DOI: 10.1534/genetics.108.098145

A Systematic Screen for Transcriptional Regulators of the Yeast Cell Cycle

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Manuscript received October 29, 2008
Accepted for publication November 21, 2008

ABSTRACT

Transcription factors play a key role in the regulation of cell cycle progression, yet many of the specific regulatory interactions that control cell cycle transcription are still unknown. To systematically identify new yeast cell cycle transcription factors, we used a quantitative flow cytometry assay to screen 268 transcription factor deletion strains for defects in cell cycle progression. Our results reveal that 20% of nonessential transcription factors have an impact on cell cycle progression, including several recently identified cyclin-dependent kinase (Cdk) targets, which have not previously been linked to cell cycle transcription. This expanded catalog of cell-cycle-associated transcription factors will be a valuable resource for decoding the transcriptional regulatory interactions that govern progression through the cell cycle. We conducted follow-up studies on Sfg1, a transcription factor with no previously known role in cell cycle progression. Deletion of Sfg1 retards cells in G_1, and overexpression of Sfg1 delays cells in the G_2/M phase. We find that Sfg1 represses early G_1, Swi5/Ace2-regulated genes involved in mother–daughter cell separation. We also show that Sfg1, a known *in vitro* cyclin-dependent kinase target, is phosphorylated *in vivo* on conserved Cdk phosphorylation sites and that phosphorylation of Sfg1 is necessary for its role in promoting cell cycle progression. Overall, our work increases the number of transcription factors associated with cell cycle progression, strongly indicates that there are many more unexplored connections between the Cdk–cyclin oscillator and cell cycle transcription, and suggests a new mechanism for the regulation of cell separation during the M/G_1 phase transition.

REGULATION of transcription is a major strategy employed by cells to control the timing and succession of the events of cell division, yet many of the specific regulatory interactions involved remain unknown (Spellman *et al.* 1998; Wittenberg and Reed 2005; Pramila *et al.* 2006). Cell cycle transcription was initially believed to be important but limited (Price *et al.* 1991) until genomewide expression profiling and chromatin immunoprecipitation (ChIP–chip) experiments revealed that cell cycle transcription is remarkably complex, involving a growing number of transcription factors and a large number of periodically transcribed genes in organisms from bacteria to yeast, plants, and animals (Cho *et al.* 1998; Spellman *et al.* 1998; Laub *et al.* 2000; Whitfield *et al.* 2002; Menges *et al.* 2003; Rustici *et al.* 2004). In the yeast *Saccharomyces cerevisiae*, cell cycle transcription involves at least 800 periodically transcribed genes (~14% of the genome) (Spellman *et al.* 1998; Pramila *et al.* 2006) and to date more than a dozen transcription factors involved in numerous regulatory complexes and feedback loops (reviewed in Breeden 2003 and Wittenberg and Reed 2005). ChIP–chip and expression profiling studies have revealed that these major cell cycle transcription factors form an interlocking cycle, with the major activators of one phase of the cell cycle inducing the expression of the key activators of the next phase (Simon *et al.* 2001; Pramila *et al.* 2006). The intricacy of cell cycle transcription reflects the fact that successful cell division requires a complex series of events that must be responsive to a variety of internal and external signals, and these signals nearly always culminate in a transcriptional response.

Our growing understanding of the complex nature of cell cycle transcription underscores how much still remains unknown about the transcriptional regulatory interactions involved, and about the interactions between transcription factors and the cyclin-dependent kinase (Cdk)–cyclin oscillator. A large number of periodically transcribed genes are not directly regulated by any of the known cell cycle transcription factors, and new cell cycle transcription factors continue to be found piecemeal (Pramila *et al.* 2002, 2006; Costanzo *et al.* 2003, 2004; De Bruin *et al.* 2004, 2006; Ashe *et al.* 2008). Other transcription factors, so far not known to be involved in cell cycle transcription, are *in vitro* Cdk substrates (Ubersax *et al.* 2003), which suggests that there still remain unidentified connections between the core cell cycle oscillator and cell cycle transcription. For example, Whi5 was identified as an *in vitro* Cdk substrate (Ubersax *et al.* 2003) and later shown to be a key repressor of the G_1 transcriptional activator SBF

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(Costanzo *et al.* 2004; De Bruin *et al.* 2004). Other recently discovered regulatory interactions further demonstrate the importance of multiple binding partners in determining the timing and specificity of cell cycle transcription. Swi5 and Ace2, two major early G_1 activators with nearly identical DNA-binding domains, can physically bind a common set of target genes, but they activate different subsets of these targets (Doolin *et al.* 2001). This regulatory specificity is achieved in part by newly discovered interactions with the transcription factors Fkh1 and Fkh2, which, in addition to their well-known role in activating G_2 transcription, can repress Swi5 activity at the promoters of Ace2-regulated genes (Voth *et al.* 2007). Recent studies such as these make it clear that cell cycle transcription is not simply a matter of successive waves of transcriptional activation; the timing of gene expression during the cell cycle is instead controlled by the combined regulatory interactions of activators, repressors, and Cdk–cyclin complexes.

The continuous discovery of new cell cycle transcription factors and the unexplained cell cycle regulation of hundreds of genes suggest that many cell cycle transcription factors were not detected in previous screens for cell cycle regulators. This is not surprising since most screens to date have relied on identifying regulators by using dramatic mutant phenotypes such as cell cycle arrest or severe growth defects (Hartwell *et al.* 1970; Reed 1980). These previous screens have likely uncovered most transcription factors whose mutation results in large effects on the cell cycle. We hypothesize that the remaining undiscovered cell cycle transcription factors will have more subtle mutant phenotypes that were not detected in previous screens.

To systematically identify cell cycle transcription factors, we used a quantitative flow cytometry assay to screen a set of 268 mutant strains with single-gene deletions of known or putative transcription factors for cell cycle defects. Our results show that nearly 20% of nonessential transcription factors play some role in cell cycle progression. Regulators that affect each of the major cell cycle phases, as well as several reported *in vitro* Cdk substrates that have not yet been linked with cell cycle transcription (Ubersax *et al.* 2003), are among the 48 transcription factors detected in our screen. The results of our screen form an important resource for future studies aimed at unraveling the regulatory logic of cell cycle transcription.

To demonstrate the utility of this resource, we further characterized one transcription factor identified in our screen, Sfg1. We find that the deletion of Sfg1 results in retardation through G_1 and that its overexpression stalls cells in the G_2/M phase. We also show that Sfg1, an *in vitro* Cdk substrate (Ubersax *et al.* 2003), is phosphorylated *in vivo* on a set of Cdk consensus phosphorylation sites that are highly conserved in distantly related yeast species. Furthermore, we show that phosphorylation of Sfg1 is required for its role in driving progression through G_1. Through both loss of function and overexpression experiments we demonstrate that Sfg1 represses a set of cell separation genes that are known to be directly activated by the early G_1 transcription factors Swi5 and Ace2. Our data suggest a model in which Sfg1 regulates mother–daughter cell separation after mitosis by counteracting the activities of Swi5 and Ace2. The study of other genes identified in our screen is likely to reveal new regulatory interactions that govern cell cycle transcription.

