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Transcriptional Analysis of the *Candida albicans* Cell Cycle

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We have examined the periodic expression of genes through the cell cycle in cultures of the human pathogenic fungus *Candida albicans* synchronized by mating pheromone treatment. Close to 500 genes show increased expression during the G1, S, G2, or M transitions of the *C. albicans* cell cycle. Comparisons of these *C. albicans* periodic genes with those already found in the budding and fission yeasts and in human cells reveal that of 2200 groups of homologous genes, close to 600 show periodicity in at least one organism, but only 11 are periodic in all four species. Overall, the *C. albicans* regulatory circuit most closely resembles that of *Saccharomyces cerevisiae* but contains a simplified structure. Although the majority of the *C. albicans* periodically regulated genes have homologues in the budding yeast, 20% (100 genes), most of which peak during the G1/S or M/G1 transitions, are unique to the pathogenic yeast.

INTRODUCTION

Proper coordination and control of cell division is critical to cells, and failure to properly manage the division process has severe consequences ranging from cell death to chromosomal aneuploidies linked in multicellular organisms to developmental defects and to diseases such as cancer. In the past decade, genome-wide studies of cell cycle clocks in humans (Cho *et al.*, 2001; Whitfield *et al.*, 2002; Bar-Joseph *et al.*, 2008), plants (Menges *et al.*, 2003), and yeast (Cho *et al.*, 1998; Spellman *et al.*, 1998; Simon *et al.*, 2001; Rustici *et al.*, 2004; Oliva *et al.*, 2005; Peng *et al.*, 2005; Pramila *et al.*, 2006) have revealed the inner programs of these timers and their connections to external regulatory controls. Several generalities have been noted from comparisons of these different regulatory circuits: 1) only a handful of genes, including those encoding components of the central DNA replication machinery, some nucleosome subunits, and a few cyclins, have retained periodical expression across these eukaryotes (Jensen *et al.*, 2006); 2) the function of protein complexes can be made periodic by controlling the expression of as few as one of their subunits (de Lichtenberg *et al.*, 2005); and 3) this "just in time assembly" can be regulated at various levels during the cell cycle, including transcriptionally, posttranslationally, or both through modulation of protein modifications and stability (Jensen *et al.*, 2006).

Transcriptional control represents a fundamental oscillator that, despite being interconnected with other systems, can function independently of cyclin-dependent kinase regulators (Orlando *et al.*, 2008). The clearest pictures of a cell cycle transcriptional network in a eukaryotic cell come from the two yeast models, *Saccharomyces cerevisiae* (Cho *et al.*, 1998; Spellman *et al.*, 1998; Pramila *et al.*, 2006) and *Schizo-*saccharomyces pombe (Rustici *et al.*, 2004; Oliva *et al.*, 2005; Peng *et al.*, 2005), and their comparison is the foundation of our understanding of cell cycle regulatory circuits (Bähler, 2005). Despite enormous efforts being devoted to understanding eukaryotic cell cycle regulation, the difficulty in synchronizing the cell cycle within a population has restricted studies to a limited number of cell types. Therefore, our basis for the comparison of cell cycle regulatory circuits has been drawn from evolutionarily divergent organisms: humans and plants belonging to different kingdoms, and the yeast *S. cerevisiae* and *S. pombe*, which are separated by as much as 1 billion y of evolution (Heckman *et al.*, 2001). These comparisons have marked out extreme points on the evolutionary path of cell division cycle regulatory networks, leaving the challenge of reconstructing the pathways linking them.

Although various methods exist for arresting the cell cycles of the budding and fission yeasts, including the use of mutants, mechanical strategies for separating cells by size, as well as treatment with pheromones and chemical cell cycle inhibitors, these have been difficult to apply to other cell types. Early efforts to analyze human cell cycle profiles had mixed success (Iyer *et al.*, 1999; Shedden and Cooper, 2002), whereas a recent study has used in silico deconvolution to retrieve clear expression profiles from primary foreskin fibroblast cells (Bar-Joseph *et al.*, 2008). Difficulties encountered in studies of the human cell cycle have been linked to the rapid loss of synchrony in the cells and the poor efficiency (50–70%) of re-entry into the cell cycle after release from a cell cycle block (Bar-Joseph *et al.*, 2008). Such difficulties in obtaining synchronous cell populations seem to be widespread.

Candida albicans, a fungal species that has been compared with *S. cerevisiae* in the analysis of the evolution of transcriptional regulatory circuits (Hogues *et al.*, 2008; Tuch *et al.*, 2008a,b), also has been difficult to synchronize for cell cycle studies (Berman, 2006). Phylogenetically, this organism is located between *S. cerevisiae* and *S. pombe*, and it possesses characteristics not seen in its close relative the budding yeast, such as true dimorphism, a commensal/opportunistic

pathogen lifestyle, a preduplication genome, and a "CTG" codon distinction (Braun *et al.*, 2005). It has been proposed that virulence in *C. albicans* is linked to its ability to undergo morphological changes between the yeast pseudohyphal and true hyphal forms. Although connections between morphology switches, cell cycle checkpoints and cell cycle regulators have been actively studied in *C. albicans*, the absence of the cell cycle-dependent transcriptional expression pattern has limited these analyses, because synchronization methods (elutriation, saturation cultures, conditional mutants, or chemical treatments) have not provided a complete picture of the *C. albicans* cell cycle. In *C. albicans*, conditions that arrest cell cycle progression often result in a polarized growth phenotype: G1-arrested cells tend to be more hyphal-like, whereas S, G2, and M arrests tend to be pseudohyphal-like (Berman, 2006). Release from blocks that generate polarized growth can result in an altered mitotic division cycle compared with unperturbed cells. We have recently created a *C. albicans MTL***a** strain overexpressing the cyclin-dependent kinase inhibitor *FAR1* (*FAR1*^OP), which, in response to α-factor induction, shows a rapid and efficient late G1 arrest of the cell division cycle (Côte and Whiteway, 2008). In the current study, we have used this strain to develop a pheromone-induced cell cycle synchronization process, and this has allowed us to establish the global cell cycle expression profile of the mating competent (opaque) form of *C. albicans*. A full description and complete data sets are available at http://www.bri.nrc.ca/candida/cycle/.

MATERIALS AND METHODS

Strain and Synchronization Conditions

Construction and phenotypic characterization of the *C. albicans* strain *FAR1*^OP (PCa034) has been described previously (Schaefer *et al.*, 2007; Côte and Whiteway, 2008). Cell cycle synchronization of *FAR1*^OP is done as follows. Opaque *FAR1*^OP cells were pregrown in synthetic complete (SC) liquid medium for 24 h at 24°C with shaking and then diluted to an $OD_{600\ nm}$ of 0.05 in 1 liter of fresh SC medium. At $OD_{600\ nm}$ of ~0.5, the culture was split in two, and α-mating pheromone was added to one culture (Côte and Whiteway, 2008). After 60 min of pheromone treatment, the cell cycle was reinitiated by washing the culture in phosphate-buffered saline, resuspending in 750 ml of fresh SC, and incubating at 24°C with shaking; the T_0 aliquot was taken after the resuspension, and then samples were taken every 30 min for 3 h. Each aliquot corresponds to 100 ml of the synchronized culture. The noninduced culture was kept in exponential growth for 115 min after the split and then stopped at $OD_{600\ nm}$ of ~0.85 by pelleting, flash freezing, and storing at −80°C until use. To confirm pheromone activity, 1-ml aliquots were taken after induction and monitored for shmoo formation as described previously (Côte and Whiteway, 2008).

