Dbf2 is essential for cytokinesis and correct mitotic spindle formation in *Candida albicans*

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SUMMARY

We have characterized the *DBF2* gene, encoding a protein kinase of the NDR family in *Candida albicans,* and demonstrate that this gene is essential for cell viability. Conditional mutants were constructed by using the *MET3* promoter to analyze the phenotype of cells lacking this kinase. The absence of Dbf2 resulted in cells arrested as large-budded pairs that failed to contract the actomyosin ring, a function similar to that described for its *S. cerevisiae* orthologue. In addition to its role in cytokinesis, Dbf2 regulates mitotic spindle organization and nuclear segregation since Dbf2-depleted cells have abnormal microtubules and severe defects in nuclear migration to the daughter cell, which results in a cell cycle block during mitosis. Taken together, these results imply that Dbf2 performs several functions during exit from mitosis and cytokinesis. Consistent with a role in spindle organization, the protein localizes to the mitotic spindle during anaphase, and it interacts physically with tubulin, as indicated by immunoprecipitation experiments. Finally, *DBF2* depletion also resulted in impaired true hyphal growth.

INTRODUCTION

The *Saccharomyces cerevisiae* mitotic exit network (MEN) is a conserved signalling network that coordinates several events associated with mitotic exit including the inactivation of cyclin-dependent kinase (CDK), the activation of cytokinesis, the initiation of G1 gene transcription, and the formation of prereplication complexes (Simanis, 2003, Seshan and Amon, 2004). The MEN comprises several regulatory proteins, including Tem1 GTPase, the two-component GTPase-activating factor (GAP) Bub2-Bfa1, a putative GTP exchange factor (GEF) Lte1, a Cdc14 protein phosphatase, four protein kinases -Cdc5, Cdc15, Dbf2, Dbf20- and a Dbf2-associated protein: Mob1. The equivalent signalling network in *Schizosaccharomyces pombe* is called the septation initiation network (SIN) and is required for coordinating cytokinesis with CDK inactivation (Simanis, 2003, Wolfe and Gould, 2005).

S. cerevisiae Cdc14 plays a key role in MEN signalling (reviewed in Seshan and Amon, 2004, Stegmeier and Amon, 2004). Cdc14 is a phosphatase that induces the inactivation of mitotic CDKs by reversing CDK-dependent phosphorylation on proteins that are important for promoting mitotic CDK inactivation (Jaspersen *et al.*, 1998, Visintin *et al.*, 1998, Jaspersen *et al.*, 1999). Along most of the cell-cycle, Cdc14 is sequestered in the nucleolus (Shou *et al.*, 1999, Visintin *et al.*, 1999). During anaphase and telophase, Cdc14 is released from this localization by the action of the FEAR network (Cdc14 early anaphase release) and the mitotic exit network (MEN) (reviewed in Bardin and Amon, 2001, McCollum and Gould, 2001, Stegmeier and Amon, 2004). The FEAR network appears to be activated during the metaphase-anaphase transition and promotes the release of Cdc14 from the nucleolus to the nucleus during early anaphase (Stegmeier *et al.*, 2002, Sullivan and Uhlmann, 2003). The MEN is activated when the nucleus migrates into the daughter cell during anaphase, and this is essential for promoting and sustaining the release of Cdc14 from the nucleolus during the late stages of anaphase (Bardin *et al.*, 2000, Pereira *et al.*, 2000). Once released in telophase, Cdc14 disperses throughout the cell and is concentrated on spindle pole bodies (SPBs) and at the bud neck (the site of cytokinesis) before returning to the nucleolus (Yoshida *et al.*, 2002, Bembenek *et al.*, 2005).

The *S. cerevisiae* protein kinases Dbf2 and Dbf20 are members of the Lats/NDR (Nuclear Dbf2-

Related) kinase family, a subclass of the AGC family of protein kinases (Manning *et al.*, 2002, Tamaskovic *et al.*, 2003, Hergovich *et al.*, 2006) that includes the mammalian and *Drosophila* Lats/Warts tumour suppressor, *S. pombe* Orb6, *S. cerevisiae* Cbk1 and *C. elegans* Sax1 (Xu *et al.*, 1995, Verde *et al.*, 1998, Geng *et al.*, 2000, Racki *et al.*, 2000, Zallen *et al.*, 2000). The NDR kinases share high sequence conservation, and have been shown to regulate mitosis, cell growth, and development in eukaryotes (Hergovich *et al.*, 2006, Hergovich *et al.*, 2008). In *S. cerevisiae*, Mob1 and the Dbf2 kinase function together as a unit. Mob1 binding to Dbf2 is essential for kinase activation by Cdc15 (Mah *et al.*, 2001). Mob1-Dbf2 kinase activity varies along the cell cycle, peaking during mitosis and being minimal in the G1 and S phases (Toyn and Johnston, 1994, Visintin and Amon, 2001). Dbf2 kinase activation is also dependent on other MEN proteins, which is consistent with the notion that Mob1-Dbf2 would function late in MEN signalling. Both Mob1 and Dbf2 localize to the cytoplasmic surfaces of SPBs, the nucleus, and to the bud neck during late mitosis (Frenz *et al.*, 2000, Luca *et al.*, 2001, Visintin and Amon, 2001, Stoepel *et al.*, 2005). The SPB localization appears to be important for Mob1-Dbf2 function, because mutations in the SPB protein Nud1 induce telophase arrest and abolish the SPB and bud neck localizations of Mob1 and other MEN proteins (Luca *et al.*, 2001, Seshan and Amon, 2004). In the nucleus, Mob1-Dbf2 colocalizes with Cdc14, and this localization is important for maintaining chromosomal passenger proteins on the mitotic spindle during anaphase and mitotic exit, thus coordinating chromosome segregation with cytokinesis and/or coordinating spindle disassembly with other mitotic exit events (Stoepel *et al.*, 2005). In addition to their role in mitotic exit, Dbf2-Mob1 and the other MEN proteins also function in cytokinesis. Bud neck localization of Dbf2 and Mob1 are dependent on each other as well as on the MEN proteins Cdc5, Cdc14, Cdc15, Nud1, and the septins Cdc12 and Cdc3 (Frenz *et al.*, 2000, Luca *et al.*, 2001, Yoshida and Toh-e, 2001), and this localization is crucial for cytokinesis.

Candida albicans is an important opportunistic fungal pathogen in humans. It can grow as a unicellular yeast or in multicellular form; i.e. as pseudohyphae or true hyphae, which are distinct morphological states (Sudbery *et al.*, 2004). Because the virulence of *C. albicans* mutants that are unable to switch between yeast and hyphal forms is considerably reduced in mouse models, the

yeast-to-hypha transition is thought to be one of the major factors contributing to virulence (Lo *et al.*, 1997, Mitchell, 1998, Braun *et al.*, 2000). Most MEN/SIN proteins are conserved from yeast to humans, but the functions of MEN/SIN-related networks have not been resolved in other eukaryotes, such as *C. albicans*. Only the function of Cdc14 has been analyzed in this organism (Clemente-Blanco *et al.*, 2006). Interestingly, *CaCDC14* is not an essential gene, in contrast to its *S. cerevisiae* ortholog (Grandin *et al.*, 1998), suggesting that regulation of the exit from mitosis and cytokinesis is different in these two yeasts. The *C. albicans* Cdc14 phosphatase is not required for exit from mitosis or cytokinesis, although it is involved in the activation and daughter-specific nuclear accumulation of the Ace2 transcription factor, which activates the cell separation program (Kelly *et al.*, 2004, Clemente-Blanco *et al.*, 2006).

Here we analysed the function of the Dbf2 kinase in *C. albicans* in the different morphological states in order to investigate whether this kinase is important for regulating exit from mitosis. We demonstrate that Dbf2 is an essential protein necessary for actomyosin ring contraction and cytokinesis in *C. albicans.* Furthermore, Dbf2 also regulates mitotic spindle organization and nuclear segregation. In addition, the absence of Dbf2 results in cells with impaired hyphal growth, suggesting that Dbf2 activity is also required for morphogenesis in this fungal pathogen.

RESULTS

DBF2 is essential for vegetative growth

A search of the *Candida* Genome Database (Arnaud *et al.*, 2007) revealed the presence of a single open reading frame (*orf19.1223*) that had significant sequence similarity to the *S. cerevisiae* NDR kinases Dbf2 and Dbf20 (45% overall identity to both Dbf2 and Dbf20). Sequence analysis of the polypeptide encoded by *orf19.1223* unveiled a structure similar to that of ScDbf2, containing a protein kinase domain (residues 240 to 557) followed by an AGC-kinase C-terminal domain profile (559-658). Interestingly, the *C. albicans* protein showed the typical characteristics of NDR kinases, such as the presence of an insert of 30-60 residues between subdomains VII and VIII of the kinase domain (aa 396 to 450) and a hydrophobic motif located at the C-terminus (Fig. 1A). Furthermore, NDR kinases contain two main regulatory phosphorylation sites, one at the activation site (Ser374

in ScDbf2) and the other at the hydrophobic motif (Thr544 of ScDbf2), which were perfectly conserved in the *C. albicans* protein. These observations suggested that the product of *orf19.1223* could be the only ortholog of the Dbf2 and Dbf20 kinases, and it was designated Dbf2. However, the *C. albicans* Dbf2 was longer than the *S. cerevisiae* counterparts (710 aa) and contained additional features absent in *S. cerevisiae* proteins, such as a Gln-rich region at the N-terminus (residues 58 to 141) and an Asn-rich region immediately after the C-terminal hydrophobic motif (653-697).

