

Cdc15 Is Required for Spore Morphogenesis Independently of Cdc14 in *Saccharomyces cerevisiae*

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ABSTRACT

In *Saccharomyces cerevisiae* exit from mitosis requires the Cdc14 phosphatase to reverse CDK-mediated phosphorylation. Cdc14 is released from the nucleolus by the Cdc14 early anaphase release (FEAR) and mitotic exit network (MEN) pathways. In meiosis, the FEAR pathway is essential for exit from anaphase I. The MEN component Cdc15 is required for the formation of mature spores. To analyze the role of Cdc15 during sporulation, a conditional mutant in which *CDC15* expression was controlled by the *CLB2* promoter was used. Cdc15-depleted cells proceeded normally through the meiotic divisions but were unable to properly disassemble meiosis II spindles. The morphology of the prospore membrane was aberrant and failed to capture the nuclear lobes. Cdc15 was not required for Cdc14 release from the nucleoli, but it was essential to maintain Cdc14 released and for its nucleo-cytoplasmic transport. However, cells carrying a *CDC14* allele with defects in nuclear export (Cdc14- Δ NES) were able to disassemble the spindle and to complete spore formation, suggesting that the Cdc14 nuclear export defect was not the cause of the phenotypes observed in *cdc15* mutants.

MEIOSIS is a specialized cell division that produces the haploid cells needed for sexual reproduction, and the completion of meiosis is normally coupled to differentiation programs that produce gametes. In *Saccharomyces cerevisiae*, sporulation is initiated when diploid cells are grown in the absence of nitrogen and the presence of a nonfermentable carbon source such as acetate (ESPOSITO and KLAPHOLTZ 1981). When triggered to enter the sporulation program, cells exit the mitotic cycle from the G1 phase. This is followed by one round of S phase and two meiotic divisions that generate four haploid nuclei, which are packaged into individual spores. As a consequence of this process, four haploid gametes (spores) are formed in the cytoplasm of the mother cell (ascus) (reviewed by NEIMAN 2005). At the onset of meiosis II, the spindle pole body (SPB) is modified by the incorporation of several meiosis-specific proteins, which together make up the meiosis II outer plaque (KNOP and STRASSER 2000; BAJGIER *et al.* 2001; NICKAS *et al.* 2003). This change in composition converts the function of the cytoplasmic face of the SPB from microtubule nucleation to membrane nucleation. The process of spore construction requires the *de novo* as-

sembly of two cellular structures, the prospore membrane (PSM) that is generated around the daughter nuclei to create prospores and a protective spore wall that surrounds the prospores. Once the meiosis II outer plaque of the SPB has been assembled, it becomes a site for the docking and coalescence of secretory vesicles to form flattened double-membrane sheets termed PSMs (MOENS 1971; NEIMAN 1998). These nascent PSMs expand during meiosis II, and at the time of nuclear division each PSM completely engulfs the nuclear lobe to which it is anchored via the SPB. At the completion of meiosis II, each PSM fuses with itself so that each daughter nucleus (and associated cytoplasm) is captured inside the PSM, creating immature spores. The prospores mature into spores through the synthesis of a specific spore wall, which initially occurs in the lumen between the two PSMs (reviewed by NEIMAN 2005).

During the mitotic cycle, nuclear division is tightly linked to cytokinesis, the final event of the cell cycle that gives rise to two daughter cells. Exit from mitosis is brought about by the inactivation of mitotic cyclin-dependent kinase (CDK) activity. In *S. cerevisiae*, the Cdc14 protein is a temporally and spatially regulated phosphatase that mediates mitotic exit by promoting CDK inactivation and by dephosphorylating CDK substrates (reviewed by BARDIN and AMON 2001; SURANA *et al.* 2002). Cdc14 is sequestered in the nucleolus

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throughout most of the cell cycle (SHOU *et al.* 1999; VISINTIN *et al.* 1999). The Cdc14 early anaphase release network (FEAR) and mitotic exit network (MEN) have been shown to act in a coordinated fashion to trigger exit from mitosis by mediating the release of Cdc14 from the nucleolus. Cdc14 is transiently released in early anaphase by the actions of the FEAR pathway, and the MEN allows levels of nuclear Cdc14 to remain high until mitotic exit is complete (reviewed by BARDIN and AMON 2001; MCCOLLUM and GOULD 2001; D'AMOURS and AMON 2004). In addition to mediating Cdc14 release at different times, both pathways have been shown to affect different processes. The FEAR pathway is critical for nuclear positioning in anaphase while the MEN is essential for mitotic exit. The MEN is a signal transduction pathway composed of the GTP-binding protein Tem1 and its putative guanine nucleotide exchange factor Lte1, the protein kinases Cdc5, Cdc15 and Dbf2/Dbf20, the Dbf2-associated factor Mob1, and the SPB protein Nud1 (reviewed by BARDIN and AMON 2001). Conditional lethal temperature-sensitive (ts) mutations in any of the MEN genes cause cells to arrest in late mitosis with elevated mitotic CDK activity when shifted to the restrictive temperature.

Recent studies have suggested that the roles of Cdc14 and the FEAR network during meiosis I are similar to their roles in mitosis. Cdc14 release by the FEAR pathway is essential for the inactivation of Clb-CDKs and exit from meiosis I (BUONOMO *et al.* 2003; MARSTON *et al.* 2003). FEAR mutants (such as *slk19Δ* and *spo12Δ*) are unable to disassemble the spindle at anaphase I. The FEAR pathway performs its meiotic role by regulating Cdc14 release from the nucleolus. Conversely, MEN genes are not required for exit from meiosis I (KAMIENIECKI *et al.* 2005). Cdc15 or Tem1-depleted cells are able to complete the two meiotic divisions with similar efficiencies to wild-type cells. Interestingly, Cdc15-depleted cells—but not Tem1-depleted cells or *lte1* mutants—fail to form mature spores, suggesting that Cdc15, but not Tem1 and Lte1, plays a role in linking exit from meiosis II to spore differentiation.

Here we analyze the role of Cdc15 during meiosis and spore morphogenesis. Cdc15-depleted cells underwent meiotic divisions with kinetics and efficiency similar to those of wild-type cells, but failed to form mature spores. Several defects were observed in these mutants: the inability to disassemble anaphase II spindles, morphological defects in PSM assembly resulting in failure to capture the nuclear lobes, and the persistence of Spo21 at the SPB at the end of meiosis II. Cdc15 was not required for Cdc14 release from the nucleolus at the end of anaphase II, but it was necessary to maintain Cdc14 in its released state and for its transport from the nucleus to the cytoplasm. The phenotypes of Cdc14-ΔNES mutants suggest that Cdc15 could be required to coordinate the meiotic cell cycle with the morphogenetic program that results in the formation of mature spores.

MATERIALS AND METHODS

Yeast strains and methods: The *S. cerevisiae* strains used in this study were constructed in the fast-sporulating SK1 background. The wild-type strain AN120 (MATa/MATα *ura3/ura3 leu2/leu2 trp1::hisG/trp1::hisG ARG4/arg4-NspI his3ΔSK/his3ΔSK hoΔ::LYS2/hoΔ::LYS2 lys2/lys2 RME1/rme1Δ::LEU2*) has been described previously (NEIMAN *et al.* 2000). HI50 is isogenic to AN120, except that it contains *CDC15* under the control of the *CLB2* promoter, which is repressed during sporulation. HI50 was constructed by PCR-mediated integration of the *CLB2* promoter using pRK69 (KAMIENIECKI *et al.* 2005) as template into the haploids AN117-4B and AN117-16D, followed by mating to produce the diploid HI50. Strain SEP41 [*his3ΔSK/his3ΔSK trp1::hisG/trp1::hisG ura3/ura3 leu2/leu2 lys2/lys2 arg4-NspI/arg4-NspI hoΔ::LYS2/hoΔ::LYS2 rme1Δ::LEU2/rme1Δ::LEU2 cdc14::kanMX4/cdc14::kanMX4 pEP36 (CDC14-ΔNES)*] contains the Cdc14 nuclear export allele (Cdc14-ΔNES; with residues 359–367 deleted) (BEMBENEK *et al.* 2005), and it was constructed by deleting the wild-type *CDC14* gene in two spores of opposite mating type derived from AN120 that carried plasmid pEP36 and then mating the transformants to generate the diploid SEP41.