MATERIALS AND METHODS

Yeast strains: All deletion strains were taken from the yeast deletion collection (Giaever *et al.* 2002) available at Open Biosystems (http://www.openbiosystems.com). Each deletion strain used was checked by PCR, testing for the absence of the ORF and the presence of the *kan* marker using the standard confirmation primers described on the Saccharomyces Genome Deletion Project web page (http://www-sequence.stanford.edu/group/yeast_deletion_project/Deletion_primers_PCR_sizes.txt) (Giaever *et al.* 2002). The wild-type strains used as controls in the flow cytometry experiments were the deletion collection parental strains: BY4743 (*MAT***a**/α, *his3*Δ*1*/*his3*Δ*1*, *leu2*Δ*0*/*leu2*Δ*0*, MET15/*met15*Δ*0*, LYS2/*lys2*Δ*0*, *ura3*Δ*0*/*ura3*Δ*0*), BY4741 (*MAT***a**, *his3*Δ*1*, *leu2*Δ*0*, *met15*Δ*0*, *ura3*Δ*0*), and BY4742 (*MAT*α, *his3*Δ*1*, *leu2*Δ*0*, *lys2*Δ*0*, *ura3*Δ). The P_{GAL1}-*SFG1* overexpression strain used in the expression profiling experiments was taken from the MORF yeast ORF collection (Gelperin *et al.* 2005), available at Open Biosystems; the host strain for this collection is Y258 (*MAT***a**, *pep4-3*, *his4-580*, *ura3-52*, *leu2-3*, *112*) (Zhu *et al.* 2001).

Plasmids: To study Sfg1 phosphorylation, we created a new vector, pMW100 (GenBank accession EU627197), based on pRS316 (Sikorski and Hieter 1989). pMW100 is identical to the CEN plasmid pRS316 with the following exceptions: it contains a new multiple cloning site, an HA tag followed by a stop codon, a *Bsr*GI site, and a "generic" 3′-UTR from *HIS3*. This vector makes it simple to clone genes with their native promoters placed upstream of an exchangeable epitope tag and a generic 3′-UTR. To create this vector, we purchased a custom synthesized 585-bp fragment from CelTek (http://www.celtek-genes.com/) containing the features listed above and flanked by a 5′ *Nae*I site and a 3′ *Not*I site. pRS316 and the custom fragment were digested with *Nae*I and *Not*I, gel purified (Qiagen gel extraction kit), and ligated with T4 DNA ligase (New England Biolabs) to create pMW100.

We amplified *SFG1* and its promoter from BY4741 genomic DNA (prepared following the procedure of Hoffman and Winston 1987), beginning 1 kb upstream of the ATG and ending with the codon immediately before the stop codon, using the following primers that contained the flanking *Not*I (5′) and *Sal*I (3′) restriction sites: 5′ (MO121) CAAGGAAAG GATTGCGGCCGCGCATTGAGTTACTACGCAGGCCTT and 3′ (MO122) GTCGGCAAAACAGGGTCGACTTGTTCTAAAA CCTTTGCCCACTGAACTTTTTTG. The resulting PCR product and pMW100 were digested with *Not*I and *Sal*I (New England Biolabs), gel purified, and ligated together. The final product, pMW104, is a CEN plasmid that produces HA-tagged Sfg1 from its native promoter. To identify conserved Cdc28 consensus sites, we used the sequences of related yeast species published by Cliften *et al.* (2003) and Kellis *et al.* (2003). To create *3xA-sfg1*, we purchased a custom-synthesized 984-bp fragment of *SFG1* from CelTek, which contained the following threonine-to-alanine codon changes: Thr14Ala, Thr28Ala,

and Thr45Ala. We replaced the wild-type sequence with the custom-synthesized sequence as follows: pMW104 and the CelTek fragment were digested with *Nru*I and *Sph*I (New England Biolabs), gel purified, and ligated together to create pMW113, which is identical to pMW104, with the exception of the three threonine-to-alanine mutations. pMW100, pMW104, and pMW113 were transformed into the relevant homozygous diploid deletion or wild-type parental strain, either Δ*sfg1* or BY4743.

To create an empty vector as a control for expression profiling experiments with the P_{GAL1}-*SFG1* overexpression strain, we obtained the MORF-*HIS5* plasmid from the MORF yeast ORF collection at Open Biosystems and digested the plasmid with *Bsr*GI (New England Biolabs) to remove the only the ORF. The empty vector was gel purified and closed by ligation, creating the empty plasmid pMW102. This plasmid was transformed into Y258 to create the yeast strain YMW100.

Flow cytometry assay: For the initial screen, cultures were inoculated from patches on YPD (1% yeast extract, 2% peptone, 2% dextrose)–agar plates into 96-well boxes containing 580 μl YPD media. After overnight growth at 30°, the cultures were diluted 2× with YPD, and 5 μl was used to inoculate 580 μl of fresh YPD. These new cultures were grown for 6 hr to obtain log-phase growth, then collected by centrifugation, washed with water, centrifuged again, and fixed overnight in 500 μl 70% ethanol. Duplicate 96-well boxes were grown each time this assay was performed. Growth for follow-up tests after the initial screen was performed similarly, except that cultures were started from individual colonies instead of patches and 12 replicates/strain were used. The remainder of the assay was done essentially as described by Haase and Reed (2002), but modified for the 96-well format: fixed cells were removed from ethanol and resuspended in 200 μl RNase A solution [2 mg/ml RNase A (Sigma), 50 mm Tris, pH 8, 15 mm NaCl], incubated at 37° for 2–3 hr, centrifuged and resuspended in pH 2.0 water and 5 mg/ml pepsin (Sigma), incubated for 90 min at 37°, and centrifuged and resuspended in 800 μl 50 mm Tris, pH 7.5. Prepared samples were stored at 4°. For flow cytometry, cells were assayed in 50 mm Tris, pH 7.5, and 1 μm SYTOX Green (Invitrogen) on a Beckman Cytomics FC500 MPL cytometer, using a 575BP filter on the FL2 detector.

Analysis of flow cytometry data: Flow cytometry results were initially processed by gating out debris on forward and side scatter and gating out G_1–G_1 doublets mimicking G_2/M cells by comparing peak *vs.* integral fluorescence using FlowJo (Tree Star, Ashland, OR). To avoid artifacts due to unseparated G_1 cells appearing as G_2/M cells in this assay, we identified G_1 doublets using the fluorescence pulse height *vs.* the area method (Wersto *et al.* 2001) and removed the G_1 doublets before further analysis of the cell cycle profiles. We found that the population of G_1 doublets did not vary significantly among deletion strains under our sample preparation procedure, and thus removal of these doublets from the analysis did not skew our results. Gated flow cytometry data were then exported from FlowJo and analyzed as follows: Histograms for each sample were generated from fluorescence measurements and then smoothed twice by taking the mean of a sliding five-channel window. The histograms were divided into cell cycle phases by treating the G_1 and G_2 peaks as Gaussian curves, with the standard deviation estimated by the following relation: standard deviation = (peak width at half maximal height)/$2 \times \text{sqrt}(2 \times \ln 2)$ (E. Weisstein, Mathworld at http://mathworld.wolfram.com/FullWidthatHalfMaximum.html; accessed April 13, 2008). Cells were counted as part of G_1 or G_2/M if they were within 3.5 standard deviations of the peak, and each sample was characterized by the percentage of all cells in G_1, S, and G_2/M. For the initial screen, each deletion strain was compared to the average cell cycle distribution of eight wild-type strains by calculating the sample *z*-score for each cell cycle phase. Any sample with at least one *z*-score >2.3 was counted as an initial hit and subjected to further testing. For follow-up tests after the initial screen, it was sufficient to use the percentage of cells in G_1 as our primary measure of cell cycle distribution: since there were only two mutants with cells accumulating in S phase, a change in percentage in G_1 ($\%G_1$) also indicated a complementary change in $\%G_2$/M in all but these two cases. The $\%G_1$ from 12 deletion isolates was compared with the $\%G_1$ from 12 wild-type isolates using an unpaired *t*-test, with the significance threshold set at $P = 0.01$.

