 strains (US and the SSY strains), which are in the W303 genetic background. The W303 strains contain all of the markers listed for W303a.

TABLE 2 Plasmids

Plasmid	Marker(s)	Reference
pRS306	*URA3*	53
pRS304	*TRP1*	53
pDRC6	pRS306 + *sum1-S379A*	This study
pDRC7	pRS306 + *sum1-S512A*	This study
pDRC14	pRS306 + *sum1-T306A-S379A-S512A(3A)*	This study
pDRC16	pRS306 + *sum1-c-S305A*	This study
pDRC17	pRS306 + *sum1-ci-A512S-S511A*	This study
pDRC18	pRS306 + *sum1-ci-A379S-S378A*	This study
pMES42	pRS306 + *sum1-T306A*	14
pMES77	pRS306 + *sum1-c*	15
pMES71	pRS306 + *sum1-ci*	15
pMES81	pRS306 + *sum1-ci-A379S*	This study
pMES82	pRS306 + *sum1-ci-A512S*	This study
pMP208	pRS416 + *SUM1-13XMyc*	13

postinduction and proteins were partially purified using nickel beads as described previously (21), except that bound protein was eluted from the beads by boiling in 2× Laemmli buffer containing 200 mM DTT and 200 mM imidazole instead of 2-mercaptoethanol and EDTA since EDTA influences the migration of proteins in Phos-tag acrylamide electrophoretic gels. Snf1, which binds to nickel beads due to the 13 contiguous histidines in its open reading frame, was monitored with an anti-His6 antiserum as a loading control. For Sum1-phosphomutant-HH analysis, Sum1-HH samples were prepared as described above from 4.4×10^8 cells collected at 0 h after transfer to sporulation medium and from 1.8×10^9 cells taken at 5.5 h after transfer to sporulation medium, since our goal was to compare equivalent amounts of Sum1 (the cellular concentration of Sum1-HH decreases by about 4-fold at 5.5 h postinduction).

Total cellular extracts were collected and prepared using NaOH to lyse the cells and trichloroacetic acid to precipitate the proteins as previously described (23). Samples were electrophoretically resolved for 1 h at 45 mA at constant current using 8% polyacrylamide gels (Smk1 and Snf1 blots) or 3 h at 45 mA at constant current for 6% polyacrylamide gels (Sum1-HH blots) or 6% polyacrylamide gels with 100 μM $MnCl_2$ and 100 μM Phos-tag (Wako Pure Chemical Industries, Osaka, Japan) (Sum1-HH blots) as described previously (24). Following electrophoresis, proteins were transferred onto Immobilon-P membranes (EMD Millipore), and the membrane was incubated for at least 1 h in phosphate-buffered saline–Tween (PBST) and I-block (Life Technologies) and in the same solution containing the antibody dilutions indicated below at 4°C overnight. HH-tagged Sum1 constructs and Smk1-hemagglutinin (HA) were stained by incubating membranes with 1:10,000 dilutions of monoclonal anti-HA antiserum (Covance) and visualized using an alkaline phosphatase-conjugated anti-mouse antibody. A 1:10,000 dilution of PSTAIRE antibody (Sigma), which stains a doublet of Cdk1 and Pho85, was used as a loading control. Blots using phospho-T306 antiserum were performed as described above, except that Tris-buffered saline–Tween (TBST) and 1% bovine serum albumin (BSA) were used to block membranes and for antibody incubations (14).

ChIP. For chromatin immunoprecipitation (ChIP) analyses, 2.5×10^8 cells were cross-linked with 1% formaldehyde for 15 min at room temperature. Reactions were quenched by the addition of glycine to a final concentration of 125 mM, and reaction mixtures were incubated at room temperature for 5 min. Cells were subsequently washed twice with cold phosphate-buffered saline and then lysed with glass beads in lysis buffer (50 mM HEPES-KOH at pH 7.5, 140 mM NaCl, 1 mM EDTA at pH 8.0, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, protease inhibitors, and 1 mM phenylmethylsulfonyl fluoride [PMSF]). After lysis, the bottom of the tube was punctured and the lysate was collected by brief centrifugation in precooled tubes. The lysate was sonicated using a Diagenode Bioruptor for 10 to 12 cycles (30 s on/1 min off) to generate an average fragment size of 400 to 500 bp. Cell debris was removed by centrifugation, and the supernatant was precleared using protein G-agarose–salmon sperm DNA beads (Millipore). One-tenth of the lysate was reserved as whole-cell extract (WCE) control. The remaining precleared supernatant was immunoprecipitated using 3 μg of anti-Sum1 (Santa Cruz) by incubation at 4°C overnight followed by incubation with 40 μl of 50% protein G-agarose–salmon sperm DNA beads for 3 h. The protein-bound beads were washed once with lysis buffer, once with lysis buffer in which the NaCl concentration had been raised to 500 mM, once with wash buffer (10 mM Tris-HCl at pH 8.0, 0.25 M LiCl, 0.5% NP-40, 0.5% [wt/vol] sodium deoxycholate, and 1 mM EDTA), and twice with 10 mM Tris at pH 8.0, 1 mM EDTA, for 5 min each time. Protein-DNA complexes were eluted using TES elution buffer (50 mM Tris-HCl at pH 8.0, 10 mM EDTA, 1% SDS). Reverse cross-linking was performed by incubating the samples at 65°C overnight followed by proteinase K digestion at 42°C for 3 h. Simultaneously, reverse cross-linking and proteinase K digestion were also performed for the WCE control samples. DNA was extracted with phenol-chloroform and ethanol precipitated. RNase A-treated DNA samples were quantified for Sum1 binding at the *SMK1* and *NDT80* promoters by real-time PCR (ABI and Roche). The sequences of forward/reverse primers are as follows: *NDT80*, GAGGGCAAAGTGTCAGAAAATCG/AGGGACCTTGGCTTTTCGAAAC; *SMK1*, GGCAAAAGGCGGGTGATTCG/TGGTATTATCTGTAAGTGTGCGATTC; *ACT1*, ATGCAAACCGCTGCTCAATCTTC/AGTTTGGTCAATACCGGCAGATTC.

For quantification of DNA, a standard curve was generated using serially diluted yeast genomic DNA. The relative enrichment was calculated by the following formula: (immunoprecipitated DNA at *NDT80* or *SMK1* promoters/*NDT80* or *SMK1* DNA in WCE)/(immunoprecipitated DNA at *ACT1*/*ACT1* DNA in WCE). Each experiment was repeated independently 3 times, and each replicate was analyzed in triplicate. The data shown are normalized to the mitotic ChIP signal (set arbitrarily at 100). For *SMK1* and *NDT80*, the mitotic enrichments for Sum1 were 32- and 30-fold, respectively.

Miscellaneous assays and procedures. For scoring meiosis, 10^7 cells were collected, fixed in 90% ethanol, stained with 2 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI), and washed with water prior to microscopy. Northern blotting assays were carried out using ^{32}P-radiolabeled PCR fragments as probes (9, 25).