Candida Cell Cycle Phase Determination

Fluorescence-activated cell sorting (FACS) analyses were done as presented previously (Côte and Whiteway, 2008), with few modifications. From the synchronization protocol described above, we used a total culture volume of 100 ml and 3 ml/aliquot. For each FACS assay, the DNA content of 50,000 single cells was monitored using an XL-MCL flux cytometer (Beckman Coulter, Fullerton, CA) and analyzed by EXPO32 software (Beckman Coulter). The cell division cycle phase was inferred by ModFit LT software (Verity Software House, Topsham, ME) using the default setting. For 4,6-diamidino-2-phenylindole (DAPI)/calcofluor staining, the cells were prepared using the synchronization protocol and were stained and observed as described previously (Côte and Whiteway, 2008). Cell quantification was done manually by assigning cells into one of the five categories: 1) buds (bud detected), 2) migrating nucleus (moving in the direction of the bud), 3) bud-neck (nucleus located at the bud neck, in division or not), 4) mother/daughter (clear difference of cell size, septation observed or not), and 5) single cells (no obvious characteristics, impossible to distinguish mother from daughter). To be consistent with previous literature (Spellman *et al.*, 1998; de Lichtenberg *et al.*, 2005), we used a similar cell cycle phase color code and kept it throughout this article. For both FACS and microscopic observations, four biological replicates were made, and representative pictures are shown in Figure 1.

Microarray Experiments

Microarray construction, RNA isolation, sample labeling, and data acquisition were as described previously (Côte and Whiteway, 2008). DNA microarrays were built on *Candida* Genome Assembly 19 (March 2004 release), but gene-lists were filtered and updated based on Assembly 21 (*Candida* Genome Database [CGD], September 2007 release; www.candidagenome.org) to remove suspicious or duplicated open reading frames (ORFs). For hybridization, we used the Slidebooster (BioChipNet, Reutlingen, Germany) for a more uniform signal and for maximizing probe/spot-specific interaction. Expression profiles presented in this study come from four biologically independent time course assays (for raw and normalized data, see Supplemental Material). To obtain interpretable signal for >99% of the *C. albicans* genes, we scanned each microarray at two different laser photomultiplier tubes. "Normal-intensity" scans allowed us to retrieve ~95% of the known and predicted *C. albicans* genes, whereas the low-intensity scans assessed abundant transcripts, such as the histone cluster, which had a previously saturated signal.

### Identification, Clustering, and Bioinformatic Analysis of Periodic Genes

Periodicity of a gene was based on 1) the sinusoidal shape of the expression modulation, 2) the period of the signal, 3) the amplitude of the periodic modulation, and 4) the reproducibility of the signal across replicates. Each normalized time course signal was processed using a modified least-squares spectral analysis (LSSA) approach. Because the initial pheromone treatment to arrest the cells influenced the early response of a number of transcripts, the first 60 min of each time series was linearly weighted in the LSSA, partially correcting for the abrupt transient response of some pheromone-sensitive genes. The periodic curve was used to measure the amplitude, whereas the peak value and curvature of the LSSA spectra established the dominant period and sinusoidal shape of the signal. Individual lenient cut-off values for the amplitude, period, sinusoidal shape, and variability were established. Genes that satisfied all four criteria produced an initial list of 800 that were then individually inspected. *Candida* genome assembly 21 (CGD, September 2007 release) was used to discard suspicious open reading frames annotated in assembly 19. Finally, 494 genes were retained as cycling (Supplemental Table 1).

Waves of expression were determined by clustering the periodic set of genes using Gaussian mixture and Pearson *r* by using GeneSpring7 (Agilent Technologies, Mississauga, ON, Canada). Four biologically coherent clusters were identified and named according to their approximate cell cycle phase as determined in Figure 1. As an example, 30 representative members of each cluster are presented in Figure 2 (for complete set, see Supplemental Table 1). The cell cycle transcription profile and analysis of each gene is available online (www.bri.nrc.ca/candida/cycle/) and is also linked in the Candida Genome Database (www. candidagenome.org).

Identification of putative *cis*-regulatory elements (motifs) in the upstream DNA sequences of ORFs was done using an exhaustive mini-motif detection method as described previously (Hogues *et al.*, 2008). To account for the nonhomogeneous distribution of some regulatory motifs in the upstream regions, various promoter lengths and offsets were scanned for motif enrichments. Determination of motif enrichment at any time point (Figure 4) was obtained by pooling all cycling genes whose peak time of expression coincided to within 10 min of the given time point. To overcome the difficult task of detecting significant enrichments of small, low-complexity motifs such as Fkh2 (AAACAAA), which are present upstream of a majority of genes, a set of closely related *Candida* species were used (*Candida tropicalis, Candida parapsilosis, Debraryomyces hansenii, Candida guillimondii, Lodderomyces elongisporus,* and *Clavispora lusitanie*). These species were selected for being evolutionarily close enough to each other to have conserved most of the coding regions but sufficiently distant from each other for their noncoding regions to have completely diverged, with loss of synteny, gene orientation, and even nucleotide and dinucleotide content (Wapinski *et al.*, 2007). Thus, the motif enrichment in any species in the clade will be statistically independent from the enrichment in the other species.

Gene Orthology Group Determinations

Genome sequences and annotations for *C. albicans, S. cerevisiae, S. pombe,* and *H. sapiens* were obtained from the following public repositories: CGD (www. candidagenome.org), *Saccharomyces* Genome Database (www.yeastgenome.org), the Sanger Institute (www.sanger.ac.uk/Projects/S_pombe), and Ensembl (www.ensembl.org/Homo_sapiens). Lists of orthologous genes were obtained from the Fungal Orthogroups Repository (www.broad.mit.edu/regev/orthogroups). Added to this list was the curated gene orthology provided by CGD and the Sanger Yeast_orthologous_groups. *H. sapiens* orthologues to *S. cerevisiae* and *S. pombe* were taken from Inparanoid (http://inparanoid.sbc.su.se). Periodic genes with their estimated peak-time and score for *S. cerevisiae, S. pombe,* and *H. sapiens* were obtained from CycleBase (www.cyclebase.org) (Gauthier *et al.*, 2008).
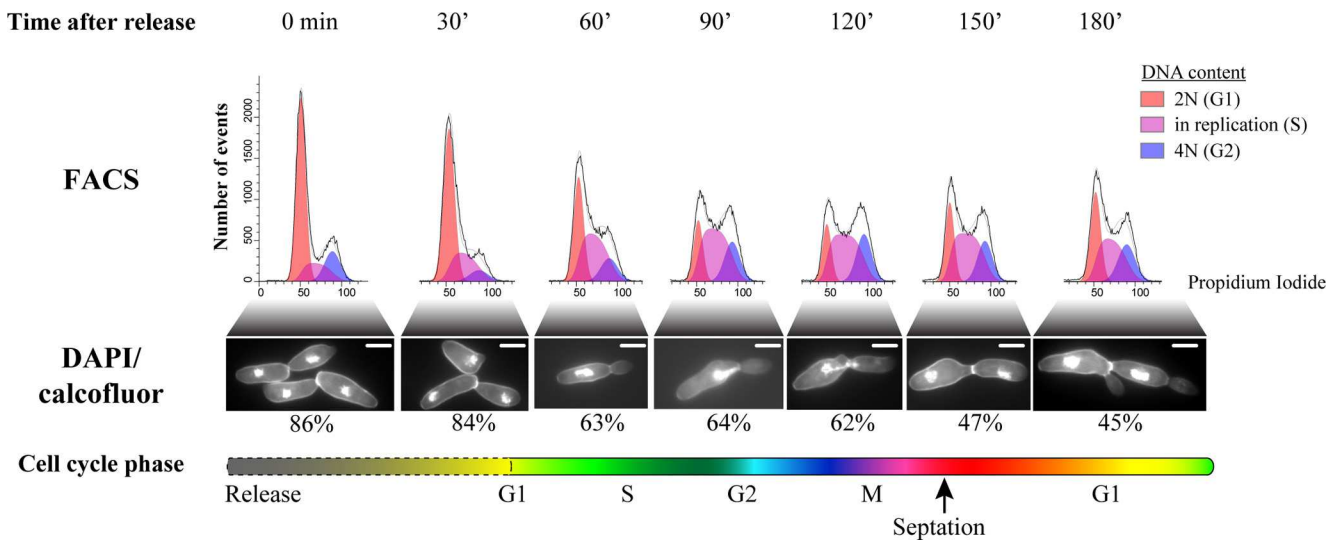
Figure 1. Determination of *C. albicans* cell cycle phases after synchronization. The *C. albicans* cell cycle was synchronized by mating pheromone treatment and release and was monitored by FACS analysis and by DAPI/calcofluor staining. FACS analysis allows the determination of DNA content per nucleus, associating the 2N and 4N peaks, respectively, as hallmarks of the G1 and G2 phases of the cell cycle. The S phase is extrapolated by ModFit LT (Verity Software House). DAPI/calcofluor staining was used to detect the beginning of mitosis, septation and bud formation (M, M/G1, and early G1, respectively). Representative pictures are shown with their associated term and percentage of the culture population. The color-coded gradient of the cell cycle phases combines both FACS and staining information. DAPI/calcolfuor bar, 3 μm.