Segregation analysis: Tetrads were produced by sporulating heterozygous diploid deletion strains taken from the existing library (Giaever *et al.* 2002). In some cases we created heterozygous diploids by crossing haploid deletion strains to the wild-type haploid strain BY4742. Individual spores, obtained by dissecting tetrads, were analyzed by flow cytometry as described above.

Western blots and phosphatase treatment: Cell pellets from 3 ml of log-phase yeast cultures were lysed by bead beating in 100 μl lysis buffer [50 mm Tris, pH 7.5, 1 mm EDTA, 0.5% Triton X-100, 300 mm NaCl, 1 mm DTT, 1× Complete-Mini EDTA-free protease inhibitor cocktail (Roche), 2.5 μg/μl pepstatin] with either 1 × PhosStop protease inhibitor (Roche) or water. After centrifugation to separate cell debris, extracts were removed from the beads and treated with 50 units calf intestinal phosphatase or water in 1× New England Biolabs buffer 3 for 30 min at 37°. Samples were then mixed with 2× Laemmli sample buffer (Bio-Rad) and heated at 95° for 5 min, following which 18 μl was loaded on either an 8–16% or a 10% polyacrylamide gel (Bio-Rad). SDS–PAGE was carried out at 150 V. Proteins were transferred to a PVDF membrane and detected with a rat monoclonal anti-HA primary antibody (clone 3F10, Roche) and a goat anti-rat HRP-conjugated secondary antibody (Jackson ImmunoResearch), as previously described (Gelperin *et al.* 2005). The blot was developed with the ECL Advance kit (GE Healthcare).

Microarray experiments: Experiments were performed using three biological replicates, with the diploid wild-type strain BY4743 compared to the homozygous diploid Δ*sfg1* deletion library strain and the MORF P_{GAL}-*SFG1* strain compared with strain YMW100 harboring the empty plasmid pMW102. For the deletion experiment, overnight cultures from single colonies were diluted 200-fold into 50 ml synthetic-complete media with 2% dextrose, grown for 6 hr to establish log-phase growth, and then harvested and snap frozen at −70°. For the overexpression experiments, strains were grown at 30° for 20 hr in −ura dropout, 2% dextrose media and then diluted 200-fold into 50 ml −ura dropout, 2% raffinose media. After 15 hr at 30°, protein expression was induced by adding 20% galactose to the medium for a final concentration of 2% galactose. After 6 hr of induction, the cells were harvested and snap frozen. RNA extraction, cDNA synthesis, fluorescent labeling, and array hybridization were performed as described (Gerke *et al.* 2006). Fluorescent spots were quantified using ScanArray Express (PerkinElmer, Waltham, MA), and the data were analyzed using the limma package in BioConductor (Smyth 2005). Linear models were fit for each gene using the equation $E(\mathbf{y}_g) = \alpha_g$, where $\mathbf{y}_g$ is a vector of log ratios for gene *g* from the three arrays, $E(\mathbf{y}_g)$ is the expected value of $\mathbf{y}_g$, and α_g is the vector of log ratios being estimated. Values of α_g were tested for significant difference from 0. Genes were ranked by significance and the top Sfg1 regulatory targets were identified by two criteria: (1) gene expression had to change in the opposite direction in the overexpression and deletion experiments (*i.e.*, up in the deletion and down in the overexpression

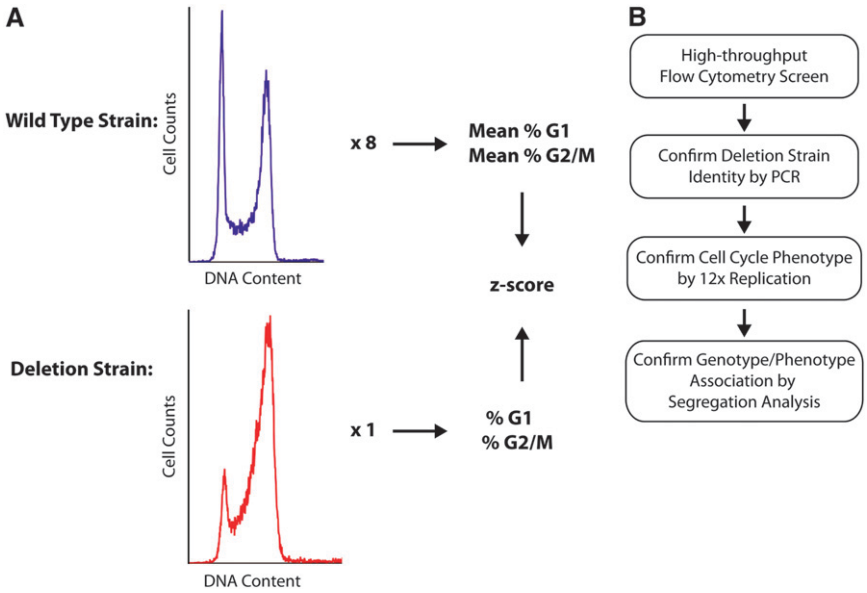

FIGURE 1.—Screen for cell cycle phenotypes among transcription factor deletions. (A) Scoring procedure for the initial high-throughput screen: strains were tested in 96-well plates, each of which contained eight replicates of the wild-type strain and a single copy of each deletion strain. Cell cycle profiles of the wild-type strains were averaged to produce a baseline cell cycle distribution. Cell cycle distributions of the deletion strains were compared against the baseline by calculating a *z*-score, as described in MATERIALS AND METHODS. (B) Outline of the procedure for identifying transcription factor deletions with altered cell cycle profiles.

or vice versa) and (2) a gene had to meet a stringent significance threshold in one experiment (unadjusted $P < \sim 5 \times 10^{-6}$) and a relaxed threshold in the other experiment (unadjusted $P < \sim 5 \times 10^{-4}$). Microarray images, spot fluorescence ratios, and lists of differentially expressed genes are available upon request.

RESULTS

Cell cycle defects in transcription factor deletion strains: We used a quantitative, high-throughput flow cytometry assay to identify mutant strains of yeast with defects in cell cycle progression. We screened 268 homozygous diploid deletion strains, each harboring a single deletion of a gene that has been annotated as a transcription factor, that contains a predicted DNA-binding domain, or that bound DNA in a large-scale *in vitro* screen (HALL *et al.* 2004) (supplemental Table S1). We used homozygous diploid deletion strains to minimize artifacts due to recessive second-site mutations. Asynchronous cultures were grown to mid-log phase in rich media, and their DNA content was measured by flow cytometry. Taking DNA content as an indicator of position in the cell cycle, we determined the distribution of cells among the major cell cycle phases for each sample and compared the cell cycle phase distributions of transcription factor deletions with the distribution of the wild-type strain (Figure 1A).

