Mitotic Phosphorylation of the Anaphase-promoting Complex Inhibitory Subunit Mnd2 Is Necessary for Efficient Progression through Meiosis I*

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The yeast anaphase-promoting complex (APC) subunit Mnd2 is necessary for maintaining sister chromatid cohesion in prophase I of meiosis by inhibiting premature ubiquitination and subsequent degradation of substrates by the APC amended ubiquitin ligase. In a proteomics screen for post-translational modifications on the APC, we discovered that Mnd2 is phosphorylated during mitosis in a cell cycle-dependent manner. We identified and characterized the sites of mitotic Mnd2 phosphorylation during the cell cycle. Collective mutation of Mnd2 phosphorylation sites to alanine had no effect on vegetative growth but a striking effect (>85% reduction) on the percentage of tetrad-forming cells compared with the wild type strain. Similar to the MND2 deletion strain, cells harboring the alanine mutant that did not form spores arrested after premeiotic S phase with a single undivided nucleus and low levels of the APC ameliorative meiotic substrate, Clb5, relative to wild type cells. In contrast, collective mutation of Mnd2 phosphorylation sites to aspartic acid resulted in partial suppression of the sporulation defect. No differences were observed in the binding between each Mnd2 isoform and the APC in vitro. However, in vivo, we observed a gradient in the abundance of APC-associated Mnd2 in each strain that was proportional to the observed differences in sporulation and Clb5 levels. Taken together, these data suggest that mitotic phosphorylation of Mnd2 is necessary for APC-mediated progression beyond the first meiotic nuclear division.

Proper progression through the eukaryotic cell cycle is achieved in large part through coordinated regulation of protein degradation mediated by E3 ubiquitin ligases (for a review, see Refs. 1–4). During mitosis and meiosis, targeted protein degradation is tightly associated with the organization of chromosomes and their equivalent distribution into two separate progeny cells during anaphase and is controlled by a multisubunit ubiquitin ligase called the anaphase-promoting complex (APC) (5–7). As an E3 ligase, the APC transfers ubiquitin onto lysine residues of substrate proteins in an iterative manner that forms polyubiquitin chains on the surface of a substrate protein (8, 9). The polyubiquitinated substrate is then recognized by the 26 S proteosome and degraded, while the ubiquitin is recycled. The APC is itself regulated, ensuring that the timing of substrate degradation is tied to the completion of other cellular processes. Indeed misregulation of the APC can lead to disastrous results including cell cycle arrest or improper chromosome segregation and cell death (10–12).

Regulation of the APC during mitosis is accomplished by several mechanisms, including the association of co-activator proteins and through phosphorylation of individual subunits within the complex. Two co-activator proteins, Cdc20 and Cdh1, facilitate the association of substrate proteins with the APC at different stages of the mitotic cell cycle (13–17). Each co-activator is regulated through mechanisms involving both phosphorylation and protein-protein interactions (15–21). Additionally specific subunits of the APC are phosphorylated in a cell cycle-dependent manner, which has been shown to enhance binding and activation by Cdc20 (22–24). APC subunit phosphorylation is controlled in large part by mitotic cyclin-dependent kinase activated by the Clb2 cyclin, which is later targeted for destruction by the APC upon exit from mitosis (25, 26). As a result, the APC exists in a hyperphosphorylated state during mitosis and a hypophosphorylated state during G1 phase. Mutation of the cyclin-dependent kinase consensus sites in the APC subunits Cdc16, Cdc27, and Cdc23 results in reduced APC activity toward Clb2 in vivo as well as a delay in the onset of anaphase (25, 26). Currently there is no evidence that mutation of known mitotic APC phosphorylation sites will have detrimental effects on mitotic cell cycle transitions.

In budding yeast the APC is regulated during meiosis not only by Cdc20 and Cdh1 co-activators but also by a meiosis-specific co-activator called Amal (21, 27, 28), which is necessary for spore wall assembly and expression of late meiotic genes (29). AMAL gene transcription and splicing occurs only in meiotic cells (29) and begins during premeiotic S phase (30). However, the co-activator is selectively inhibited early in meiosis by the APC subunit Mnd2 (30, 31).

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‡ The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; APC, anaphase-promoting complex; HPLC, high pressure liquid chromatography; MALDI, matrix-assisted laser desorption ionization; TOF, time of flight; MS, mass spectrometry; GST, glutathione S-transferase; ACON, acetoacetil-CoA; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; SPN, sporulation medium; DAPI, 4',6-diamidino-2-phenylindole; WT, wild type;YPD, yeast peptone dextrose; YP, yeast peptone.
Mnd2 is found in complex with the APC during both the mitotic and meiotic cell cycles (32–34). Yeast lacking Mnd2 proceed normally through the mitotic cell cycle but are unable to complete sporulation, arresting before the first meiotic nuclear division with uncondensed chromatin (35). Recent evidence has shown that Mnd2 specifically inhibits APC activation by Ama1 during prophase I. In cells lacking Mnd2, uninhibited APC\textsuperscript{Ama1} prematurely targets securin (Pds1) for degradation, which results in the premature separation of sister chromatids due to unrestrained separase (Esp1) activity (30), followed by cell cycle arrest. Uninhibited APC\textsuperscript{Ama1} was also found to control the degradation of Clb5 during meiosis. Although inhibited early in meiosis, APC\textsuperscript{Ama1} becomes active during anaphase I despite the presence of Mnd2, suggesting that the inhibitory function of Mnd2 is regulated post-translationally. Furthermore, it has been suggested that regulation of Mnd2 might be linked to its phosphorylation status (30).