RESULTS

Sum1 is phosphorylated on multiple Cdk1 phospho-consensus sites during meiotic prophase. Although we have shown previously that mutating the putative Cdk1 phosphosites in Sum1 affects meiotic progression (15), we wanted to demonstrate by direct biochemical experiments that these sites are phosphorylated during meiosis and that phosphorylation affects Sum1 activity. To compare Sum1-ci to wild-type Sum1, His8-HA (HH)-tagged forms of these proteins were purified from cells collected at different times after transfer to sporulation medium using Ni beads, and proteins were analyzed by immunoblot analyses. A mutant background containing a deletion in the *NDT80* open reading frame was used in these experiments so that the *SUM1-HH* and the *sum1-ci-HH* strains were trapped at pachytene at the end of the time course. The constitutively produced Snf1 protein, which binds to Ni beads due to a naturally occurring polyhistidine tract, was used as a control for protein recovery. These analyses show that the wild-type Sum1 protein undergoes complex changes during meiotic development (Fig. 1A). First, it is maximally phosphorylated by Ime2 on T306 within 1 h (prior to S phase), as shown by phospho-specific pT306 immunoreactivity. Next, the electrophoretic mobility of Sum1 starts to decrease at 3 h (as DNA replication is being completed), and then its level declines until cells accumulate in pachytene. In contrast to the wild-type pro-

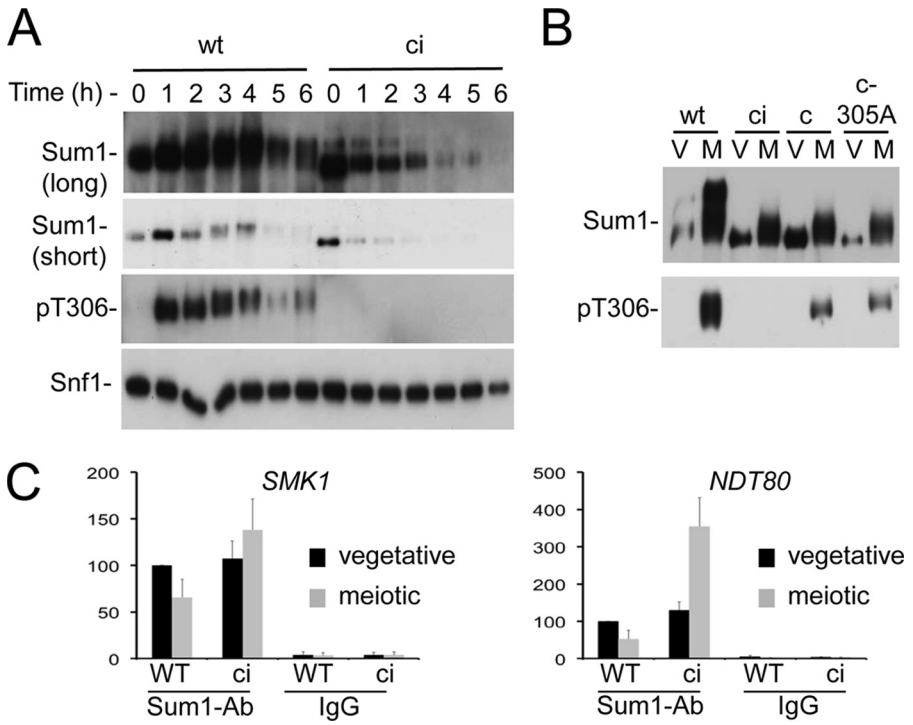


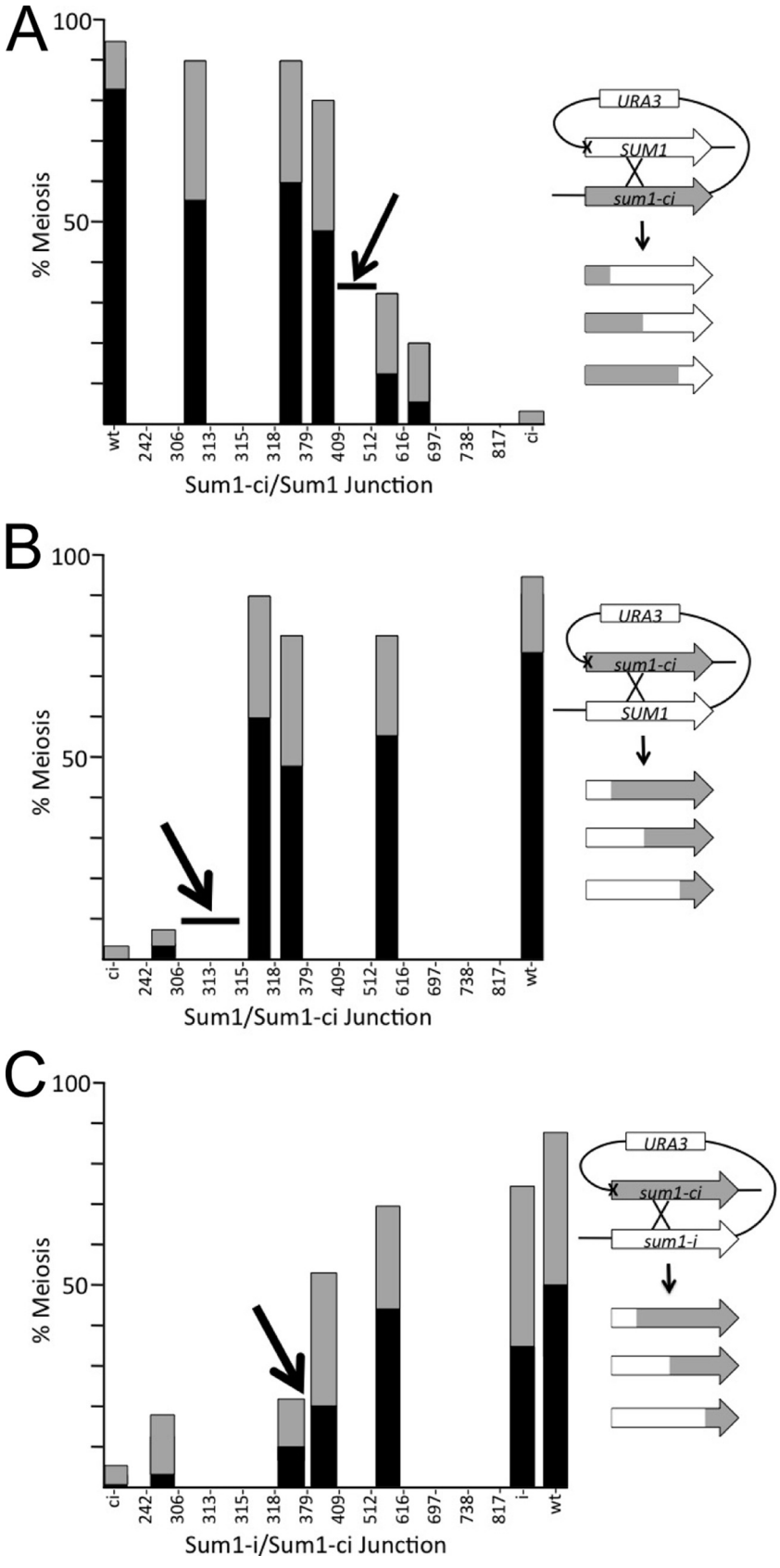
FIG 1 Changes to the Sum1 and Sum1-ci proteins during meiotic development. (A) Sum1-HH (wt) and Sum1-ci-HH (ci) proteins were enriched on Ni beads from *ndt80*Δ cells collected at the indicated times after transfer to sporulation medium, and the proteins were analyzed by electrophoresis and immunoblot analyses using an HA antibody (Sum1). A long and a short exposure of the same blot are shown to facilitate the comparison. The same samples were analyzed with a phospho-specific antiserum for the residue that is phosphorylated by Ime2 (pT306) and a polyhistidine antiserum as a loading control (Snf1). (B) Sum1-HH proteins containing the indicated mutations from vegetative (V) or meiotic (M) *ndt80*Δ cells were analyzed as in panel A except that Phos-tag acrylamide was included in the running gel. The Sum1 proteins from 4 times as many meiotic as vegetative cells were analyzed to facilitate comparison. (C) Occupancies of wild-type Sum1 and Sum1-ci were analyzed at the *SMK1* and *NDT80* promoters in vegetative and meiotic *ndt80*Δ cells using a Sum1 antibody or a negative-control IgG as indicated. The occupancy of Sum1 in vegetative cells was arbitrarily set at 100.

tein, Sum1-ci is not phosphorylated by Ime2 and it also does not exhibit a detectable decrease in mobility in these electrophoretic assays. Surprisingly, the decline in Sum1-ci levels is faster and more substantial than that for the wild type (see the long and short exposures of these data in Fig. 1A). These findings suggest that the Cdk1 and Ime2 phospho-consensus sites in Sum1 do not trigger its degradation. Since Sum1-ci blocks meiotic development at pachytene, these experiments also suggest that the Sum1-ci that is bound to MSE DNA is more stable than the unbound protein (see Discussion).