The deletion strains were first processed through a rapid initial screen, in which we tested single samples of deletion strains against the averaged cell cycle distribution of eight replicates of the wild-type strain grown on the same 96-well plate (Figure 1A). This initial screen was performed in duplicate, and any deletions that exhibited altered cell cycle distributions in both replicates were subjected to three further verification steps: (1) confirmation of the identity of the deletion strains by PCR; (2) confirmation of the reproducibility of the cell cycle defect by testing 12 isolates of the deletion strain against 12 wild-type isolates; and (3) performance of tetrad analysis to test whether the cell cycle phenotype cosegregated with the transcription factor deletion (Figure 1B).

We found that nearly 20% of the transcription factor deletions included in our screen exhibited verifiable defects in cell cycle progression. A total of 114 deletions showed altered cell cycle distributions in our rapid initial screen, and in 64 cases the phenotype could be verified by comparing 12 independent replicates per mutant strain with 12 replicates of the wild-type strain. To determine whether the cell cycle phenotype was associated with the transcription factor deletion in these 64 strains, we performed segregation analysis by sporulating the corresponding heterozygous diploid deletion strains and testing the resulting spores from 6 to 10 tetrads/strain for cell cycle defects. We found that 47 transcription factor deletions segregated with verifiable cell cycle defects. In the case of one transcription factor deletion, Δ*cse2*, we could not complete the segregation analysis since Δ*cse2* strains are defective in meiosis (data not shown; Saccharomyces Genome Database at http://www.yeastgenome.org/; accessed April 11, 2008). However, the homozygous diploid cell cycle phenotype was both dramatic and reproducible, and furthermore, *CSE2* is a known cell cycle regulator that is involved in mitotic sister-chromatid separation (XIAO *et al.* 1993). Including *CSE2*, we thus identified in our screen a total of 48 transcription factors with an impact on cell cycle progression (Table 1). These 48 transcription factors, together with 5 other known cell cycle transcription factors whose deletion phenotypes were inconclusive in our screen (see below), comprise 20% of all the

TABLE 1

Transcription factors with cell cycle deletion phenotypes

ORF	Name	Accumulated phase	% G_1 ratio (deletion/ wild type)	*P*-value	*In vitro* Cdc28 substrate?[a]	Cell cycle regulated?[b]	Functional role
YMR072W	ABF2	G_1	1.18	1.90E-05		Yes	Mitochondrial DNA-binding protein
YGL071W	AFT1	G_1	1.77	1.80E-11			Transcription factor involved in iron utilization
YDR173C	ARG82	G_1	1.14	1.00E-03			Inositol triphosphate kinase and regulator of the Mcm1 transcription factor
YKR099W	BAS1	G_1	1.16	3.49E-08	Yes		Purine, pyrimidine, histidine biosynthesis
YLR074C	BUD20	G_1	1.42	3.10E-08		Yes	Nuclear C2H2 zinc-finger protein involved in bud site selection
YLR226W	BUR2	G_1	1.56	5.02E-14			Potential DNA-binding protein, cyclin for Bur1 kinase
YPL049C	DIG1	G_1	1.33	1.00E-02			Repressor of MAPK-stimulated genes
YIL131C	FKH1	G_2	0.95	4.00E-03		Yes	G_2 cell cycle transcription factor
YNL068C	FKH2	G_2	0.9	3.21E-09	Yes	Yes	G_2 cell cycle transcription factor
YNL199C	GCR2	G_1	1.28	6.90E-13			Transcriptional activator of glycolysis genes
YER040W	GLN3	S	0.65	2.17E-10			Regulator of low-nitrogen genes, substrate of TOR kinases
YBL021C	HAP3	G_1	1.25	2.71E-10			Regulator of respiratory gene expression
YCR065W	HCM1	G_2	0.7	1.50E-06	Yes	Yes	S-phase cell cycle transcription factor
YDR174W	HMO1	G_1	1.39	8.72E-09			DNA-binding protein involved in rDNA transcription
YNL227C	JJJ1	G_1	1.23	1.14E-05			C2H2 zinc-finger protein involved in ribosome biogenesis
YMR021C	MAC1	G_1	1.47	3.81E-07			Copper-sensing transcription factor
YIR033W	MGA2	G_2	0.8	7.48E-11			Transcriptional regulator of essential fatty acid desaturase OLE1
YDR296W	MHR1	G_1	1.41	1.09E-14			Transcription factor required for mtDNA recombination
YER068W	MOT2	G_1	1.56	6.98E-21			Negative regulator of pheromone-responsive genes, member of CCR4 transcriptional complex
YMR070W	MOT3	G_2	0.78	9.19E-06			Repressor of hypoxic genes and ergosterol biosynthesis genes
YBR195C	MSI1	G_2	0.93	7.74E-05			Subunit of chromatin assembly factor CAF-I, potentially involved in transcriptional regulation and S-phase progression
YDR176W	NGG1	G_1	1.33	3.93E-08	Yes		Member of ADA, SAGA, and SLIK complexes, involved in glucose repression of GAL genes
YBR279W	PAF1	G_1	1.17	5.77E-10			RNA Pol II-associated factor required for transcription of a subset of cell cycle genes
YDR323C	PEP7	G_1	1.23	5.08E-10			Potential DNA-binding protein; involved in vesicle targeting
YDL106C	PHO2	G_1	1.88	4.63E-07			Regulator of phosphate metabolism genes
YFR034C	PHO4	G_1	1.41	6.61E-09			Response to phosphate availability
YBR267W	REI1	G_1	1.49	4.91E-09		Yes	Zinc-finger protein involved in ribosome biogenesis and bud growth
YLR176C	RFX1	G_1	1.09	8.37E-05			Regulates DNA-damage-inducible genes
YPR065W	ROX1	G_1	1.13	6.00E-04			Repressor of hypoxic genes
YDL020C	RPN4	G_2	0.63	4.03E-09			Regulator of proteasome genes
YBL025W	RRN10	G_1	2.44	1.45E-16			Involved in rDNA transcription
YOR315W	SFG1	G_1	1.15	4.52E-06	Yes	Yes	Nuclear protein overexpression induces filamentous growth
YLR403W	SFP1	G_1	1.77	2.42E-22			Transcription factor involved in ribosomal gene regulation and the G_2 DNA-damage checkpoint
YOL004W	SIN3	G_2	0.52	5.12E-15			Component of histone acetylase complex

(*continued*)

TABLE 1

(Continued)

ORF	Name	Accumulated phase	% G_1 ratio (deletion/ wild type)	*P*-value	*In vitro* Cdc28 substrate?[a]	Cell cycle regulated?[b]	Functional role
YHR206W	SKN7	S	0.94	1.16E-04			Stress response
YJL127C	SPT10	G_2	0.68	3.63E-12			Activator of histone gene transcription
YBR081C	SPT7	G_1	1.23	3.12E-06			Component of SAGA and SALSA complexes
YDR310C	SUM1	G_2	0.86	3.60E-04			Repressor of sporulation genes during mitosis
YJL176C	SWI3	G_1	1.3	2.36E-06			Component of SWI/SNF required for transcription of HO and other genes
YER111C	SWI4	G_2	0.67	9.23E-07	Yes	Yes	G_1 cell cycle transcription factor
YDR146C	SWI5	G_1	1.18	2.52E-05	Yes	Yes	M/G_1 cell cycle transcription factor
YLR182W	SWI6	G_2	0.28	5.93E-16	Yes	Yes	G_1 cell cycle transcription factor
YOL072W	THP1	G_1	1.18	2.14E-06			Nuclear pore protein involved in transcription and mRNA export
YCR084C	TUP1	G_1	1.24	4.90E-04		Yes	Multi-functional transcriptional repressor
YNL229C	URE2	G_1	1.31	6.97E-14			Negative regulator of GLN3-activated transcription
YDR359C	VID21	G_2	0.66	1.83E-09			Component of histone acetylation complex
YOR083W	WHI5	G_2	0.83	2.48E-13	Yes	Yes	Transcriptional repressor of the G_1 transcription factor SBF
YNR010W	CSE2	G_1	1.31	8.74E-07			Member of mediator complex and necessary for proper sister-chromatid separation

Cell cycle phenotypes are reported as the ratio of the mean $\%G_1$ deletion/mean $\%G_1$ wild type, using the mean values of 12 isolates/strain as described in MATERIALS AND METHODS. *P*-values were determined as described in MATERIALS AND METHODS and are dependent upon both the magnitude and the variability of the phenotype.

