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*NAR Breakthrough Article*

# A new transcription factor for mitosis: in *Schizosaccharomyces pombe*, the RFX transcription factor Sak1 works with forkhead factors to regulate mitotic expression

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## ABSTRACT

**Mitotic genes are one of the most strongly oscillating groups of genes in the eukaryotic cell cycle. Understanding the regulation of mitotic gene expression is a key issue in cell cycle control but is poorly understood in most organisms. Here, we find a new mitotic transcription factor, Sak1, in the fission yeast *Schizosaccharomyces pombe*. Sak1 belongs to the RFX family of transcription factors, which have not previously been connected to cell cycle control. Sak1 binds upstream of mitotic genes in close proximity to Fkh2, a forkhead transcription factor previously implicated in regulation of mitotic genes. We show that Sak1 is the major activator of mitotic gene expression and also confirm the role of Fkh2 as the opposing repressor. Sep1, another forkhead transcription factor, is an activator for a small subset of mitotic genes involved in septation. From yeasts to humans, forkhead transcription factors are involved in mitotic gene expression and it will be interesting to see whether RFX transcription factors may also be involved in other organisms.**

## INTRODUCTION

Eukaryotic cell division is driven by an oscillation in the activity of cyclin-dependent kinase (CDK) (1). The CDK has many effectors, but amongst these are CDK-regulated transcription factors, which drive the expression of hundreds of genes up and down at different times in the cell cycle. Many of the cell cycle transcription factors form an interlocked loop, such that the dominant transcription factor of a particular cell cycle phase will, directly or indirectly, induce the transcription factor of the next phase, while repressing the factor of the previous (2–4).

Critical amongst the cell cycle regulated genes are those needed for mitosis, arguably the most complex cell cycle event. In *Saccharomyces cerevisiae*, mitotic gene regulation involves a transcription factor complex containing both activators and repressors. The core of the complex consists of a forkhead transcription factor bound to DNA immediately adjacent to a dimer of the MADS-box transcription factor Mcm1 (5–7), with important protein–protein contacts made between the Fkh2 and Mcm1 proteins. On the other side of the Mcm1 dimer can often be found a binding site for a repressor (Yox1 or Yhp1) (8). Despite being at a spatially separate site, the Yox1/Yhp1 competes with Fkh2 for binding to the Mcm1 dimer (9,10). The Yox1–Mcm1 complex is repressive, but, in its ground state, so is the Fkh2–Mcm1 complex. This is at least in part due to the recruitment of the Sin3 histone deacetylase by Fkh2 during G1 phase (11). Gene activation occurs when a co-activator protein, Ndd1, becomes phosphorylated by CDK (6,12–15) and polo kinase (16) in a cell cycle dependent manner. Phosphorylated Ndd1 binds to the forkhead associated domain (FHA domain) of Fkh2 and this phospho-Ndd1/Fkh2/Mcm1 complex is apparently the ultimate activator of mitotic gene expression. Recruitment of Sin3 and the associated histone deacetylase activity also requires the Fkh2 FHA domain but not its phospho-threonine binding activity (11).

Forkhead transcription factors have likewise been implicated in mitotic gene expression in other organisms, from the yeast *Schizosaccharomyces pombe* to mammals, but other components of the system are different, and the basis of regulation is not understood. For instance, in mammals, though the forkhead transcription factor FoxM1 is

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involved (17–20), it is unclear whether this protein finds its target genes by direct sequence-specific binding, or whether it is targeted solely by protein–protein interactions (21–23). In *S. pombe*, two different forkhead transcription factors, Fkh2 and Sep1, have been implicated in mitotic control (24–34), with some evidence that Fkh2 is a repressor of mitotic genes and Sep1 the activator (24–25,30–31,33). But it is unclear how the putative switch from Fkh2 repression to Sep1 activation is achieved. Furthermore, although *S. pombe* has a homolog of Mcm1 called Mbx1, it does not seem necessary for mitotic gene control (25,31). No *S. pombe* homolog of Ndd1 is apparent. Finally, while it has been hypothesized that Sep1 is the activator of the mitotic genes, this is difficult to reconcile with genetic results. A *sep1*Δ mutant is alive with a relatively mild phenotype, namely a defect in cell–cell separation (35–37). A stronger phenotype might have been expected for a lack of mitotic gene expression; for instance, the *ndd1*Δ mutant of *S. cerevisiae* is inviable (38).