To investigate whether the changes in electrophoretic mobility of Sum1 are due to phosphorylation, comparable levels of Sum1 and Sum1-ci from mitotically growing and meiotic (*ndt80*Δ-trapped) cells were electrophoretically resolved as in Fig. 1A except that Phos-tag acrylamide, which specifically retards the migration of phosphorylated proteins, was included in the running gel. As shown in Fig. 1B, inclusion of Phos-tag acrylamide substantially retarded the electrophoretic mobility of the wild-type protein from meiotic cells. In contrast, the Sum1-ci and Sum1-c meiotic proteins show more modest electrophoretic retardation. These findings indicate that multiple Cdk1 phospho-consensus sites in Sum1 are phosphorylated during meiotic prophase.

The Sum1-ci protein is persistently bound to MSEs in meiotic cells. To address whether the phosphosite substitutions in Sum1-ci influence its interaction with DNA, Sum1 and Sum1-ci occupancy at middle meiotic promoters was analyzed using a chromatin immunoprecipitation (ChIP) assay. Consistent with published studies (14), occupancy of wild-type Sum1 at the *SMK1* promoter, which contains a single MSE, was higher in vegetative cells than in pachytene-arrested cells (Fig. 1C, left panel). Similar results were observed at the *NDT80* promoter, which contains 2 MSEs (the entire *NDT80* promoter is present in the *ndt80*Δ allele used in this study) (Fig. 1C, right panel). In contrast to the wild-type Sum1 protein, occupancy of Sum1-ci at the *SMK1* promoter was indistinguishable in vegetative and pachytene-arrested cells. Occupancy of Sum1-ci at the *NDT80* promoter was higher in pachytene than in vegetative cells. These findings demonstrate that the phosphosite substitutions in Sum1-ci increase its occupancy at MSEs specifically in meiotic prophase. The more substantial increase in binding of Sum1-ci to the *NDT80* promoter than to the *SMK1* promoter suggests that the meiosis-specific changes in Sum1 occupancy can be modulated in a promoter-specific fashion. Taken together, the data suggest that the phosphorylation of Sum1 that occurs in meiotic prophase reduces Sum1 occupancy at MSE DNA.

A genetic strategy to analyze regulatory phosphosites in Sum1. To identify the Cdk1 phospho-consensus sites in Sum1 that influence Sum1 activity, we introduced phosphorylatable (S or T) residues back into the *sum1-ci* phosphosite mutant starting from its carboxy terminus and assayed the ability of these mutants to undergo meiosis (Fig. 2A). For this purpose, we used a genetic strategy that we term directional phosphosite analysis (DPA) that



involves an intermediate strain containing a chromosomal *sum1-ci/URA3/SUM1-nostart* duplication (*nostart* eliminates the initiator ATG of *SUM1*, which allows *sum1-ci* to be scored when the duplication is present). Homologous recombination events between *sum1-ci* and *SUM1* generate a series of *sum1-ci/SUM1* chimeric recombinants while evicting *URA3* and therefore can be selected using 5-fluoroorotic acid (5-FOA). We defined the fusion junction of the *sum1-ci/SUM1* chimeras in 5-FOA-resistant colonies by DNA sequencing (*x* axis of Fig. 2A). The *SUM1/sum1-ci* chimeras were mated to a *sum1-ci* tester haploid (*sum1-ci* is recessive), and the resulting heterozygous diploids were assayed for meiosis and spore formation after they had been incubated in sporulation medium for 24 h (after which further increases in meiosis did not take place).

Phenotypic assays of the DPA-generated strains showed that reversion of the 3 nonphosphorylatable Cdk1 consensus sites closest to the carboxyl end of Sum1-ci (reverting residues 817, 738, and 697 from A to S) modestly increased the fraction of cells that completed meiosis. Reversion of the next site (616) caused a further incremental increase in meiosis. In contrast, a large increase in meiosis and spore formation was observed when two additional sites in *sum1-ci* (residues 409 and 512) were reverted (see arrow in Fig. 2A). These observations suggest that the carboxy-terminal Cdk1 phospho-consensus residues 817, 738, 697, and 616 do regulate Sum1 but that their quantitative influence is modest. The further substantial increase in meiosis that is observed when the *sum1-ci/SUM1* chimeric junction crosses residue 409 to introduce 2 additional phosphorylatable amino acids at positions 409 and 512 could occur because Sum1 is downregulated in a threshold-dependent manner (i.e., substantial downregulation occurs when phosphate is added to a defined number of sites). Alternatively, residues 409 and/or 512 might be more potent regulatory sites than the more C-terminal sites. Below, we will demonstrate that residue 512 is a potent regulatory site while residues 409, 616, 697, 738, and 817 exert only a modest influence on Sum1. To simplify the presentation of this data, we will here refer to the sites that modestly increase meiosis as "minor" sites and the sites that substantially increase meiosis as "major" sites.

To generate a set of *SUM1/sum1-ci* chimeras that introduce phosphorylatable residues into Sum1-ci starting from its amino terminus, we created a *SUM1/URA3/sum1-ci-nostart* intermediary strain (Fig. 2B). Chimeric recombinants were isolated and analyzed as described above. Analyses of these series 2 chimeras showed that reversion of the most amino-terminal Cdk1 phosphoacceptor (making residue 242 phosphorylatable) modestly increased meiosis similarly to the minor C-terminal sites. The next mutated residue in this series is the Ime2 phosphoacceptor (residue T306), which we previously showed is capable of promoting high levels of meiosis (15). As expected, an increase in the fraction of meiotic cells was observed when the Sum1/Sum1-ci chimeric interval passed T306 (arrow in Fig. 2B) and all of the chimeras containing junctions that were C-terminal to this residue (and which therefore contain the phosphorylatable T residue at position 306) underwent high levels of meiosis and formed spores.

It is possible that Cdk1 phospho-consensus residues that exert a major regulatory influence on Sum1 exist in the interval bounded by the most amino-terminal and carboxy-terminal major sites. The function of these sites would not have been revealed in the series 1 and 2 DPA experiments. To test for regulatory sites in this interval, a series-2-like DPA was carried out in which the amino-terminal Ime2 site was mutated in both chimeric partners (*sum1-i/sum1-ci-nostart*) (Fig. 2C). Analysis of this series of chimeras confirmed that modest increases in meiosis take place when the amino-terminal Cdk1 site (residue 242) in *sum1-ci* is reverted. Similarly, reversion of the next 3 sites (313, 315, and 318) led to further modest increases in meiosis. However, when the next Cdk1 phospho-consensus residue (379) was reverted, a substantial increase in the pattern of meiosis was observed (arrow in Fig. 2C). These data suggest that S379 is a major regulatory site. These data also suggest that the amino-terminal phospho-consensus sites (242, 313, 315, and 318) exert a minor influence on Sum1 but that they can operate in a quasiadditive fashion similar to the C-terminal minor sites.

Collectively, these experiments indicate that there are 3 sites that are sufficient to promote high-level prophase exit and meiosis when reverted in *sum1-ci*. The major regulatory site closest to the amino terminus is the Ime2 phosphoacceptor at position 306 that is contained in the sequence R-P-S-T-A (26). The next major site is the Cdk1-phosphoacceptor consensus site at position 379 that is contained in the sequence S-S-P (Cdk1 phosphoacceptor consensus sites underlined). The major regulatory site closest to the carboxy terminus could be residue 409 or 512. Since a chimeric recombinant in this interval was not isolated in the DPA experiments described above, we could not distinguish between these possibilities. In experiments described below, we will show that residue 512, which is contained in the sequence S-S-P, is a major regulatory site.