[a] UBERSAX, *et al.* (2003).

[b] PRAMILA, *et al.* (2006).

transcription factors that we tested. This indicates that a substantial fraction of nonessential transcription factors play some role in cell cycle progression, which is consistent with the extensive cell-cycle-regulated transcription found in yeast and with the fact that many cellular processes are connected to the cell cycle.

Our screen was sensitive, as shown by the fact that among our 48 hits were 6 of the 8 nonessential, major cell cycle transcriptional activators: Swi4, Swi6, Hcm1, Fkh1, Fkh2, Swi5, as well as a repressor of G_1 transcription, Whi5 (Table 1). In at least three cases, our flow cytometry results are supported by previous reports in the literature. PRAMILA *et al.* (2006) showed that a Δ*hcm1* mutant spends longer in G_2/M, consistent with our result (Table 1). Deletion of the G_1 transcription factors Swi4 (GRAY *et al.* 1997) and Swi6 (WIJNEN *et al.* 2002) have each been reported to result in an accumulation of cells in G_2/M, which we also observed. In the case of the Δ*swi4* mutant, the G_2/M phenotype is due to the fact the Δ*swi4* cells are defective in budding but not in DNA replication (GRAY *et al.* 1997). In the case of one major cell cycle transcription factor, Ace2, we were unable to obtain a cell cycle profile, since Δ*ace2* mutants form large aggregates of unseparated cells (VOTH *et al.* 2005), which did not pass through the flow cytometer. Four other known cell cycle transcription factors exhibited reproducible cell cycle phenotypes in the homozygous diploid deletions, but failed the segregation analysis test in the heterozygous diploids: Δ*mbp1*, Δ*stb1*, Δ*yox1*, and Δ*yhp1* all exhibited altered flow cytometry profiles, yet we failed to find any consistent cell cycle phenotype in the segregation analysis, even after two separate rounds of sporulation and tetrad dissection. It is possible that multiple secondary mutations confounded the segregation analysis in these cases, since all four spores from each tetrad generally exhibited cell cycle profiles that were different from those of the wild-type haploid strain. Thus the failure to include four major known cell cycle transcription factors in our final list of 48 hits was due to secondary mutations confounding the tetrad analysis, and not due to a lack of sensitivity in our quantitative flow cytometry assay.

Six other transcription factor deletions also exhibited cell cycle defects that may be genuine but could not be confirmed because of confounding secondary mutations: Δ*arr1*, Δ*dal82*, Δ*nbp2*, Δ*pib2*, Δ*rph1*, and Δ*mig3*. All four spores from most tetrads exhibited cell cycle profiles different from the control strain, again suggesting that any cell cycle defect that might be segregating with the deletion is being masked by multiple secondary mutations. In spite of these effects, secondary mutations had only a small impact on the overall effectiveness of our screen: 6 of 64 original hits were exclusively due to secondary mutations and were easily revealed in the segregation analysis; deletion strains of four known cell cycle transcription factors contained confounding mu-

TABLE 2

Gene ontology biological process terms for identified cell-cycle-associated transcription factors

Gene ontology biological process term	No. of genes
Organelle organization and biogenesis	27
Transcription	16
Cell cycle	12
RNA metabolic process	10
Response to stress	10
DNA metabolic process	9
Response to chemical stimuli	6
Protein modification process	6
Transport	5
Ribosome biogenesis and assembly	4
Pseudohyphal growth	3
Cytokinesis	2
Lipid metabolic process	2
Meiosis	2
Conjugation	2
Heterocycle metabolic process	2
Amino acid and derivative metabolic process	2
Membrane organization and biogenesis	2
Aromatic compound metabolic process	2
Anatomical structure morphogenesis	2
Vesicle-mediated transport	2
Cytoskeleton organization and biogenesis	1
Cell budding	1
Signal transduction	1
Cellular homeostasis	1

tations; and 6 other hits may be genuine but could not be verified due to secondary mutations. We were thus able to effectively eliminate false positives, and 90% of the remaining hits (53 of 59) were either verified in our tests or already known to be cell cycle regulators.

Cell cycle transcription factors are involved in cell-size control and multiple cellular processes: The 48 transcription factors identified in our screen cover all phases of the cell cycle (Table 1) and operate in a variety of functional pathways (Table 2). The most common cell cycle phenotype that we observed was an accumulation of cells in G_1 (Figure 2A), consistent with the fact that many processes have an effect on the decision to exit G_1 and commit to DNA replication. We also found deletions that caused cells to accumulate in the other phases of the cell cycle. Our results indicate that the phenotype assayed in our screen is reasonably specific for cell cycle regulators and that we did not simply detect mutants with general stress or metabolism defects that trigger the G_1 START checkpoint. In fact, deletions of many major stress and metabolism transcription factors, including Δ*gcn4*, Δ*leu3*, Δ*met31*, Δ*met32*, Δ*msn2*, Δ*msn4*, and Δ*yap1*, failed to show cell cycle phenotypes in the rich media conditions used in our screen, suggesting that the cell cycle defects detected in our screen are specific to cell cycle control, and not the result of a general impact on stress or metabolism pathways.

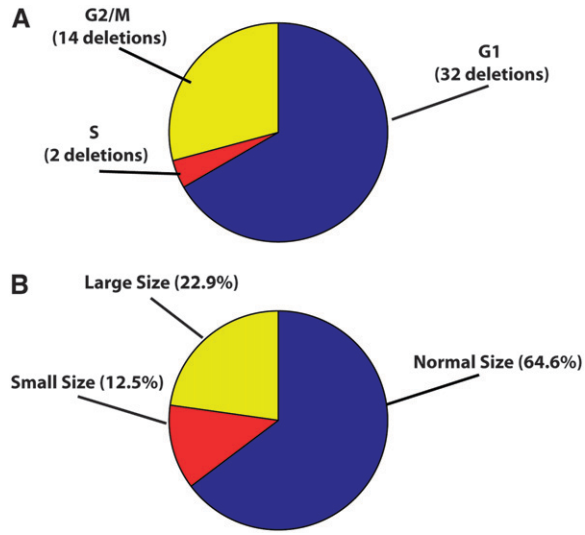

Figure 2.—Distribution of cell cycle phenotypes and association with cell-size control. (A) Distribution of cell cycle mutants by phase. Most hits exhibited an accumulation of cells in G_1, although all phases of the cell cycle were represented among the hits. (B) Cell cycle transcription factor mutants are enriched in cell-size mutants categorized as the largest or the smallest 5% of the strains in Jorgensen *et al.* (2002).