For sporulation, overnight liquid cultures were grown in YEPD or SC media (SHERMAN *et al.* 1986) to saturation. Cultures were then diluted 1:300 in YAcetate (1% yeast extract, 2% peptone, 2% potassium acetate), grown to midlog phase, and transferred to 2% potassium acetate at a final concentration of 3×10^7 cells/ml (NEIMAN 1998). Aliquots of the cultures were removed at different times. Yeast strains were transformed by the lithium acetate protocol (GIETZ and WOODS 2002).

Plasmids: Plasmid pC763 carries a *XhoI*–*Bam*HI fragment containing the *CDC14* gene obtained from plasmid pUG121 (GRUNBERG *et al.* 2000), cloned into the same sites of pRS426. pC774 carries *SIC1* under the control of the *SPO20* promoter cloned in pRS424. To construct it, a PCR fragment carrying nucleotides –731 to –1 of the *SPO20* promoter was used to replace the *TEF1* promoter in plasmid pRS424-TEF to generate pRS424-Spo20pr. The *SIC1* coding region was then isolated from plasmid YipPGK-*SIC1* (TOYN *et al.* 1997) by digestion with *Eco*RI and cloned into the similarly digested pRS424-SPO20pr plasmid. Plasmid pEP36 contains the *CDC14-ΔNES* allele (BEMBENEK *et al.* 2005) cloned into vector pRS426, and it was constructed by PCR amplification of a DNA fragment in which amino acids 359–367 were deleted, and then replacing the *Eco*RI–*Xho*I from plasmid pEP17 (containing the wild-type *CDC14* gene) with the amplified fragment.

Microscopy techniques: Analysis of meiotic progression and visualization of *in vivo* fluorescent proteins and indirect immunofluorescence were accomplished using a Leica DMXR microscope equipped for Nomarski optics and epifluorescence. Images were captured using a Photometrics Sensys CCD camera. For visualization of GFP fluorescence, samples were fixed with formaldehyde 4% for 10 min, washed once in PBS, and placed in mounting media (Vectashield; Vector Laboratories, Burlingame, CA) containing DAPI (4',6'-diamino-2-phenylindole). For some figures, an Axioplan2 microscope (Carl Zeiss, Thornwood, NY) was used. Images were then acquired using a Zeiss mRM Axiocam and deconvoluted using Zeiss Axiovision 3.1 software. Transmission electron microscopy was performed as previously described (COLUCCIO *et al.* 2004); images were captured on a JEOL 1200 EX microscope at 80 kV.

Indirect immunofluorescence was performed as previously described (SALAH and NASMYTH 2000). Primary antibodies were used at 1:100 dilution and incubated for 1 hr. Samples were washed five times with PBS-BSA 1% and incubated with secondary antibodies (1:200) for 1 hr. After incubation,

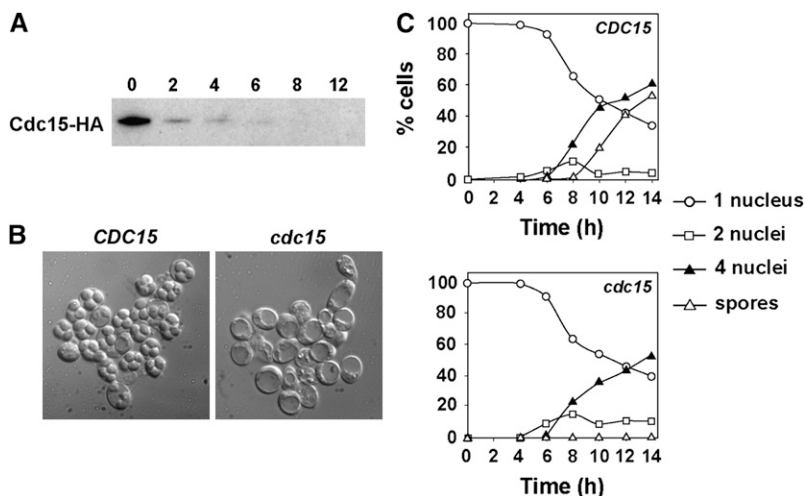


FIGURE 1.—*CDC15* is not required for DNA replication or meiotic segregation. (A) Levels of Cdc15 protein in cells with the P_{CLB2} -*CDC15* construct. Cdc15 was immunoprecipitated from 1 mg of protein extracts before Western blot analysis. Antibodies against the N-terminal HA epitope tags were used for detection and immunoprecipitation. Samples were collected at the indicated number of hours after transfer to sporulation medium (0, 2, 4, 6, 8, and 12 hr). (B) Depletion of *CDC15* during sporulation blocks the formation of spores. DIC images of wild type (*CDC15*) or P_{CLB2} -*CDC15* mutant (*cdc15*) incubated for 24 hr in sporulation medium. (C) Time course of appearance of binucleate cells (squares) or tetranucleate cells (triangles) in wild type (*CDC15*) or P_{CLB2} -*CDC15* mutant (*cdc15*). Strains were incubated in sporulation medium for the indicated times (hours) before aliquots were collected and the cells were stained with DAPI. At least 200 cells were counted for each time point.

antibodies were removed by washing thoroughly with PBS buffer. Samples were mounted with DAPI mounting media. The primary antibodies used were anti-Tubulin (Sigma 75168, St. Louis); anti-Cdc14 (YE17 sc12045, Santa Cruz Biotechnology, Santa Cruz, CA); anti-Cdc11 (Y415 sc7170, Santa Cruz Biotechnology); or anti-GFP (Living colors 8371, Becton Dickinson). Different secondary antibodies coupled to Alexa Fluor 488, 594, or 633 (Invitrogen Molecular Probes, Invitrogen, Carlsbad, CA) were used.

Protein methods: Protein extracts were prepared using TCA precipitation as described (KLEIN *et al.* 1999). Samples were separated in NUPAGE Novex 10–12% Bis-Tris gels (Invitrogen) according to the recommendations of the supplier. For Western blot, 25 μ g of protein extracts were transferred to P-Hybrid membranes (GE Healthcare) using an X-Cell Sure Lock mini-cell system (Invitrogen). Anti-Cdc14 antibody (YE17 sc12045, Santa Cruz Biotechnology; 1:500) or anti-Pgk1 antibody (mouse monoclonal 22C5, A6457 Invitrogen Molecular Probes, 1:3000) were used. Horseradish peroxidase-conjugated anti-goat antibody (R21459, Invitrogen Molecular Probes) or anti-mouse antibody (NA931V GE Healthcare) were used as secondary antibodies. Detection of proteins was performed using the ECL kit (GE Healthcare). For detection of Cdc15-HA, protein extracts from cells incubated in sporulation medium for different times were prepared in B70 buffer (ZACHARIAE *et al.* 1998), adding protease inhibitors (Complete X, Roche). Cdc15-HA was immunoprecipitated from 1 mg of protein extract using the μ MACS-HA isolation kit (Miltenyi Biotec) according to supplier recommendations. Immunoprecipitated material was separated by SDS-PAGE and incubated anti-HA antibody (Covance, 1:1000) at 4° and developed with horseradish peroxidase-conjugated anti-mouse antibody as secondary antibody.

RESULTS

Cdc15 function is required at the end of meiosis II:

To analyze the role of Cdc15 during meiosis, we used a conditional mutant in which the expression of *CDC15* was under the control of the *CLB2* promoter (P_{CLB2} -*CDC15*), which is expressed during vegetative growth but is repressed during sporulation. This allele significantly reduced Cdc15 protein after 4 hr of transfer to

sporulation medium, and the protein was undetectable by Western blot after 6 hr of incubation (Figure 1A). For this reason, this strain behaved as a conditional mutant, which was viable during vegetative growth but was unable to form spores. This construct was introduced into the fast-sporulating SK-1 background, and we observed that mature spores were never seen in this strain, even after prolonged incubation times in sporulation medium (Figure 1B). This suggested that Cdc15 is essential for completion of sporogenesis.