In a screen for cell cycle-dependent post-translational modifications that occur on the APC during mitosis, we discovered that Mnd2 is one of five major phosphorylated subunits in the complex. Here we show the identity of Mnd2 phosphorylation sites and their relative changes during the cell cycle using mass spectrometry. To determine the functional significance of Mnd2 phosphorylation in vivo, we generated yeast strains harboring Mnd2 phosphorylation site mutants and then tested these mutants for their ability to progress through mitosis or meiosis. Vegetative growth of mnd2 mutant cells was unperturbed. However, sporulation of a mnd2\textsuperscript{S/T-A} strain was severely compromised, whereas reciprocal mutation to aspartic acid partially suppressed the sporulation defect. The defects in sporulation of the alanine mutant were consistent with those of the deletion strain, including arrest before the first meiotic nuclear division and reduced ability to accumulate Clb5. The sporulation defect of the alanine mutant strain can be explained by the relatively low levels of mnd2\textsuperscript{S/T-A} found in APC co-precipitations compared with the aspartic acid mutant and wild type Mnd2 isoforms in vivo. However, in vitro binding between the APC and each Mnd2 isoform was found to be almost identical, and treatment of the APC with phosphatase did not eliminate Mnd2 co-precipitation, suggesting that phosphorylation may be necessary to maintain the stability of Mnd2 before entry into meiosis. Taken together, these data support a role for mitotic phosphorylation in the regulation of Mnd2 as a APC\textsuperscript{Ama1} inhibitor in early meiosis.

### Experimental Procedures

**Plasmid and Strain Construction**—Standard methods for the growth, maintenance, and transformation of yeast and bacteria and for the manipulation of DNA were used. All constructed plasmids were verified by DNA sequencing. Yeast strains used in this study are listed in Table 1. Strain W1588-4c is a derivative of W303 in which the weak rad5 mutation has been repaired (36). Strains expressing Cdc27 and Mnd2 with C-terminal 3×FLAG epitopes were constructed by integrating PCR amplicons amplified from p3FLAG-KanMX plasmid (a gift from Dr. Toshio Yosukiyama, Fred Hutchinson Cancer Center) into the yeast genome as described previously (37). The Apc1-TAP strain was generated by integration of PCR amplicons from pBS1539 as described previously (38). Construction of phosphorylation site mutations in Mnd2 was accomplished using the QuickChange multisite-directed mutagenesis kit (Stratagene) according to the manufacturer’s specifications (oligonucleotide sequences are available upon request). Mutations were made and sequenced in pIVEX-FLAG-MND2 in which the MND2 open reading frame was originally cloned into the NotI/Sall sites (34). After mutagenesis, the XhoI fragment from pIVEX-FLAG-MND2 or pIVEX-FLAG-mnd2 phosphorylation site mutant (containing all 14 mutations within the last 702 base pairs of the open reading frame) was subcloned into the XhoI site of pMT004, a pRS404 vector containing the first 405 base pairs of the MND2 open reading frame (up to the naturally existing XhoI site) and 500 base pairs of the upstream promoter region. The resulting wild type, S/T-A mutant, and

### Table 1

| Strain          | Relevant genotype                                      | Source          |
|-----------------|--------------------------------------------------------|-----------------|
| W1588-4c\textsuperscript{a} | MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1           | R. Rothstein   |
| DLY300-1353     | MATa cdc27-15 bar1-2 his3-1                            | J. Pringle      |
| RSY333          | MATa cdc27-15 bar1-2 his3-1                            | R. Strich       |
| RSY335          | MATa cdc27-15 bar1-2 his3-1                            | R. Strich       |
| YKA152\textsuperscript{a} | CDC27-3FLAG-KanMX4 bar1-2                              | This Study      |
| YKA155\textsuperscript{a} | CDC27-3FLAG-KanMX4 bar1-2                              | This Study      |
| YKA156\textsuperscript{b} | CDC27-3FLAG-KanMX4 bar1-2                              | This Study      |
| YKA181\textsuperscript{a} | APc1-TAP+ura3 CDC27-3FLAG-KanMX4                        | This Study      |
| YKA191\textsuperscript{a} | APc2-TAP+ura3 CDC27-3FLAG-KanMX4                        | This Study      |
| YKA311\textsuperscript{a} | mnd2Δ::ura3                                              | This Study      |
| YKA312\textsuperscript{a} | mnd2Δ::KanMX4 trp1::pRS404-mnd2(S/T-A)-TRP1            | This Study      |
| YKA313\textsuperscript{a} | mnd2Δ::KanMX4 trp1::pRS404-mnd2(S/T-D)-TRP1            | This Study      |
| YKA314\textsuperscript{a} | mnd2Δ::KanMX4 trp1::pRS404-mnd2(S/T-D)-TRP1            | This Study      |
| YKA315\textsuperscript{a} | mnd2Δ::KanMX4 trp1::pRS404-mnd2(S/T-A)-TRP1            | This Study      |
| YKA316\textsuperscript{a} | mnd2Δ::KanMX4 trp1::pRS404-mnd2(S/T-D)-TRP1            | This Study      |
| YKA318\textsuperscript{a} | mnd2Δ::KanMX4 trp1::pRS404-mnd2(S/T-D)-TRP1            | This Study      |
| YKA321\textsuperscript{a} | CDC27-3FLAG-KanMX4 mnd2Δ                                 | This Study      |
| YKA331\textsuperscript{a} | CDC27-3FLAG-KanMX4 mnd2Δ                                 | This Study      |
| YKA332\textsuperscript{a} | CDC27-3FLAG-KanMX4 mnd2Δ                                 | This Study      |
| YKA333\textsuperscript{a} | CDC27-3FLAG-KanMX4 mnd2Δ                                 | This Study      |

\textsuperscript{a} Derived from W1588-4c, which is a derivative of W303 in which the weak rad5 mutation has been repaired (see “Experimental Procedures”).

\textsuperscript{b} Derived from DLY3033.

\textsuperscript{c} Derived from RSY333.
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S/T-D mutant plasmids were linearized with HindIII and integrated into the trp locus of YKA311 in which the open reading frame for MND2 in the high efficiency sporulating yeast strain RSY333 was replaced with KanMX4 by PCR-mediated transformation (29). Diploid strains were created by transformation with YCp50·HO (HO mothalic switching endonuclease) and selecting for transformants on synthetic dextrose lacking uracil (SD-URA). Successful conversion to diploid was confirmed by flow cytometry.