Transcription factor deletions with previously reported cell-size defects generally exhibited cell cycle phenotypes in our study. Twenty-one of the 268 transcription factors that we tested were previously reported to be among either the smallest 5% or the largest 5% of all viable systematic deletion strains (Jorgensen *et al.* 2002). Of these 21 genes, deletions of 17 exhibited cell cycle phenotypes in our screen, and one other, *ACE2*, is a known cell cycle transcription factor (Figure 2B). Compared to all transcription factor deletions that we tested, the deletions with cell cycle phenotypes were enriched in cell-size regulators ($P = 4.7 \times 10^{-11}$, hypergeometric distribution). Our results are consistent with the tight connection that exists between control of cell cycle progression and control of cell size, and they highlight the significance of transcriptional regulators in these control mechanisms.

Among the transcription factors identified in our screen are several genes with additional evidence supporting a cell cycle role, yet their effect on cell cycle progression was not previously recognized. For example, several transcription factors are themselves periodically regulated during the cell cycle (Table 1), such as Abf2, a high-mobility-group class DNA-binding protein involved in mtDNA replication (Macalpine *et al.* 1998), Bud20, a nuclear protein of unknown function required for a normal budding pattern (Ni and Snyder 2001), and Sfg1, a putative transcription factor that induces noninvasive filamentous growth when overexpressed (Fujita *et al.* 2005). Our hits are enriched for *in vitro* cyclin-dependent kinase targets (Table 1), relative to all transcription factors tested in a screen for Cdc28/Clb2

substrates ($P = 0.016$, binomial distribution) (Ubersax *et al.* 2003). This further suggests that our screen has identified cell-cycle-specific transcription factors. Among these Cdk substrates are transcription factors with no known direct cell cycle role: Sfg1, mentioned above; Bas1, a transcriptional regulator of nucleotide biosynthesis genes (Daignan-Fornier and Fink 1992); and Ngg1, a transcription cofactor involved in chromatin remodeling (Saleh *et al.* 1997). Further study of these transcription factors may reveal novel protein–DNA and transcription factor–Cdk regulatory interactions important for cell cycle progression.

Sfg1 represses cell separation genes: Sfg1 stood out among our hits as a poorly characterized gene that shares two characteristic traits of known cell cycle transcription factors: its transcript is expressed periodically during the cell cycle (Pramila *et al.* 2006) and it is an *in vitro* substrate of Cdc28 (Ubersax *et al.* 2003). A Δ*sfg1* strain exhibits an accumulation of cells in G_1 in our flow cytomery assay (Figure 3A), and overexpression of *SFG1* induces the opposite effect, the accumulation of cells in G_2. (Figure 3B). The expression of *SFG1* mRNA closely parallels that of the two major early G_1 transcription factors, *SWI5* and *ACE2*, which peak in M phase (Spellman *et al.* 1998; Pramila *et al.* 2006). Similar to these two transcription factors, the promoter of *SFG1* contains a Fkh1/Fkh2-binding site, and proper cell cycle expression of *SFG1* is abolished in a *fkh1 fkh2* double mutant (Zhu *et al.* 2000), suggesting that *SFG1*, *ACE2*, and *SWI5* are induced simultaneously in G_2/M by either Fkh1 or Fkh2. Overexpression of *SFG1* leads to noninvasive filamentous growth and an *sfg1* mutant is deficient in pseudohyphal growth (Fujita *et al.* 2005), suggesting that this gene plays some role in regulating the cell fate decision between vegetative and filamentous growth. We therefore sought to further study the functional role of Sfg1.

To identify putative regulatory targets of Sfg1, we used genomewide expression profiling to examine gene expression in a Δ*sfg1* strain and a P_{GAL}-*SFG1* overexpression strain. In three replicate experiments with the Δ*sfg1* strain, 25 of the 30 (83%) most significantly changed genes increased in expression, suggesting that Sfg1 may be a transcriptional repressor. We therefore looked for genes whose expression increased in the deletion and decreased in the overexpression strain. Seventeen genes passed a significance threshold (see materials and methods) in both the deletion and overexpression experiments and were thus considered putative Sfg1 regulatory targets (Table 3). While these genes were identified because they are repressed by Sfg1, 11 of these 17 genes are known to be activated directly by Swi5 and/or Ace2 (Kovacech *et al.* 1996; King *et al.* 1998; McBride *et al.* 1999; Doolin *et al.* 2001; Voth *et al.* 2005, 2007; see also Table 3). Most of these putative Sfg1 target genes also fell into a coherent functional class. Ten of 17 are involved in mother–daughter cell separation or cell-wall integrity; one more, Ash1, is a transcription factor that regulates *MUC1*, a key glycoprotein critical for pseudohyphal growth (Chandarlapaty and Errede 1998). These results strongly suggest that Sfg1 plays a regulatory role connecting cell cycle progression with pseudohyphal growth, during which cells do not separate. Sfg1 may act as a direct repressor of Ace2/Swi5-activated cell separation genes and of *ASH1* (also activated by Swi5 or Ace2; McBride *et al.* 1999), thus preventing both cell separation and *MUC1* induction until a decision is made either to proceed with vegetative growth or to switch to filamentous growth. Our expression profiling results explain the previously discovered effect of Sfg1 on pseudohyphal growth: Sfg1 can repress cell separation genes as well as *ASH1*; thus overexpression of *SFG1* results in filamentous growth, but without the ability to grow invasively, which requires the full pseudohyphal transcriptional program.

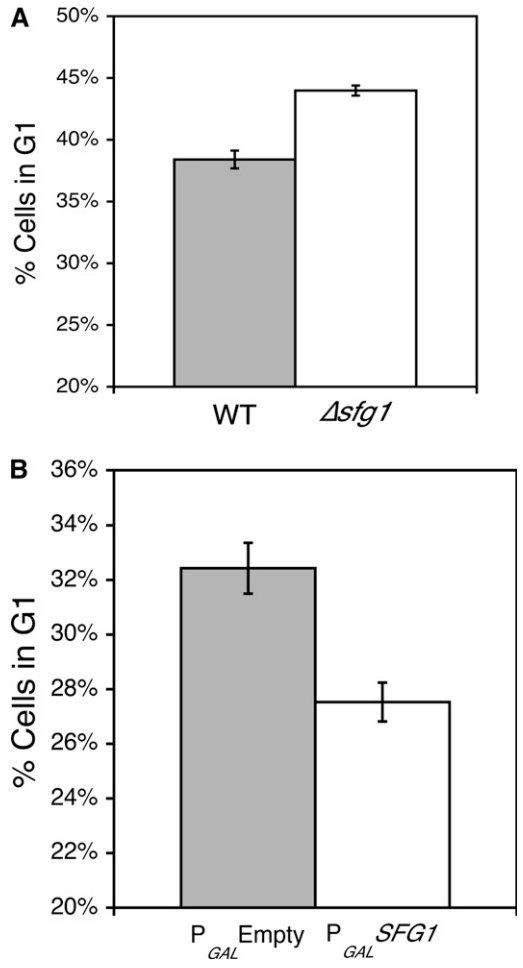

Figure 3.—Impact of Sfg1 on the cell cycle. (A) Flow cytometry results demonstrate that a Δ*sfg1* strain exhibits an accumulation of cells in the G_1 phase of the cell cycle relative to the wild-type (WT) strain. (B) Overexpression of *SFG1* results in the accumulation of cells in G_2/M relative to a strain harboring an empty plasmid. The difference in wild-type $\%G_1$ is due to differences in growth media–rich media with 2% dextrose in A and synthetic uracil dropout media with 2% galactose in B. Error bars indicate standard error.