Confirmation of DPA phosphosite phenotypes. To establish whether the major phosphosites implicated by DPA are sufficient to promote Sum1 downregulation and meiosis, A306, A379, or A512 was individually reverted to a phosphorylatable residue in *sum1-ci*, and strains homozygous for these alleles were assayed for meiosis. These experiments demonstrate that reversion of any of the three sites is sufficient to promote relatively high levels of meiosis and spore formation (compare the 4 rightward bars in Fig. 3A). However, the fraction of cells that completed only one of the two divisions (to produce dyad spores) was higher for all three major-site reversion strains than for the wild-type control strain. In addition, while half-maximal levels of meiosis in the wild-type strain were completed by 7 h, half-maximal levels of meiosis for the *sum1-ci* strain that contained the T306, 512S, or 379S reversion took longer than 8, 9, and 10 h, respectively. In addition, the

FIG 2 Directional phosphosite analysis (DPA) of Sum1 chimeras. Starting strains in each case consist of duplicated *SUM1* alleles flanking a *URA3* marker which are diagrammed in a looped configuration (right). The X indicates recombination, which can occur throughout the indicated region of homology to generate the chimeric products indicated below. Recombinants in *SUM1* were selected using 5-FOA, and the recombination interval was identified by DNA sequencing (*x* axis). In each case, the data bar is positioned between the phosphoacceptor residues that were either absent (*sum1-ci*) or present (*SUM1*). Recombinants were crossed by a *sum1-ci* tester strain, and the fraction of cells that completed a single meiotic division (gray; 2 DAPI-stained foci) or more than one division (black; 3 or 4 DAPI-stained foci) was scored (*y* axis). The arrows point to inflection intervals discussed in the text. (A) Analysis of nonphosphorylatable/phosphorylatable Sum1-ci/Sum1 chimeras. (B) Analysis of phosphorylatable/nonphosphorylatable Sum1/Sum1-ci chimeras. (C) Analysis of phosphorylatable/nonphosphorylatable Sum1-i/Sum1-ci chimeras.

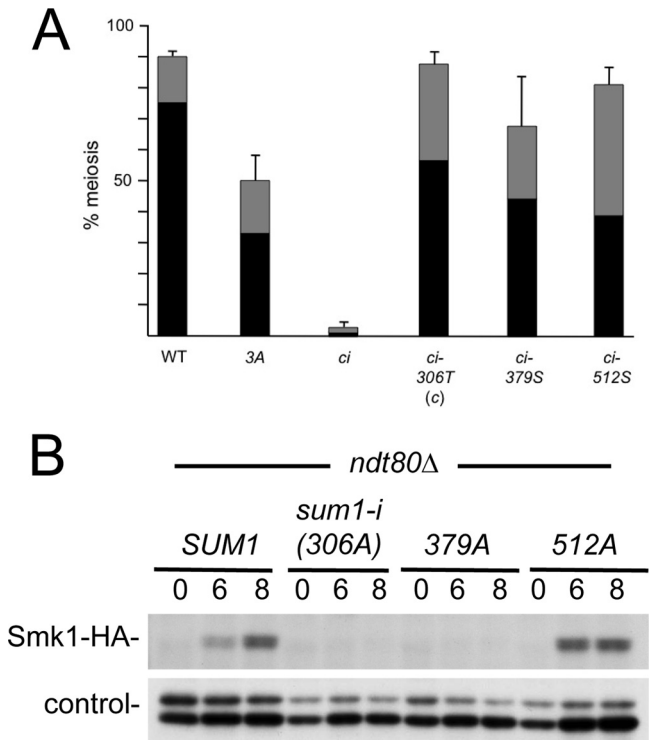


FIG 3 Mutants in *SUM1* reveal 2 classes of CDK phospho-consensus sites. (A) Homozygous strains of the indicated genotypes were assayed for meiosis at 24 h postinduction (gray, 2 DAPI-stained foci; black, 3 or 4 DAPI-stained foci; 200 cells counted per experiment, $n = 3$). The *3A* mutant contains phosphosite substitutions of the 3 major phosphoacceptors (T306A, S379A, and S512A). The *sum1-ci-306T* mutant is identical to *sum1-c*, as indicated (*c*). (B) The indicated *SUM1* mutants in the *ndt80*Δ background were transferred to sporulation medium, cells were withdrawn at the indicated times (hours), and Smk1-HA was measured by immunoblot analyses as a surrogate assay for Sum1 removal. The PSTAIR antibody, which recognizes a doublet of Cdk1 and Pho85, was used as a loading control.

meiosis that took place in these strains was asynchronous (meiotic kinetics in these backgrounds can be found in Fig. 6B). These data show that while the individual major sites significantly increase the probability that exit from pachytene/entry into meiosis will take place, multiple phosphoacceptor sites in Sum1 collectively shape the meiotic kinetics of the system.

While the phenotypic similarity of the *sum1-ci* reversion strains might suggest that these sites are influencing the activity of Sum1 in comparable fashions, further analyses suggest that these mutants have different properties. In particular, while the *sum1-i* mutant (T306A) undergoes relatively high levels of meiosis and spore formation in an otherwise wild-type background, this substitution prevents the *NDT80*-independent disassociation of Sum1 from DNA (14). *SMK1* is a tightly regulated Sum1-repressible middle meiotic gene, and the production of Smk1-HA in an *ndt80*Δ strain provides a readout for changes in Sum1 activity that take place in the absence of Ndt80 competition. A *sum1-S379A ndt80*Δ mutant fails to produce Smk1, similarly to the *sum1-i ndt80*Δ mutant, while a *sum1-S512A ndt80*Δ mutant produces Smk1 (Fig. 3B). These findings suggest that phosphorylated T306 and S379 are required for the Ndt80-independent removal of Sum1 from DNA but that phosphorylated S512 influences Sum1 through another mechanism.

In contrast to the major Cdk1 phospho-consensus sites, reversion of individual minor sites caused only a modest increase in meiosis. To assess the collective potency of the entire set of minor sites, nonphosphorylatable substitutions in the 3 major sites (*sum1-T306A*, *S379A*, and *S512A*) were introduced into wild-type *SUM1*, leaving only the minor sites (referred to as the "*3A*" mutant in Fig. 3). The frequency of meiosis in this triple mutant was substantial (50%), suggesting that while minor sites individually promote modest levels of meiosis, collectively these sites can promote high levels of meiosis and spore formation. However, the maximal level of meiosis observed in the *sum1-3A* mutant was reached slowly and asynchronously (half-maximal levels of meiosis in *sum1-3A* took longer than 14 h to achieve compared to 7 h for the wild type; see Fig. 6B for details). These findings suggest that under conditions where major sites cannot be phosphorylated, Cdk1 can increase the fraction of cells that enter meiosis in a graded manner through minor site residues.

The recombination checkpoint does not prevent Ime2 or Cdk1 from phosphorylating Sum1. The recombination checkpoint pathway plays key roles in processing the double-strand breaks (DSBs) that are introduced during prophase, and it can also block meiotic progression at pachytene in response to persistent recombination intermediates. A *dmc1*Δ mutant, which blocks processing of DSBs, therefore undergoes checkpoint-mediated arrest at pachytene (27). A *dmc1*Δ *sum1*Δ mutant does not block at pachytene and segregates (broken) chromosomes (28, 29). This observation has led to the suggestion that Sum1 is a regulated target of the checkpoint (28). However, a different model in which Ndt80 is the target is suggested by the observation that the checkpoint inhibits Ndt80 by anchoring it in the cytoplasm (7, 30–32) and that *NDT80* is expressed precociously in a *sum1*Δ mutant (8). Therefore, deletion of *SUM1* might cause checkpoint bypass by allowing *NDT80* to be expressed before a functional checkpoint response can be generated (29). Cdk1 is a well-studied target of the recombination checkpoint, and Ime2 is functionally related to Cdk1. These connections make it important to establish whether Sum1 phosphorylation is controlled by the recombination checkpoint.

We were unable to detect any differences in the levels and electrophoretic mobilities of Sum1 in *ndt80*Δ *dmc1*Δ cells (where the recombination checkpoint is active) and *ndt80*Δ cells (where the recombination checkpoint is inactive). In addition, the relative amounts of Sum1 that are phosphorylated by Ime2 in these two backgrounds are indistinguishable (Fig. 4A). Smk1-HA is expressed in both an *ndt80*Δ strain and an *ndt80*Δ *dmc1*Δ strain (Fig. 4B). These data demonstrate that Sum1 repression can be lifted when the checkpoint is active. Taken together, these findings suggest that phosphorylation of the Cdk1 and Ime2 phosphoacceptor sites in Sum1 is not regulated by the recombination checkpoint. These data are consistent with Ndt80 being the major target that couples recombination intermediates to suppression of middle meiotic gene expression.