Time Warping/Rescaling (for Phase/Phase Comparisons among Sc, Ca, and Sp)

Because organisms have different lengths of their cell cycles, we used a common reference time point and linearly corrected the cell cycle period accordingly. In brief, we used cytokinesis and histone genes as landmarks of the M/G1 (zero point) and the "middle S phase" of the cell cycle, respectively (de Lichtenberg *et al.*, 2005; Jensen *et al.*, 2006).

RESULTS

### Candida albicans *Cell Cycle Synchronization*

Synchronization of cell populations has been an important tool in the investigation of cell cycle transcriptional control networks (Spellman *et al.*, 1998). Only partial synchronization has been established for the model fungal pathogen *C. albicans* (Berman, 2006), although two recent studies have constructed strains able to be arrested in G1 by mating pheromone (Bennett and Johnson, 2006; Côte and Whiteway, 2008). In the present study, we have used α-factor arrest of an opaque *C. albicans* MTL**a** FAR1[OP] strain (Côte and Whiteway, 2008), which shows a rapid and efficient pheromone-induced late G1 block of the cell division cycle. Arrest and release of the cell cycle were monitored by FACS analysis and microscopic observations (DAPI/calcofluor) every 30 min for 3 h (Figure 1). This combination of two independent methods permits us to associate cell cycle phases with our time points (Figure 1, color gradient) and to determine essential characteristics of the synchronized population of *C. albicans*. Mating pheromone treatment efficiently blocks at the prereplication stage, and >86% of *C. albicans* cells show an unbudded, uninuclear morphology (Figure 1, T_0). Similar morphologies are observed after 30 min after release, suggesting a lag after release that is commonly found in synchronization experiments (Spellman *et al.*, 1998). The 60-min time point exhibits the first landmark of re-entry into the cell cycle: nuclei are largely in the replication phase (S phase; Figure 1, FACS) and buds are clearly visible at cell poles (DAPI). Despite having similar FACS

profiles, the 90- and 120-min time points possess characteristic visual differences. Cells at the earlier time point possess a nucleus that begins to move toward the bud, whereas the latter point is enriched in cells that exhibit nuclear division occurring at the bud neck. These two time points can be assigned to the G2 and M phases of the cell cycle. One hundred fifty minutes after release, separation between mother (bigger) and daughter (smaller) cells is observed for nearly 50% of the cell population. Similar to T_{60}, cells at the 180-min time point exhibit nuclei in the G1/S replication phase and buds are clearly visible at cellular poles. The length of a complete cell cycle of opaque *C. albicans* cells under these conditions is ~2 h. As also seen during human cell synchrony experiments (Bar-Joseph *et al.*, 2008), a substantial fraction of cells are unable to re-enter the cell cycle and the overall loss of synchrony prevents the analysis of sequential cycles.

C. albicans *Cell Cycle: Four Waves of Expression Coordinated by Four Transcription Factors*

RNA was isolated from synchronized *C. albicans* cells after their release from the mating pheromone G1 block and at subsequent 30-min intervals. RNA from unsynchronized cells was also isolated and used as a control, and gene expression profiles were monitored using custom-designed DNA microarrays (see *Materials and Methods*). Mating pheromone treatment also triggered expression of pheromone-induced genes, and these are not considered in the present study. The combined data from four different synchronous cultures identified >1400 genes with expression levels that differ by at least 1.4-fold during the cell cycle. Further quantitative and visual inspection refined this group to a high confidence set of 494 periodically expressed genes (complete list in Supplemental Table 1).

Peak expression of the first transcriptional wave is observed between 30 and 60 min after the release from cell cycle arrest (Figure 2, red cluster). Consistent with the G1/S
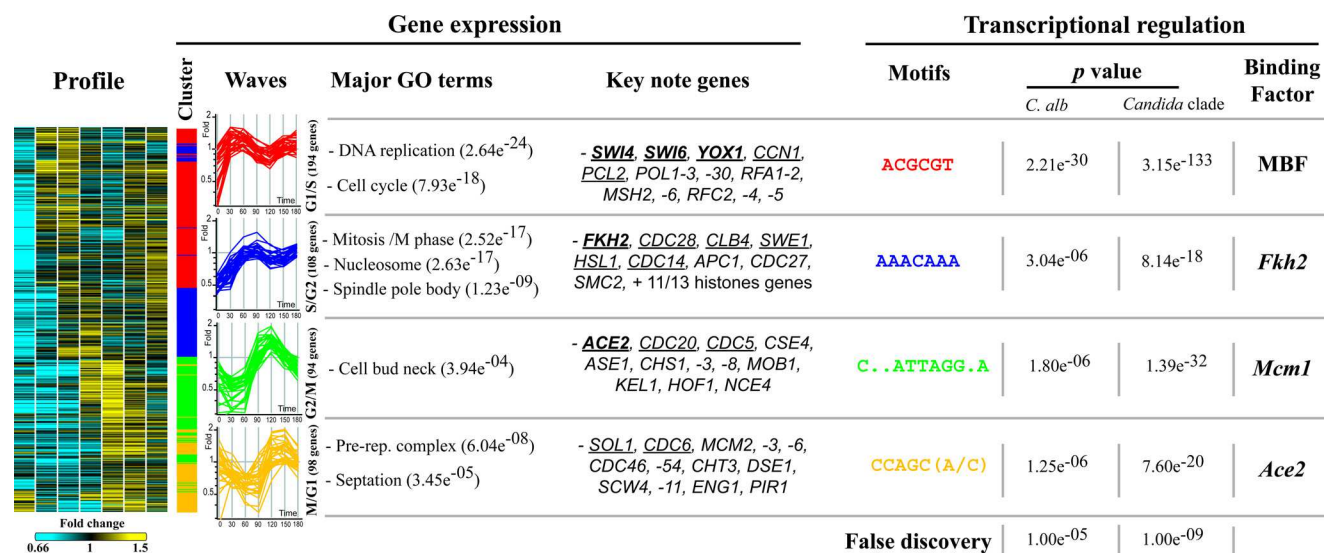
Figure 2. Gene expression profile of the *C. albicans* cell cycle. Left to right, phaseogram representing transcript level ratios of the 494 cell cycle-regulated genes (*y*-axis) collected at each of the seven time points (30-min intervals; *x*-axis). Levels of expression ratios are color coded as follows: yellow (induced), blue (repressed) and gray (no data). The colored bar adjunct to the expression profile reflects our clustering of genes into four major waves of expression (red, G1/S; blue, S/G2; green, G2/M; and orange, M/G1). Representative expression patterns of 30 genes per cluster are illustrated further on the right. The associated table describes for each cluster—the major gene ontology terms, keynote genes, transcription regulatory motifs found, their significance (in *C. albicans* only and in *Candida* clade), and the transcription factor known to bind these motifs. In the keynote genes section, cell cycle regulators are underlined and transcription factors are in bold and underlined. A complete list of *C. albicans* periodically regulated genes can be found in Supplemental Table 1. For p value calculation details, see *Materials and Methods*. *Candida* clade includes the following species: *C. albicans, C. tropicalis, C. parapsilopsis, D. hansenii, C. guillimondii, L. elongisporus,* and *C. lusitanie*.