In order to study the function of *DBF2* in *C. albicans* , both alleles of the gene were deleted using the *URA3* blaster method in strain CAI4 (Fonzi and Irwin, 1993). The first allele was easily replaced with the *dbf2::hisG-URA3-hisG* construct (strain JJ1) and heterozygous *DBF2*/*dbf2::hisG* strains were obtained (strain JJ2). Replacement of the second allele was never achieved, since all the transformants analyzed contained a copy of the wild-type *DBF2* allele (Fig. 1B), suggesting that *DBF2* is an essential gene in *C. albicans,* in contrast to its *S. cerevisiae* counterpart (Toyn and Johnston, 1994). To check this observation, a conditional strain was constructed in which the single remaining copy of *DBF2* was placed under the control of the *MET3* promoter on the BWP17 background (Care *et al.*, 1999). P_{MET3}-*DBF2* cells grew normally under non-repressing conditions, but followed a slow growth pattern when the promoter was repressed by the addition of methionine and cysteine to the culture medium (Fig. 1C). This absence of a lethal phenotype in Dbf2-depleted cells could be due to a residual promoter activity remaining under the repressed conditions. Thus, to analyze the levels of Dbf2 in repressed cells, the P_{MET3}-*DBF2* allele was tagged with the 3xHA epitope at the C-terminus and the levels of Dbf2-HA were monitored by Western blot analysis of cells grown in liquid medium under repressed conditions. As shown in Fig. 1D, the amount of Dbf2-HA decreased rapidly after promoter shut-off, and was undetectable by 60 minutes after the addition of methionine and cysteine to the culture medium. Moreover, P_{MET3}-*DBF2-HA* cells were unable to grow on plates under repressing conditions (Fig. 1C), indicating that *DBF2* is essential for cell viability in *C. albicans*. We therefore generated two conditional alleles (P_{MET3}-*DBF2* and P_{MET3}-*DBF2-HA*) with different degrees of severity in the phenotype. These differences were not due to the HA tag because Dbf2-HA was functional, as judged by the absence of any discernable

phenotype when expressed as the sole source of this protein (*dbf2Δ/DBF2-HA*; not shown). However, we cannot rule out the possibility that the HA might be partially compromising the activity of the Dbf2 protein, resulting in a more severe phenotype under repressing conditions. Together, these observations indicate that *DBF2* is essential for cell viability in *C. albicans.*

Dbf2 is required for primary septum assembly

In *S. cerevisiae,* the Dbf2 kinase is a component of the MEN pathway that plays redundant roles with Dbf20, being necessary for the activation of actomyosin ring contraction during mitotic exit and cytokinesis (Frenz *et al.*, 2000, Corbett *et al.*, 2006). To test whether the Dbf2 kinase might play a similar role in the regulation of actomyosin ring contraction in *C. albicans*, the phenotype of P_{MET3}-*DBF2* cells was analyzed by microscopic inspection. In contrast to the wild-type strain, in which yeast cells readily separated after cytokinesis, we found that P_{MET3}-*DBF2* cells formed chains of cells that failed to separate after promoter shut-off (Fig. 2A). Moreover, the bud neck in Dbf2-depleted cells was wider than that of wild-type cells, especially those close to the ends of the cell chains (arrows in Fig. 2A). Consistent with this observation, the neck diameter/cell diameter ratio in P_{MET3}-*DBF2* cells after repression was higher than in wild-type cells (0.3 ± 0.03 *versus* 0.5 ± 0.05; n=100; Fig. 2A). This phenotype is reminiscent of that described for *S. cerevisiae* mutants in MEN components, such as *cdc15-lyt1* or *dbf2-1* (Jiménez *et al.*, 1998), which fail to contract the actomyosin ring and assemble the primary septum.

To analyze whether Dbf2-depleted cells were able to complete cytokinesis and assemble the separation septum, we used electron microscopy. Wild-type and P_{MET3}-*DBF2* cells grown in the absence of both methionine and cysteine had a similar morphology and were able to assemble normal separation septa (Fig. 2B, panels 1 and 2, respectively). However, after 6 h of repression chains of cells were present in P_{MET3}-*DBF2* cells, in which the separation septum was not assembled (Fig. 2B, panels 3 and 5). Thus, these results indicate that Dbf2 is necessary for cytokinesis and septum formation.

Dbf2 is required for actomyosin ring contraction

The failure to assemble the separation septum under *DBF2* repression conditions could be due to defects in the assembly of the actomyosin ring or its contraction. To distinguish between these two possibilities, Mlc1 was tagged with YFP in the P_{MET3}-*DBF2* strain to analyze actomyosin ring assembly. Mlc1 is the myosin regulatory light chain, which localizes to the bud tip and the bud neck during cytokinesis (Crampin *et al.*, 2005). In wild-type cells, Mlc1-YFP formed a ring at the bud neck in large-budded cells that was clearly visible (Fig. 3A, left panels). When P_{MET3}-*DBF2 MLC1-YFP* cells that had been incubated for 6h under repressing conditions were analyzed, the fluorescence at the bud neck of the chains of cells was also clearly detected (Fig. 3A, right panels), indicating that the actomyosin ring had been assembled under these conditions. However, the actomyosin ring persisted in the bud neck of the cells of the chain, even after the daughter cell had already budded (arrows), suggesting possible defects in ring contraction or disassembly.

To analyze actomyosin ring contraction in greater detail, time-lapse experiments were performed on wild-type and P_{MET3}-*DBF2* cells carrying *MLC1-YFP*. In wild-type cells, Mlc1 was incorporated at the bud neck region in large budded cells, contracted during cytokinesis and then disassembled, with an average time from ring assembly to disassembly of around 15 ± 1 min (n=8; data not shown and Fig. 3C). By contrast, in P_{MET3}-*DBF2* cells Mlc1-YFP was incorporated into the actomyosin ring before cytokinesis (Fig. 3B, asterisks) but remained stable and uncontracted for a long time, even after the next ring had been assembled (Fig. 3B, arrows). Mlc1-YFP rings remained assembled and failed to contract for more than 75 minutes in the mutant strain (n=15 cells), the time analyzed in the experiment (Fig. 3B-C). These results therefore indicate that Dbf2 is essential for actomyosin ring contraction during cytokinesis.

Dbf2-depleted cells arrest after DNA replication

To study whether Dbf2-depleted cells had any cell cycle defects, DNA contents and cell size were monitored before and after repression of the *MET3* promoter. In comparison with wild-type cells, asynchronous cultures of induced P_{MET3}-*DBF2* and P_{MET3}-*DBF2-HA* cells showed a significant increase of the G1 population (Fig. 4A, time 0h), correlated with a smaller cell size

(Fig. 4A, lower panels), suggesting that non-physiological levels of Dbf2 give rise to a premature mitotic exit. After three hours of promoter repression, the DNA content and cell size of P_{MET3}-*DBF2* and P_{MET3}-*DBF2-HA* cells were similar to those of the wild-type cells (Fig. 4A, time 3h). Further incubation under repressing conditions produced defects in cell cycle progression. Thus, after 6 hours of growth in *MET3* repressing medium P_{MET3}-*DBF2-HA* cells arrested as cells with duplicated DNA (4c) (Fig. 4A, time 6h). As expected, growth of P_{MET3}-*DBF2* cells was not blocked since a DNA content higher than 4c was observed due to the cytokinesis phenotype described above (Fig. 2A). The different phenotype of P_{MET3}-*DBF2* and P_{MET3}-*DBF2-HA* cells is mainly due to the fact that cells carrying P_{MET3}-*DBF2* do not block at the end of mitosis, despite the defect in cytokinesis, while those bearing the P_{MET3}-*DBF2-HA* allele arrest as pairs of large-budded cells without a septum (see below). Interestingly, the size of P_{MET3}-*DBF2* and P_{MET3}-*DBF2-HA* cells gradually increased during incubation in repressing conditions and after 6h they were bigger than wild-type cells (Fig. 4A, lower panels). These results also show that despite their different severity, the P_{MET3}-*DBF2* and P_{MET3}-*DBF2-HA* alleles have similar effects on cell-cycle progression. To test whether the differences in cell size and the relative abundance of G1/G2 cells observed in cells grown under non-repressing conditions might be attributed to different protein levels, Western analysis was used to compare Dbf2 protein abundance in strains carrying *DBF2*-HA under the control of its native promoter or the *MET3* promoter. The results indicated the Dbf2 protein levels were 4 to 5-fold higher in P_{MET3}-*DBF2-HA* cells than those observed for *DBF2*/*DBF2*-HA or *dbf2Δ*/*DBF2*-HA cells grown under non-repressing conditions (Fig. 4B). Together, these observations suggest that Dbf2 might play a role in regulating cell-cycle progression in *C. albicans*.

To further confirm these observations, P_{MET3}-*DBF2-HA* and heterozygous *DBF2*/*DBF2-HA* cells were synchronized by incubation with hydroxyurea (HU) for 2 h and then released in medium containing methionine and cysteine to repress the *MET3* promoter. Aliquots were collected every 15 min after release and the DNA content of the samples was analyzed by flow cytometry. The results indicated that after HU release, Dbf2-depleted cells arrested after DNA replication (Fig. 4C), in contrast to control cells, which were able to continue cell-cycle progression. As a control, the same strains were released into medium without methionine after HU arrest to allow the

expression of *DBF2*. In this case, cell-cycle progression was similar to that of the control cells (Supplementary Figure S1). These results therefore indicate that Dbf2 might play an essential function in cell-cycle progression after DNA replication in *C. albicans*.