**Cell Cycle Arrests**—To arrest cells in G₁ phase, cultures were first grown for two generations in YPD medium as measured by absorbance at 600 nm (corresponding to growth from A₆₀₀ = 0.2–0.8) followed by addition of synthetic α-factor peptide (University of North Carolina peptide synthesis facility) to a final concentration of 50 μg/liter. For M phase arrests in the temperature-sensitive strain YKA156, cells were grown for two generations at the permissive temperature (27 °C) and arrested by shifting the temperature to 37 °C, which results in late M phase arrest (39–41). The degree of cell cycle arrest was monitored by phase-contrast microscopy, and cells were harvested after >90% of the culture reached the desired morphology (unbudded for G₁ arrest and equivalently budded for M arrest). Harvested cells were washed once with deionized water, centrifuged once more, and frozen at −80 °C overnight prior to protein purification.

**Affinity Purifications and Phosphatase Assays**—Yeast cells were lysed at 4 °C in prechilled lysis buffer containing 25 mM HEPES-NaOH, pH 7.5 (Fisher), 400 mM NaCl (Sigma), 10% glycerol (Fisher), 0.5 mM dithiothreitol (Sigma), 0.1% Triton X-100 (Sigma), 50 mM NaF (Sigma), 1.3 mM activated sodium ortho-vanadate (Sigma), 50 mM β-glycerophosphate (Spectrum), 0.5 mM phenylmethylsulfonyl fluoride (Roche Applied Science), and Complete protease inhibitor tablets (Roche Applied Science). All purification steps were conducted at 4 °C. Approximately 10¹⁸ cells were lysed with 0.5-mm glass beads in 200 ml of lysis buffer using a bead beater (Biospec Products Inc.) operated for 6–12.5-min pulses with 5-min rest intervals. The resulting extract was cleared by centrifugation at 92,000 × g for 1 h. The cleared extract was incubated on a rotisserie for 3 h with a 200-μl bead volume of EZview Red anti-FLAG M2 affinity matrix (Sigma) equilibrated with lysis buffer. After incubation, the affinity matrix was harvested by centrifugation and washed 4 × 10 min with 25 ml of lysis buffer. For experiments in which the APC was separated by HPLC, the beads were washed another 4 × 10 min with 25 ml of detergent-free buffer containing 25 mM sodium phosphate buffer, pH 7.5, 400 mM NaCl, 10% glycerol, and all phosphatase and protease inhibitors described earlier. The APC was eluted from the affinity matrix by two 10-min incubations at 30 °C and 950-rpm shaking with a bead-equivalent volume of 0.5 mg/ml 3X FLAG peptide (Sigma-Aldrich) dissolved in either lysis buffer or detergent-free sodium phosphate buffer.

Phosphatase assays on full-length protein were conducted on the APC directly bound to the affinity matrix after washing with detergent-free sodium phosphate buffer. The affinity matrix was first conditioned 4 × 7 min with 1 ml of phosphatase buffer containing 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 2 mM MnCl₂. Next the matrix was split, and one-half was treated with 1000 units of λ-phosphatase dissolved in 1 bead-equivalent volume of phosphatase buffer at 30 °C for 30 min. Both halves were then washed 2 × 7 min with 1 ml of detergent-free sodium phosphate buffer, and the APC was eluted as described above. Matrix-assisted laser desorption ionization (MALDI) target phosphatase assays were conducted as described previously (42).

**GST Fusion Protein—**MND2 wild type, S/T-A, or S/T-D genes were subcloned into pGEX-4T1 by PCR from the pRS404 vectors harboring each gene isoform. The resulting N-terminal GST fusion constructs were overexpressed in BL21 pLysS cells according to the manufacturer’s protocol (Stratagene). Cells were lysed by sonication in 1X phosphate-buffered saline and cleared by centrifugation. GST fusion proteins were purified from the cell lysate by batch incubation with glutathione-Sepharose™ 4B (Amersham Biosciences). Each GST fusion protein was digested with trypsin to verify length and sequence by MALDI-time of flight (TOF) MS.

**Chromatography**—Full-length APC subunits eluted from the affinity matrix were fractionated by reverse-phase HPLC using an Agilent 1100 series HPLC instrument coupled to a C₄ Mass Spec reverse-phase column (Vydac), an 1100 series UV spectrophotometer (Agilent), and a Foxy 200 fraction collector (ISCO Inc.). Conditions for HPLC separation were as follows: buffer A, 5% acetonitrile (ACN), 0.1% trifluoroacetic acid; buffer B, 95% ACN, 0.09% trifluoroacetic acid; gradient, 5% B for 5 min, 5–65% B in 65 min, and 65–95% B in 10 min. Fractions were collected every minute, frozen at −80 °C, and lyophilized.

In-gel digestion with porcine trypsin was conducted as described previously (43). Separation of tryptic peptides was accomplished by capillary HPLC using an Agilent 1100 series capillary liquid chromatography system with PepMap C₁₈ column (LC Packings) connected in line with a Probot MALDI target microfraction collector (LC Packings) (44).

**Mass Spectrometry**—Lyophilized C₄ HPLC fractions containing full-length Mnd2 were reconstituted in 5 μl of 85% ACN, 0.1% trifluoroacetic acid, and one-tenth of the sample was prepared for MALDI-TOF MS by the dried droplet method with a saturated solution of sinapic acid (Fluka) in 50% ACN, 0.1% trifluoroacetic acid. The reconstituted sample was then analyzed directly on a Reflex III MALDI-TOF mass spectrometer (Bruker Daltonics Inc.) operating in linear mode. Linear mode MALDI-TOF mass spectra were obtained immediately after calibrating the mass spectrometer using a mixture of bovine serum albumin (Sigma) and cytochrome c (Sigma) that was spotted in close proximity to the sample. Liquid chromatography microfractions were analyzed on an ABI 4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems Inc.) using a saturated solution of recrystallized α-cyano-4-hydroxycinnamic acid in 50% ACN, 0.1% trifluoroacetic acid as the matrix.