Clb5 is the cyclin that activates Cdk1 for Sum1 phosphorylation. The cyclins Clb5 and Clb6 promote S phase in both the mitotic and meiotic cell cycles. While other B-type cyclins (Clb1 to Clb4) are able to support S phase in mitotically growing cells (33–35), this is not the case during meiotic development, and *clb5*Δ *clb6*Δ cells transferred to sporulation medium fail to undergo meiotic DNA replication (36). However, these cells also fail to activate the meiotic DNA replication checkpoint, and they progress

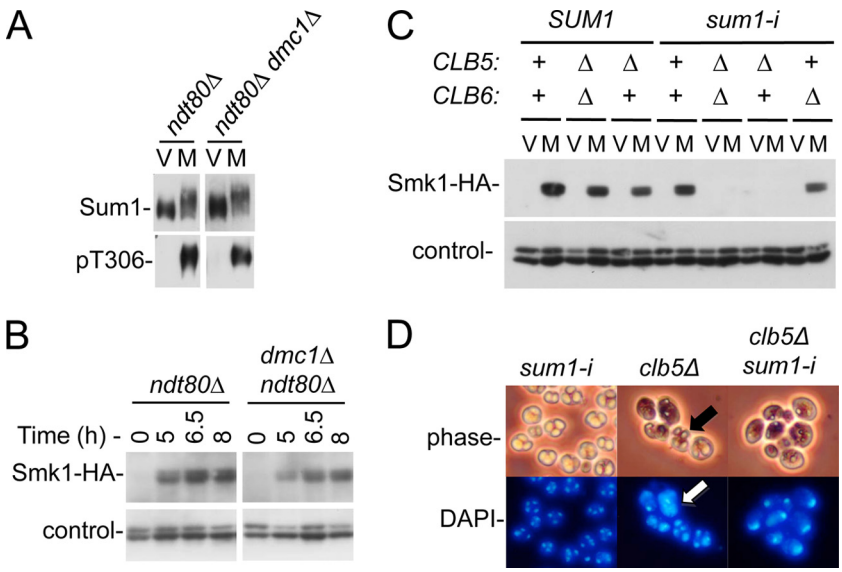


FIG 4 The recombination checkpoint does not influence the phosphorylation of Sum1 by Ime2 or Cdk1, and Clb5 is the cyclin that activates Cdk1 for Sum1 phosphorylation. (A) Sum1-HH purified from the indicated strains that were grown vegetatively (V) or incubated in SPO medium for 5.5 h (M) was analyzed by Phos-tag electrophoresis and immunoblot analysis using a Sum1 antiserum or a phospho-specific antiserum for pT306. Sum1 from 4.5 times more meiotic than vegetative cells was analyzed. (B) Cells of the indicated genotype were collected at the indicted times after transfer to sporulation medium and analyzed by immunoblotting for Smk1-HA protein as a surrogate assay for Sum1 downregulation. PSTAIR immunoreactivity was used to control for loading. (C) Strains of the indicated genotypes were assayed for the production of Smk1-HA during vegetative growth (V) and following incubation for 7 h in sporulation medium (M). PSTAIR immunoreactivity was used to control for loading. (D) Strains of the indicated genotype were transferred to sporulation medium, fixed and stained with DAPI at 24 h postinduction, and photographed using phase-contrast microscopy (phase) or fluorescence microscopy (DAPI). Note the appearance of spore-like structures (black arrow) and the diffuse DNA masses (white arrow) in the *clb5*Δ strain compared to the single DNA masses and the absence of spore-like structures in the *clb5*Δ *sum1-i* strain.

through the program, segregate unreplicated DNA in catastrophic nuclear segregations, and assemble spore-like structures. Middle genes are induced with only a modest delay in *clb5*Δ *clb6*Δ cells, suggesting that Sum1 repression is downregulated in this background (36). To address the possibility that Sum1 can be regulated by the Clb5 and/or Clb6 form of Cdk1, we tested whether *clb5*Δ and/or *clb6*Δ cells remove Sum1-mediated repression by using the *SMK1* middle meiotic gene as a readout. In *SUM1 clb5*Δ *clb6*Δ strains, Smk1 is produced (Fig. 4C). In contrast, in *sum1-i clb5*Δ *clb6*Δ cells, Smk1 is not produced (Fig. 4C). Moreover, *SUM1 clb5*Δ *clb6*Δ cells segregate DNA and form spore-like structures, while *sum1-i clb5*Δ *clb6*Δ cells do not segregate DNA and do not form spore-like structures. These findings suggest that in the *sum1-i* background, where Ime2 cannot phosphorylate Sum1, the Clb5 and/or Clb6 form of Cdk1 is required to downregulate Sum1.

We next tested the induction of Smk1 and nuclear segregation in the *sum1-i* background lacking either *CLB5* or *CLB6*. In the *clb6*Δ *sum1-i* strain, Smk1 was produced and nearly wild-type levels of nuclear segregation and spore formation took place, suggesting that *CLB6* is not required for the removal of Sum1 repression (Fig. 4C). In contrast, Smk1 was not produced in the *clb5*Δ *sum1-i* strain. Nuclear segregation took place in the *clb5*Δ *SUM1* strain, and by 10 h postinduction, 84% ± 2% of the cells had completed nuclear segregation as evidenced by 2 or more masses of DNA. The DNA pattern became more diffuse over time, and at later times, spore-like structures that contained DNA were apparent in a subset of the cells (Fig. 4D). In contrast, nuclear segregation almost never took place in the *sum1-i clb5*Δ strain. In this background, 99% ± 1% of cells contained a single mass of DNA at 10 h ($n = 3$ experiments, 100 cells counted per experiment). Even at later times (24 h), nuclear segregation was rare in the *sum1-i clb5*Δ strain (less than 2%) and spore-like structures were never observed (Fig. 4D). These observations show that when Ime2 cannot phosphorylate Sum1 on T306, *CLB5* becomes essential for exit from pachytene and meiosis. These data indicate that Clb5 is the cyclin that activates Cdk1 for Sum1 phosphorylation. Cyclins influence whether Cdk1 is subject to checkpoint-mediated inactivation by the Swe1 inhibitory kinase, and the Clb5-bound form of Cdk1 is insensitive to Swe1 (37, 38). These findings therefore provide an explanation for why the phosphorylation of Sum1 by Cdk1 is not inhibited by the recombination checkpoint.

Major Cdk1 motif function requires adjacent S residues. The major Cdk1 motifs contain an S residue at the −1 position, while the minor Cdk1 phospho-consensus sites do not. Lo et al. have reported that Cdc7 promotes pachytene exit by phosphorylating Sum1 (19). Cdc7 can phosphorylate the amino-terminal S residue in S-S-P motifs when the S closest to the P has been phosphorylated by Cdk1 (S-pS-P is part of a low-K_m Cdc7 phospho-consensus site) (39, 40). To establish whether the −1 S residues at major sites control Sum1, an S378A substitution was introduced into the *sum1* allele that is exclusively downregulated by the 379 Cdk1 phosphosite (*sum1-ci,378A,379S*) and an S511A substitution was introduced into the *sum1* allele that is exclusively downregulated by the S512 Cdk1 phosphosite (*sum1-ci,511A,512S*). As shown in Fig. 5, changing the −1 S to A in both of these mutants substantially reduced the fraction of cells that completed meiosis. These findings are consistent with Clb5/Cdk1 acting at major phosphoregulatory motifs by increasing the phosphorylation of residues 378 and 511 by Cdc7.