Dbf2 is also required for proper nuclear segregation

To characterize the arrest phenotype of cells carrying the P_{MET3}-*DBF2-HA* allele in more detail, cells were grown to mid-logarithmic phase in minimal medium and then transferred to repressing medium. Aliquots were collected at different time points after promoter shut-off and stained with DAPI to analyze nuclear morphology. Microscopic inspection of DAPI-stained cells indicated that the depletion of the Dbf2 kinase resulted in defects in nuclear segregation, as suggested by the presence of cells with abnormal numbers of nuclei or abnormal nuclear segregation (Fig. 5A). Several different defects were observed. In some cases, the two nuclei generated after mitosis remained in the mother cell instead of entering the bud (cells 1-2); in other cells, the orientation of the daughter nuclei was correct but the separation of the two DNA masses was incomplete (cells 3-5), and even a few cells with three or more DNA masses were observed (cell 6). The percentage of cells displaying these defects was quantified along time. First, cells were classified according to the number of nuclei in each cell body. The depletion of Dbf2 resulted in an accumulation of cells with abnormal numbers of nuclei (none, 2 or 3) as compared with wild type, which increased with the incubation time (Fig. 5B). Cells were also classified according to the nuclear morphology and orientation of the mitotic spindle in four groups: normal orientation and separation of DNA masses (Fig. 5C, group a); correct orientation along the bud-mother axis but defects in separation of the DNA (Fig. 5C, group b); defects in orientation but normal separation (Fig. 5C, group c), and defects in orientation and separation (Fig. 5C, group d). In contrast to the wild-type cells, Dbf2-depleted cells had greater percentages of defects in alignment, separation, or both, which increased with the incubation time. Interestingly, when nuclear segregation was analyzed in P_{MET3}-*DBF2* cells incubated in medium containing methionine and cysteine, the same defects in nuclear segregation were observed (Fig. 5D, arrowheads). These results therefore indicate that the Dbf2 kinase is necessary for proper nuclear segregation during mitosis.

Dbf2-depleted cells have defects in mitotic spindle organization

Since nuclear segregation requires proper organization and orientation of the mitotic spindle, we analyzed its morphology in wild-type and mutant cells by immunofluorescence using anti-tubulin antibodies. Wild-type and P_{MET3}-*DBF2-HA* cells that had been incubated in repressing medium for 6 h were fixed and stained with anti-tubulin antibody and DAPI and samples were inspected under the microscope. We found that the absence of Dbf2 produced defects in spindle organization (Fig. 6). Wild-type cells exhibited microtubules that could be clearly seen inside the nucleus and also astral microtubules, as previously described (Finley and Berman, 2005). In contrast, P_{MET3}-*DBF2-HA* cells presented aberrant microtubules, in which abnormal tubulin structures were observed in addition to the mitotic spindle (Fig. 6, arrows), or extremely elongated spindles. These observations suggest that the nuclear segregation defects observed in P_{MET3}-*DBF2-HA* cells could arise from defects in the proper organization of the mitotic spindle, and that Dbf2 plays an important role in spindle organization in *C. albicans*.

Dbf2 localization is cell cycle-regulated

To study the *in vivo* localization of Dbf2, a *DBF2-GFP* strain was constructed by inserting the GFP-coding sequence at the C-terminus of the *DBF2* gene. In *S. cerevisiae*, Dbf2 localizes to the cytoplasmic surfaces of SPBs, the nucleus, and the bud neck during late mitosis (Frenz *et al.*, 2000, Luca *et al.*, 2001, Visintin and Amon, 2001, Stoepel *et al.*, 2005). Microscopic inspection of *C. albicans* yeast cells bearing *DBF2-GFP* revealed that the fluorescent signal was also cell cycle-regulated (Fig. 7A). In small unbudded G1 cells, the GFP signal was detected as an intense dot, possibly corresponding to the SPB. At the G1/S transition (small budded cells), Dbf2-GFP remained associated with the SPB but also started to accumulate in a linear structure in the nuclei of the cells. During mitosis, a very faint Dbf2-GFP signal along the mother-bud axis was observed, suggesting that Dbf2 could be associated with the mitotic spindle during this part of the cell cycle. Finally, Dbf2-GFP localized to the bud neck at the end of mitosis and cytokinesis. Thus, these observations indicate that Dbf2 localization is cell cycle-regulated in *C. albicans*.

To confirm that Dbf2 localized to the mitotic spindle during mitosis, anti-GFP antibodies were

used to localize the fusion protein in nuclear spreads from *DBF2-GFP* cells. As a control, anti-tubulin antibodies were used in an independent assay. Tubulin was initially detected as a dot in premitotic cells (the SPB), after which it expanded as a linear structure corresponding to the mitotic spindle (Fig. 7B). This pattern was similar to the localization described for Tub1-GFP in *C. albicans* (Finley and Berman, 2005). When the localization of Dbf2-GFP was analyzed using anti-GFP antibodies, we found that the pattern was similar to that observed for microtubules (Fig. 7B). Thus, in pre-mitotic nuclei Dbf2 appeared as a dot while in mitotic cells it localized as a linear structure that expanded the nuclei, similar to the pattern observed for tubulin. As a final confirmation that Dbf2 indeed localized to the SPBs and mitotic spindle, *DBF2* was tagged with YFP in a strain carrying *TUB2-CFP* to visualize these structures. Microscopic inspection of these cells confirmed that Dbf2 colocalized with the SPBs in G1 cells and with the mitotic spindle during mitosis (Fig. 7C). After nuclear segregation, Dbf2 was found at the bud neck. Together, these results therefore indicate that Dbf2 localizes to the mitotic spindle during mitosis in *C. albicans*. This localization is in good agreement with the defects in spindle assembly and nuclear segregation observed for Dbf2-depleted cells.

Co-immunoprecipitation experiments were used to confirm the physical association of Dbf2 and tubulin. Protein extracts from a wild-type or a *DBF2-HA* strain were incubated with anti-HA antibodies and the immunoprecipitated material was analyzed by Western blotting using anti-tubulin or anti-HA antibodies. The results indicated that the anti-HA antibody was able to immunoprecipitate tubulin from protein extracts prepared from *DBF2-HA* cells (Fig. 7C), and that the Dbf2-tubulin interaction was specific, since no material was immunoprecipitated from cells lacking Dbf2-HA. Thus, these results indicate that Dbf2 not only colocalizes with microtubules but that, directly or indirectly, it is physically associated with tubulin.

Dbf2 is a phosphoprotein and its phosphorylation during hyphal growth is partially dependent on the Hgc1 cyclin

Western analysis of Dbf2 unveiled the presence of forms with different mobilities (Fig. 1D). To determine whether the slower-migrating forms were due to phosphorylation, protein extracts

from yeast cells or hyphae were treated with λ phosphatase (λ PPase). The addition of λ PPase to the extracts produced a shift in Dbf2 to the faster-migrating form (Fig. 8A) both in yeast and hyphae, indicating that Dbf2 had indeed been phosphorylated under both growth conditions.

To gain further insight into the signals that regulate Dbf2 phosphorylation in response to hypha-inducing signals, we analysed its phosphorylation in an *hgc1*Δ mutant strain. *HGC1* encodes a G1-like cyclin that plays an important role in hyphal morphogenesis (Zheng *et al.*, 2004). In exponentially growing *hgc1Δ DBF2-HA* cells at 28ºC (yeast growth conditions), the phosphorylated band was also present (Fig. 8B). However, when these cells were incubated in hypha-inducing conditions (YPD plus 10% FCS at 37ºC), the amount of the phosphorylated form was reduced compared to the wild-type strain (ratio phosphorylated/non-phosphorylated of 0.5 in *hgc1*Δ cells versus 0.99 in the wild type; Fig. 8B), indicating that Dbf2 phosphorylation during hyphal growth is at least partially dependent on the Hgc1 cyclin.

Dbf2 is required for normal hyphal morphogenesis

Since the phosphorylation of Dbf2 in hyphae is dependent on Hgc1, we examined whether hyphal growth was also affected in Dbf2-depleted cells. To this end, we analysed the morphology of the P_{MET3}-*DBF2* cells under hypha-inducing conditions. Cells were incubated in liquid YPD medium containing methionine, cysteine, and 10% of FCS at 37ºC, and samples were fixed and stained with DAPI and Calcofluor White to analyze hyphal morphology and nuclear position. Cells without Dbf2 showed substantial defects in hyphal morphology as compared to wild-type cells (Fig. 8C). Their hyphal tubes were wider than those found in wild-type cells, and frequently displayed constrictions resembling the bud neck of yeast cells (Fig. 9C, arrows). In addition, Calcofluor staining revealed that no septum had been assembled between the different nuclei of the hyphae. These results indicate that Dbf2 is essential for proper hyphal morphogenesis and for septum assembly during this growth pattern.

DISCUSSION

In eukaryotes, NDR kinases perform essential roles in the regulation of mitosis, cell growth, and

development (Hergovich *et al.*, 2006, Hergovich *et al.*, 2008). In *S. cerevisiae*, the protein kinases Dbf2 and Dbf20 function as redundant proteins, acting as downstream components of the MEN pathway, which is required for exit from mitosis and cytokinesis (reviewed in Bardin and Amon, 2001, McCollum and Gould, 2001, Stegmeier and Amon, 2004). BLAST searches revealed the presence of a single protein with significant sequence similarity to Dbf2 and Dbf20 in the *C. albicans* genome, which also contains all the features of the NDR kinase family and was accordingly named CaDbf2.