**In Vivo ³²P Labeling**—Yeast strains were labeled with monosodium [³²P]phosphate in vivo as described previously (26). Briefly saturated overnight 5-ml cultures were diluted to A₆₀₀ = 0.2 in 50 ml of YPD and allowed to grow to A₆₀₀ = 0.6 at which time Me₂SO was added to 1%. At A₆₀₀ = 0.8, each culture was arrested in M phase by addition of nocodazole to 15 μg/ml or in G₁ phase by addition of α-factor as described above (see “Cell Cycle Arrests”). After arresting for 3 h, cells were har-
vested by centrifugation, washed once with sterile water, and resuspended in 35 ml of synthetic complete medium lacking phosphate and containing Me₆SO, nocodazole, and 1 mM of NaH₂¹⁸PO₄ (ICN Inc.). After labeling for 1 h at 30 °C, cells were collected by centrifugation, washed once with water, transferred to a 2-ml screw cap tube, and frozen at −80 °C.

Cells were lysed by adding cell pellet-equivalent volumes of lysis buffer and 0.5-mm glass beads to the pellet and vortexing 6 × 2 min at maximum power with 5-min rest intervals on ice. All following steps were conducted at 4 °C. The extract was cleared by centrifugation in a microcentrifuge at 16,100 × g for 20 min, and the supernatant was transferred to a fresh 2-ml screw cap tube followed by affinity purification as described above. The APC was eluted as described above and then precipitated to remove excess volume and salt by adding 10 volumes of cold acetone and incubating at −20 °C overnight. The precipitate was collected by centrifugation at 16,100 × g for 20 min, air-dried at room temperature, and then resuspended in 1× lithium dodecyl sulfate loading buffer (Invitrogen Inc.) containing 100 mM dithiothreitol. The sample was separated on a precast 4–12% BisTris acrylamide gel (Invitrogen), dried, and exposed to a storage phosphor screen for analysis by PhosphorImager (Storm, Amersham Biosciences).

**Growth Curves, Sporulation Assays, and Western Blotting—**
To measure relative growth rates of strains harboring mnd2 phosphorylation site mutants or complete open reading frame deletions, saturated overnight cultures grown in YPD medium were diluted 20-fold into 5 ml of fresh YPD medium and grown for ∼1 or 3 h at 30 or 37 °C, respectively. Each culture was then diluted to A₆₀₀ = 0.025 (∼3 × 10⁴ cells/ml) in three separate tubes with 5 ml of fresh YPD medium and grown at 30 or 37 °C for 8–9 h. Growth was monitored every 2 h by measuring the absorbance at 660 nm with a SmartSpec 3000 spectrophotometer (Bio-Rad).

To induce sporulation, single colonies picked from YP-glycerol agar plates were spread over the entire surface of a fresh YPD agar plate and grown for 24 h at 30 °C, ensuring that overnight growth saturated the YPD plate. All following steps were conducted at 30 °C. The YPD plates were replica-plated to pre-sporulation agar plates (1% potassium acetate, 1% yeast extract, 2% peptone) until >95% of the cell population were unbudded (23–24 h) as counted using a hemacytometer followed by replica-plating to sporulation medium (SPM) agar plates (1% potassium acetate, 0.1% yeast extract, 0.05% dextrose). The percentage of tetrads was monitored by light microscopy using a hemacytometer. Cells were collected by gently scraping a fraction of the cells followed by resuspension in water, centrifugation, snap freezing the pellet in a dry ice/ethanol bath, and storage at −80 °C (for Western blots). Additionally an aliquot of the scraped cells was resuspended in 70% ethanol (for fluorescence-activated cell sorting or fluorescence microscopy).

Protein levels were analyzed by Western blotting after breaking cells with glass beads in trichloroacetic acid lysis buffer (10 mM Tris-HCl, pH 8.0, 25 mM ammonium acetate, 1 mM Na₂EDTA, 10% trichloroacetic acid). The trichloroacetic acid-precipitated protein pellet was dissolved in resuspension solution (3% SDS, 100 mM Tris-HCl, pH 11.0), and the total protein concentration of each sample was quantified by absorbance at 750 nm using a DC protein assay kit (Bio-Rad). For normalized comparison, 40 μg of total protein was loaded in each lane. Primary antibodies to Cb5 (Santa Cruz Biotechnology sc-20170; 1:1000) or custom rabbit Mnd2 N terminus antibodies (α-Mnd2) (Sigma Genosys) were used in PBS-T (1× phosphate-buffered saline, 0.1% Tween) with 5% nonfat dried milk.

Detection of bound primary antibody was accomplished with an ECL-Plus chemiluminescence detection kit (Amersham Biosciences) and horseradish peroxidase-conjugated goat antirabbit secondary antibody (Chemicon International). The custom antibody was verified to bind each Mnd2 isoform equivalently for Western blot detection.

**Fluorescence Microscopy and Flow Cytometry—**
Cells fixed in 70% ethanol were harvested by centrifugation, washed twice, and resuspended in 1× phosphate-buffered saline at 25 °C. Approximately 10 μl of the cell suspension was adsorbed onto a poly(l-lysine) glass microscope slide for 5 min after which the excess suspension was removed and replaced with 10 μl of DAPI solution (2 μg/ml) for 5 min. After removal of the excess DAPI solution, each sample was washed twice for 2 min with 30 μl of 1× phosphate-buffered saline and allowed to dry at room temperature. Finally 10 μl of mounting solution (90% glycerol, 1 mg/ml p-phenylenediamine, 20 ng/ml DAPI) was added to the top of each sample, covered with a coverslip, and compressed with weight for at least 1 h. Cells were imaged by differential interference contrast or by fluorescence detection of DAPI-stained nuclei. Measurements of cellular DNA content were collected on a FACScan flow cytometer as described previously (34).