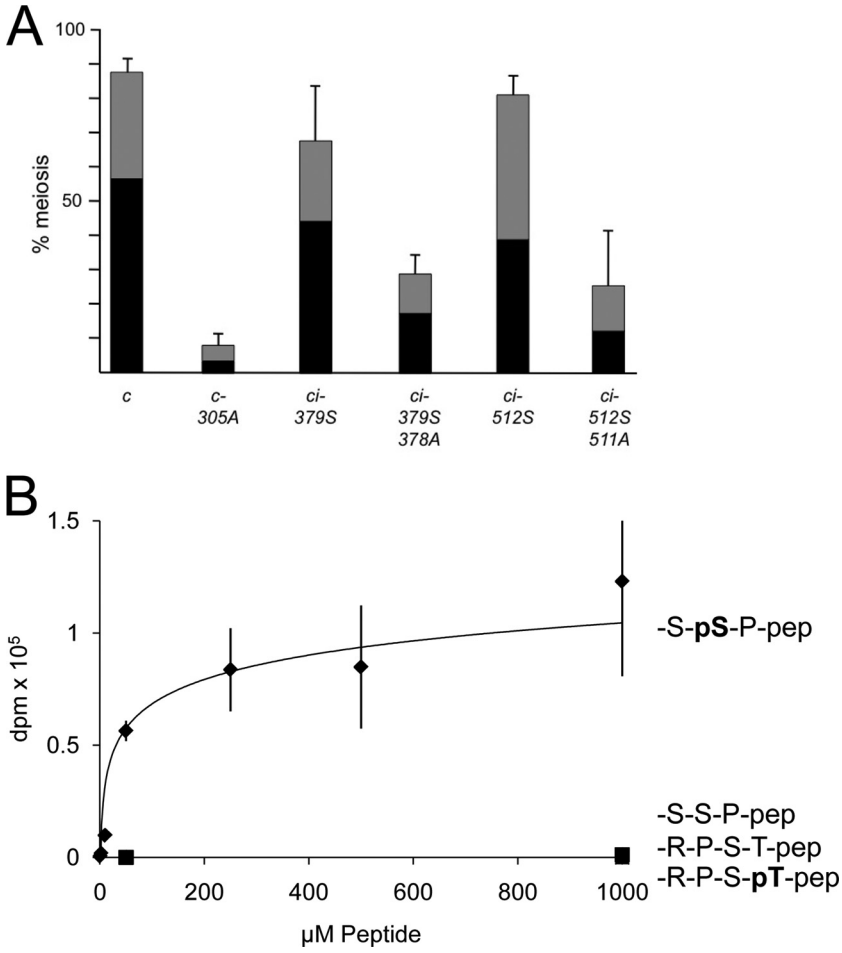


FIG 5 Major phosphoregulatory site function requires −1 S residues. (A) Homozygous *sum1* strains containing the indicated substitutions were assayed for meiosis (gray, 2 DAPI-stained foci; black, 3 or 4 DAPI-stained foci; $n = 3$, 200 cells counted per experiment). (B) The indicated peptides were assayed in phosphotransferase reactions using purified Cdc7/Dbf4 (-S-S-P-pep, the 372–384 Cdk1 phospho-consensus peptide; -S-pS-P-pep, the 372–384 peptide phosphorylated on the Cdk1 site; R-P-S-T-pep, the 299–312 Ime2 phosphoacceptor peptide; R-P-S-pT-pep, the 299–312 peptide phosphorylated on the Ime2 site).

The Ime2 phospho-consensus motif is R-P-X-S/T-A/V (26, 41–43). The Ime2 phosphoacceptor in Sum1 conforms to this motif and also contains an S at the −1 (X) position (the Sum1 sequence surrounding residue T306 is R-P-S-T-A). We tested whether this potential Cdc7 phosphoacceptor is required for Ime2 to promote meiosis by introducing an S305A substitution into the *sum1-c* background (*sum1-c,305A*). As seen in Fig. 5A, the *sum1-c,305A* mutant shows sharply reduced meiosis compared to *sum1-c*. Wild-type Sum1-HH and Sum1-c,305A-HH proteins purified from *ndt80*Δ cells trapped in pachytene were phosphorylated on T306 (Fig. 1B). The lack of pachytene exit in the *sum1-c,305A* strain is therefore not due to decreased phosphorylation of T306 by Ime2. These data are consistent with studies demonstrating that the −1 position has little influence on the substrate selectivity of Ime2 (26, 42). Taken together, these findings show that the −1 S residue is required for Ime2 to exert a major influence on Sum1 and are consistent with Ime2 preparing Sum1 for secondary phosphorylation by another kinase (e.g., Cdc7).

The genotype/phenotype data raise the possibility that the phosphorylation of residues 379 and 512 by Cdk1 and of 306 by Ime2 prepares adjacent −1 S residues (378, 511, and 305) for secondary phosphorylation by Cdc7. To address these possibilities, peptides containing the phosphorylated and unphosphorylated forms of residues T306 and S379 were compared in phosphotransferase assays containing purified Cdc7 complexed to the Dbf4 activating subunit. The S378 site was phosphorylated by Cdc7/Dbf4 with an apparent K_m of 60 μM but only when residue 379 was phosphorylated. We did not detect Cdc7-dependent phosphate incorporation into either the phosphorylated or unphosphorylated Ime2 site peptides. These data indicate that the modification of Sum1 by Cdk1 can promote the activity of Cdc7/Dbf4 for the adjacent (−1) S residue. While the genetic data are consistent with Ime2 also playing a "priming" function at T306 for S305, the phospho-T306 peptide that we tested is not a Cdc7/Dbf4 substrate.

The ability of Ime2 and Clb5/Cdk1 to promote prophase exit requires Cdc7. The Hollingsworth group previously generated a form of Cdc7 that is sensitive to the purine analog PP1, and they showed that treatment of *cdc7-as3-myc* cells with PP1 blocks cells in prophase (44). They also showed that the deletion of *SUM1* allows chromosome segregation to take place in *cdc7-as3-myc* cells treated with PP1 (19). However, the chromosome segregation that takes place in these strains is abnormal and the spores that are produced are nonrecombinant dyads since *CDC7*-dependent pro-

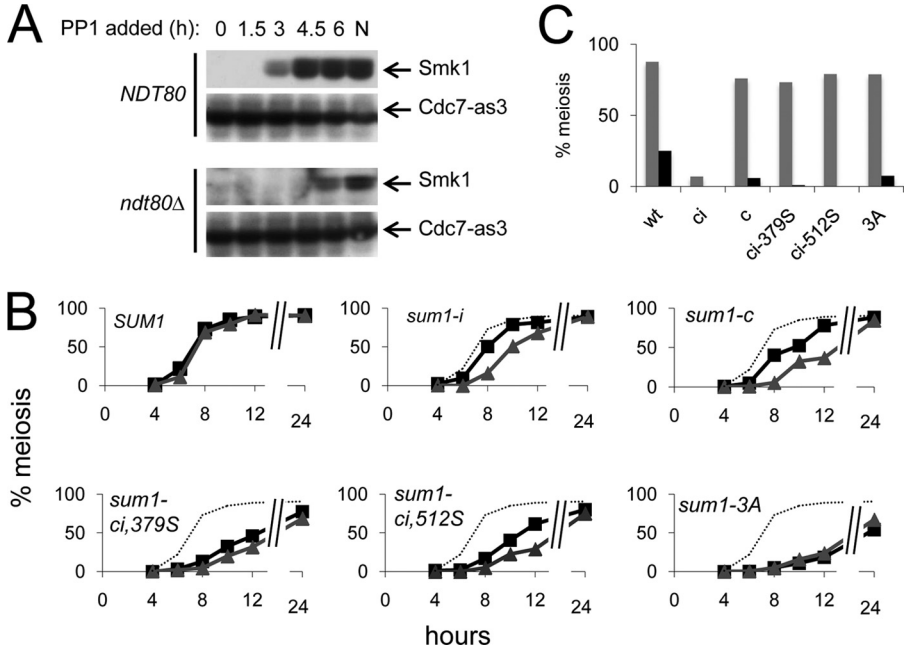


FIG 6 Cdc7 is required for the *NDT80*-dependent and *NDT80*-independent pathways for Sum1 removal and shows synthetic interactions with *sum1* phosphosite mutants. (A) *cdc7-as3-myc SMK1-HA* cells were transferred to sporulation medium, inhibitor (PP1) was added at the indicated time, and cells were withdrawn at 7 h postinduction and tested for Smk1-HA production as a surrogate assay for Sum1 repression (N, no PP1 added). Cdc7-as3-Myc was measured using a Myc antibody to control for protein levels. (B) Cells of the indicated *SUM1* genotype in the *CDC7* wild-type (squares) and *cdc7-as3-myc* (triangles) backgrounds were transferred to sporulation medium lacking inhibitor, withdrawn at the indicated time postinduction, and assayed for the completion of meiosis (combined MI and MII values). (C) The indicated *SUM1* alleles in the *cdc7-as3-myc* background were treated with PP1 at 3 h postinduction (black bars) or not treated (gray bars), and cells were collected at 24 h and scored for meiosis (MI and MII). In all cases, further increases in meiosis did not take place after the 24-h time point.