To determine the moment at which Cdc15 exerts its function during meiosis, the kinetics of the appearance of bi- and tetranucleate cells was measured in wild-type and *cdc15*-depleted cells incubated in sporulation medium for different time intervals. Microscopic inspection of the DAPI-stained cells indicated that bi- and tetranucleate cells were present in the P_{CLB2} -*CDC15* mutant. When progression through meiosis was quantified, the results indicated that the kinetics of appearance of bi- and tetranucleate cells in P_{CLB2} -*CDC15* mutant was slightly delayed and they showed a reduction in the percentage of cells with four nuclei after 14 hr of incubation compared to the wild-type strain (Figure 1C). Mature spores started to appear after 10 hr of incubation and accounted for ~80% of the culture by 24 hr of incubation in the wild-type strain. However, P_{CLB2} -*CDC15* mutants arrested at the end of meiosis after the four nuclei had separated, and mature spores were never observed in the mutant. No differences in the timing of DNA replication as assayed by FACS analysis were observed between the wild-type and *cdc15* mutant strain (data not shown). Together, these results indicate that *CDC15* is not required for DNA duplication or meiotic segregation, but that it does function at the end of meiosis II.

Cdc15 is required for postanaphase II spindle disassembly: To analyze the nature of the defect of P_{CLB2} -*CDC15* mutants in more detail, we monitored the kinetics of spindle assembly and disassembly during the

different phases of meiosis using indirect immunofluorescence. The appearance of meiosis I spindles and their morphology were similar in wild-type and $P_{CLB2}\text{-}CDC15$ mutants, suggesting that Cdc15 function is not essential during the first meiotic division. The assembly of meiosis II spindles was also similar in both strains, but the $P_{CLB2}\text{-}CDC15$ mutant was delayed in meiosis II spindle disassembly as judged by the persistence of cells with anaphase II spindles (Figure 2A). At the end of meiosis II,

wild-type cells disassembled the spindle and initiated the morphogenetic program that led to the formation of mature spores. In contrast, the $P_{CLB2}\text{-}CDC15$ mutant failed to normally disassemble the anaphase II spindles, and in many cells these collapsed or broke down into several fragments (arrowheads in Figure 2A).

Quantitation of the number of cells with different types of spindles confirmed these results. No apparent differences were observed for meiosis I spindles (Figure 2B), which disassembled with similar kinetics in both strains. Assembly of the meiosis II spindle in the $P_{CLB2}\text{-}CDC15$ strain took place at approximately the same time as the wild-type strain. However, there was a slight delay in meiosis II progression in Cdc15-depleted cells, as judged by the accumulation of meiosis II spindles (Figure 2B). The percentage of cells with meiosis II spindles was higher in the $P_{CLB2}\text{-}CDC15$ mutant than in the wild type, suggesting either a defect in spindle disassembly or a delay in progression through meiosis II. However, the most significant differences were observed after meiosis II, since most of the wild-type cells had disassembled the spindle and showed no tubulin staining after 10–12 hr of incubation in sporulation medium (70% of the cells), while only 8% of the *cdc15* depleted cells showed no staining. In contrast, ~50% of the $P_{CLB2}\text{-}CDC15$ mutant cells showed abnormal tubulin staining. Thus, these results indicate that Cdc15 is required for proper spindle disassembly after meiosis II.

PSM formation is aberrant in *cdc15* mutants: Since the depletion of Cdc15 results in defects at the end of meiosis, we analyzed whether the morphogenetic program leading to spore morphogenesis was affected in these cells. During the second meiotic division, the outer plaque of the SPB acts as a nucleation center for the assembly of the PSM. Spo21 is a sporulation-specific component of the outer plaque, and it localizes to the meiotic SPB (KNOP and STRASSER 2000; BAJGIER *et al.* 2001). As meiosis II progresses, the PSM engulfs the adjacent nuclear lobe and later fuses with itself, creating

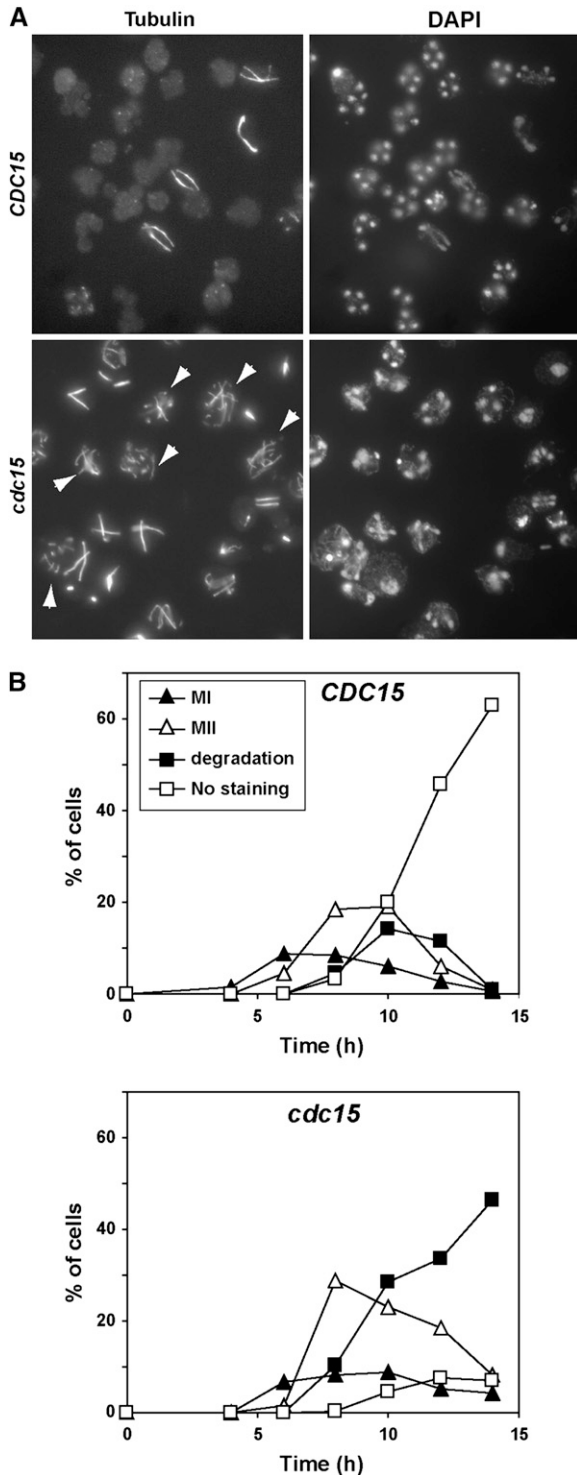


FIGURE 2.—Cdc15-depleted cells have defects in meiotic spindle disassembly. (A) Nuclear and spindle morphology in wild-type and *cdc15* mutants. Wild type (*CDC15*) and $P_{CLB2}\text{-}CDC15$ mutant (*cdc15*) were incubated in sporulation medium; samples were collected at different time intervals and stained with anti-tubulin antibody (left) and DAPI (right). The image shows an example of cells that had been incubated in sporulation medium for 10 hr. (B) Quantification of the different spindle morphologies during the sporulation process in wild-type (*CDC15*) and $P_{CLB2}\text{-}CDC15$ (*cdc15*) cells. Cells from the meiotic time course shown in Figure 1C were stained with anti-tubulin antibody and classified according to the spindle morphology. Meiosis I includes cells in prophase, metaphase I, and anaphase I, whereas cells with metaphase II or anaphase II spindles were classified as meiosis II. Postmeiotic cells that had already disassembled the meiotic spindle and were negative for tubulin staining were classified as “no staining.” Cells in meiosis II with broken or collapsed spindles were classified as “degradation.”

immature spores. To test whether spore morphogenesis was altered in Cdc15-depleted cells, we first checked if SPB outer plaque assembly occurred normally in mutant cells using a functional Spo21-GFP fusion (BAJGIER *et al.* 2001). We found that Spo21 localized to the four SPBs of anaphase II cells, and no significant differences in the percentage of cells displaying Spo21 staining were detected between wild-type and *P_{CLB2}-CDC15* mutants (Figure 3). However, the Spo21-GFP fluorescence disappeared at the time of spindle disassembly in wild-type cells, while in *cdc15* mutants it persisted and remained associated with the four SPBs even after some of the microtubules had started to fragment. The percentage of *cdc15* postmeiotic cells showing Spo21 at the SPBs when the spindle collapsed was ~45%.