In this report, we have analyzed the function of the Dbf2 protein kinase in the *C. albicans* cell cycle and different forms of growth. The results obtained indicate that Dbf2 shares some functions with its *S. cerevisiae* counterparts Dbf2/Dbf20 during exit from mitosis, although there are also some interesting peculiarities of the *C. albicans* protein. As in *S. cerevisiae* Dbf2/Dbf20, CaDbf2 was necessary for actomyosin ring contraction and cytokinesis to be activated at the end of mitosis, and it localized to the SPBs early in mitosis and at the bud neck prior to cytokinesis (Frenz *et al.*, 2000, Luca *et al.*, 2001, Visintin and Amon, 2001, Stoepel *et al.*, 2005, Corbett *et al.*, 2006, and this work). In spite of these similarities, there were also important differences. In *S. cerevisiae*, Dbf2 and Dbf20 are partially redundant proteins, since the single mutants are viable and only the double *dbf2 dbf20* mutant is lethal (Toyn and Johnston, 1994, Corbett *et al.*, 2006), while in *C. albicans DBF2* is essential for cell viability. More interestingly, in *C. albicans* Dbf2 localized to the mitotic spindle and was necessary for the proper organization of the spindle in mitotic cells and for correct nuclear segregation. Finally, Dbf2 activity was also required for proper hyphal morphogenesis.

Dbf2 regulates nuclear segregation during mitosis

Analyses of synchronized cultures of cells carrying the P_{MET3}-*DBF2-HA* allele showed that mutant cells uniformly arrested as pairs of large budded cells with duplicated DNA masses. Interestingly, we observed that these cells were often defective in completing correct segregation of the two daughter nuclei, and even presented defects in the orientation of the mitotic spindle along the mother-bud axis. This abnormal DNA segregation could be due to untimely nuclear segregation

or, alternatively, to defects in mitotic spindle assembly or orientation during mitosis. Consistent with these possibilities, we found that the absence of Dbf2 resulted in defects in properly organizing and orienting the mitotic spindle, and in some cases in disassembling it at the end of mitosis. Accordingly, these observations suggest that Dbf2 has an important function in nuclear segregation at the end of mitosis.

Analysis of the localization of Dbf2 during the cell cycle supports this idea. The subcellular localization of Dbf2 varied during the cell cycle, similar to the results described for *S. cerevisiae* (Frenz *et al.*, 2000, Luca *et al.*, 2001, Visintin and Amon, 2001, Stoepel *et al.*, 2005). According to our results, in *C. albicans* Dbf2 localized to the SPBs early on in mitosis and at the bud neck after DNA separation at the end of mitosis, as has been previously reported for the *S. cerevisiae* homolog. However, our results indicate that *CaDBF2* also localized to the mitotic spindle during mitosis. Moreover, co-immunoprecipitation experiments revealed a physical interaction between Dbf2 and tubulin, although we do not know whether this interaction is direct or indirect. Interestingly, in *S. cerevisiae* the Dbf2 kinase has also been reported to interact with tubulin (Graumann *et al.*, 2004). This localization to the mitotic spindle could explain the defects in spindle organization and nuclear segregation observed in Dbf2-depleted cells.

Taken together, our observations suggest that the spatial regulation of Dbf2 may be important for progression through the cell cycle. Moreover, the essential function of Dbf2 in *C. albicans* cells could be the regulation of spindle integrity involved in chromosome separation during mitosis and/or spindle disassembly during mitotic exit. This function is in good agreement with the general role of the MEN in coordinating multiple mitotic exit events, such as CDK inactivation, cytokinesis, and the initiation of G1-specific transcription proposed for *S. cerevisiae* (Seshan and Amon, 2004). Interestingly, in *S. cerevisiae* it has been shown that the Mob1-Dbf2 complex regulates passenger protein localization, and it has been suggested that this function could be important for maintaining genomic stability during mitosis or for contributing to spindle disassembly (Stoepel *et al.*, 2005). Accordingly, in *C. albicans* the spindle-associated Dbf2 might contribute to spindle disassembly at the end of mitosis by regulating the interaction or function of essential spindle-associated proteins.

Dbf2 is essential for actomyosin ring contraction and cytokinesis

Successful completion of the cell cycle requires that mitosis and cytokinesis be co-ordinated in all cells. However, yeasts also have to achieve cell separation, which demands the deposition of new cell wall material in addition to contractile ring-guided plasma membrane synthesis. Using the P_{MET3}-*DBF2* allele, we found that Dbf2 was also necessary for actomyosin ring contraction and septum deposition. The absence of Dbf2 resulted in cells that were able to incorporate the myosin regulatory light chain Mlc1 to the bud neck region during cytokinesis, indicating that the contractile ring was normally assembled, but that its contraction had failed to be activated. Interestingly, the observation that Dbf2 localizes to the bud neck region at the end of mitosis suggests that this kinase could be important in the activation of ring contraction, thus coordinating exit from mitosis and cytokinesis.

In budding yeast the localization patterns of Cdc5, Cdc15 and Dbf2 at the bud neck following mitotic exit have led to the suggestion that these proteins would serve to promote cytokinesis and/or cell separation. In fact, it has been shown that Cdc15 and other MEN proteins play a critical role in the control of cytokinesis (Jiménez *et al.*, 2005). Furthermore, phenotypic examination of strains carrying the thermosensitive mutations *tem1-3*, or *cdc15-1*, or the double *dbf2*Δ *dbf20*Δ mutant indicated that these mutant strains have little or no defects in actomyosin ring assembly although ring contraction often fails (Corbett *et al.*, 2006). It has also been shown that in cells carrying the *cdc15-lyt1* allele, septation and cell separation fail and the cells undergo additional budding cycles without actomyosin ring contraction (Jiménez *et al.*, 1998). These observations therefore indicate that one of the functions of the Dbf2 kinase in *C. albicans* is activation of actomyosin ring contraction at the end of mitosis, as described in other yeasts.

Our results and previous findings indicate that the essential function of Dbf2 in *C. albicans* does not appear to be linked to Cdc14 function or localization, unlike the situation in *S. cerevisiae*. The observation that *C. albicans cdc14*Δ mutant cells are viable suggests that the Cdc14 phosphatase plays a non-essential role in mitotic exit. *cdc14*Δ mutants are able to complete the cell cycle and assemble a separation septum between mother and daughter cells, although they fail to degrade the separation septum and remain attached in large clumps, indicating that in *C. albicans*

the phosphatase Cdc14 is a key activator of cell separation (Clemente-Blanco *et al.*, 2006). Although we have not analyzed whether Dbf2 is necessary for Cdc14 release from the nucleus, the above observations suggest that the essential function of this kinase during the cell cycle might not the release of Cdc14 from the nucleus. Accordingly, our results indicate that the regulation of exit from mitosis and its coordination with spindle disassembly and cytokinesis have different requirements in these two yeasts.

Dbf2 and hyphal morphogenesis

We found that Dbf2-depleted cells had impaired true hyphal growth, suggesting that Dbf2 contributes to filamentous growth. In response to serum, the cells gave rise to abnormal germ tubes. Interestingly, we found that Dbf2 is a phosphoprotein during yeast and hyphal growth, and that its phosphorylation during hyphal growth is dependent on the Hgc1 cyclin. *HGC1* encodes a G1 cyclin-related protein that forms a complex with Cdc28p and plays a key role in hyphal morphogenesis. *HGC1* expression is under the control of hypha-inducing signals, ensuring that Hgc1 is expressed at all times during the cell cycle as long as inducing conditions are maintained. In response to serum, *hgc1*Δ cells have severe defects in hyphal morphogenesis (Zheng *et al.*, 2004). In this context, Dbf2 contains only one Cdc28 consensus site (S/TPXK/R) at its N-terminus. It is therefore possible that the Hgc1/Cdc28-dependent phosphorylation of CaDbf2 might be indirect and mediated through other unknown kinases.

EXPERIMENTAL PROCEDURES

Strains construction and growth conditions

The strains used in this work are listed in Table 1. The oligonucleotides used in this work are listed in Supplementary Table 1. Cells were grown in YPD or in synthetic minimal (SC) medium at 28ºC. The *MET3* promoter was switched off by the addition of methionine and cysteine at a final concentration of 2.5 mM to the different media. Hypha formation was induced by supplementing the media with 10% Fetal Calf Serum (FCS) at 37ºC. Strains were transformed using the lithium acetate protocol (Walther and Wendland, 2003). All transformants were checked

for correct genome integration by Southern blot or PCR.

The *DBF2* disruption cassette used for Fig. 1B was constructed using the *URA3* Blaster method (Fonzi and Irwin, 1993). 1-kb fragments from the flanking regions of the *CaDBF2* gene were cloned into a vector containing the *hisG-URA3-hisG* cassette. The plasmid was digested with *Not*I/*Xho*I before transformation. The strains used for the subsequent work (disruption and P_{MET3}-promoter cassettes) were constructed using pFA plasmids (Gola *et al.*, 2003) and specific primers.

The *DBF2-HA* allele was created using plasmid pCaHA-*DBF2*. To construct this plasmid, a 1 kb fragment containing the C-terminal end of the gene without the stop codon was PCR-amplified from genomic DNA. The PCR product was cloned in-frame with the HA coding region of plasmid pCaHA (Peter Sudbery, University of Sheffield, UK) using the *Xho*I and *Eco*RV restriction sites introduced in the primers. The plasmid was linearized with *Cla*I for transformation. The generation of C-terminal fusions of *DBF2, TUB2* and *MLC1* to different fluorescent proteins was performed as previously described (Gola *et al.*, 2003). The functionality of the tagged alleles was confirmed in heterozygous strains carrying the tagged alleles as the sole source of the protein.

Microscopy

Cells were observed as wet mounts using a Nikon Eclipse 90i microscope equipped with epifluorescence, Nomarski optics and a Hamamatsu ORCA ER camera. Images were captured and analyzed with Metamorph software. Alternatively, a Personal Deltavision microscope was used and the images were analyzed with SoftwoRx software. For fluorescence microscopy, cells carrying integrated versions of fluorescently tagged proteins were mounted on glass slides and observed with epifluorescence. Septum formation was determined by staining the cells with Calcofluor White, as described (Sherman, 1991). For time-lapse experiments, cells were prepared as described previously (Clemente-Blanco *et al.*, 2006).