phase of the cell cycle determined by FACS and morphological assessment (Figure 1), cluster 1 (194 genes) shows very significant Gene Ontology (GO) term enrichment for "DNA replication," "cell cycle processes," and "sister chromatin cohesion." Representative genes encode DNA polymerase subunits (such as *POL1, -2,* and *-3*), the DNA clamp loader PCNA orthologue (*POL30*), DNA replication factor elements (*RFA1* and *-2, RFC2, -4, -5,* and *-52*), as well as ribonucleotide reductase (*RNR1*). We also detect enhanced expression of the genes *CCN1* and *PCL2* that encode G1-specific cyclins. Inspection of the upstream regulatory regions of the G1/S cluster shows a strong enrichment for a MluI cell cycle box (MCB) binding site, "ACGCGT" ($p = 2.21 \times 10^{-30}$) that is recognized in yeast by members of the ankyrin-repeat transcription factor family (Bähler, 2005). In *C. albicans*, two members of this family, *ORF19.4545* (*SWI4*) and *ORF19.4725* (*SWI6*), are also transcriptionally activated at G1/S, whereas the third family member, *ORF19.5855* (*MBP1*), is not transcriptionally modulated in the cell cycle.

Cluster 2 (108 genes), whose expression peaks somewhat later than cluster 1 (Figure 2, blue cluster), around the S/G2 phase of the cell cycle, features many genes implicated in "chromosome organization" (*SMC2, APC1, CDC27,* and *TUB2* and *-4*), "spindle formation" (*CDC14, ESP1, MPS1,* and *SPC98*), and "cell cycle processes" (see below). This cluster also has a tight nucleosome subcluster grouping of 11/13 of the histone-encoding genes identified in budding (Spellman *et al.*, 1998) and fission yeast (Rustici *et al.*, 2004) as S phase landmarks. The *FKH2* transcription factor, the *SWE1* kinase and its antagonist *HSL1* and the G2-specific B-type cyclin *CLB4* are other notable cell cycle regulatory genes coexpressed in this cluster. Also induced in this phase is the central cyclin-dependant kinase (Cdk1) of the cell cycle, encoded in *C. albicans* by *CDC28* (Figure 2, keynote genes). Enrichment in this cluster for the known Fkh2 mini-motif

(AAACAAA) ($p = 3.04 \times 10^{-6}$) is close to our detection threshold ($p = 1.00 \times 10^{-5}$), primarily due to the low complexity and very frequent occurrence of this motif in *C. albicans* intergenic regions. However, when closely related *Candida* species (*C. tropicalis, C. parapsilopsis, D. hansenii, C. guillimondii, L. elongisporus,* and *C. lusitaniae*; along with *C. albicans*, these seven species will be referred as the "*Candida* clade") also are scanned for their motif enrichment upstream of evolutionarily conserved genes in this cluster, the Fkh2 mini-motif becomes clearly detectable with significant confidence ($p = 8.14 \times 10^{-18}$ compared with $p = 1.00 \times 10^{-9}$ for threshold) (Figure 2). To illustrate the increased sensitivity of the multispecies motif detection approach, when we applied this strategy to the previously characterized G1/S cluster, the MCB motif ACGCGT is retrieved with greatly enhanced significance ($p = 3.15 \times 10^{-133}$ compared with $p = 2.21 \times 10^{-30}$ for *C. albicans* alone) (Figure 2).

The G2/M phase cluster 3 (94 genes) is particularly enriched for "cytokinesis" and "mother-daughter cell separation" functions (Figure 2, green cluster). Components of the mitotic exit network (*MOB1*), cytokinesis related genes (*INN1* and *HOF1*), mitotic spindle associated genes (*KIP2* and *ASE1*), the anaphase-promoting complex regulatory subunit (*CDC20*), and the polo-like kinase (*CDC5*) belong to this group. Cluster 3 also contains the early G1-specific transcription factor *ACE2*, known to be an essential regulator of the cell cycle transcriptional network in *S. cerevisiae* and *S. pombe* (Bähler, 2005). Although *MCM1* is itself constitutively expressed, Mcm1 binding sites are significantly enriched in this phase ($p = 1.39 \times 10^{-32}$ in the *Candida* clade).

The final cell cycle cluster, the M/G1 group (98 genes), contains genes associated with "prereplication complexes," "cell bud," or "cell wall" organization (Figure 2, orange cluster). Almost all Mcm2-7 complex subunits (*MCM2, -3* and *-6, CDC46* and *-54*) and the DNA prereplication subunit

CDC6 are coexpressed during this phase. Cell bud or cell wall are represented by daughter specific expression 1 gene (*DSE1*), protein containing internal repeats (*PIR1*), cell wall protein (*SCW4* and *-11*) and the *S. cerevisiae* chitinase 1 orthologue *CHT3*. As well, part of the "exit from mitosis" gene network, Dumbell Forming 2 gene (*DBF2*) is present in this cluster. *SOL1*, the *C. albicans* orthologue of a key Cdk1 G1/S transition inhibitor Sic1, is also periodically transcribed at this time. Ace2, which is expressed during the preceding G2/M phase and acts through the mini-motif CCAGC^A/$_C$ (*Candida* clade p value; $p = 7.60 \times 10^{-20}$), seems to be a major transcriptional regulator of the M/G1 *C. albicans* cluster.

Conservation of Cell Cycle-regulated Genes

We have compared our *C. albicans* periodic gene list to those of *S. cerevisiae* (Cho *et al.*, 1998; Spellman *et al.*, 1998; Pramila *et al.*, 2006), *S. pombe* (Rustici *et al.*, 2004; Oliva *et al.*, 2005; Peng *et al.*, 2005), and *H. sapiens* (Whitfield *et al.*, 2002; Bar-Joseph *et al.*, 2008), as compiled in the public database CycleBase (Gauthier *et al.*, 2008). In total, 2168 orthologous gene groups, with at least one member in each of the four species, were constructed from published lists of interspecies orthologues (Figure 3; for details, see *Materials and Methods*). Within this limited set of orthologous gene groups, each species has ~200 members that are significantly modulated during their cell cycle (Figure 3). Of the 494 periodically expressed *C. albicans* genes, 394 belong to orthologous gene groups (they fall into 232 such groups), whereas 100 are *Candida* specific (see Supplemental Table 1). This group of *Candida*-specific genes is of clear interest, but because most (>70%) of these genes are uncharacterized, with functional classes or GO terms poorly identified, we have focused initially on homologous sets of genes (Figure 3). Of the 232 groups that are periodic in *C. albicans*, 103 are also periodic in *S. cerevisiae* and 43 are common to all yeasts, but only 11 have retained some form of periodic expression across all four species (Figure 3). This group of common cycling transcripts features genes such as *CCN1*, *CDC5*, *CDC20*, *CDC6*, *RNR1*, *ACE2*, the sister chromatid cohesion complex subunit *MCD1*, and histone genes. The statistical significance for the overlap of cycling genes between pairs of species is highest for *C. albicans* with *S. cerevisiae* ($p = 1.15 \times 10^{-50}$), lowest for *S. pombe* with *H. sapiens* ($p = 1.06 \times 10^{-3}$), and similar for *C. albicans* with *H. sapiens* ($p = 6.9 \times 10^{-15}$) and *C. albicans* with *S. pombe* ($p = 6.7 \times 10^{-19}$) (Figure 3B).