The formation of PSM was monitored using a Spo20-GFP fusion protein (NAKANISHI *et al.* 2004). Spo20p is a sporulation-specific SNAP-25 homolog that is required for the developmentally regulated branch of the secretory pathway that leads to spore morphogenesis (NEIMAN 1998). In wild-type cells, each nucleus was usually surrounded by a PSM, which displayed a regular shape, and the four compartments in each cell were similar in size (Figure 4A). However, in *P_{CLB2}-CDC15* mutants there was considerable variation in the number, size, and shape of the PSMs. In particular, cells often displayed fewer than four PSMs, and the membranes that did form were frequently misshapen and failed to capture nuclei (Figure 4, B and C). The defects in PSM formation and nuclear capture were quantified to gain an idea of the extent of the phenotype. The results indicated that the morphology of the PSM was aberrant in a large proportion of the cells and that the defect increased over time, ~90% of abnormal PSMs being observed at 9 hr (Figure 4D). Also, the number of PSMs per cell varied more than in wild-type cells, and the percentage of cells with four PSMs was lower than in wild-type cells (Figure 4E). Finally, nuclear capture was also deficient in *P_{CLB2}-CDC15* cells, since only 45% of the PSMs were able to correctly surround a nucleus in mutant cells as compared to the wild type (~83%). The failure to capture nuclei was also evident in ultrastructural studies of the *P_{CLB2}-CDC15* cells, and examples of PSMs without a nucleus and uncaptured nuclei were often seen (Figure 4, G and H). This was in contrast to wild-type cells, in which the nuclei were almost always seen to be within PSMs (Figure 4F). Interestingly, the electron microscopy analysis also revealed that prospores that formed in the *P_{CLB2}-CDC15* strain failed to initiate spore wall assembly, as indicated by the lack of spore wall material in the lumen of the PSM. Taken together, these results clearly suggest that *CDC15* function is required for normal PSM formation and nuclear capture after the assembly of the membrane-nucleating center on the SPB.

Septin organization is normal in Cdc15-depleted cells:

Since the defect produced by the depletion of Cdc15

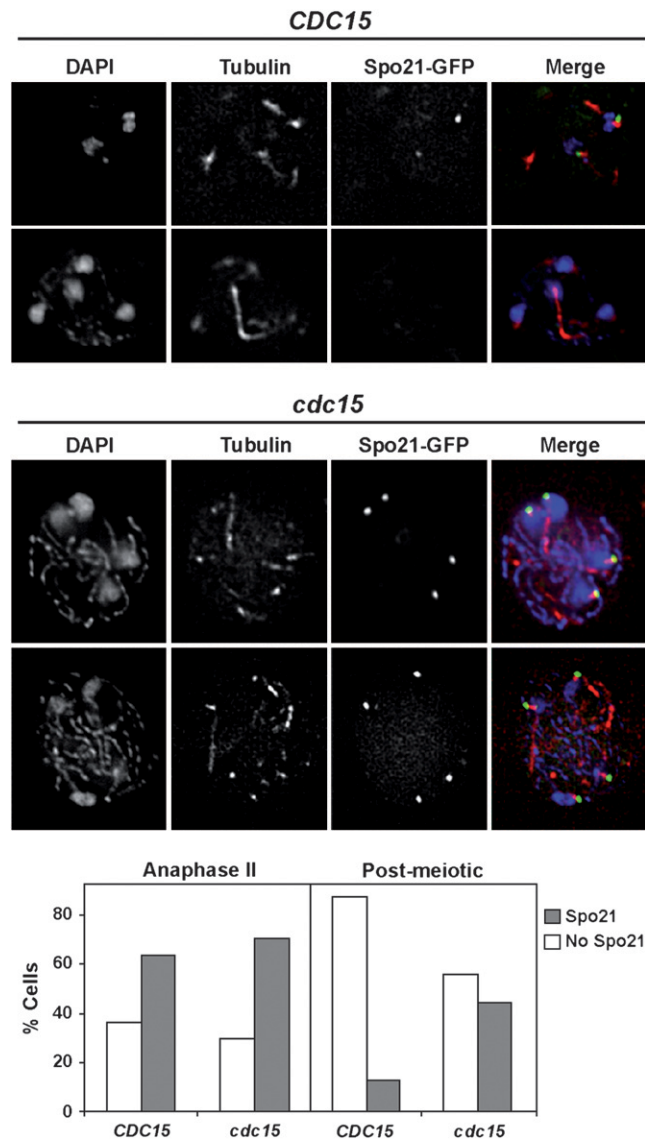


FIGURE 3.—*cdc15* mutants have defects in SPB modification. Spo21 localizes to the SPB in Cdc15-depleted cells during sporulation. Wild type (*CDC15*, top) or *P_{CLB2}-CDC15* mutant (*cdc15*, bottom) expressing *SPO21-GFP* (from plasmid pSPO21) were transferred to sporulation medium. After 8 hr of incubation, cells were collected and stained with anti-tubulin antibody and DAPI. Images of Spo21-GFP (green), tubulin (red), and DAPI (blue) and the merging of the three channels are shown. The graph shows the percentage of wild-type (*CDC15*) or *cdc15* mutant cells with Spo21 staining (open rectangles) or without staining (shaded rectangles) in anaphase II or postmeiotic cells. At least 100 cells were counted for each category.

resulted in a block in the late steps of the sporulation process, we also analyzed the localization of septins in wild-type and *cdc15* mutants. During meiosis, septins are assembled as sheets that line the PSM (DE VIRGILIO *et al.* 1996; FARES *et al.* 1996; TACHIKAWA *et al.* 2001). The localization of Cdc11 was analyzed by indirect immunofluorescence using anti-Cdc11 antibodies. In wild-type cells, Cdc11 was found to form rounded structures that