Here, we have done Chromatin-Immunoprecipitation sequencing (ChIP-seq) and gene expression analysis with Fkh2 and Sep1 to understand their roles in mitosis. In agreement with previous results, we find that Fkh2 is a repressor of mitotic genes and Sep1 is an activator. However, Sep1 appears to bind and activate only a small subset of the mitotic genes, mainly those involved in septation. We find that another transcription factor, Sak1, an essential gene and a member of the highly-conserved RFX family, is intimately involved in mitotic gene control and may be the ultimate transcriptional activator of most or perhaps all of the mitotic genes.

## MATERIALS AND METHODS

### Yeast methods and strains

General fission yeast methods and media were used (39). For physiological experiments, cells were grown in complete Yeast Extract Supplemented (YES) media or Edinburgh Minimal Media (EMM) (MP Biomedical) with the required supplements at 32 or 25°C. Inactivation of the alleles of temperature sensitive strains (alleles: *cdc10-v50*, *nuc2–663*, *cdc25–22*) was achieved by shifting the culture to 35°C for 4 h. *sak1–891-ts* strain was grown at 26°C as permissive temperature and was restricted at 36°C for the times indicated. Cell cycle experiments were conducted via the transient inactivation of the *cdc25–22* allele essentially as previously described (40). Samples removed at various times were fixed in 70% ethanol for microscopic analysis or harvested for immuno-blot analysis. Overexpression of genes was achieved by using the pJR2–3XL vector (41). Cells were grown in Minimal Media (supplemented) with 5μg/ml thiamine (repressed state) to early log phase. Cells were washed two times with equal volume of media free of thiamine and then grown in EMM without thiamine. Samples were removed and fixed (70% ethanol) for microscopic examination and flow cytometry at various times as indicated. Measurement of cell length was conducted on fixed cells after rehydration and stained using DAPI (4',6-diamidino-2-phenylindole) and Calcofluor white (Sigma). Flow cytometry was carried out essentially as described by Sabatinos *et al*. (42). Sep1 ChIP's were performed after growing Sep1-TAP (JLP1670) cells in YES medium at 32°C to mid-log phase. The culture was split in two, and either 1mM Bortezomib (LC Labs) in dimethyl sulfoxide (DMSO) or an equal volume of DMSO was added. Cells were harvested for immunoblotting from each culture at 0, 15, 30 and 45 min after addition of drug or solvent. The 45 min drug treated sample was fixed for ChIP analysis.

### Strain and DNA constructs

All DNA constructs used for constructing C-terminally tagged strains or strains bearing a gene deletion were made using the polymerase chain reaction (PCR) based method (43). pJR2–3XL: *sak1*$^{+}$ (JL_AG_Plasmid_1) and pJR2–3XL: *fkh2*$^{+}$ (JL_AG_Plasmid_2) were made by amplifying the open reading frame of each gene by using primers bearing appropriate restriction sites for cloning into the pJR2–3XL vector. To generate the *fkh2–3* strain, ∼400 bp upstream of *fkh2* gene open reading frame (ORF) along with the tandem affinity purification (TAP) tag was cloned using the StrataClone PCR cloning kit (Agilent Technologies). Mutagenesis for generating R100A, S128A and N151A was done using QuickChange Lightning Multi-Site Directed Mutagenesis Kit (Agilent Technologies). The mutated sequence was sub-cloned into the pJK210 vector (ATCC). This plasmid construct was digested using MfeI and transformed into *fkh2-TAP>>KanMX6 ura4-D18* generating a tandem repeat of *fkh2–3-TAP* and *fkh2-TAP*. The inserted vector was counter-selected using 5-FOA and the selected colonies were screened by sequencing for the mutations. The strains (Supplementary Table S3), oligonucleotides and plasmids (Supplementary Table S4) used in this study are listed in the Supplementary Material.