**RESULTS**

**Mnd2 Is Hyperphosphorylated in Vivo during Mitosis—**
Recent mass spectrometric analyses have shown that at least five of 11 different human APC subunits are phosphorylated on a total of 43 different Ser or Thr residues, many of which are differentially phosphorylated in a cell cycle-dependent manner (45). In comparison, only three of 13 APC subunits in yeast have been shown to be phosphorylated in vivo (25, 26), and although specific sites have been inferred from canonical cyclin-dependant kinase consensus sequences, there has not been a focused proteomics scale analysis of APC subunits thus far. Therefore, to reveal the presence of previously unknown post-translational modifications on the APC (including phosphorylation), we measured the full-length molecular weights of individual APC subunits by MALDI-TOF MS and compared these measurements to the calculated masses of each subunit based on sequence information found in the *Saccharomyces* Genome Database (www.yeastgenome.org/). When conducting these analyses with APC purified from yeast arrested in M phase with nocodazole we discovered a 392-Da positive shift (m/z 43,217) in the apex mass of the subunit Mnd2 with respect to the calculated molecular mass (m/z 42,825 Da), suggesting that Mnd2 was post-translationally modified in M phase. We repeated this experiment with cells arrested in G₁ phase and found only a modest positive mass shift of 94 Da (m/z 42,919) relative to the calculated molecular weight (Fig. 1A). The mass deviation of Mnd2 between G₁ and M phases (m/z₉₁ - m/z₇₄ = 298) was the largest of any subunit we measured (data not
shown) and suggested that the modification was cell-cycle-dependent. Next we determined whether Mnd2 could be metabolically labeled with NaH$_2$$_{32}$PO$_4$ in yeast arrested in M phase with nocodazole or in G1 with /H$_9$251-factor. A comparison of FLAG affinity purifications from metabolically labeled yeast with or without a C-terminal 3× FLAG tag on the APC subunit Cdc27 is shown (Fig. 1B). Similar to previous reports, APC subunit phosphorylation was much higher in M phase compared with G$_1$ phase. In nocodazole-arrested cells, we observed six distinct bands unique to the tagged strain that did not appear in the purification from a strain lacking a 3× FLAG tag. Alignment of the phosphoimage with a Coomassie-stained large scale purification of the APC from nocodazole-arrested cells revealed the identity of the known phosphorylated subunits Cdc16, Cdc27, and Cdc23. In addition, we observed phosphoproteins that aligned with Apc1, Apc2, and Mnd2. To verify the identity of these three phosphoproteins, we introduced epitope tags onto the C termini of Apc1 (Apc1-TAP in Cdc27-3× FLAG background), Mnd2 (Mnd2-3× FLAG), and Apc2 (Apc2-TAP in Mnd2-3× FLAG background), which would create a gel mobility shift in the $^{32}$P-labeled phosphoprotein band in question if the identity was assigned correctly. Each tagged strain was metabolically labeled in the presence of nocodazole, and the resulting FLAG affinity purification was compared with the original Cdc27-3× FLAG $^{32}$P-labeled purification. We observed a shift in gel mobility for the phosphoproteins provisionally assigned to Apc1 and Mnd2 (Fig. 1C). However, no shift was observed for the band assigned to Apc2 (data not shown).

To determine whether the apex mass shift observed between the Mnd2 MALDI mass spectra was completely or partially due to phosphorylation and to estimate the number of phosphorylation sites involved, we compared the full-length molecular weights of Mnd2 in M phase by MALDI-TOF MS before and after treatment with /H$_9$261-phosphatase. Nocodazole induces a mitotic checkpoint arrest by inhibiting microtubule polymerization, which may induce phosphorylation of proteins that would not normally be phosphorylated during an unperturbed mitotic progression. Therefore, in this experiment, we
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opted to use a strain that harbors a temperature-sensitive mutation in the mitotic exit network kinase Cdc15. We have used this strain successfully in the past to characterize phosphorylation of the APC co-activator Cdh1 (46). APC purifications from yeast arrested in G1 or M phase cells were split, and half of each purification was treated with λ-phosphatase. The apex mass of Mnd2 from M phase cells was 43,221 Da before phosphatase treatment and shifted to a mass of 42,837 Da after phosphatase treatment; this is within 12 Da of the calculated mass of the unmodified protein and indicated that the entire mass shift observed in M phase is due to phosphorylation (Fig. 1D). In comparison, the apex mass of Mnd2 from G1 phase cells was 42,926 Da before phosphatase treatment and was shifted to 42,825 Da after phosphatase treatment, indicating that Mnd2 is less phosphorylated during G1 phase compared with M phase. Dividing the average mass difference between the measurements made before and after phosphatase treatment (Δm = m_p − m_ap) by the mass of a single phosphorylation modification (HPO3− = 80 Da) indicated that Mnd2 was phosphorylated on at least four to five different sites (384 ± 80 Da) during M phase and only one site on average (101 ± 80 Da) during G1 phase. Furthermore the 283-Da mass difference observed between the modified states of Mnd2 measured in the phosphatase assay corresponded very well with the measured mass difference of 298 Da determined with Mnd2 purified from nocodazole-arrested cells. Although these data indicate a specific number of phosphate modifications on Mnd2, it has been our experience that these values are usually underestimates of the total number of phosphorylation sites of a protein possibly due to modification site heterogeneity and also due to the resolution of MALDI-TOF MS in the linear mode. Attempts to measure the intact protein mass by electrospray ionization MS were not successful. We conclude that Apc1 and Mnd2 are phosphorylated subunits of the budding yeast APC. We further measure the intact protein mass by electrospray ionization MS to their high Asp/Glu content, which can affect peptide ionization in the mass spectrometer, as well as phosphorylation, which can also affect peptide ionization and inhibit tryptic digestion at cleavage sites that neighbor a phosphorylated residue.

We used linear mode MALDI-TOF MS, which allows the detection of high mass protein species, to search for the large trypptic peptide Mnd2-(170–255) as well as any misnamed trypptic peptides that contained Mnd2-(287–301). Using this approach, four major species of interest were detected at m/z 6618, 6688, 8393, and 9838, none of which could be assigned to the unmodified Mnd2 sequence. Comparison of the spectra with and without phosphatase treatment showed that each of the four species was phosphorylated as indicated by negative shifts in the apex mass after phosphatase treatment (Fig. 2C). Using the apex mass of the ion species after phosphatase treatment, we were able to assign each peptide to Mnd2 as well as calculate the average number of occupied phosphorylation sites within each peptide (Table 2). The trypptic peptides assigned to m/z 6618, 6688, and 8393 each contained multiple missed cleavage sites and included the tryptic region Mnd2-(287–301) with one or two occupied phosphorylation sites. Because we were able to detect each of the tryptic peptides flanking the Mnd2-(287–301) region in the reflectron mode MS analysis and confirm that they were unmodified, we concluded that the occupied phosphorylation sites are Ser292 and Ser300. The ion at m/z 9838 was assigned to the largest trypptic peptide, Mnd2-(170–255), and contained an average of four occupied phosphorylation sites. The mass accuracy of this assignment was within 7 Da of the expected mass, likely reflecting an average of a mixture of the unmodified peptide (m/z 9519) and a mono-oxidized peptide (m/z 9535) because no other peptides with up to 10 missed cleavages could be closely matched to this ion. Calculation of the apex mass shift after phosphatase treatment indicated that Mnd2-(170–255) contains an average of four phosphorylated sites out of eight potential serine or threonine phosphorylation sites. A comparison of these phosphopeptides in G1 phase showed that Mnd2 maintains partial phosphorylation (Fig. 2D). In summary, we found that Mnd2 is phosphorylated on at least eight different sites during mitosis and at least four sites during G1 phase.