For transmission electron microscopy, cells were fixed with 3% glutaraldehyde, post-fixed with 2% potassium permanganate, and acetone-dehydrated, as described (González-Novo *et al.*, 2008). Samples were embedded in Spurr resin (Taab Laboratories), stained with osmium tetroxide and observed under a Zeiss EM900 microscope.

Immunofluorescence and nuclear spreads

To detect tubulin microtubules, indirect immunofluorescence was employed as described previously (Pablo-Hernando *et al.*, 2007). Anti-tubulin primary antibody (Sigma 75168, St. Louis) was used at 1:100 dilution and was incubated O/N at 4ºC. The secondary antibody conjugated to Alexa 568 was used at 1:400 dilution. Samples were mounted with DAPI mounting medium (Vectashield, Vector Labs, CA).

Nuclear spreads and immunofluorescence were prepared as previously described (San-Segundo and Roeder, 1999). Briefly, 10 ml of exponentially growing cells was collected and resuspended in 1 ml of 2% KAc, 1M sorbitol, pH 7. For spheroplast formation, 10µl 1M DTT, 50 µl 20T Zymoliase (10mg/ml; Seikagaku Corporation) and 50µl of a 1:10 dilution of Glusulase (Dupont) were added and the mixture was incubated at 28ºC until the cell wall was degraded. Spheroplasts were resuspended in ice-cold MES sorbitol buffer (0.1M MES, 1M sorbitol, 1mM EDTA, 0.5mM $MgCl_2$, pH 6.4) and 4% p-formaldehyde was added. The mixture was placed on polylysine-treated slides, washed twice with 0.4% Photo-Flo200 (Sigma) and dried before immunofluorescence was performed. Primary antibodies against tubulin (Sigma 75168, St. Louis) and anti-GFP (Living colors 8371, Becton Dickinson) were used at 1:100 dilution. The secondary antibody conjugated to Alexa 568 was used at 1:400 dilution. Samples were mounted with DAPI mounting medium.

Western blotting and Immunoprecipitation

For western blotting, cells were collected, washed twice with ice-cold water and resuspended in 300 µl of RIPA buffer (10mM sodium phosphate, 1% Triton X-100, 0.1% SDS, 10mM EDTA, 150mM NaCl, pH7) supplemented with 2mM PMSF and phosphatase inhibitors. Cells were immediately broken with glass beads in a Fast-prep device (FP120 Bio101 Savant) with 3 pulses of 20 seconds at the 5.5 speed setting. Total extracts were cleared with a 15-minute spin at top speed at 4ºC. 15 µg of proteins was boiled for 5 minutes, separated on 8% SDS-PAGE and transferred to PVDF membranes (Hybond-P, GE Healthcare). Anti-HA (12CA5, Roche), anti-tubulin (Sigma 75168, St. Louis) and anti-PSTAIRE (sc-53, Santa Cruz Biotechnology) antibodies were used at

1:1000 dilution. Secondary antibodies conjugated with horseradish peroxidase were used at 1:4000. Membranes were developed with the ECL system (GE Healthcare). Phosphatase treatment was carried out incubating 15 µg of total protein extracts prepared in lysis buffer without phosphatase inhibitors with 1000 U of λ-Phosphatase (New England Biolabs) for 30 minutes at 28ºC.

For immunoprecipitation, 1 mg of protein from extracts obtained from strains carrying *DBF2-HA* was employed to immunoprecipitate Dbf2-HA using the µMACS™ HA Tagged Proteins Isolation Kit (Miltenyi Biotec). Eluted proteins were boiled for 5 minutes and loaded on 8% SDS-PAGE gels.

Cell synchronization and flow cytometry

When S-phase synchronization was desired, 200mM hydroxyurea was added to mid-log-phase cultures, and the mixture was incubated at 28ºC for 2 hours. To analyze DNA contents, S phase-arrested cultures were washed twice with water to eliminate the hydroxyurea, and transferred to fresh media. Samples were taken every 15 minutes, prepared as described previously (Jiménez *et al.*, 1998), stained with propidium iodide and analyzed on a FACScan® device (Becton Dickinson, Mountain View, CA). DNA content was determined using the fluorescence channel (FL-2) and cell size with the forward scattered channel (FSC).

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FIGURE LEGENDS

Figure 1. *DBF2* is an essential gene in *C. albicans*. (A) Comparison of domain organisation of *S. cerevisiae* Dbf2 (ScDbf2) and Dbf20 (ScDbf20) kinases with the *C. albicans* Dbf2 ortholog (CaDbf2). Shaded box: kinase domain; white box: insert between subdomains VII and VIII; light grey box: AGC-kinase C-terminal domain (CTD); dark grey boxes indicate Gln-rich (Q) and Asn-rich (N) regions in CaDbf2. The position of the regulatory phosphorylation sites is indicated. (B) Confirmation of the integration of the *hisG-URA3-hisG* cassette into the *DBF2* locus by Southern analysis. Genomic DNAs were extracted from strains CAI4, JJ1 (*DBF2/dbf2::hisG-URA3-hisG*), JJ2 (*DBF2/dbf2::hisG*) and four independent transformants obtained after deletion of the second allele (T1 to T4). (C) Growth of wild-type (WT), P_{MET3}-*DBF2* and P_{MET3}-*DBF2-HA* cells on minimal medium without (ON) or with (OFF) methionine and cysteine. Plates were incubated for 3 days at 28ºC. (D) Dbf2 abundance after promoter shut-off. Overnight cultures of P_{MET3}-*DBF2-HA* cells grown in minimal medium were inoculated in repressing medium. Samples were collected at 30-minute intervals after repression and were assayed by western blotting for Dbf2-HA levels. Cdc28 (Anti-PSTAIRE) was used as loading control.

Figure 2. Dbf2 is necessary for primary septum assembly. (A) Morphology of wild-type (WT) and P_{MET3}-*DBF2* cells grown in repressing medium for 6 hours. Arrows indicate wide bud necks. Scale bars: 5 µm. The lower graph represents the ratio neck width/cell diameter in wild-type and Dbf2-depleted cells (P_{MET3}-*DBF2*). 100 cells were measured for each strain. (B) Electron microscopy of wild-type (CAF2) and P_{MET3}-*DBF2* mutant (LL2) cells. Wild-type (1) and P_{MET3}-*DBF2* mutants (2-3) cells were grown in minimal medium to midlog phase (1-2). After methionine and cysteine addition to the culture medium, cells were incubated for 6 h before preparation for electron microscopy (3). Panels 4 and 5 show magnifications of the regions indicated with a rectangle in 1 and 3, respectively. P_{MET3}-*DBF2* mutants fail to assemble a normal septum after promoter shut-off (arrows in 3). Scale bars: 500 nm (1-3); 250 nm (4-5).

Figure 3. Dbf2 is necessary for actomyosin ring contraction. (A) Localization of Mlc1-YFP

during yeast growth. Wild-type (left panels) or P_{MET3}-*DBF2* cells (right panels) expressing Mlc1-YFP (CAG50 and CAG51, respectively) grown for 6 h in the presence of methionine and cysteine were prepared for microscopy. Images of Mlc1-YFP and differential interference contrast (DIC) are shown. Arrows indicate different actomyosin rings in a chain. Bars, 5 µm. (B) Time-lapse fluorescence microscopy of P_{MET3}-*DBF2* carrying Mlc1-YFP (CAG51). Cells were grown for 6 h in repressing medium before being mounted on glass slides containing 2% agar. Pictures were taken every 5 minutes and selected time-points are shown. A DIC image of the initial time point is shown to the left for reference. Asterisks indicate assembled rings and arrows mark the position of the next ring in the chain. Bars, 5 µm. (C). Kymograph representations of Mlc1-YFP ring contraction in P_{MET3}-*DBF2* living cells grown in the absence (ON) or presence (OFF) of methionine and cysteine in the culture medium (one frame/2 minutes).

Figure 4. **Dbf2 is necessary for cell cycle progression**. (A) Flow cytometry analysis of the DNA content and cell size of wild-type (WT), P_{MET3}-*DBF2* and P_{MET3}-*DBF2-HA* strains after promoter shut-off. Exponentially growing cultures of wild-type (LL4), P_{MET3}-*DBF2* (LL2) and P_{MET3}-*DBF2-HA* (LL5) were transferred to methionine and cysteine-containing medium to repress the P_{MET3} promoter. Samples were taken at the indicated times after promoter shut-off and analyzed for DNA contents (upper panels) or cell size (lower panels). (B) Dbf2 abundance varies with the different promoters. Overnight cultures of P_{MET3}-*DBF2-HA, DBF2*/*DBF2-HA* or *dbf2*Δ/*DBF2-HA* cells grown in minimal medium were inoculated in minimal medium (ON) or minimal medium containing methionine and cysteine (OFF) and grown for 3 hours. Samples were collected and were assayed by Western blotting for Dbf2-HA levels. Cdc28 (Anti-PSTAIRE) was used as a loading control. (C) Flow cytometry analysis of the DNA content of *DBF2*/*DBF2-HA* (WT) or P_{MET3}-*DBF2-HA* cells arrested by hydroxyurea treatment. Exponentially growing cultures of *DBF2*/*DBF2-HA* (LL4) or P_{MET3}-*DBF2-HA* (LL5) cells were grown in YPD medium containing methionine and cysteine for 1 h before the addition of hydroxyurea. Cells were incubated with HU for 2 h and then released in YPD medium containing methionine and cysteine. Samples were taken at the indicated intervals after the release (minutes) and processed for flow cytometry analysis.