TABLE 3

Putative Sfg1 target genes determined by whole-genome expression profiling

ORF	Name	Swi5 target gene?[a]	Ace2 target gene?[a]	Annotated function[b]
YBR158W	*AMN1*	Yes	Yes	Required for daughter cell separation and mitotic exit
YER124C	*DSE1*	No	Yes	Protein involved in cell-separation and cell-wall metabolism
YGL028C	*SCW11*	Yes	Yes	Cell-wall glucanase
YHR143W	*DSE2*	No	Yes	Daughter-cell-specific secreted glucanase involved in cell separation
YJL078C	*PRY3*	Yes	Yes	Cell-wall protein of unknown function
YKL164C	*PIR1*	Yes	No	Cell-wall protein required for cell-wall stability
YKL185W	*ASH1*	Yes	No	Transcriptional activator of mucin *MUC1*, required for pseudohyphal growth
YLR286C	*CTS1*	Yes	Yes	Endochitinase required for cell separation
YNL327W	*EGT2*	Yes	Yes	Endoglucanase required for cell separation
YNR067C	*DSE4*	No	No	Daughter-cell-specific glucanase required for cell separation.
YHL028W	*WSC4*	No	Yes	ER membrane protein involved in the secretory pathway
YDR432W	*NPL3*	No	No	RNA-binding protein involved in mRNA nuclear export
YGR138C	*TPO2*	No	No	Plasma membrane spermine transporter
YNL058C	*YNL058C*	No	No	Unknown function, potential CDK1 target
YNL066W	*SUN4*	No	No	Cell-wall glucanase possibly involved in septation
YNR033W	*ABZ1*	No	No	Para-aminobenzoate sunthase
YOL031C	*SIL1*	No	No	ER nucleotide exchange factor

[a] According to references cited in the text.
[b] SGD project Saccharomyces Genome Database (http://www.yeastgenome.org; accessed April 11, 2008).

Regulation of Sfg1 by phosphorylation: Sfg1 is a strong *in vitro* substrate of the cyclin-dependent kinase Cdc28 (Ubersax *et al.* 2003). To test for *in vivo* Sfg1 phosphorylation, we created a phosphorylation-deficient mutant (3xA-sfg1) by making threonine-to-alanine substitutions at three highly conserved, N-terminal, Cdc28 consensus sites (Figure 4A). We found that wild-type Sfg1 increased its mobility on SDS–PAGE after phosphatase treatment, indicating that Sfg1 is phosphorylated *in vivo* (Figure 4B). In contrast with the wild-type protein, phosphatase treatment had no impact on 3xA-sfg1 migration: both bands migrate with the phosphatase-treated wild-type protein, suggesting that the mutant is not phosphorylated (Figure 4B). These results show that Sfg1 is phosphorylated *in vivo* at one or more of the three conserved Cdc28 consensus sites clustered at the N terminus, consistent with the previous report that Sfg1 is an *in vitro* cyclin-dependent kinase substrate.

To explore the functional significance of Sfg1 phosphorylation, we tested whether the *3xA-sfg1* mutant can complement the cell cycle phenotype of the Δ*sfg1* strain. We transformed low-copy-number CEN plasmids harboring either wild-type *SFG1* or *3xA-sfg1* under the control of the native *SFG1* promoter into the homozygous diploid Δ*sfg1* strain. Using our flow cytometry assay, we compared the resultant strains to the wild-type and the Δ*sfg1* strain harboring an empty CEN plasmid. Wild-type *SFG1* expressed from its native promoter on a CEN plasmid partially suppressed the cell cycle defect in the Δ*sfg1* strain, while the *3xA-sfg1* mutant had no effect (Figure 4C). The lack of complete complementation with wild-type *SFG1* is unsurprising and could be due to effects caused by the HA tag or effects of expressing *SFG1* from a plasmid. Our results show that the effect on the cell cycle profile of abolishing phosphorylation of Sfg1 resembles that of a loss-of-function mutation and suggest that post-translational regulation of Sfg1 is essential for its function as a cell cycle regulator.

DISCUSSION

We used a sensitive and quantitative assay to detect cell cycle defects in transcription factor deletion strains to identify previously undetected transcriptional regulators of the yeast cell cycle. Our results demonstrate that 20% of all nonessential transcription factors are required for normal cell cycle progression under the growth conditions used in our screen. We verified the cell cycle phenotypes for deletions of these transcription factors, testing for the reproducibility of the cell cycle defect, the identity of the deletion strain, and segregation of the phenotype with the deletion. These 48 transcription factors are therefore very likely to be genuine regulators of cell cycle progression.

Our screen was a sensitive test for cell cycle transcriptional regulators. With a quantitative flow cytometry assay we could reliably detect deviations in cell cycle phase distribution as small as 5% relative to the wild-type profile (Table 1). We were able to find cell cycle defects in deletions of each of the known cell cycle transcription factors assayed, although in four instances the segregation analysis was problematic. It is possible that our screen missed regulators whose deletion phenotype was masked by second-site suppressor mutations. However,