Yeast Harboring Mnd2 Phosphorylation Site Mutations Grow Normally—Cell cycle-dependent phosphorylation is an important component of APC-mediated cell cycle progression. Yeast...
FIGURE 2. MS identification of Mnd2 phosphopeptides purified from M phase cells. A, tandem MS spectrum of m/z 2823.1 corresponding to the monophosphopeptide Mnd2-(140–162). B, comparison of phosphorylation at Ser156 in M phase (cells arrested with temperature shift) and G1 phase. In this experiment, m/z 2823.1 was not found; rather a shorter form (Mnd2-(153–162)) containing only one missed cleavage was found that was confirmed by tandem MS. C, overlaid linear mode MALDI-TOF mass spectra of high molecular weight tryptic Mnd2 phosphopeptides from M phase-arrested cells before (solid line) and after (dashed line) treatment with alkaline phosphatase (AP). The average estimation of occupied phosphorylation sites is indicated by the shift after treatment with alkaline phosphatase. Note that the alkaline phosphatase-treated masses are all oxidized. D, same as C but from G1 phase-arrested cells. Note that the alkaline phosphatase-treated peptide Mnd2-(170–255) contains two oxidized methionine residues rather than one, resulting in a slight shift in the mass spectra compared with that in C. pS, phosphoserine.
of WT, station is required for sporulation, we compared the phenotypes of premeiotic S phase but before the first meiotic nuclear division. Mitotic Mnd2 phosphorylation is required for progression of mitosis. APC processivity (33, 34). Growth rates for all but the apc10Δ strain were indistinguishable at both 30 and 37 °C (Fig. 3). This observation was not surprising because the mnd2Δ strain displays no significant difference in growth rate compared with the expected average masses (m) shown. The mass accuracy is defined as the difference between the phosphorylated peptides and the expected average mass (mexp - m) calculated from the expected phosphorylation mass. pS, phosphoserine; Calc. avg., calculated average; MetOx, oxidized methionine. ±1 S.D. (left) and ±2 S.D. (right). Data were averaged from triplicate experiments.

Mitotic Mnd2 Phosphorylation Is Required for Progression through the First Meiotic Nuclear Division—Yeast lacking Mnd2 are incapable of sporulation and arrest after the completion of premeiotic S phase but before the first meiotic nuclear division (30, 31, 35). To determine whether Mnd2 phosphorylation is required for sporulation, we compared the phenotypes of WT, mnd2Δ, mnd2Δ-(S/T-A), and mnd2Δ-(S/T-D) strains upon induction of sporulation. When comparing the kinetics of tetrad formation for each strain, we found that 85% of WT cells formed complete tetrads within 42 h after plating to sporulation medium; this was nearly identical to the sporulation percentage for cells harboring MND2 at its natural genetic locus (Fig. 4A). In contrast, mnd2Δ strains were unable to form tetrads. Only 13% of cells harboring mnd2Δ-(S/T-A) formed tetrads, whereas the percentage of tetrads increased to 42% of cells harboring mnd2Δ-(S/T-D). In addition, as observed by light microscopy, there was no appreciable accumulation of cells containing only two spores (dyads), which would have indicated arrest between the first and second nuclear division. The differences in tetrad formation between the WT and mutant strains was not due to a failure in meiotic entry because each strain completed premeiotic S phase as measured by flow cytometry (Fig. 4B).

Quantitation of tetrad formation cannot be used as an indicator for the ability of cells to complete meiotic nuclear divisions. Indeed meiotic nuclear divisions and spore formation are not completely connected pathways, and it is possible to achieve nuclear divisions without forming spores, which has been reported for subpopulations of ama1Δ cells (29). Cells that do not undergo the first meiotic nuclear division arrest with a single nucleus that can be visualized using fluorescence microscopy of DAPI-stained nuclei. To determine whether meiotic nuclear divisions were compromised in the mnd2Δ

### TABLE 2
Assignment of Mnd2 phosphopeptides

| Observed masses | Δm (= m~p~ - m~dp~) | Avg. no. HPO~3~ | Assigned Mnd2 peptide sequence | Add. mod. | Calc. avg. mass (m) | Error (m~exp~ - m) |
|----------------|---------------------|----------------|-----------------------------|---------|-------------------|-----------------|
| 1808.8         | 1                   | 1              | 259TVPPDDLMLRTPSLR259       |         | 1808.8            | 0.09            |
| 2823.1         | 1                   | 1              | 144AQANIDNNEEPDFQHPSPSEEDPR1462|         | 2823.1            | 0.01            |
| 6618           | 87                  | 1              | 24NPYDIDSGDDESEVILMNPFDDED  | 1 MetOx | 6531              | 0               |
| 6688           | 157                 | 2              | 28NPYDIDSGDDESEVILMNPFDDED  | 1 MetOx | 6531              | 0               |
| 8393           | 168                 | 2              | 271SLQQPVEEAIHRDNFYIDNNDGED | 1 MetOx | 8223              | 2               |
| 9838           | 306                 | 4              | 0–1 MetOx                   |         | 9525              | 7               |