Transcriptionally regulated subunits of protein complexes are typically expressed just before their time of action (de Lichtenberg *et al.*, 2005), but the identity of the periodically expressed proteins within a complex can differ significantly among organisms (Jensen *et al.*, 2006; de Lichtenberg *et al.*, 2007). Our analysis of the cell cycle-regulated protein complexes and their subunits in *C. albicans* supports this idea. DNA polymerase δ, the Msh2-Msh6 G-T mismatch repair complex, and the cohesin complex and its associated cofactors are examples highlighting this concept (Figure 4A). Many other examples, already detailed by Jensen and coworkers (such as DNA replication and repair machinery, histones, the sister chromatid cohesion complex, and spindle formation proteins) further support the flexibility by which species-specific subunits control the periodic transcriptional regulation of protein complexes (Jensen *et al.*, 2006). Among the specific relationships observed in the interspecies analysis, the genes that show periodic expression only in *C. albicans* and in human cells reveal an unexpected organization at the protein complex level (Figure 4B). Rather than being randomly distributed, the 19 genes fall into specific complexes such as the chromosome condensation complex
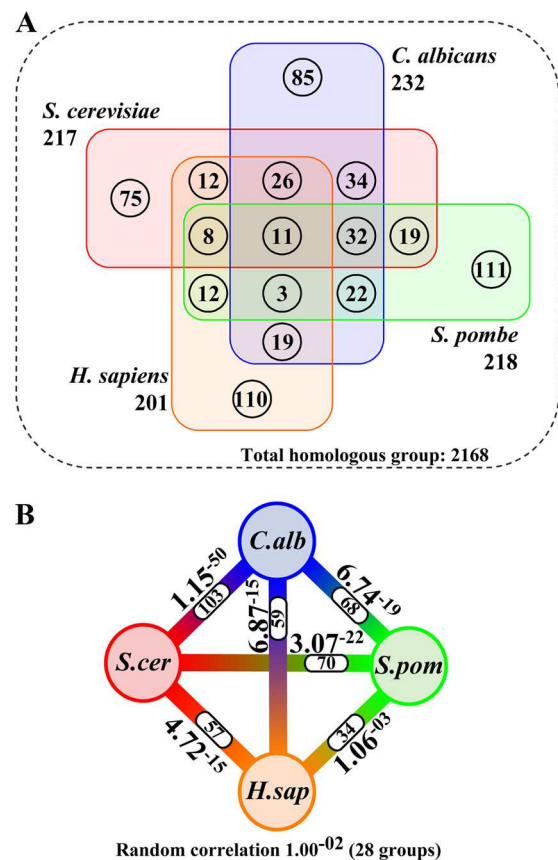
Figure 3. Periodical expression is significantly conserved in pairwise but not in common-to-all comparisons. (A) Four-way diagram representing the periodically expressed groups of genes that have homologues in all the four species. To be periodic, a group had to have at least one of its protein members periodically expressed in at least one species. For common-to-all periodically expressed groups, see associated text. (B) Periodically conserved pairwise significance correlation. Despite phylogenetic distances, each pairwise combination of species shares a highly significant group of homologous genes that are periodically regulated (random correlation, 28 groups; $p = 1.00 \times 10^{-02}$). A noticeable exception to this general observation is the human-fission yeast pair that share only 34 groups in common ($p = 1.06 \times 10^{-03}$). Pairwise conserved groups of genes are indicated in the white rectangle.

(condensin), an origin of DNA replication associated complex (GINS) and the DNA replication factor C complex (*RFC1-5*). It is also interesting that the transcriptional regulation of the *C. albicans* Cdk1 homologue *CDC28*, also seen in *H. sapiens* Cdk1 orthologue Cdc2 (Whitfield *et al.*, 2002), is not observed in budding and fission yeast where both Cdk1 representatives are constitutively expressed (Figure 4A). Intriguingly, it also seems that the *Cdc28* protein level fluctuates during the cell cycle because G1 elutriated cells show a maximal concentration around the G2/M phase (Wang *et al.*, 2007). Finally, the *SMC5/6* DNA repair complex, the chromatin remodeling complex COMPASS, and the DNA replication firing complex *CDC7/DBF4* highlight additional cell cycle-dependent transcription that would not be predicted based on the budding yeast data (Figure 4B).

Timing of the Cell Cycle Transcription Factor Network

Analysis of periodic gene expression in *S. cerevisiae* and *S. pombe* has shown that specific sequentially activated tran-
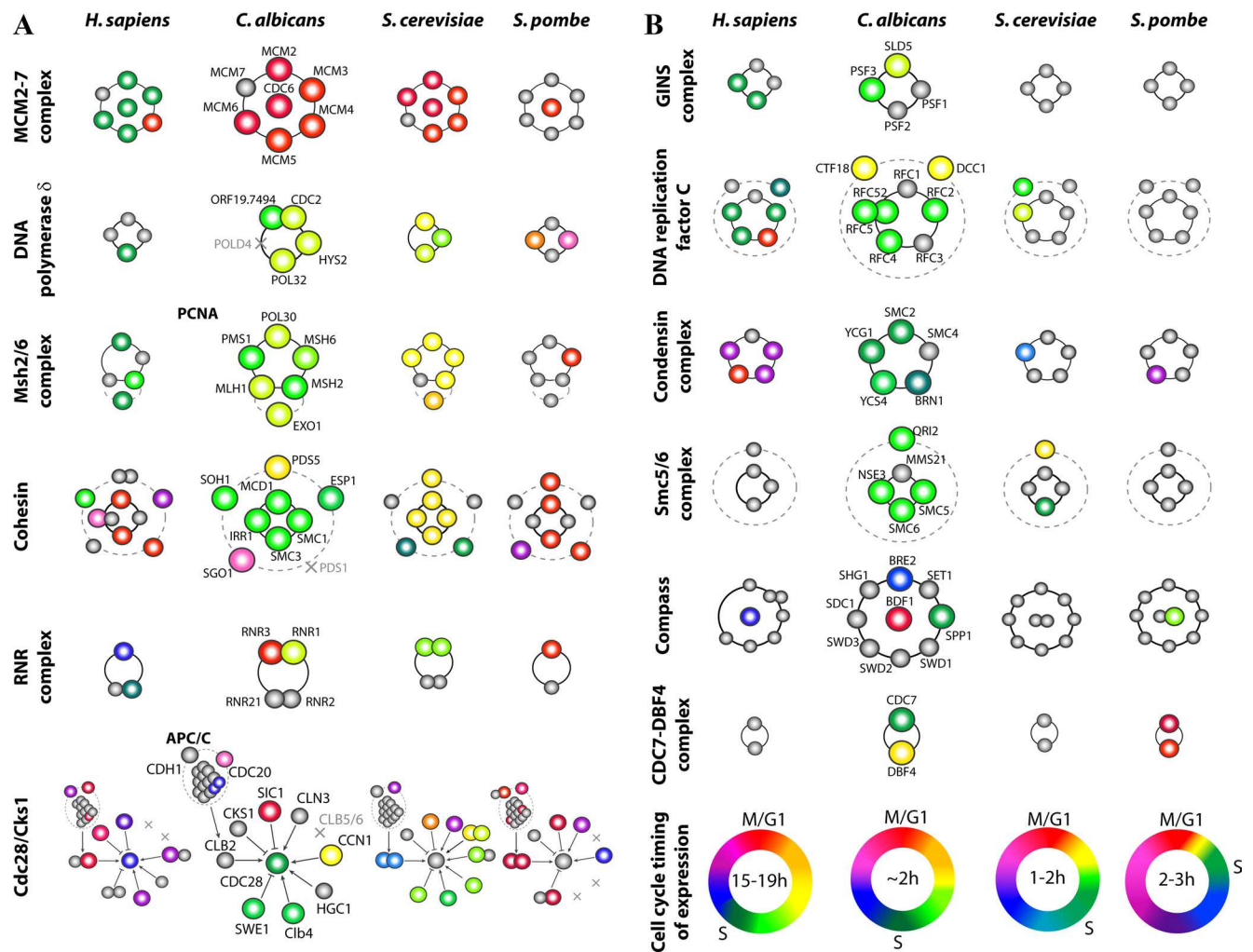
Figure 4. Dynamic regulation of protein complexes through various subunits in each organism. Selected core and derivative protein complexes (solid and dashed rings, respectively) and their subunits (circles); aligned on the same line are sequence orthologues believed to perform the same function. Transcriptionally regulated subunits are colored according to their approximate time of peak expression (B, bottom) or in gray if they are constitutively expressed. (A) Transcriptional dynamics of subunits evolutionarily conserved in at least three of the four organisms. (B) Unexpected differential regulation of protein complexes among the three yeast species. Peak time of transcriptional expression is color coded according to each cell cycle phase and uses the color code of Jensen *et al.* (2006). For protein complex subunit names and peak of expression, see Supplemental Table 4.