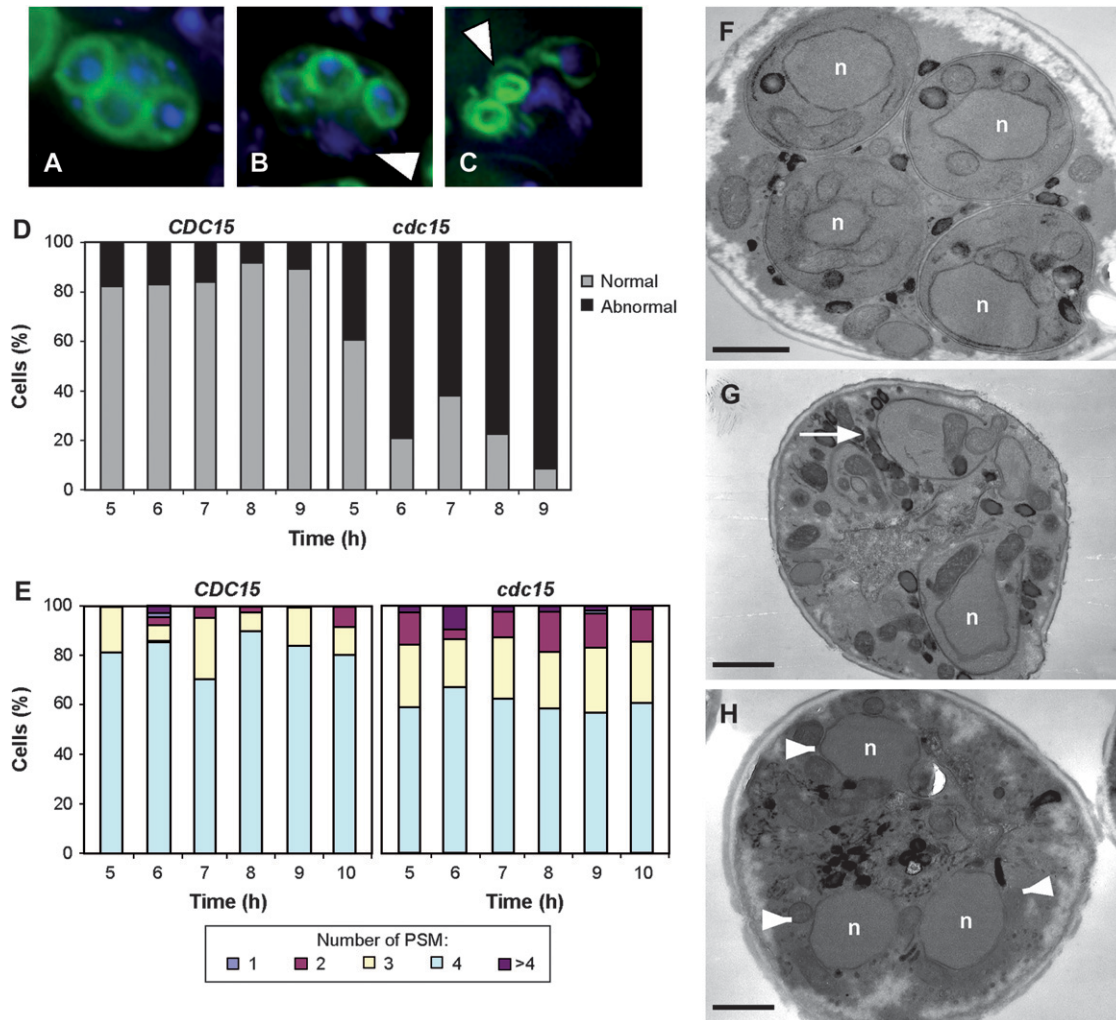


FIGURE 4.— P_{CLB2} - $CDC15$ cells display PSM defects. Fluorescence micrographs of sporulating wild-type (A) and P_{CLB2} - $CDC15$ cells (B and C). PSMs are visualized in green using GFP-Spo20⁵¹⁻⁹¹, and nuclei are shown in blue using DAPI. Arrowheads in B indicate unpackaged nuclei in postmeiotic cells and abnormal PSMs in C. (D) Quantification of the PSM morphology during sporulation in wild type ($CDC15$) and P_{CLB2} - $CDC15$ mutant ($cdc15$). At least 200 cells in which the PSM was visible were counted for each strain. The graph shows the percentage of cells with normal morphology (shaded bars) or aberrant shapes (solid bars) in cells with visible PSMs. Cells with at least one abnormal PSM were counted as aberrant. (E) Quantification of the number of PSMs during sporulation in wild type ($CDC15$) and P_{CLB2} - $CDC15$ mutant ($cdc15$). At least 200 cells in which the PSM was visible were counted for each strain. (F–H) Electron micrographs of sporulating wild type (F) and P_{CLB2} - $CDC15$ cells (G and H). White arrow in G indicates a growing PSM that is not encapsulating a nucleus (indicated with n in all images). Arrowheads in H indicate multiple, unpackaged nuclei in a postmeiotic cell. Bars, 1 μ m.

surrounded the nuclear lobes after anaphase II, later becoming localized in a pattern that closely resembled the PSM (Figure 5). No apparent differences were found in the P_{CLB2} - $CDC15$ mutant at any stage of the sporulation process, and the localization was very similar to that observed for wild-type cells (Figure 5). Similar results were obtained with a GFP fusion to the sporulation-specific septin Spr28 (DE VIRGILIO *et al.* 1996) (data not shown). Thus, these results suggest that Cdc15 is required for late steps of spore morphogenesis, but that it is not necessary for the assembly of the septin structure that underlies the PSM.

Cdc15 is not required for Cdc14 release from the nucleolus but is involved in its nucleo-cytoplasmic

transport: During vegetative growth, Cdc15 is required for exit from mitosis. This kinase is an upstream component of the MEN, a signaling pathway whose activation results in the release of the protein phosphatase Cdc14 from the nucleolus. Cdc14 remains sequestered in the nucleolus during most of the cell cycle (SHOU *et al.* 1999; VISINTIN *et al.* 1999). To test whether Cdc15 might perform a similar function during meiosis, we analyzed Cdc14 localization as the cells progressed through meiosis by indirect immunofluorescence. We found that in wild-type cells, Cdc14 exhibited a localization pattern similar to that described previously (BUONOMO *et al.* 2003; MARSTON *et al.* 2003); that is, Cdc14 resided in the nucleolus throughout meiotic prophase I and

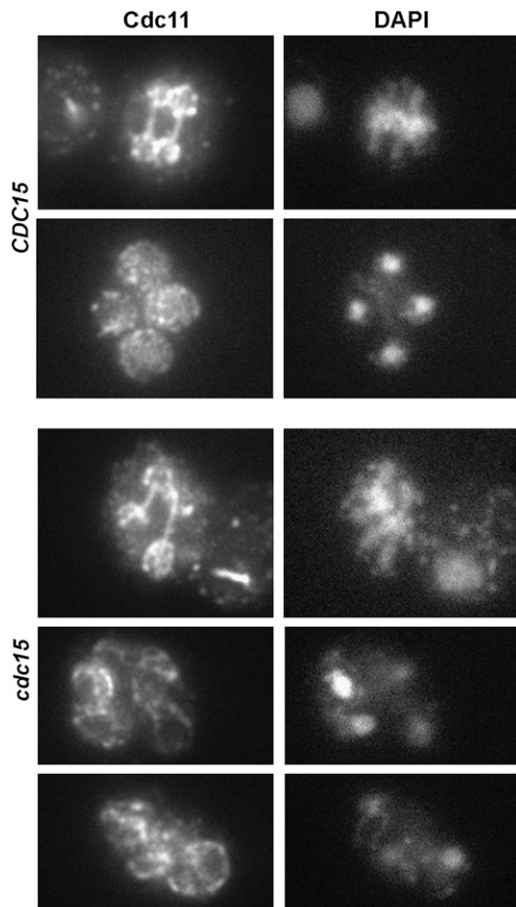


FIGURE 5.—Septins are normally assembled in *cdc15* mutants. Localization of Cdc11 in wild type and *P_{CLB2}-CDC15* mutant. Cells were incubated in sporulation medium. Samples were collected at different times and stained with anti-Cdc11 antibody and DAPI and inspected for septin morphology. Images of Cdc11 (left) and DAPI (right) are shown.

metaphase I (Figure 6, A and B), and it was released from the nucleolus to the nucleus upon entry into anaphase I. During metaphase II, Cdc14 was resequenced into the nucleoli before being finally released uniformly into the nucleus and the cytoplasm upon anaphase II entry (Cdc14 was released in >80% of the wild-type cells). Finally, as sporogenesis progressed Cdc14 staining was observed in the cytoplasm (40% of the cells), but it also disappeared from a large proportion of the cells (40%).

In *P_{CLB2}-CDC15* mutants, Cdc14 localization during meiosis I and metaphase II was similar to that observed in the wild-type strain (Figure 6, A and B). However, during anaphase II important differences between the wild-type and mutant were detected, since in most of the mutant cells Cdc14 was released from the nucleoli, as happens in the wild-type strain, but failed to reach the cytoplasm and accumulated in the nucleus of the cells (~80% of the cells presented Cdc14 nuclear staining). In addition, at later steps Cdc14 was resequenced into four intense foci in postmeiotic cells (~80%) instead of

being exported. These results therefore indicate that Cdc15 is not required for Cdc14 release from the nucleoli during meiosis, but it is necessary to maintain Cdc14 in its released state and for its transport from the nucleus to the cytoplasm of the cells in anaphase II.