Figure 5. Dbf2 is necessary for proper nuclear segregation. (A) Wild-type or P_{MET3}-*DBF2-HA* cells grown for 6 h in repressing medium were stained with DAPI. Representative cells are shown. Scale bars: 5 μm. (B) Quantification of the number of nuclei present in wild-type or mutant cells at different times (hours) of incubation in methionine and cysteine-containing medium. At least 100 cells were counted in each time-point. (C) Quantification of the different nuclear morphologies. Cells were classified according to nuclear morphology: (a) wild-type; (b) normal orientation along the mother-bud axis but defects in nuclear separation; (c) defects in orientation but normal separation, and (d) defects in orientation and separation. (D) P_{MET3}-*DBF2* cells also show defects in nuclear segregation. P_{MET3}-*DBF2* cells grown for 6 h in repressing medium were stained with DAPI. A merged image of DIC and DAPI channels is shown. Arrowheads indicate cells with abnormal nuclear segregation. Bars, 5 μm.

Figure 6. Dbf2 is necessary for proper spindle organization. Wild-type or P_{MET3}-*DBF2-HA* cells grown for 6 h in methionine and cysteine-containing medium were fixed and stained with anti-tubulin antibodies and DAPI. Merged images of the two channels are shown. Arrows indicate abnormal tubulin staining. Bars, 5 μm.

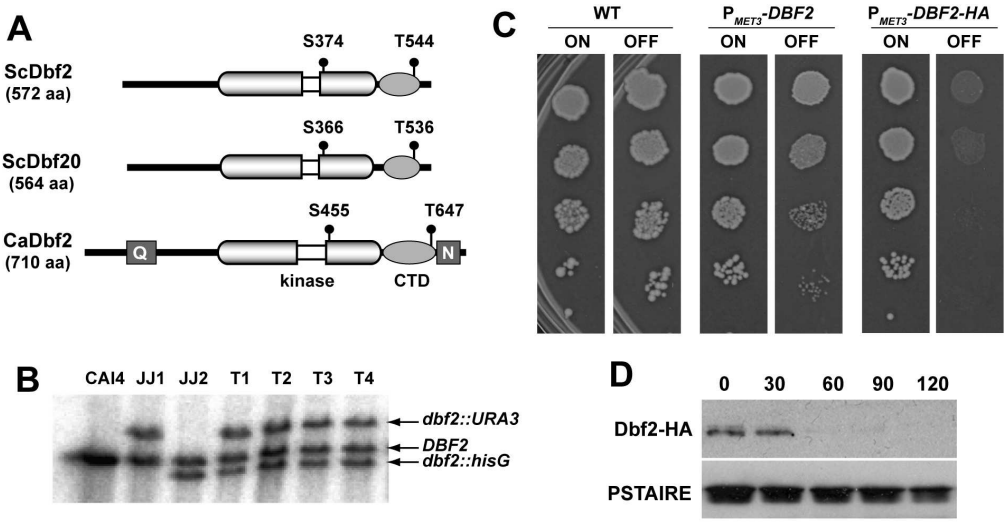
Figure 7. Localization of Dbf2-GFP during the cell cycle. (A) Exponentially growing cells expressing *DFB2-GFP* (LL3) were prepared for microscopy. Images of Dbf2-GFP, Calcofluor White (CF) and differential interference contrast (DIC) are shown. Representative photographs with different cell cycle stages are shown (see text). Arrows indicate position of Dbf2. Bars, 2.5 μm. (B) Localization of tubulin and CaDbf2 in spread nuclei. Spread nuclei from a strain carrying *DFB2-GFP* stained with anti-tubulin (left column) or anti-GFP (right column) antibodies. Bar, 1 μm. (C) Colocalization of Dbf2-YFP and Tub2-CFP. Exponentially growing cells expressing *DFB2-YFP TUB2-CFP* (CAG53) were prepared for microscopy. Images of Tub2-CFP (red) and Dbf2-YFP (green) channels, and the merged image are shown. Bars, 2.5 μm. (D) Dbf2 and tubulin co-immunoprecipitate. Protein extracts prepared from wild-type or *DBF2-HA* cells were incubated

in the presence of anti-HA antibody. Immunoprecipitated material was probed with anti-HA or anti-tubulin antibody.

Figure 8. Dbf2 is essential for hyphal morphogenesis. (A) Mitotic extracts of P_{MET3}-*DBF2-HA* cells grown asynchronously under yeast or hyphae-inducing conditions were treated (+) or not (-) with λ Phosphatase for 30 minutes. Samples were separated by SDS-PAGE and probed with anti-HA antibodies (12CA5). (B) Hgc1 is required for complete Dbf2-phosphorylation during hyphal growth. The wild-type and the *hgc1*Δ mutant (LL7) carrying *DBF2-HA* were grown under yeast or filament-inducing conditions. Samples were collected for western blot analysis with anti-HA antibodies. Anti-PSTAIRE was used as a loading control. (C) Morphology of wild-type and P_{MET3}-*DBF2* hyphae. Wild type (LL4) and P_{MET3}-*DBF2* cells (LL2) grown for 3 h in filament-inducing conditions were fixed and stained with DAPI and Calcofluor White. Arrowheads indicate constrictions of the filaments. Scale bars, 5 μm.

Table 1. Strains used in this study.

STRAIN	GENOTYPE	SOURCE
BWP17	*ura3::imm434/ura3::imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*	(Enloe *et al.*, 2000)
CAI4	*ura3::imm434/ura3::imm434*	(Fonzi and Irwin, 1993)
WYZ12	BWP17 *hgc1Δ::ARG4/ hgc1Δ::HIS1*	(Zheng *et al.*, 2004)
CAG 50	BWP17 *MLC1/MLC1-YFP-URA3*	This study
CAG 51	LL2 *MLC1/MLC1-YFP-URA3*	This study
CAG52	BWP17 *dbf2::HIS1/ DBF2*-3HA	This study
CAG53	BWP17 *TUB2/TUB2-CFP-URA3 DBF2/DBF2-YFP-ARG4*	This study
JJ1	CAI4 *DBF2/dbf2::hisG-URA3-hisG*	This study
JJ2	CAI4 *DBF2/dbf2::hisG*	This study
JC320	BWP17 *TUB2/TUB2-CFP-URA3*	This study
LL2	BWP17 *dbf2::HIS1/ARG4-* P_{MET3}*-DBF2*	This study
LL3	BWP17 *DBF2/DBF2-GFP-HIS1*	This study
LL4	BWP17 *DBF2/DBF2-3HA-URA3*	This study
LL5	BWP17 *dbf2::HIS1/ARG4-* P_{MET3}*-DBF2-3HA-URA3*	This study
LL7	WYZ12 *DBF2/DBF2-3HA-URA3*	This study



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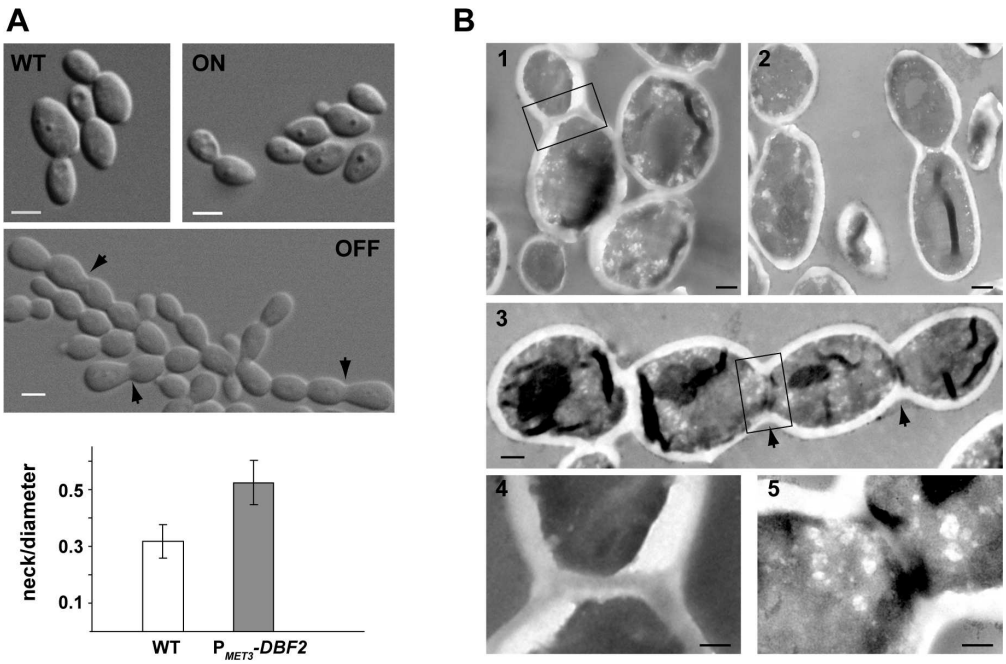