scription factors form networks that are responsible for the proper gene activation at various phases of the cell cycle (Simon *et al.*, 2001; Rowicka *et al.*, 2007). Therefore, at each fraction of the cell cycle, we examined the promoters of the periodic genes that reach their peak of expression at that time for *S. cerevisiae*, *S. pombe*, and *C. albicans* (Figure 5; see Supplemental Table 2 for raw data). Each cell cycle was centered at the M/G1 transition instead of the standard G1/S transition because the lag during recovery from pheromone arrest in *C. albicans* could influence its accurate assignment.

Our motif detection strategy, when applied to published *S. cerevisiae* and *S. pombe* periodical profiles, retrieved known consensus binding sites and regulatory patterns for the major expression motifs linked to the cell cycle coordination of each organism (Bähler, 2005). The two fundamental cell division processes—DNA replication, whose genes are regulated by the ankyrin-repeat-domain transcription factors; and cell division, controlled by Ace2 family members—are the most temporally conserved regulatory motifs (Figure 5,

green and red lines, respectively). A third general motif, which is bound by members of the Forkhead transcription factor group, is also detected but with a shifted window of action in *S. pombe*, because of the extended G2 phase in this organism (Figure 5, blue line). When similar analyses are performed in *C. albicans*, regulatory motifs similar to those found in *S. cerevisiae* and *S. pombe* are identified. However, although the temporal regulatory pattern of these cell cycle motifs globally resembles that of budding yeast, the regulated genes show differences.

Therefore, although these three major *cis*-regulatory elements are conserved from *S. cerevisiae* to *S. pombe*, each organism has unique characteristics (Figure 3). The G1/S transition, which represents the critical step in cell commitment to starting a division cycle, shows clear differences among the three fungi. The MCB consensus binding site (ACGCGT), bound by Mlu1 box-binding factor (MBF) complex (*MBP1/SWI6*), has the strongest consensus binding site conservation across the three ascomycetes and is the only conserved regulatory element of the G1/S transition (Figure
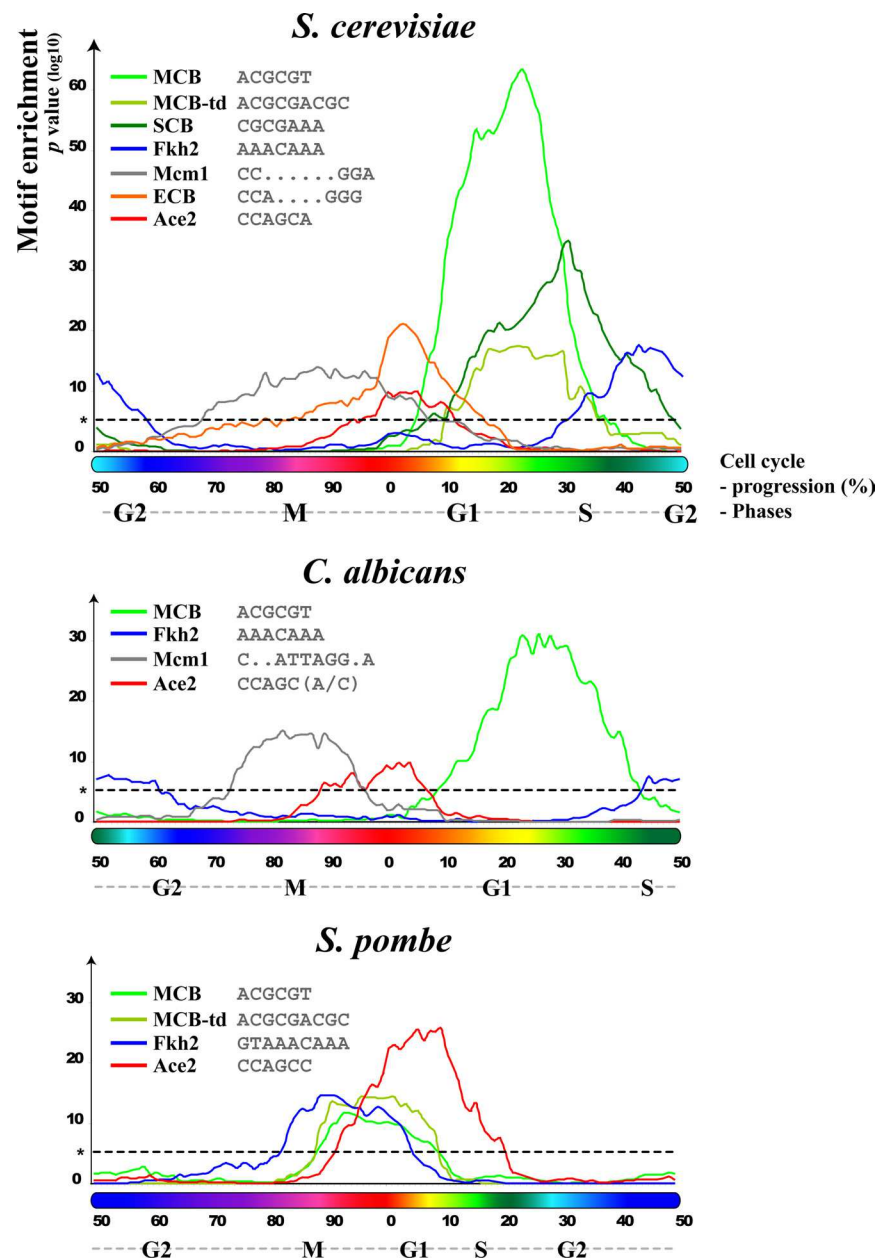
Figure 5. Timing coordination of the yeast cell cycle transcriptional regulatory network. Each curve represents a specific motif enrichment detected in periodic genes that reach their peak of expression within a 10% window around every time point. Motif confidence (p value; see *Materials and Methods*) was determined for every fraction of the cell cycle (resized from 1 to 100%). A color code for each motif is used for every organism: Ace2 in red, G1/S regulatory elements in various greens (MCB, regular green; MCB-td, light green; and SCB, dark green), Fkh2 in blue, Mcm1 in gray, and ECB in orange. The *y*-axis has the confidence scale in $\log_{10}$, and the *x*-axis the cell cycle progression (in percentile). Finally, the approximate divisions of cell cycle phases are displayed for each organism. Cut-off threshold, $p > 10^{-05}$, is indicated by the black dashed line.