Masses of the dephosphorylated peptides were used for assigning Mnd2 sequence to each MS ion with the exception of m/z 1808.8 and m/z 2823.1 whose error was calculated from the expected phosphorylated mass. pS, phosphoserine; Calc. avg., calculated average; MetOx, oxidized methionine. ±1 S.D. (left) and ±2 S.D. (right). Data were averaged from triplicate experiments.
Mnd2 Phosphorylation Is Necessary for Meiosis I Progression

Phosphomutant strains, we scored the percentages of non-tetrad-forming (residual) cells that contained a single undivided nucleus after 48 h on sporulation medium. In strains harboring mutant or wild type Mnd2, the percentage of non-tetrad cells containing a single undivided nucleus was greater than 90% (Fig. 4C). Because tetrads are formed at low levels in strains harboring mnd2-(S/T-A), we also quantified the viability of tetrad spores. A high percentage of spore viability indicates that cells are capable of proper nuclear divisions that distribute a complete complement of the haploid genome to each spore. Low spore viability is observed when nuclear divisions are compromised such that individual spores are left with an incomplete complement of the haploid genome. Following tetrad dissection, 95% of WT spores were viable compared with 89 and 84% spore viability for mnd2-(S/T-D) and mnd2-(S/T-A) cells, respectively (Fig. 4C).

The proteins Pds1 and Clb5 fail to accumulate appreciably in sporulating mnd2Δ cells due to unrestrained APC\(^{\text{Ama1}}\) activity during meiosis (30, 31). To ascertain whether the phosphorylation status of Mnd2 affects its function as an APC\(^{\text{Ama1}}\) inhibitor, we compared the protein stability of Clb5 in cell extracts taken from a sporulation time course. Sporulating cells were collected at 0, 24, and 48 h, and the percentages of tetrad-forming cells and cells with replicated DNA were quantified to verify synchrony and meiotic entry of each strain (Fig. 4D, top). Clb5 levels were low to immeasurable in the mnd2Δ strain compared with the WT strain (Fig. 4D, bottom). Very little accumulation of Clb5 was observed for the mnd2-(S/T-A) strain, but accumulation of Clb5 was noticeably higher in the mnd2-(S/T-D) strain. A similar trend was observed for Pds1 (data not shown). Taken together, these data show that phosphorylation of Mnd2 is critical for efficient progression through the first meiotic nuclear division and for normal accumulation of the APC\(^{\text{Ama1}}\) substrate, Clb5. We conclude that phosphorylation of Mnd2 is necessary for inhibition of APC\(^{\text{Ama1}}\) in early meiosis.

Mnd2 Phosphomutants Display Differential Co-precipitation with the APC in Vivo but Not in Vitro—Although mitotic phosphorylation of Mnd2 is necessary for progression through early meiosis, it is clearly not essential for normal cell growth, making it difficult to explain the reason why Mnd2 phosphorylation occurs during the mitotic cell cycle. Previous reports suggest that Mnd2 can only inhibit the APC when it is present on the complex (30). Therefore, one possible explanation for our results is that phosphorylation of Mnd2 affects its binding to the APC. First we compared the levels of each Mnd2 isoform after affinity purification of the APC from WT, mnd2Δ, mnd2-(S/T-A), or mnd2-(S/T-D) strains (Fig. 5A). Comparative Western blot analysis revealed that the alanine mutant was undetectable, similar to an mnd2Δ strain. In contrast, the aspartic acid mutant was detectable but at a lower level than the WT isoform. Together the relative difference in abundance of each Mnd2 isoform was directly proportional to the observed relative difference in sporulation and Clb5 levels for each MND2 isoform strain (compare with Fig. 4, A and D). Interestingly the abundance of WT Mnd2 in these experiments was low when compared with a WT strain expressing Mnd2 from its endogenous genetic locus (data not shown) in which case Mnd2 is stoichiometric with other APC subunits (48). This suggests that substoichiometric levels of Mnd2 are sufficient to inhibit APC\(^{\text{Ama1}}\) and allow normal meiotic progression because our WT integrant strain exhibited identical sporulation kinetics compared with the WT strain where Mnd2 was expressed from its endogenous genetic locus.

In contrast to our in vivo data, we found that binding between APC\(^{\text{mnd2Δ}}\) and recombinant WT GST-Mnd2, GST-mnd2-(S/T-A), or GST-mnd2-(S/T-D) was almost constant in comparison with a control in which only GST was present (Fig. 5B). In fact, binding of the aspartic acid isoform was slightly weaker than either the WT or alanine isoform, which is counter to what would be expected based on the more proficient sporulation of yeast harboring the aspartic acid mutant. These in vitro binding results support our earlier data, which showed that Mnd2 remains associated with the APC before and after phosphatase treatment (Fig. 1D). Taken together, these data suggest that mitotic phosphorylation is not required for association of Mnd2 with the APC and that binding between the APC and mnd2-(S/T-A) or mnd2-(S/T-D) is similar in vitro. Furthermore the difference in results for the in vivo co-precipitation assay versus the in vitro binding assay suggests that mitotic Mnd2 phosphorylation may function in an unknown regulatory mechanism that is necessary to ensure proper levels of APC-bound Mnd2 prior to meiotic entry when APC\(^{\text{Ama1}}\) becomes active.

**DISCUSSION**

We used a mass spectrometric approach to discover and identify mitotic phosphorylation sites on the APC subunit Mnd2. The phosphorylation status of Mnd2 appears to be generally similar to that of other phosphorylated yeast APC subunits (Cdc16, Cdc27, and Cdc23) in that it was higher in mitosis compared with G1 phase. Interestingly none of the phosphopeptides observed contain the canonical cyclin-dependent kinase recognition sequence (S/T)P suggesting the involvement of other kinases. Mutation of Mnd2 phosphorylation sites had no effect on mitotic cell cycle progression; this is not surprising considering that Mnd2 has no required function during vegetative cell growth. However, there was a striking difference in the ability of Mnd2 phosphorylation site mutants to complete meiosis. The similarities in sporulation defect for the alanine and deletion mutant strains strongly suggest that phosphorylation is critical for Mnd2 function early in the meiotic program. Both strains completed premeiotic S phase, and like the deletion strain, the majority of alanine mutants failed to undergo the first meiotic nuclear division. In addition to a meiotic sporulation defect, we also found that mnd2-(S/T-A) mutants have lower levels of the APC\(^{\text{Ama1}}\) substrate, Clb5, which suggests that mitotic phosphorylation of Mnd2 is necessary to maintain its APC inhibitory function rather than an effect unrelated to APC activity. Mutation of each phosphorylation site to aspartic acid, which may mimic the phosphorylated state of Mnd2, partially suppressed the sporulation defect and further supported the conclusion that phosphorylation is necessary for Mnd2 function in early meiosis.
Mnd2 Phosphorylation Is Necessary for Meiosis I Progression