cesses required for a proper MI reductional division have been inhibited. In an attempt to define the interval when Cdc7 activity is required for Sum1 downregulation, we assayed Smk1-HA in *cdc7-as3-myc* cultures that had been treated with PP1 at different times. PP1 addition prevented *cdc7-as3-myc* cells from producing Smk1-HA when added early in the program, but it had no effect on Smk1-HA production when added later than about 3 h postinduction (Fig. 6A, upper panel). We also tested the *NDT80*-independent removal of Sum1 repression using this same assay and found that the addition of PP1 to *cdc7-as3-myc ndt80*Δ cells prevented Smk1-HA accumulation when added up to 6 h postinduction (Fig. 6A, lower panel). Taken together, these data narrow down the time when Cdc7 influences Sum1 to the 3- to 6-h interval, when prophase-specific events are taking place. Ime2 phosphorylates Sum1 shortly after induction (within the first hour after cells have been transferred to sporulation medium, as seen in Fig. 1), and some active Clb5/Cdk1 must be present prior to 3 h since Clb5/Cdk1 promotes S phase, which is mostly completed by the 3-h time point. These observations are consistent with Sum1 being phosphorylated first by Ime2, then by Clb5/Cdk1, and finally by Cdc7.

cdc7-as3-myc has been reported to be a weak hypomorph in the absence of inhibitor (44). We compared the meiotic kinetics of *cdc7-as3-myc* in combination with various *sum1* alleles in the absence of PP1. While *cdc7-as3-myc* did not detectably affect the meiotic kinetics of a wild-type (*SUM1*) strain under the conditions tested, it did retard the kinetics of the *sum1-ci* mutants containing the single major site reversions, which themselves progress through the divisions slowly as discussed above. In contrast, the slow meiotic kinetics of the *sum1-3A* mutant was unaffected by *cdc7-as3-myc* (Fig. 6B). These synthetic genetic interactions are consistent with the Ime2 and the major Clb5/Cdk1 phosphoacceptor motifs functioning in a *CDC7*-dependent manner. We tested whether the set of *sum1*-phosphosite, *cdc7-as3-myc* strains are sensitive to the addition of PP1 at 3 h (Fig. 6C). In all cases, PP1 reduced the ability of cells to carry out the meiotic divisions. These results suggest that Cdc7 can influence Sum1 through mechanisms in addition to the direct priming site interactions described above.

Sum1 repression of *NDT80* does not require Hst1 deacetylase activity. The deletion of *RFM1* or *HST1* bypasses the *sum1-ci* block to meiosis (15). One explanation for these results is that Rfm1/Hst1 is required for Sum1 to repress *NDT80* transcription. Another explanation is that Rfm1/Hst1 influences the ability of Sum1 to disassociate from DNA. In order to understand how the phosphorylation of Sum1 controls pachytene exit, it is important to determine whether Rfm1/Hst1 is required to repress *NDT80*. We find that *NDT80* mRNA is undetectable in *ume6*Δ or in *sum1*Δ vegetative cells (in which early or middle meiosis-specific genes are derepressed, respectively) but that it is present in the *ume6*Δ *sum1*Δ background, consistent with previous reports (9) (Fig. 7). We also find that *NDT80* mRNA is undetectable in *ume6*Δ *hst1*Δ or in *ume6*Δ *hst1-N291A* cells (the *N291A* substitution inactivates Hst1 catalytic activity). These findings indicate that the Sum1 protein is able to extinguish *NDT80* expression in the absence of Hst1 deacetylase activity and suggest that it is the occupancy of Sum1 at MSE DNA that determines whether *NDT80* can be transcribed.

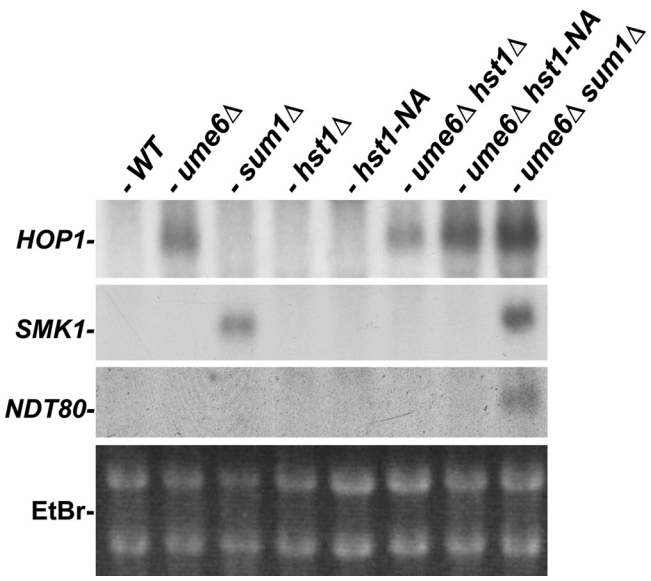


FIG 7 *NDT80* expression is repressed by Sum1 in an Hst1-independent manner. Cells of the indicated genotype were grown to mid-log phase in rich medium, total RNA was prepared, and the indicated transcript was assayed by Northern blotting analyses. Probes specific for the Ume6-repressible early meiosis-specific gene *HOP1*, the Sum1-repressible *SMK1* gene, and the Ume6/Sum1-coregulated *NDT80* gene are shown. The ethidium-stained rRNAs are shown as loading controls. EtBr, ethidium bromide.

DISCUSSION

In this study, the multiply mutated *sum1-ci* phosphosite allele and "loop-in/loop-out" recombination were used to generate sets of Sum1 proteins in which the 11 minimal CDK phospho-consensus motifs (S/T-P sites) were changed to A-P in a directional fashion. Phenotypic analyses of these mutants allowed the regulatory significance of these motifs to be inferred and set the stage to interrogate how multisite phosphorylation controls Sum1. This genetic strategy (DPA) allows relatively large sets of directionally mutated alleles of any gene to be efficiently generated and should be generally useful for studying yeast proteins that are controlled by multisite modification. The yeast system and DPA may also be useful for producing sets of directionally mutated proteins from other organisms that can be analyzed with biochemical methods (e.g., phosphotransferase assays) to identify amino acids that are modified by an enzyme of interest.

Surprisingly, for every S/T-P site that was tested, *sum1* backgrounds were uncovered that underwent decreased levels of meiosis when that site was rendered nonphosphorylatable. These findings are unlikely to reflect nonspecific consequences of these substitutions, since *SUM1* defects are expected to increase (not decrease) meiosis. Consistent with these genetic data, biochemical assays show that a large number of S/T-P motifs in Sum1 are phosphorylated in meiotic prophase (Fig. 1). In addition, phosphorylated forms of 5 of these residues have been identified in mass spectrometry studies (19, 45). The collection of *sum1* phosphosite mutants also allowed us to show that Clb5 is the cyclin that activates Cdk1 for Sum1 phosphorylation. Taken together, these observations suggest that Clb5/Cdk1 phosphorylates most and perhaps all of the S/T-P sites in Sum1 and that these phosphates collectively shape pachytene exit.

While the genotype/phenotype data indicate that all 11 S/T-P sites can regulate Sum1, they fall into 2 classes based on their quantitative ability to promote meiosis (Fig. 8). Nine of the sites individually promote only modest levels of meiosis, yet these minor sites can act in a quasiadditive fashion to promote substantial levels of meiosis. Nonetheless, the meiosis that takes place in a mutant where only the 9 minor phosphoacceptor sites are present (*sum1-3A*) is asynchronous and slow (Fig. 6B). *CLB5* expression is dramatically induced by Ndt80 as a middle gene despite the fact that *CLB5* functions earlier in the program to promote S phase (46). The minor Clb5/Cdk1 sites in Sum1 may not only promote initiation of the *NDT80* positive autoregulatory loop but also modulate the duration of middle meiotic gene expression after *NDT80* has been induced.