A Cdc14 allele with a nuclear export defect affects entry into meiosis but not spore formation: To examine if Cdc14 nuclear export is important for sporogenesis, we examined the sporulation in strains containing a Cdc14 allele in which the nuclear export signal (NES) had been deleted. The Crm1-dependent nuclear export of Cdc14 is important for the coordination of mitotic exit and cytokinesis during vegetative growth, since cells carrying a Cdc14- Δ NES allele have an abnormal morphology and defects in cytokinesis (BEMBENEK *et al.* 2005). We therefore constructed a homozygous diploid strain carrying the *CDC14- Δ NES* allele in the SK1 background. This strain was induced to sporulate, and progression through meiosis was monitored by DAPI staining and microscopic inspection of spores. The results indicated that the Cdc14- Δ NES allele produced a marked delay in the onset of sporulation and a reduction in the percentage of mature spores in comparison with the wild-type strain, in which mature spore production was never >20% (Figure 7A). This delay could be due to the defects in cytokinesis described for this mutant allele (BEMBENEK *et al.* 2005) and the consequent defects in exiting the mitotic cycle from the G1 phase to enter the meiotic program. However, cells that started meiosis were able to form mature spores, as judged by the fact that the percentages of tetranucleate cells and mature spores were similar.

To test whether the Cdc14- Δ NES also produced a defect in nuclear export during sporulation, samples were stained with anti-Cdc14 antibody for microscopic inspection. In Cdc14- Δ NES cells that had entered the meiotic cycle, Cdc14 localization during meiosis I and metaphase II was similar to that observed in the wild type. However, in anaphase II cells important differences between the wild type and mutant were detected. From those cells that had entered the meiotic program, only ~25% of them showed a clear cytoplasmic staining for Cdc14, while in the rest the Cdc14 nuclear export was impaired (Figure 7B). Furthermore, staining with anti-tubulin antibody failed to indicate defects in meiosis II spindle disassembly in Cdc14- Δ NES cells (data not shown), even though Cdc14 was retained in the nucleus in a large number of the cells. These observations suggest that the absence of complete Cdc14 nuclear export is not the basis for the *cdc15* sporulation defect.

Overexpression of MEN components does not complement the absence of Cdc15: The components of the MEN pathway show multiple genetic interactions with one another during vegetative growth, including high-copy suppression of the conditional lethal growth defect of some mutations (JASPERSEN *et al.* 1998). For example, overexpression of *CDC14* can rescue the

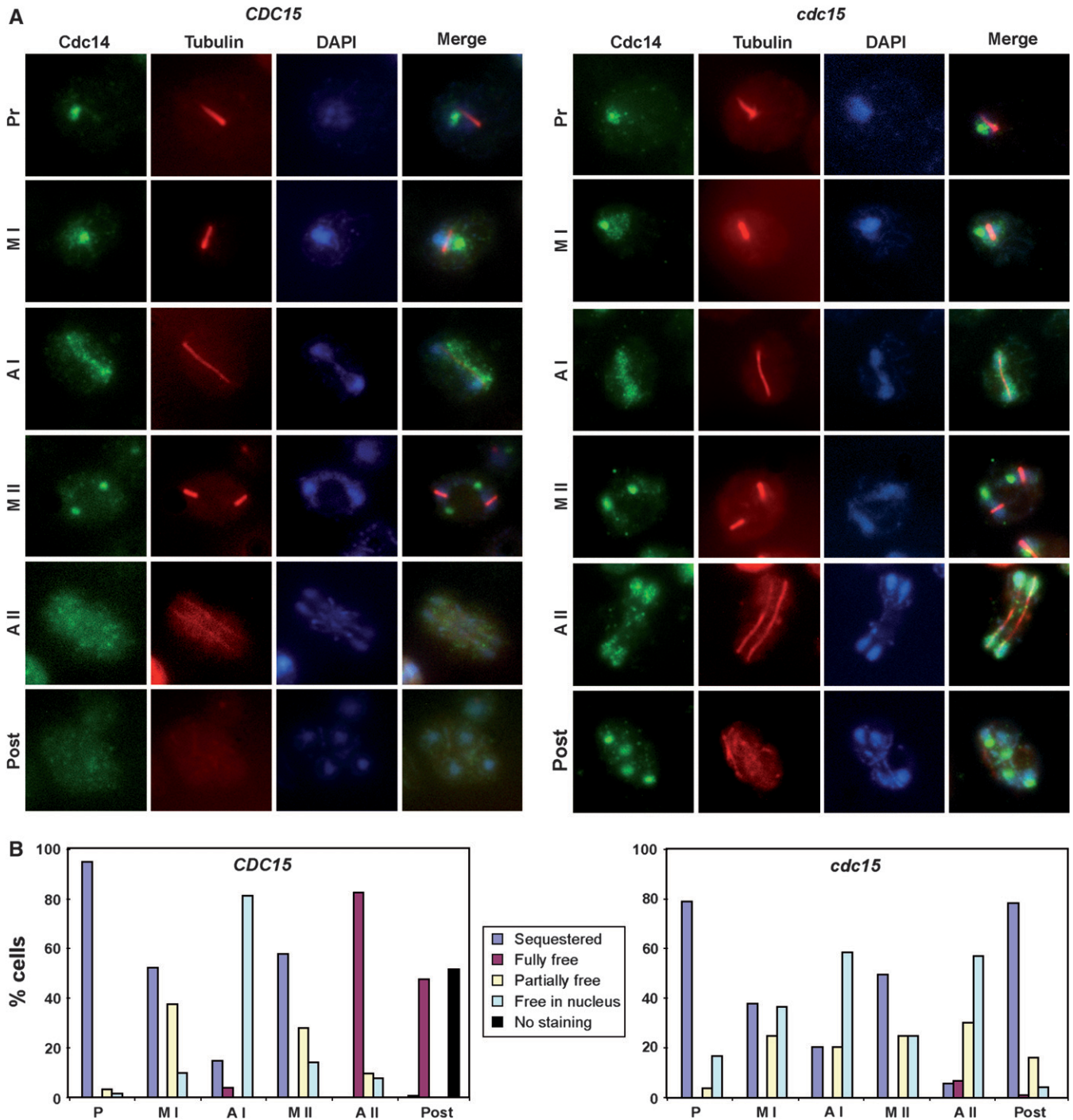


FIGURE 6.—Localization of Cdc14 during sporulation. (A) Wild type (*CDC15*, left) or *P_{CLB2}-CDC15* mutant (*cdc15*, right) were transferred to sporulation medium. At different times after the induction of sporulation, cells were collected and stained with anti-Cdc14 and anti-tubulin antibodies and DAPI. Anti-goat Alexa Fluor 488 and anti-mouse Alexa Fluor 633 were used as secondary antibodies. Cdc14 (green), tubulin (red), and DAPI (blue) images are shown, as well as the merged image. Representative images of the different meiotic phases are shown for each strain. (B) Localization of Cdc14 during the different stages of the sporulation in wild-type (left) or *P_{CLB2}-CDC15* (right) cells. Localization of Cdc14 in each phase of the sporulation process was classified as sequestered in the nucleolus (blue), fully (maroon) or partially (yellow) released from the nucleolus, free in the nucleus (light blue), or cells showing no staining (black). For wild-type cells, the different localization is presented as a percentage of metaphase I (MI; $n = 109$), anaphase I (AI; $n = 92$), metaphase II (MII; $n = 149$), anaphase II (AII; $n = 155$), or spores ($n = 113$) with visible Cdc14. For *P_{CLB2}-CDC15* cells, a similar percentage for metaphase I (MI; $n = 112$), anaphase I (AI; $n = 74$), metaphase II (MII; $n = 128$), anaphase II (AII; $n = 132$), or postmeiotic cells ($n = 64$) is shown.

phenotype of *cdc15-2* mutants (GRANDIN *et al.* 1998). In addition, Sic1 is a specific inhibitor of cyclin-Cdc28 complexes whose overexpression can also complement a *cdc15* mutation (MENDENHALL 1993; SCHWOB *et al.* 1994; JASPERSEN *et al.* 1998). We tested whether overexpression of Cdc14 phosphatase was enough to bypass the Cdc15 requirement during sporulation. Since *CDC14* expression increases during sporulation (CHU *et al.* 1998), we used a plasmid containing *CDC14* cloned under the control of its own promoter. Increase in Cdc14 protein levels during sporulation in these strains was assessed by Western blot (Figure 7C). Even though Cdc14 protein levels were higher in the strains overexpressing *CDC14*, no complementation of the spore formation defect or the spindle disassembly defect of *P_{CLB2}-CDC15* mutants was observed (data not shown). Additionally, we tested whether overexpression of the *SIC1* inhibitor had any effect on *P_{CLB2}-CDC15* mutants during meiosis. To increase the expression level of this gene during sporulation, *SIC1* was placed under the control of the *SPO20* sporulation-specific promoter, which is induced midway through sporulation (NEIMAN 1998). We found that *SIC1* overexpression was also unable to rescue the sporulation or spindle disassembly phenotypes of *P_{CLB2}-CDC15* mutants (data not shown). Taken together with the results of the *CDC14*-NES experiments described above, these data suggest that the critical function of *CDC15* in sporulation is not mediated through Cdc14 and the downregulation of Cdc28 activity.