(A) % Tetrad vs. Time (hrs)
(B) % Cells with Replicated DNA vs. Time (hrs)

C

|          | DIC | DAPI | % Tetrad | % Residual Cells with Single Nucleus | Spore Viability |
|----------|-----|------|----------|-------------------------------------|-----------------|
| WT       |     |      | 80%      | 100%                                | 95%             |
| S/T-D    |     |      | 37%      | 93%                                 | 89%             |
| S/T-A    |     |      | 7%       | 91%                                 | 84%             |
| mnd2Δ    |     |      | 0%       | 84%                                 | ---             |

(D) % Tetrad vs. Time (hrs)

Loading Control

Normalized Band Intensity vs. Time (hrs)
The difference in sporulation of strains harboring different Mnd2 isoforms does not appear to be due to a difference in binding between the APC and the Mnd2. In vitro, WT Mnd2, mnd2-(S/T-A), and mnd2-(S/T-D) are each capable of binding the APC with similar affinities. Furthermore phosphorylation of WT Mnd2 is not necessary for its association with the APC because treatment of M phase or G1 phase APC with phosphatase did not eliminate Mnd2 binding. In vivo, the co-precipitation of each Mnd2 isoform with the APC was strikingly different and correlated well with the observed differences in sporulation and Clb5 levels for each strain. Taken together, these data do not support a role for mitotic Mnd2 phosphorylation in the direct regulation of APC\textsuperscript{Ama1} ubiquitin ligase activity. Rather mitotic Mnd2 phosphorylation appears to be involved in a mitotic process that is independent of Ama1 but that has significant implications on the cell cycle once Ama1 is expressed at the beginning of meiosis, similar to the defects observed when Mnd2 was absent.

Despite its overall similarities to the deletion strain, some cells harboring mnd2-(S/T-A) are still capable of complete sporulation resulting in the formation of viable haploid spores. Thus, eliminating mitotic Mnd2 phosphorylation at the sites we discovered did not completely abolish the inhibitory function of Mnd2. Furthermore the high percentage of spore viability in the phosphomutant strains also suggests that mitotic phosphorylation of Mnd2 is only necessary before the first meiotic nuclear division because mutant cells that do form tetrads must be capable of completing normal meiotic nuclear divisions and spore formation. This observation is consistent with existing data that show that Mnd2 is necessary for the completion of recombination before the first meiotic nuclear division (30, 31). Conversely cells harboring mnd2-(S/T-D) were not capable of completely suppressing the sporulation defect of mnd2Δ cells; this may be due to insufficient mimicry of phosphorylation by the side chain of aspartic acid.

Two different hypotheses could explain the difference in APC/Mnd2 association observed in our in vivo co-precipitation experiments compared with our in vitro APC binding assay. 1) Mitotic phosphorylation may be necessary to block the association of “seclusion factors” that could function to inhibit the association of Mnd2 with the APC in vivo. 2) Phosphorylation may be necessary to inhibit the degradation of Mnd2 during mitosis. Currently there is no evidence in support of the first hypothesis, although it has never been tested directly. With regard to the second hypothesis, others have found that phosphorylation can protect proteins from various forms of degradation including direct enzymatic proteolysis (49, 50) as well as ubiquitin-mediated proteolysis (51, 52). Consistent with this hypothesis, a decrease in the stability of Mnd2 would be expected to mimic an mnd2Δ sporulation defect due to uninhibited APC\textsuperscript{Ama1}, similar to what we saw with the mnd2-(S/T-A) mutant. In addition, evidence for Mnd2 regulation by degradation has been suggested previously (30, 31) where it was shown that the inhibitory function of Mnd2 is regulated in part by degradation that occurs late in meiosis after anaphase II and that may require Mnd2 phosphorylation (30, 31). A mechanism similar to that which occurs in late meiosis may also exist during mitosis and would provide an interesting explanation for the role of mitotic Mnd2 phosphorylation.

The kinase(s) that phosphorylate Mnd2 remain unknown. Indeed the phosphorylation of Mnd2 during mitosis may not be caused by the same kinases responsible for its phosphorylation during meiosis. Currently the primary kinases known to phosphorylate the APC are cyclin-dependent kinase (Cdc28 in yeast) and polo-like kinase Cdc5. However, none of the Mnd2 phosphopeptides that we observed contained either a canonical cyclin-dependent kinase recognition sequence (S/T)P or the polo box domain recognition sequence (S/pS/pT)P where pT is phosphothreonine. Notably many of the phosphorylation sites implicated by our MS analysis fell within regions of high Asp/Glu residue content, which could suggest phosphorylation by casein kinase 2 (S3).
Mnda2 Phosphorylation Is Necessary for Meiosis I Progression

In addition to our discovery of Mnda2 phosphorylation, we also showed that Apc1 is phosphorylated during mitosis. Preliminary experiments showed that Apc1 is phosphorylated on a number of sites that are contained within a canonical cyclin-dependent kinase recognition sequence as well as some sites that are not. Further work will focus on characterizing these sites at different cell cycle stages and determining their role in APC-mediated mitotic and meiotic progression.

We showed that phosphorylation sites occupied differentially during the mitotic cell cycle can provide critical regulatory roles for the APC during meiosis. To our knowledge, this is the first case in which mitotic APC phosphorylation site mutants have been tested in meiosis. The fact that phosphorylation sites between the two types of nuclear division are shared in this way suggests that known mitotic phosphorylation sites within other APC subunits may also be required for meiosis. Future experiments that characterize the dynamics of APC phosphorylation during mitosis and meiosis will likely be invaluable to our understanding of APC regulation.

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