Two CDK sites in Sum1 are individually sufficient to promote relatively high levels of meiosis (major sites), similar to the Ime2 site. Both of these major sites contain S residues adjacent to the Cdk1 phosphoacceptors, and these adjacent residues are required for major site function. In addition, a peptide containing a major site can be phosphorylated by Cdc7 on its −1 S residue (S378) *in vitro* but only when the Clb5/Cdk1 phosphoacceptor (S379) is phosphorylated (Fig. 5B). Moreover, *sum1* mutants that depend on a major Cdk1 site show genetic interactions with *cdc7-as3-myc* (Fig. 6B). Based on these findings and the previously documented role of Cdc7 in regulating Sum1 (19), we propose that the phosphorylation of major sites (residues 379 and 512) by Clb5/Cdk1 promotes the secondary phosphorylation of −1 S residues (378 and 511) by Cdc7. According to this hypothesis, the combined phosphorylation of these residues promotes substantial downregulation of Sum1, which in turn permits pachytene exit and progression through meiosis. Inherent differences in the activity of Clb5/Cdk1 for different S/T-P sites and/or phosphatases that oppose these modifications may also contribute to quantitative differences in the ability of these sites to control meiotic progression.

The *CDC7* sensitivity of the Ime2-dependent *sum1-c* mutant and the requirement of S305 for T306 to promote meiosis raise the possibility that Ime2 also activates Sum1 for secondary phosphorylation by Cdc7. However, a peptide phosphorylated on the Ime2 site (containing pT306) was not phosphorylated by Cdc7. It has previously been shown that Cdc7 can phosphorylate the Mcm4 protein on multiple residues and that these phosphomodifications require a segment of Mcm4 that recruits Cdc7 that is not adjacent to the Cdc7 phosphoacceptor residues (47). It remains possible that the phosphorylation of Sum1 on T306 activates S305 for phosphorylation by Cdc7 but that a feature in the protein that is not present in the relatively short Ime2 phospho-consensus peptide used in our assays is required for the reaction to take place. Further biochemical experimentation will be required to establish whether this is the case. Irrespectively, these observations raise the possibility that the variable position (X) in the R-P-X-S/T-A/V Ime2 phospho-consensus site (26, 42) plays a role in diversifying Ime2 sites into sets that are functionally connected to Cdc7 and those that are not.

Clb5/Cdk1 is the major form of CDK that controls premeiotic S phase (36). Ime2 is required for destruction of the Sic1 inhibitor of Clb5/Cdk1 in meiotic cells (48). Cdc7 is essential for the firing of replication origins throughout S phase (49, 50). The Ime2 site in Sum1 is phosphorylated prior to S phase, and the Clb5/Cdk1 sites start to be detectably phosphorylated around S phase and to become increasingly hyperphosphorylated as cells transit through

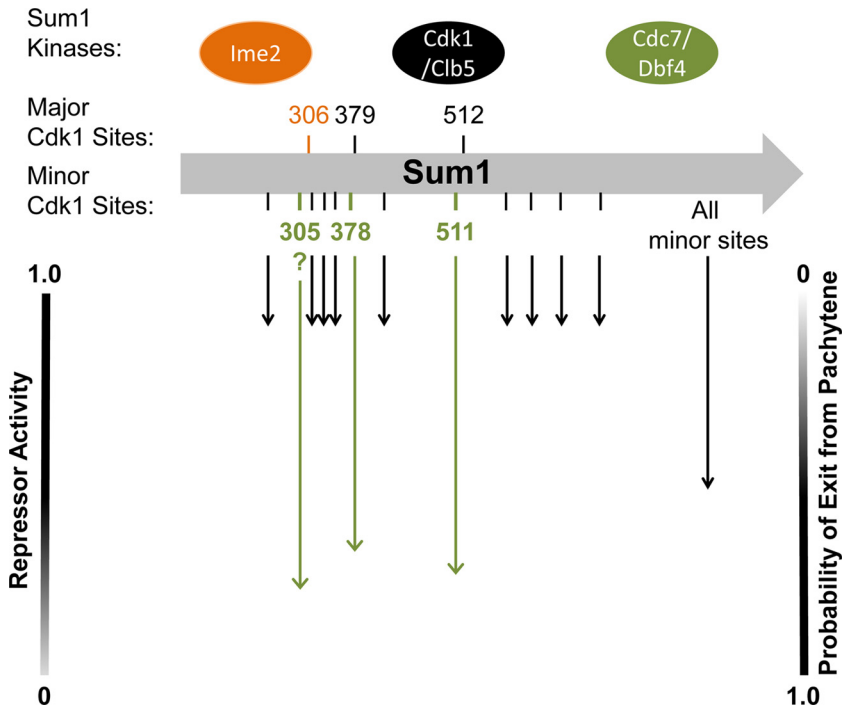


FIG 8 Regulatory phosphosites in Sum1. The minimal Cdk1 phospho-consensus S/T-P sites (at residues 242, 313, 315, 318, 379, 409, 512, 616, 697, 738, and 817) that were changed to A-P in *sum1-c* are indicated with black lines. The Ime2 phosphoacceptor (at residue T306) that was changed to an A residue in *sum1-i* is shown in orange. Sites in green are potential Cdc7 phosphoacceptors that require prior phosphorylation of adjacent sites by Clb5/Cdk1. The question mark at the 305 site reflects uncertainty about whether this residue is a Cdc7 target. The leftward shaded column indicates Sum1 activity which is inversely related to the probability of prophase exit and completion of meiotic development as indicated by the rightward shaded column. The downward arrows reflect relative potencies of each of the phosphoacceptor sites.

prophase. However, Sum1 repression is not removed until late in prophase. The priming interaction data suggest that Cdc7 is the last of the 3 kinases to deposit phosphate on Sum1. Ime2 and Clb5/Cdk1 therefore appear to generate a state in which Cdc7 can downregulate Sum1. Cdc7 controls multiple steps in meiotic prophase, including double-strand break formation, recruitment of monopilin to kinetochores, and separase cleavage (16–18, 20). Further studies on the regulation of Sum1 by Clb5/Cdk1, Ime2, and Cdc7 may give insight into how Cdc7 is regulated during meiotic development and how this enzyme coordinates the steps leading up to MI.

The key regulatory target of Sum1 that governs whether exit from pachytene can take place is the *NDT80* promoter, since once active Ndt80 is produced, it can competitively displace Sum1 (8, 15). The ability of *hst1*Δ or *rfm1*Δ to bypass the *sum1-ci* block (15) has led to the proposal that Sum1 phosphomodifications could activate *NDT80* transcription by promoting dissociation of the Rfm1/Hst1 complex from Sum1. Our results demonstrate that Sum1 can repress the transcription of *NDT80* in an *HST1*-independent fashion. These data suggest that it is the physical presence of Sum1 at MSEs, and not the enzymatic activity of the Hst1 protein deacetylase, that represses gene transcription. It is notable that the occupancy of wild-type Sum1 at MSEs decreases but is not eliminated when *ndt80*Δ cells are trapped at pachytene (Fig. 1). The intermediate ChIP signal observed in the *ndt80*Δ background suggests that that the hyperphosphorylated form of Sum1 that is present in pachytene may exist in a state of substantial ON/OFF flux with MSE DNA. One explanation that is consistent with all of these data is that Hst1 promotes a chromatin state that is not permissive for Sum1 exchangeability and that the phosphorylation of Sum1 in meiotic prophase reduces localized Hst1 activity and thereby increases the off-rate of Sum1 from chromatin. The level of Sum1 decreases as cells transition through prophase (Fig. 1A) and the reduced level of Sum1 in pachytene is predicted to further favor a "switch-like" transition in which exchangeability leads to *NDT80* derepression, since the pool of Sum1 that would be available to reoccupy MSEs is low. In this model, downregulation of the Hst1 deacetylase would not directly alter transcriptional output, but it would permit changes in Sum1 occupancy at the *NDT80* promoter to take place. This model of regulated exchangeability of a repressor may be relevant to how other sirtuins control commitment and cell fate decisions.

ACKNOWLEDGMENTS

We thank Nancy Hollingsworth and David Stuart for sharing plasmids and yeast strains. We also thank Volodymyr Yefymenko for technical assistance and Erica McGovern, Gregory Omerza, and Chong Tio for thoughtful comments on the manuscript.

This work was supported by a research grant from the NIH to E.W. (R01GM094244).

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