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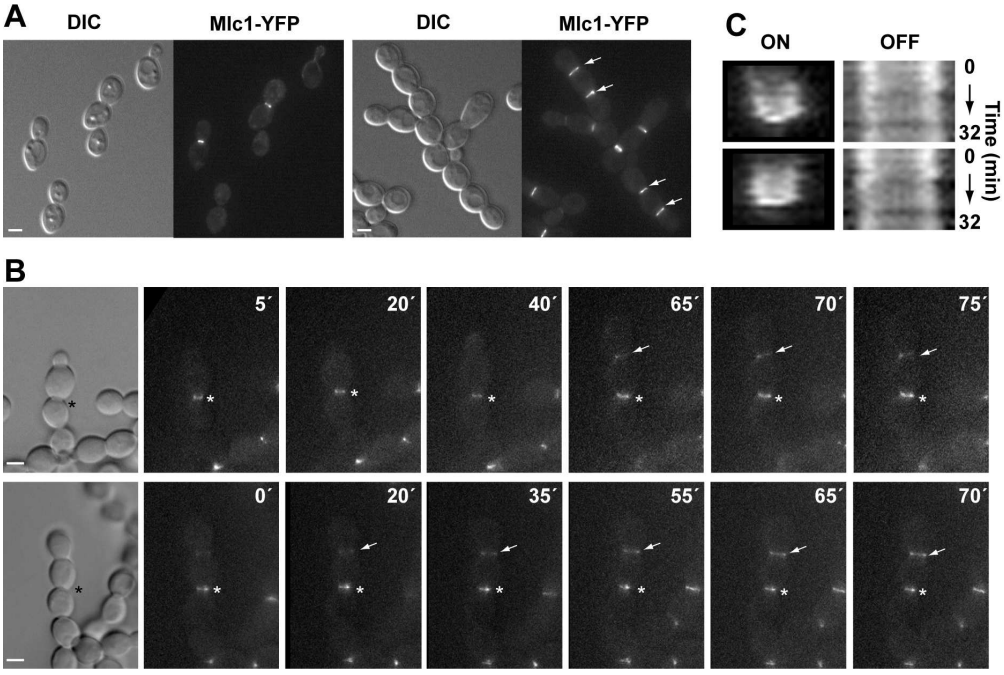

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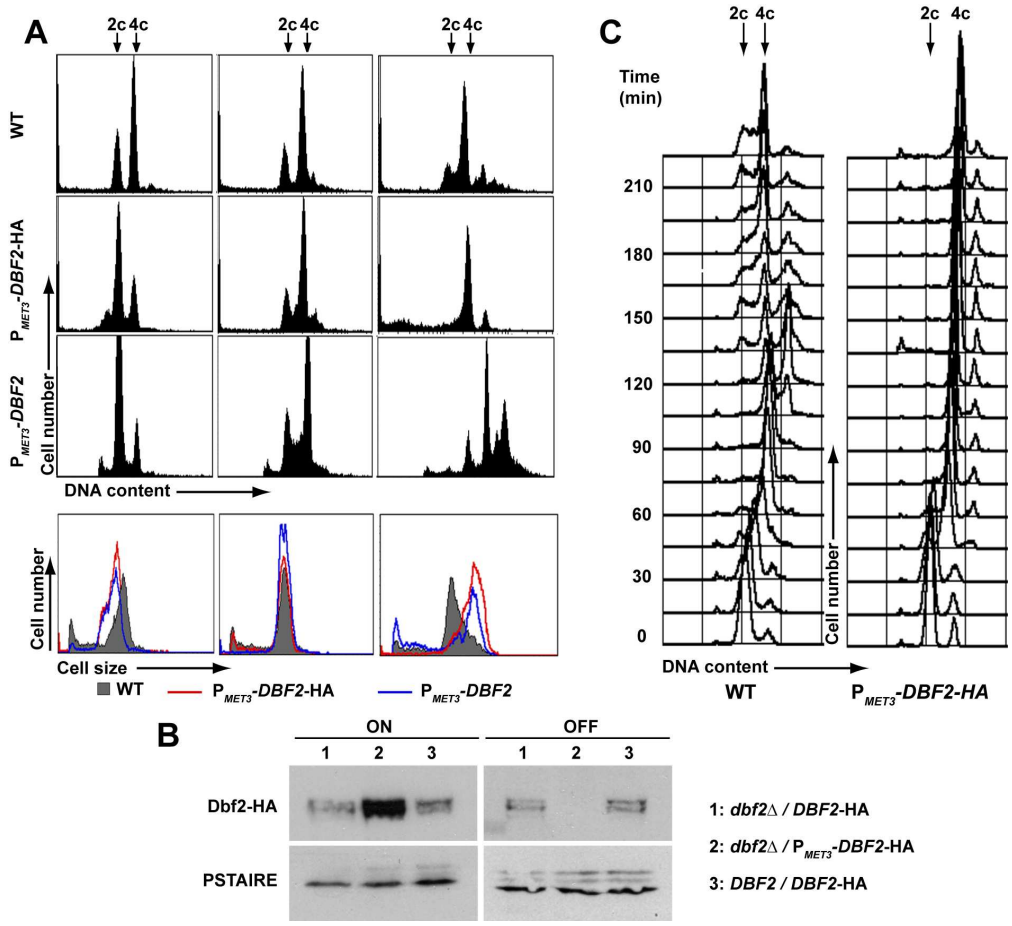

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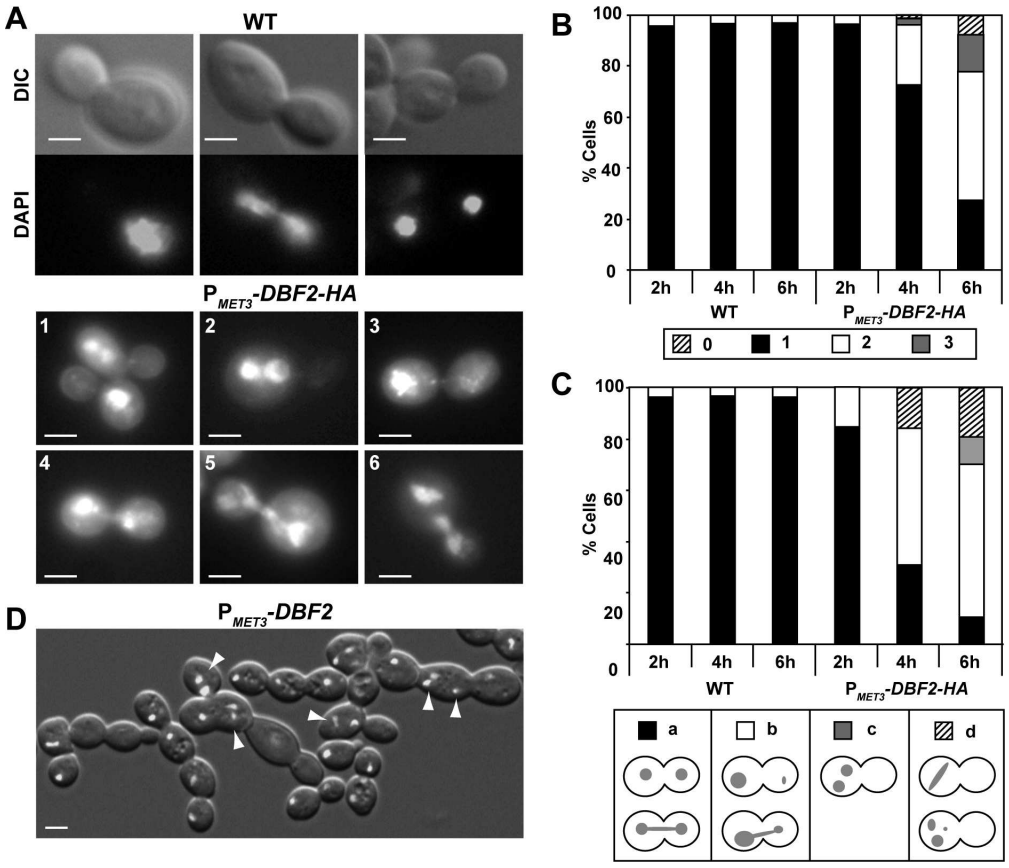

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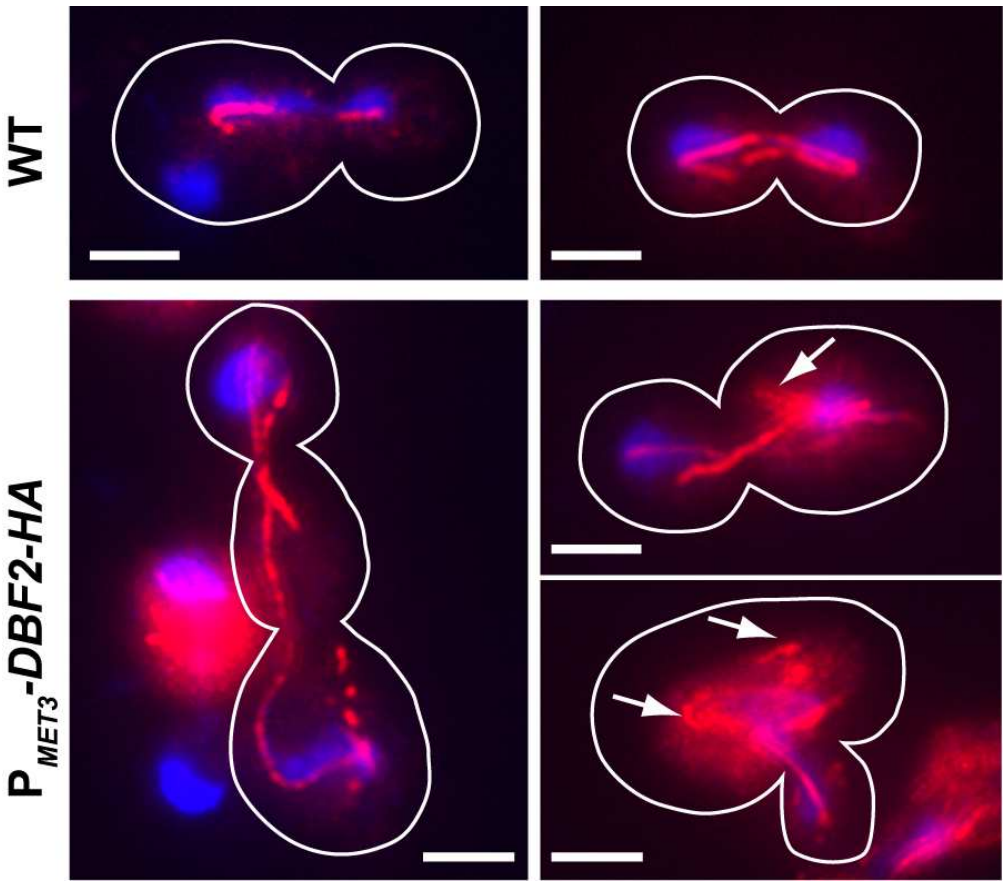