5, light green lines). A derivative form of the MCB element composed of two ACGCG elements in tandem (MCB-td), initially identified in *S. pombe* (Rustici *et al.*, 2004; Oliva *et al.*, 2005), is detected in *S. cerevisiae* but not in *C. albicans*. MCB-td possesses its own regulatory pattern and is detected in the promotors of 22 *S. cerevisiae* genes and 28 *S. pombe* genes, of which 5 are in common: two have functions related to DNA damage checkpoint pathway (*NRM1/nrm1* and *SWE1/mik1*), two are members of the cohesion complex (*IRR1/psc3* and *SMC3/psm3*), and one is implicated in anchoring spindle pole body in nuclear membrane (*MPS2/ctp1*) (for complete set, see Supplemental Table 3). In addition to MCB and MCB-td, a third G1/S regulatory element is detected specifically in *S. cerevisiae*: the Swi4/Swi6 cell cycle box (SCB; Figure 5, dark green line). Consistent with a recent report (Rowicka *et al.*, 2007), we found that the SCB peak of regulation is slightly shifted after the MCB one (32 and 24% of the cell cycle, respectively). In-depth scanning of both the

S. pombe and *C. albicans* cell division cycle profiles failed to identify significant traces of a SCB-type motif in either organism (Supplemental Table 2).

In both *S. cerevisiae* and *C. albicans*, but not in *S. pombe*, we were able to detect a candidate binding motif implicating Mcm1p as a fourth transcription factor controlling a cell cycle transition (Figure 5, gray lines). Despite showing a clear motif associated with the G2/M transition in both *S. cerevisiae* and *C. albicans*, *MCM1* gene expression itself is not periodically regulated during their respective cell cycles (Spellman *et al.*, 1998; this study). Because Mcm1p works in conjunction with other factors, part of its temporal regulation might arise from these proteins (Tuch *et al.*, 2008a). The Mcm1 regulatory motif seen in *S. cerevisiae* and *C. albicans* is intriguing. Not only are key nucleotides of the Mcm1p consensus binding sequences different between the two yeasts, as reported recently (Tuch *et al.*, 2008a), but their regulatory patterns are also different: the signal is used through a broad

window in the *S. cerevisiae* cell cycle (from 68 to 12%), whereas it is restricted to a narrower point of action in *C. albicans* (from 72 to 96%). As mentioned, the Mcm1p factor is known to be associated with various other transcription factors during the yeast cell cycle; for example, in association with Yox1p, Mcm1p is known to recognize the early cell cycle box (ECB) motif (Pramila *et al.*, 2002). In our motif detection, we were able to find a second Mcm1-type element in *S. cerevisiae* that fits the known function of the Mcm1–Yox1 protein complex (Figure 5, orange line). Unfortunately, we were unable to find significant enrichment sequence for the low-complexity Yox1p consensus sequence ($^C/_T$TTATT) (McInerny *et al.*, 1997; Spellman *et al.*, 1998).

DISCUSSION

The results of this study provide a survey of the periodical expression profile of *C. albicans* opaque cells. We used pheromone-induced cell cycle arrest of mutant *C. albicans* cells overexpressing the cyclin-dependent kinase inhibitor *FAR1* (Schaefer *et al.*, 2007; Côte and Whiteway, 2008) to generate synchronous cultures and to determine the pattern of gene expression throughout the cell cycle. The addition of *C. albicans* to the comparison of transcription analyses in synchronized populations of the budding yeast *S. cerevisiae* (Spellman *et al.*, 1998; Cho *et al.*, 1998; Pramila *et al.*, 2006), the fission yeast *S. pombe* (Rustici *et al.*, 2004; Oliva *et al.*, 2005; Peng *et al.*, 2005), and human fibroblasts (Whitfield *et al.*, 2002; Bar-Joseph *et al.*, 2008) highlights properties common to ascomycetes yeasts, and characteristics common to yeasts and humans.

The temporal activity of specific transcription regulators can be inferred by the coordinated expression of genes whose promoters carry a regulatory motif recognized by the transcription factor. Using a mini-motif detection approach described previously (Hogues *et al.*, 2008), we were able to retrieve and follow the individual transcriptional regulatory networks of *S. cerevisiae*, *C. albicans*, and *S. pombe*. Our data suggest that *C. albicans* uses four transcription factors to regulate its gene expression waves; these factors act on unique target motifs and function in a temporally distinct manner. In this, *C. albicans* differs from the two other yeasts that possess largely overlapping signals and multiple transcription factors acting at similar times.

Transcriptional Regulation Difference in Yeasts: from Binding Sequences to Timing

Overall, the M/G1 transition shows the highest level of similarity among the three fungi in terms of transcriptional control (Figure 5). Nonetheless, the motifs in *S. cerevisiae* (CCAGC<u>A</u>) and in *S. pombe* (CCAGC<u>C</u>), bound in both cases by Ace2, represent highly species-specific signatures not seen in the phylogenetically intermediate *C. albicans* that relies on the consensus core five-nucleotide motif (CCAGC) for its Ace2p specificity. In addition to the Ace2 regulatory circuit, *S. cerevisiae* has a second M/G1 regulatory circuit involving the ECB (Mai *et al.*, 2002) and the Mcm1p/Yox1p transcriptional regulatory complex (Pramila *et al.*, 2002). Neither *S. pombe* nor *C. albicans* seem to possess this additional circuit, although both have Yox1 homologue and *C. albicans* has a Mcm1 orthologue (Tuch *et al.*, 2008a).

The G2/M transition in all three yeasts involves a forkhead-family member. In *S. pombe*, this transition seems to be regulated by Forkhead proteins alone (Rustici *et al.*, 2004; Oliva *et al.*, 2005), whereas in *S. cerevisiae* (Bähler, 2005) and in *C. albicans* a forkhead family member seems to work in conjunction with the Mcm1 protein. In addition to function-

ing at the G2/M control point, forkhead proteins also act at S/G2. This transition point, however, seems fundamentally different in *S. pombe* relative to the other two fungi, because there is no apparent factor and motif associated with the S/G2 transition in the fission yeast (Rustici *et al.*, 2004).

The critical G1/S regulatory step also has clear differences among yeast. *S. cerevisiae* uses structurally related ankyrin motif-containing transcription factors to interact with two regulatory motifs: the SBF complex (Swi4/Swi6) binds the SCB element, whereas the MBF complex (Mbp1/Swi6) recognizes the MCB element (Simon *et al.*, 2001). However, in depth analysis of the *S. pombe* and *C. albicans* genes regulated at the G1/S transition failed to find a significantly enriched SCB motif, and genes in *S. cerevisiae* that are controlled independently by SBF (*TOS1*, *TOS4*, *PCL2*, and *GIN4*) or MBF (*RAD27*, *IRR1*, and *MCD1*) are all under the regulation of the unique MCB element in *C. albicans*. The genes coding for the ankyrin-repeat proteins Orf19.4545 and Orf19.4752 are transcriptionally regulated at the G1/S transition and are involved in cellular proliferation and thus likely encode the MBF for *C. albicans*. However, these genes are annotated as *SWI4* and *SWI6* due to their closest primary sequence orthologues in *S. cerevisiae*. The *C. albicans MBP1* gene *ORF91.5855* is not periodically regulated at all and is not significantly involved in proliferative control (Bachewich, personal communication), suggesting that Mbp1 function in *C. albicans* is encoded by *ORF19.4545/SWI4*. Further work will be required to confirm which of Orf19.4545p or Orf19.5855p represents the actual MCB binding protein, but it is likely that, as observed in other cases (Schaefer *et al.*, 2007), the functional orthologue in *C. albicans* of a *S. cerevisiae* gene may not be the closest structural orthologue.