DISCUSSION

In mitotically dividing cells, exit from mitosis requires the inactivation of mitotic CDKs. Cells lacking Cdc14 function arrest in late anaphase with high CDK activity (VISINTIN *et al.* 1998). For most of the cell cycle, Cdc14 resides in the nucleolus owing to its association with the Cfi1/Net1 inhibitor (SHOU *et al.* 1999; VISINTIN *et al.* 1999). The dissociation of Cdc14 from the nucleolus during anaphase is controlled by the FEAR and MEN networks (reviewed by D'AMOURS and AMON 2004; STEGMEIER and AMON 2004). At the onset of anaphase, the FEAR network initiates the release of Cdc14, and the phosphatase spreads throughout the nucleus. During later stages of anaphase, the MEN promotes further release of Cdc14 and maintains the phosphatase in its released state, and during telophase the phosphatase is also found in the cytoplasm. Cdc14 activated by the MEN is mainly responsible for promoting exit from mitosis, because this function does not occur in the absence of MEN function (VISINTIN *et al.* 1997; JASPERSEN *et al.* 1998).

The functions of Cdc14, FEAR, and MEN during meiosis are less well known. The transition from meiosis I to meiosis II requires that meiotic CDK activity should be sufficiently lowered to allow the disassembly of the

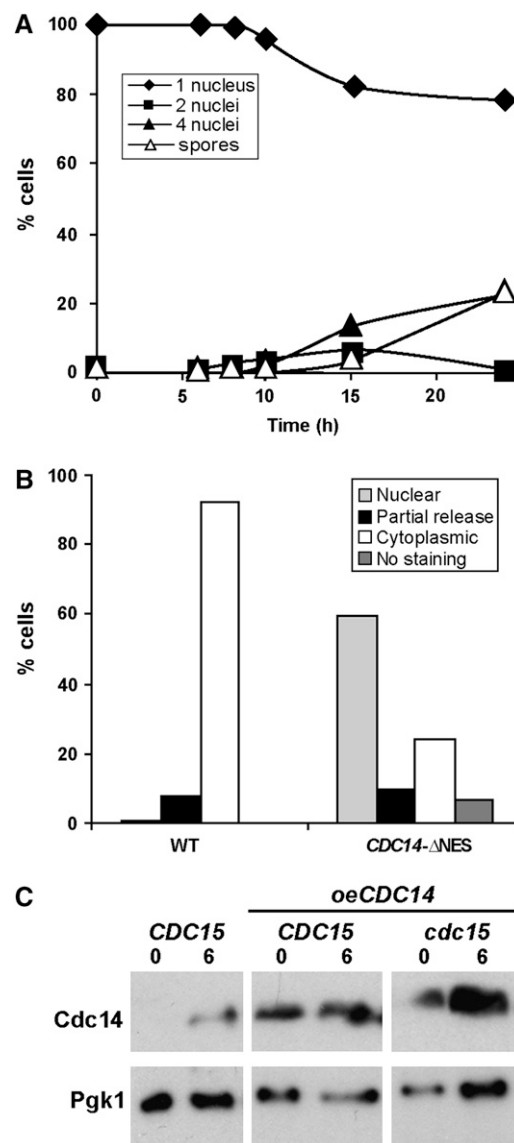


FIGURE 7.—Importance of Cdc14 nuclear export in sporulation. *Cdc14-ΔNES* strains have defects in sporulation. Time course of appearance of binucleate (squares) and tetranucleate cells (triangles) and spores (white triangles) in cells carrying *Cdc14-ΔNES* (*SEP41*). Cells were incubated in sporulation medium for the indicated times (hours) before aliquots were collected and stained with DAPI. At least 200 cells were counted for each time point. (B) Localization of Cdc14 and *Cdc14-ΔNES* in anaphase II cells. Cells were collected and stained with anti-Cdc14 and anti-tubulin antibodies and DAPI. Localization of Cdc14 in the fraction of cells that had entered meiosis was classified as nuclear (lightly shaded bar), cytoplasmic (open bars), partially released (solid bars), or without staining (darkly shaded bar). At least 100 cells in anaphase II were counted. (C) Analysis of Cdc14 protein levels in the wild-type strain (*CDC15*) and in the wild-type and *cdc15* mutant strains overexpressing *CDC14* (*CDC15* *oeCDC14* and *cdc15* *oeCDC14*, respectively). Samples were collected at 0 and 6 hr after transfer to sporulation medium to prepare protein extracts. Western blot was performed using anti-Cdc14 antibody. Anti-Pgk1 was used as loading control.

meiosis I spindle but not so much as to permit the assembly of prereplicative complexes onto the DNA, which would result in a new S phase. It has been shown that exit from meiosis I requires only the FEAR pathway, since mutants in this pathway are impaired in meiosis I spindle disassembly, whereas MEN inactivation does not affect exit from the first meiotic division (KAMIENIECKI *et al.* 2000; BUONOMO *et al.* 2003; MARSTON *et al.* 2003; KAMIENIECKI *et al.* 2005). Notably, even though chromosome segregation during meiosis I is not efficient in FEAR network mutants, they are able to progress through meiosis II and form two spores (dyads) instead of four (tetrads) (SHARON and SIMCHEN 1990; MARSTON *et al.* 2003). As for the MEN, previous work has shown that cells bearing the *P_{CLB2}-CDC15* or the *P_{CLB2}-TEM1* alleles or a deletion of *LTE1* all proceed through the meiotic nuclear divisions, but only *P_{CLB2}-CDC15* cells cannot form spores (KAMIENIECKI *et al.* 2005). Our more detailed characterization of the *P_{CLB2}-CDC15* phenotype indicates that despite the normal kinetics of chromosome behavior, Cdc15 is required for proper exit from meiosis II. In particular, the mutant segregates the four nuclei with similar kinetics to that of the wild type, but fails to properly break down the spindle at the end of meiosis II and fails to export Cdc14 from the nucleus to the cytoplasm. These results suggest Cdc15 has an important function during exit from meiosis II. Similar to its role during exit from mitosis, Cdc15 was required for full Cdc14 release and cytoplasmic accumulation at the end of meiosis II. No differences in Cdc14 localization were observed during meiosis I between the wild type and the *cdc15* cells, suggesting that MEN function is not necessary for Cdc14 release at the end of the first meiotic division, as previously described (KAMIENIECKI *et al.* 2005). In contrast, the main differences were found at the end of meiosis II, where Cdc14 was partially released to the nucleus in *cdc15* cells but was never transported to the cytoplasm. Interestingly, Cdc14 concentrated in four intense foci at the end of meiosis II in *cdc15* cells, similar to what has been described for the *cdc15-2* mutant during mitosis (STEGMEIER *et al.* 2002; YOSHIDA *et al.* 2002). These observations suggest that Cdc14 release from the nucleoli at the end of the second meiotic division is independent of the MEN. It is very likely that the FEAR network is required to initiate Cdc14 release from the nucleoli during anaphase II, but that this function has not been detected before because of its essential function at the end of meiosis I. However, Cdc15 is necessary to maintain Cdc14 in a released state until the end of meiosis II and for its nucleo-cytoplasmic transport. It is interesting that two screens aimed at identifying reduction-of-function mutants that bypass the mitotic exit defect of MEN mutants identified proteins involved in nucleo-cytoplasmic transport, such as the karyopherins Kap104 and Mtr10 (ASAKAWA and TOHE 2002; SHOU and DESHAIES 2002). Furthermore, Crm1-