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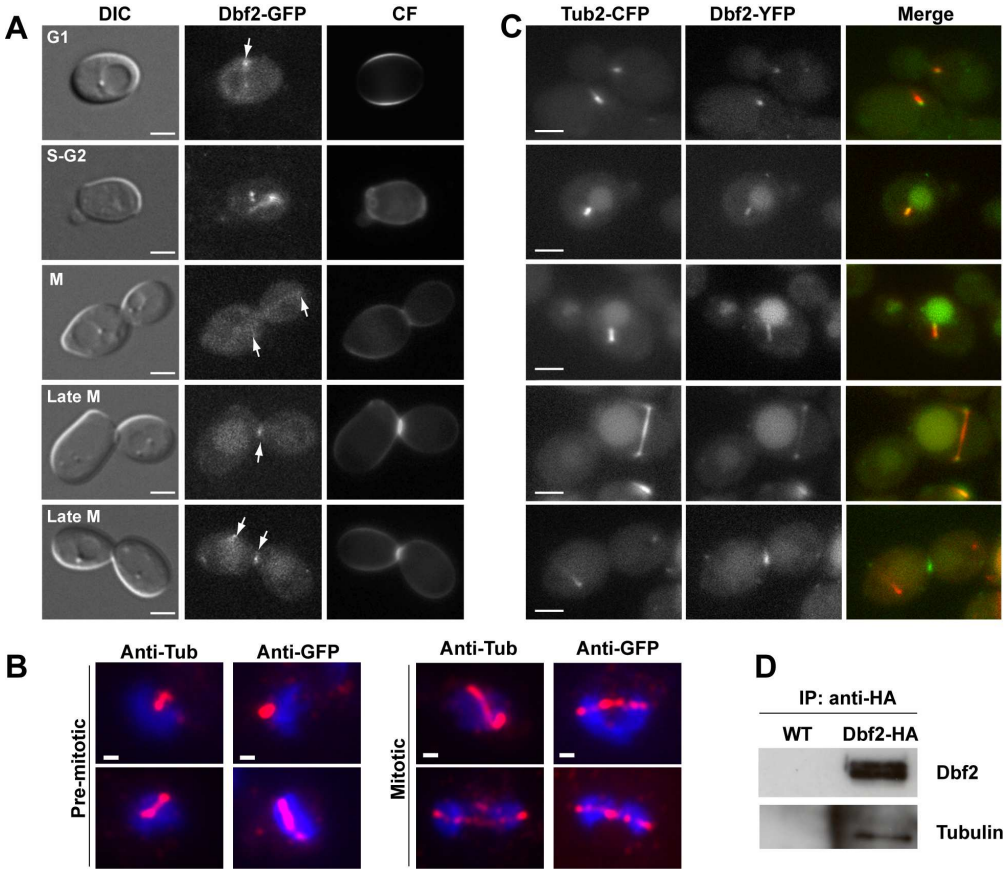

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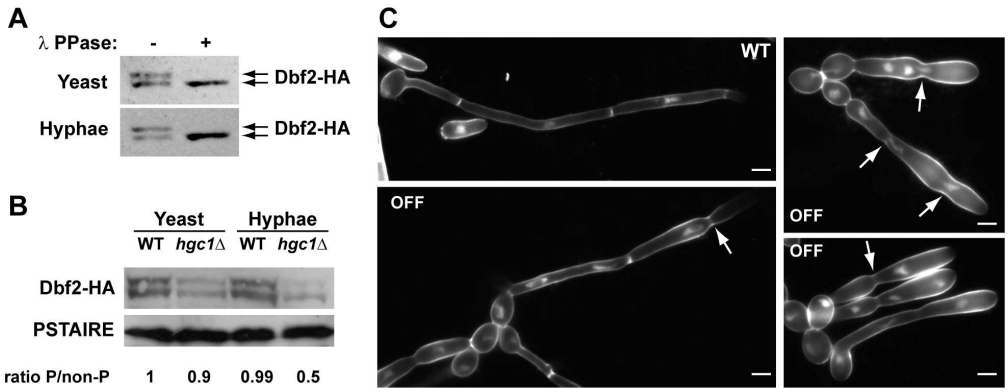

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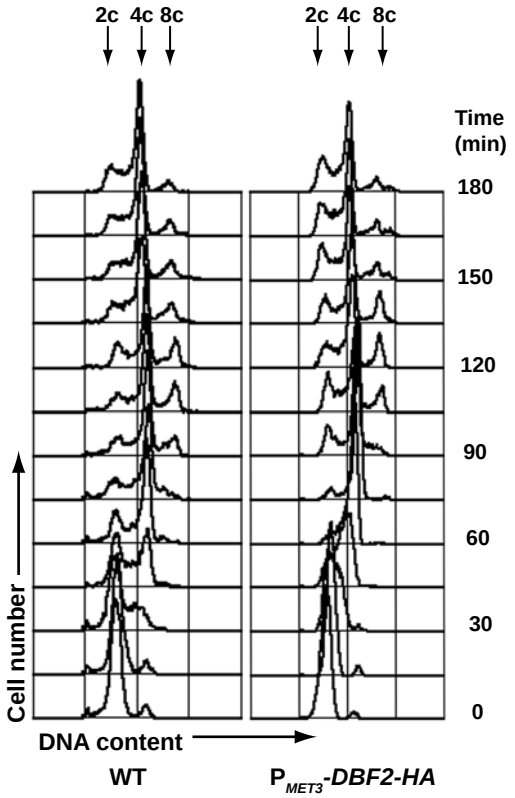
2c 4c 8c
2c 4c 8c
Time (min)
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150
120
90
60
30
0
Cell number
DNA content
WT
P MET3 -DBF2-HA

Figure S1

Figure S1. Flow cytometry analysis of the DNA content of wild-type (WT) or P_{MET3}-*DBF2-HA* that had been arrested by hydroxyurea treatment. Exponentially growing cultures of wild-type (LL4) or P_{MET3}-*DBF2-HA* (LL5) cells were arrested in S phase with hydroxyurea (2 h). Cells were then released from HU arrest and incubated in minimal medium without methionine and cysteine to allow the expression of the *MET3* promoter. Samples were taken at the indicated intervals after the release (minutes) and processed for flow cytometry analysis.

Table S1. Oligonucleotides used in this study

Name	Sequence (5´ - 3´)
Dbf2S1	TTTTAACCTTTTTCCATTTTCACTTGATTATTATTAGGTTTATTTATAAACAA ATCTTAACTTCTTCCCCTTTTCACCATTTTTAGTTTCCATAAGATATATAAAT CGAAGCTTCGTACGCTGCAGGTC
Dbf2S2	AAACTAAATCAAGCCGGATCTCTACGAGTTTACAAGTCTTATATAGTTTTC TAATCATTATACAACATCCTAAATTAATCAAACAAACACCATTTAATATAT CTGATATCATCGATGAATTCGAG
Dbf2S2pMET	TTACTTGAAATAGAAACATTTTCCATAGAATAAGAAATGTCAGTAACATCC TGTTGATGTGGTTGATAATGATGTGATTGATGTTTTGGTGATCTATTGAAAA AATTTGTCATGTTTTCTGGGGAGGGTATTTAC
Dbf2XFPS1	TGGTGAAATAAACTTATTGAATATGGTCGAAAATGGAAATGGAATTGGAA ATGGAAATTCTCGATCAAGTAGATTAAATCCATTAGCTACATTGTATGGTG CTGGCGCAGGTGCTTC
Dbf2HAup	CTCGAGGATTCTAAAGGTCATATT
Dbf2Halo	GATATCATACAATGTAGCTAATGG
H2	CAACGAAATGGCCTCCCCTACCACAG
UpVer23	AATTGTGGTGTTTTTCTAA
LoVer23	CAGGTTGTGGAGAATTAC
Dbf2XFP	CTACTACTACTACAACTAAG
Dbf2H1	GATTATTGGTCATTAGGTTGTA
pCaHA3	TTAACCGGCATAGTCTGG
UpSoDbf2	AACCTTAGCAATTTGAAAT
LoSoDbf2	AACCTAATAATAATCAAGTG
Mlc1XFPup	GTTGACTGACTCTGAAGTTGATGAGTTATTAAAAGGGGTCAATGTAACTTC TGATGGAAATGTGGATTATGTTGAATTTGTCAAATCAATTTTAGACCAAGG TGCTGGCGCAGGTGCTTC
Mlc1XFPlo	AAATAAACGGTATCCAATTCGAACAAGACTATACAATAACTATAATTTGTA AAACTTGTAGTATATATATTTCAATGGTTAATTGTAAATTTTCTTTTATTCT GATATCATCGATGAATTCGAG
Tub2XFPup	AGAAGGTGAATTCACTGAAGCTAGAGAAGACTTGGCTGCTTTAGAGAGAG ATTATATTGAAGTTGGTACTGATTCTTTCCCTGAAGAAGAAGAAGAATATG GTGCTGGCGCAGGTGCTTC
Tub2XFPlo	CCCTCCTCTTAACCATTTGACACACCAAGAGAGTCAATTCCAAAAGTAAAA ATTAAAAATCGGGCTTGGGAGTTCGGGTATATATGGTATATATATATATAAGT CTGATATCATCGATGAATTCGAG