The Tandem MCB Element

A further difference in the regulatory networks of the three ascomycetes is the presence of a tandem MCB motif, MCB-td (ACGCGACGCG, Figure 5). Initially identified in *S. pombe* (termed MCB2 in Rustici *et al.*, 2004 or Dbd10 in Oliva *et al.*, 2005), MCB-td is found in *S. cerevisiae* but not in *C. albicans* (Figure 5). The MCB-td–dependent set of genes show a high level of conservation between the budding and the fission yeast. Within this set, *NRM1*, encoding a negative regulator of MBF, has been recently characterized in *S. pombe* and *S. cerevisiae* as a corepressor that associates with MBF to limit its window of action to the late G1 phase of the cell cycle (de Bruin *et al.*, 2006). Further studies in *S. pombe* (de Bruin *et al.*, 2008) have shown that, in response to genotoxic stress such as a DNA replication block, the DNA structure checkpoint kinase Cds1p phosphorylates Nrm1p, leading to its dissociation from MBF and the reactivation of MBF-dependent genes, many of which have a function associated with DNA replication and repair (Figure 6). In *S. cerevisiae*, this Nrm1 negative feedback loop regulates MBF but not SBF (de Bruin *et al.*, 2006, 2008). Because *NRM1* expression seems directed by the tandem MCB motif, its regulation may be distinct from the bulk of the G1/S transition genes; MCB-td might act as a threshold-dependent MCB-saturation monitor, which induces *NRM1* to repress MBF function once MBF levels have reached a critical level. Although the concept of threshold-dependent transcriptional regulation is not new (Mizutani *et al.*, 2006) and has been extensively studied through morphogen regulation during metazoan patterning development (Tabata and Takei, 2004), further work needs to be done in budding and fission yeasts to examine its role during the mitotic cycle.

Loss of *NRM1* function in *S. cerevisiae* and *S. pombe* leads to resistance to arrest of cell cycle progression upon DNA
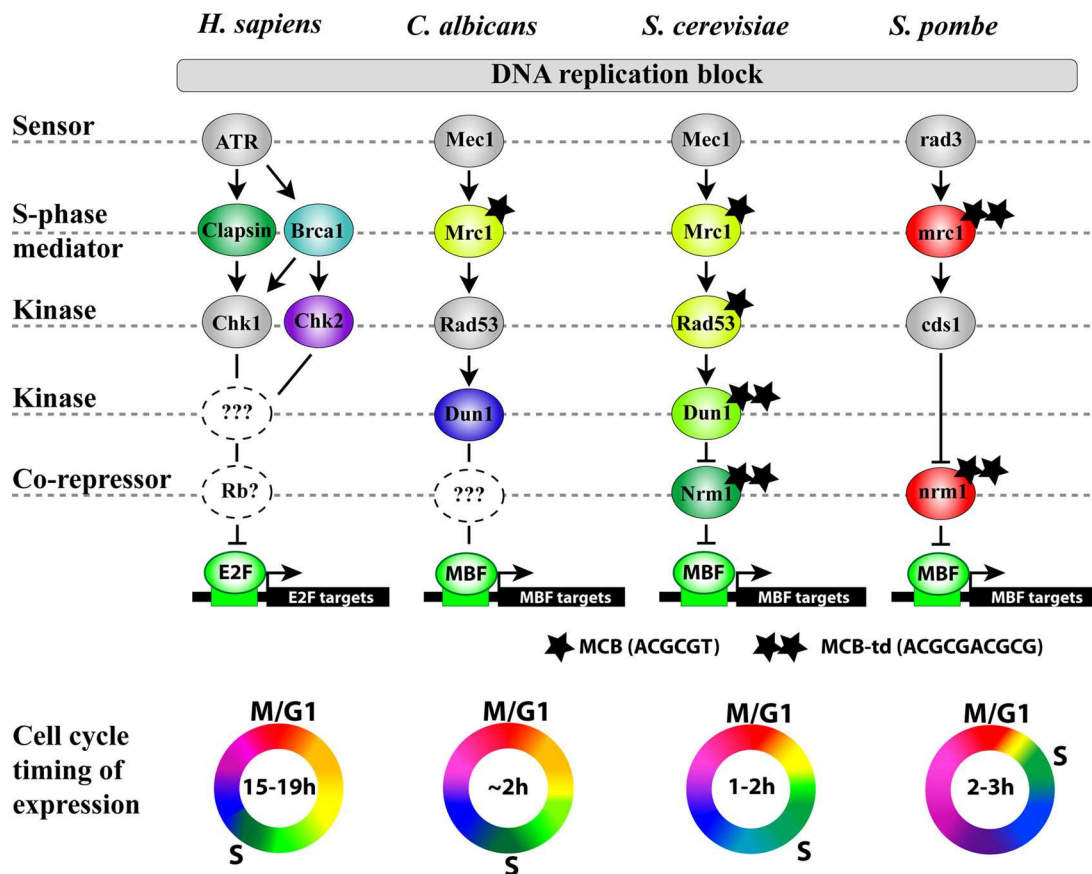
Figure 6. DNA replication checkpoint pathways in different organisms. DNA replication block-induced response pathway, including sensors and transducers, is conserved from yeast to humans. Components of the pathway (circles) on the same line are sequence orthologues believed to perform the same function. Dashed circles represent potential components yet to be directly linked to the response pathway. In the three fungi, MCB elements found at DNA replication checkpoint cascade components are marked by a black star: a single star for a MCB element and two stars for MCB-tandem motif. Periodically transcribed subunits are colored according to their approximate time of peak expression (bottom) or in gray if they are constitutively expressed.

damage and to compromised meiosis (de Bruin *et al.*, 2006). However, *C. albicans* cells, which lack an obvious *NRM1* homologue, are able to respond to DNA damage (Shi *et al.*, 2007); thus, DNA structure checkpoints are apparently coordinated independently of this circuit (Figure 6). For example, the cell cycle-dependent expression of *RNR1*, a known target for Nrm1-dependent repression in the budding yeast (de Bruin *et al.*, 2006), is conserved between *S. cerevisiae* and *C. albicans*; however, it is *RNR3*, expressed late in the *C. albicans* cell cycle, and not *RNR1* that is induced upon DNA damage (Bachewich *et al.*, 2005). Other genes in the budding and fission yeast that are MCB-td dependent and encode proteins implicated in regulation of Nrm1p, such as the DNA checkpoint kinases Dun1 and Mrc1 (Fu *et al.*, 2008), are both present and periodically expressed in *C. albicans* (Figure 6). This arrangement might reflect a requirement for *C. albicans* to allow flexibility in its genomic stability (Legrand *et al.*, 2007).

In human cells, the DNA structure checkpoint is likely to be mediated through the Chk1/Chk2 kinase, the closest homologue of the fission yeast Cds1 and the budding yeast Rad53 (Fu *et al.*, 2008). However, our understanding of the human DNA replication-block response pathway is incomplete relative to that in the budding and fission yeasts, because no direct evidence has yet linked the mitotic E2F corepressor Retinoblastoma family genes

(Genovese *et al.*, 2006) to reactivation of DNA repair E2F-dependent gene expression upon DNA damage (de Bruin and Wittenberg, 2009) (Figure 6). Because this process is well conserved across eukaryotes, both at the primary structure level and at the timing of expression, it is probable that DNA structure checkpoints linked to G1/S transcriptional regulation are a functionally conserved process (Figure 6) (Chen and Sanchez, 2004). Because *C. albicans* has a known tolerance for genome instability (Forche *et al.*, 2008), understanding the periodical coordination of DNA structure checkpoints might reveal new insight into genome instability in human cells and perhaps ultimately in cancer cells.

CONCLUSIONS

C. albicans cell cycle-dependent gene expression is composed of ~500 genes that show transcriptional modulation during one of four general waves of expression corresponding to the G1/S, S/G2, G2/M, and M/G1 transitions. Each expression wave is specifically associated with gene ontology terms, keynote genes, transcriptional regulatory motifs, and a candidate central transcription factor (ankyrin-repeat domain protein complex, Fkh2p, Mcm1p, or Ace2p) resembling a simplified version of the *S. cerevisiae* cell cycle expression program. However, the

phylogenetically intermediate *C. albicans* shows the striking absence of the G1/S termination regulatory circuit controlled by the transacting Nrm1 protein and the *cis*-acting tandem Mlu1 element MCB-td that is found in both *S. cerevisiae* and *S. pombe*. This example illustrates the plasticity of cell cycle coordination and the importance of characterizing these circuits in intermediate species to understand the patterns of evolution of cell cycle control mechanisms.

ACKNOWLEDGMENTS

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