dependent nuclear export of Cdc14 is necessary to coordinate mitotic exit and cytokinesis (MAURER *et al.* 2001; BEMBENEK *et al.* 2005). This suggests that Cdc15-mediated control of Cdc14 export is important for mitotic exit and cytokinesis. Our unpublished observations support the idea that Cdc15 is also involved in nucleo-cytoplasmic transport during sporulation. A screening for high-copy suppressors of the sporulation defect of *cdc15* mutants led to the isolation of *YRB2*. Yrb2 is a Ran-binding GTPase involved in nuclear protein export transport during mitosis (TAURA *et al.* 1997) and interacts with the Crm1/Xpo1 exportin. Overexpression of *YRB2* or *CRM1* weakly suppressed the phenotypes of *P_{CLB2}-CDC15* cells. Although progression through the chromosomal events of mitosis and meiosis displays different requirements for the FEAR and MEN networks (STEGMEIER *et al.* 2002; BUONOMO *et al.* 2003; MARSTON *et al.* 2003; KAMIENIECKI *et al.* 2005), the data presented here reveal a common requirement for the MEN kinase Cdc15 for promoting Cdc14 export at the end of the nuclear division.

Unlike mitotic cells, however, the principle functions of Cdc15 in sporulation appear not to be tied to Cdc14 localization. The observation that Cdc14- Δ NES cells are able to complete spore formation in the absence of Cdc14 nuclear export (Cdc14- Δ NES) suggests that Cdc15-mediated Cdc14 export is not essential for spore morphogenesis. Although we cannot rule out that a small fraction of Cdc14 not detected by immunofluorescence could be present in the cell cytoplasm in Cdc14- Δ NES cells, the observation that overexpression of the wild-type Cdc14 does not rescue Cdc15 depletion also suggests that the function of Cdc15 in spore formation is independent of Cdc14. Furthermore, cells carrying the *ts cdc14-3* allele have defects in chromosome segregation, but are able to complete the sporulation program and form dyads (SHARON and SIMCHEN 1990). Interestingly, studies of several *cdc15* mutant alleles have suggested that Cdc15 might play some additional functions that are independent of its effects on Cdc14 activation during mitosis (SHIRAYAMA *et al.* 1996; JIMÉNEZ *et al.* 1998; JASPERSEN and MORGAN 2000). In particular, Cdc15 function is critical for the formation of the division septum and might also be involved in the direct control of cytokinesis. During sporulation, the main morphogenetic events are PSM formation and spore wall assembly, and the cytokinetic process in meiosis is the closure of the PSM (reviewed by NEIMAN 2005). In this way, *P_{CLB2}-CDC15* cells display abnormally shaped and reduced numbers of PSMs, suggesting a defect in membrane assembly. It is also noteworthy that the prospores that are formed in the *P_{CLB2}-CDC15* cells do not appear to initiate spore wall formation. This phenotype may indicate a defect in PSM closure (TACHIKAWA *et al.* 2001). Thus, in both vegetative and sporulating cells Cdc15 could be required to complete a cytokinesis event.

It is unclear whether the sporulation morphogenetic defects seen in *P_{CLB2}-CDC15* cells would be linked to the meiosis II exit phenotypes or whether they would represent a second function for Cdc15. Several observations suggest that Cdc15 might perform an additional function in spore morphogenesis. First, PSM defects cannot be a consequence of the cell cycle block at the end of meiosis II because PSM synthesis begins shortly after the onset of meiosis II (NEIMAN 2005). The prospore defects in Cdc15-depleted cells are already evident early in meiosis II, suggesting that Cdc15 might have a function in this stage. Second, perturbation of the cell cycle machinery does not always result in PSM defects or spore wall defects. This can be seen in several cases: the Swm1 subunit of the anaphase promoting complex/cyclosome (APC/C) is required for the efficient turnover of specific APC substrates and for the degradation of Clb2 at the end of mitosis (UFANO *et al.* 2004; PAGE *et al.* 2005). *swm1Δ* mutants are able to complete PSM closure and arrest at a later stage in spore development (UFANO *et al.* 1999). Also, mutants in components of the FEAR network (such as *cdc14-3*, *spo12Δ*, or *slk19Δ*) have defects in exit from meiosis I but are able to complete the morphogenesis program and form asci with two spores (dyads) (SHARON and SIMCHEN 1990; MARSTON *et al.* 2003). Finally, the Spo13 protein plays a key role in preventing sister centromere separation in meiosis I (LEE *et al.* 2002; SHONN *et al.* 2002). Even though *spo13Δ* mutants have defects in nuclear segregation, they are able to produce two viable diploid spores (KLAPHOLZ and ESPOSITO 1980a; KLAPHOLZ and ESPOSITO 1980b).

One important question still to be resolved is how MEN is activated at the end of meiosis II and whether the entire MEN functions in exit from meiosis or whether this is a unique function for Cdc15. As noted, mutation of the MEN components *TEM1* and *LTE1* does not cause sporulation defects (KAMIENIECKI *et al.* 2005), although the function of the downstream kinases Dbf2 and Dbf20 during meiosis remains to be tested. In addition, unlike the situation with *cdc15-ts* mutations in mitotic cells (GRANDIN *et al.* 1998), overexpression of Cdc14 failed to rescue the *P_{CLB2}-CDC15* phenotype. These results suggest that the sporulation function of *CDC15* might be independent of the MEN pathway. Alternatively, it could be speculated that two upstream activators Tem1 and Lte1 might be dispensable during meiosis due to the intrinsic differences between mitotic and meiotic division. Although we have been unable to localize Cdc15 during sporulation, either using a Cdc15-GFP or by indirect immunofluorescence, this kinase localizes to the SPB in mitosis (CENAMOR *et al.* 1999; XU *et al.* 2000; MENSSEN *et al.* 2001; VISINTIN and AMON 2001). Thus, it is tempting to surmise that Cdc15 would also reside in the SPBs during meiosis, and that its activation could be related to some specific modification of the outer plaque of the SPB that occurs at the

beginning of meiosis II. In fact, Cdc15-depleted cells have defects in the disassembly of the meiotic outer plaque at the end of anaphase II.

In *S. pombe*, the analog of the MEN is referred to as the septation initiation network (SIN) (for reviews, see BARDIN and AMON 2001; KRAPP *et al.* 2004). The SIN has recently been shown to be required for sporulation in *S. pombe* (KRAPP *et al.* 2006). Interestingly, mutants lacking SIN function proceed through meiosis with normal kinetics but display abnormal forespore membranes, the *S. pombe* equivalent of the PSM, which fail to package nuclei and form mature spores. No unusual organization of microtubules at late time points was noted in this study, but it is tempting to speculate that the MEN and the SIN might play conserved roles in meiotic exit in the two yeasts. One SIN component characterized in this work was the SPB component *cdc11*, which serves as the organizing center for the SIN. *NUD1*, the *S. cerevisiae* homolog of *cdc11*, plays an analogous role for the MEN, and it too has been reported to have a sporulation defect (GORDON *et al.* 2006). Strains harboring a *ts* allele of *NUD1* proceed normally through meiosis but failed to form spores efficiently at the restrictive temperature. Although this is a less severe phenotype than *P_{CLB2}-CDC15* or *cdc11-ts* mutants in *S. pombe*, it is conceivable that the *nud1* sporulation defect might result from a failure to adequately activate Cdc15. Taken together, these different results suggest that the phenotypes seen in the *P_{CLB2}-CDC15* strain may indicate a general requirement for the MEN in meiosis II exit and a specific Cdc15 function in spore development.

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