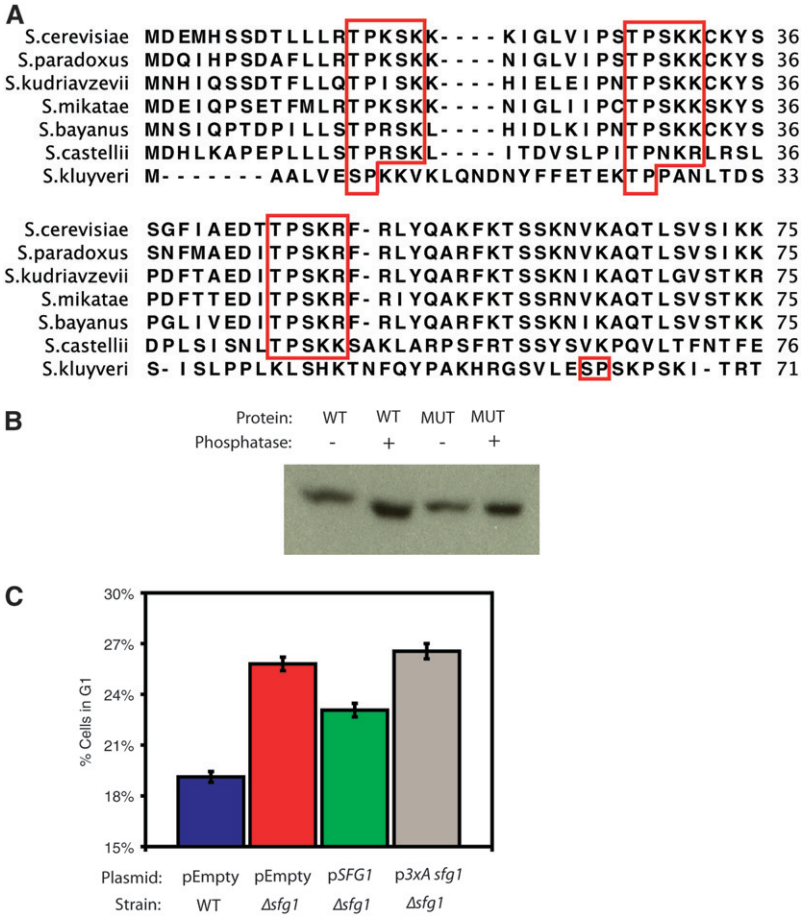

FIGURE 4.—Sfg1 is an *in vivo* cyclin-dependent kinase target. (A) Sfg1 contains three conserved Cdc28 consensus sequences (S/T-P-X-X-K/R) with partial consensus sequences (S/T-P) conserved in *Saccharomyces kluyveri*. (B) Phosphatase-treated wt-Sfg1 from cell extracts migrates faster on SDS–PAGE, indicating that it is phosphorylated *in vivo*; untreated 3xA-sfg1 migrates with phosphatase-treated Sfg1, indicating that abolition of the three Cdc28 consensus sites inhibits *in vivo* phosphorylation. (C) Abolition of Sfg1 phosphorylation sites recapitulates the cell cycle defect of Δ*sfg1*, assayed by flow cytometry in synthetic uracil dropout media. The plasmids listed correspond to the following plasmids described in MATERIALS AND METHODS: pEmpty, pMW100; p*SFG1*, pMW104; p*3xA-sfg1*, pMW113. Error bars indicate standard error.

the number of missed regulators is likely to be few, since we were able to detect small changes in deletions of known cell cycle regulators, even in cases of extensive functional redundancy such as Fkh1 and Fkh2. Furthermore, most obvious secondary mutations produced cell cycle profiles different from the wild-type strain, suggesting that it is rare for secondary mutations to completely suppress a transcription factor deletion phenotype. Thus we likely did not miss cell cycle regulators due to lack of sensitivity; instead, we probably missed regulators that have not yet been identified as transcription factors or that are not represented in the current deletion library.

The transcription factors that we identified in our screen are likely to have specific cell cycle functions. We identified all of the known, nonessential cell cycle transcription factors included in our screen, although secondary mutations confounded the segregation analysis for four of these regulators. Several other genes among our hits are not well characterized, but have some further evidence supporting a cell cycle role (Table 1). *BUD20* is a poorly characterized gene that is required for a normal budding pattern (NI and SNYDER 2001), and its message is cell cycle regulated (PRAMILA *et al.* 2006). Ngg1 is an *in vitro* Cdc28 substrate (UBERSAX *et al.* 2003) that contains conserved phosphorylation consensus sites, but the functional significance of an Ngg1–Cdc28 interaction is unknown. Mga2 is a regulator of the periodically expressed gene *OLE1* (ZHANG *et al.* 1999); however, Mga2 has no known cell cycle role. Although our flow cytometry assay did not directly test for an effect on cell-cycle-regulated transcription, our set of hits is a valuable pool of high-priority candidate regulators of currently unexplained cell cycle transcription. These results open new possibilities for investigating key questions concerning cell cycle transcription, such as how specific periodic genes are regulated and how transcription is connected to the Cdk–cyclin oscillator.

We conducted follow-up studies on Sfg1, a transcription factor with no previously documented role in cell cycle progression. Our genomewide expression profiling results indicate that Sfg1 represses a set of genes involved in early G_1 mother–daughter cell separation that is activated by Ace2 and Swi5. This explains the previously reported effects of Sfg1 on cell separation and pseudohyphal growth (FUJITA *et al.* 2005) and suggests a new model of how these genes are regulated. In the early phase of G_1, two activators, Ace2 and Swi5, and a repressor, Sfg1, are simultaneously induced by Fkh1/Fkh2 (ZHU *et al.* 2000; PRAMILA *et al.* 2006). Ace2, Swi5, and Sfg1 then act on a common set of target genes

to control the timing of cell separation. Deletion of Fkh1 and Fkh2, Ace2, Swi5, or various cell separation genes leads to inhibited cell separation, resulting in filamentous growth phenotypes similar to the overexpression phenotype of Sfg1 (Kovacech *et al.* 1996; King and Butler 1998; Pan and Heitman 2000; Pic *et al.* 2000; Zhu *et al.* 2000; Bidlingmaier *et al.* 2001; Doolin *et al.* 2001; Fujita *et al.* 2004, 2005). Furthermore, inappropriate activation of cell separation genes in the Δ*sfg1* strain is consistent with the finding that an *sfg1* mutant is defective in filamentous growth, since cells must remain attached in this growth mode (Fujita *et al.* 2005). Thus the deletion or overexpression phenotypes of *SFG1*, *SWI5*, *ACE2*, *FKH1*, *FKH2*, and known cell separation genes are consistent with our proposed model.

The likely function of this regulatory pathway is to control the timing of cell separation, so that it occurs after the cell fate decision between vegetative and pseudohyphal growth. During pseudohyphal growth, cells complete cytokinesis but remain connected at the cell wall and thus grow as filaments of attached cells (Gimeno *et al.* 1992). Sfg1 may act as a repressor to prevent both cell separation and the induction of the key pseudohyphal growth protein Muc1 until nutrient conditions in the environment have been assessed, keeping open the option to completely separate and begin the next cell cycle or to switch to pseudohyphal growth with its own attendant transcriptional program. However, the exact mechanism of Sfg1 function is still unclear. Sfg1 could bind DNA directly, adjacent to Swi5 or Ace2 at the promoters of target genes, or it could repress transcription via a protein–protein interaction with these transcription factors. To date, we have been unable to detect direct binding between Sfg1 and promoters of cell separation genes. However, it is possible that Sfg1 interacts with these promoters only briefly during the cell cycle (and thus the interaction is undetectable in the asynchronous samples that we tested), or it could act indirectly via another DNA-binding protein. We also could not detect any interaction between Sfg1 and Ace2 or Swi5 in co-immunoprecipitation experiments (data not shown); however, if such interactions did exist, they could be transient or mediated by other cofactors. The role that Cdc28 regulation plays in this process is also unknown. Sfg1 is phosphorylated by Cdc28-Clb2 *in vitro* (Ubersax *et al.* 2003), and we found that it is phosphorylated *in vivo* on at least one of three highly conserved Cdc28 consensus sites. A mutant Sfg1 that lacks these consensus phosphorylation sites recapitulates the cell cycle phenotype of Δ*sfg1* (Figure 4C), and not the overexpression phenotype (Figure 3B), which suggests that phosphorylation of Sfg1 may be required for active repression of cell separation genes, allowing cell separation to occur only after exit from mitosis and the inactivation of Cdc28/Clb2, when Sfg1 can be dephosphorylated.

These studies add to the emerging picture of complex transcriptional regulation at each phase of the cell cycle. While ChIP–chip studies of the major known cell cycle transcription factors suggested that waves of cell cycle transcription are induced by an interlocking, and possibly self-perpetuating cycle of transcription factors (Simon *et al.* 2001), it now appears that most major regulatory points of cell cycle transcription involve both repressors and activators, as well as interactions with Cdk–cyclin complexes. We anticipate that many of these interactions will include transcription factors identified in our screen.

This work was supported by American Cancer Society Research Scholar grant 06-039-01-GMC to B.A.C. M.A.W. is supported by National Research Service Award postdoctoral fellowship no. GM080964.

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Communicating editor: A. P. Mitchell