### Cell lysate preparation and immunoblots

Cell lysates were prepared by re-suspending $10^8$ cells in 100 μl of lysis buffer (1× FA lysis buffer, 2× protease inhibitor cocktail (Roche cOmplete), 1 mM phenylmethylsulfonyl fluoride (PMSF)). 0.5 mm zirconia beads were added and a FastPrep-24 was used to lyse cells. The filtered lysate was used for analysis. Immunoblotting was performed using anti-TAP, PAP (Sigma P1291) and monoclonal anti-Cdc2 (Abcam ab5467) antibodies.

### ChIP and ChIP-seq

ChIP was done on two biological replicates for each experiment (GEO# GSE60712). ChIP was carried out as described (44) with the following variations from Keogh *et al*.: (i) Fixed cells were re-suspended in 400 μl lysis buffer (split between two 2 ml screw cap tubes). 0.5 mm zirconia beads were added and a FastPrep-24 was used to lyse cells. (ii) ChIP was performed on 1600 μl (60 ml initial culture) of prepared chromatin (TAP-tagged protein or untagged) and 20 μl of beads (GE Sepharose 6 Fast Flow) were used for IP. Pre-clearance of the beads with ascites fluid was not performed. IP was carried out for 2–3 h or overnight at 4°C. (iii) The following variations were introduced in the wash buffers used: wash buffer 1: 1× FA-lysis buffer, 0.1% sodium dodecyl sulphate (SDS), 1 M NaCl; wash buffer 2: 1× FA-lysis buffer, 0.1% SDS, 500 mM NaC. (iv) For each wash

buffer, two rinses (invert 30×) and one 4-min wash on rotator was conducted. Beads were collected in between each step by centrifugation at 1000 rpm for 30 s. While changing to the next wash buffer, beads were transferred to a fresh microcentrifuge tube (total three tube changes). (v) Purified IP'ed DNA was re-suspended in 40-μl DNase free water. A total of 10 μl of this IP'ed DNA was used for qPCR's (Roche Lightcycler 480 SYBR Green I Master, Machine: Eppendorf Realplex2 Mastercycler ep Gradient S) and Illumina sequencing libraries were made from the remaining 30 μl.

### ChIP-seq library preparation

(i) End Polish (final conc.): IP'ed DNA, 1X T4 DNA ligase buffer (NEB), 3U T4 DNA Polymerase (NEB), 10U T4 Polynucleotide Kinase (NEB), 0.4 mM dNTP's. Reaction was kept at 20°C for 30 min. It was subsequently cleaned up with QIAquick PCR Purification Kit and eluted in 20 μl water. (ii) A-tail addition (final conc.): previously eluted DNA, 5U Klenow Fragment (3′-5′ exo-) (NEB), 33.33 μM dATP, 1× NE Buffer 2 (NEB). Total volume was 30 μl. The reaction was incubated at 37°C for 30 min. The enzyme was inactivated by heating at 75°C for 20 min and was ramped down (rate = 10%) to 4°C. (iii) Adaptor Ligation (final conc.): previous volume, 750U T4 DNA Ligase (Rapid) (Enzymatics), 1× T4 DNA Ligase Buffer, 37.5 nM Illumina adaptor (sequences and detailed method for adaptors is provided in the GEO dataset; GSE60712) (45). The reaction was kept at 25°C for 1 h and inactivated at 65°C for 10 min. The reaction was cleaned up twice using 0.8× volume of Agencourt Ampure beads (first elution volume: 40 μl $H_2O$, second elution volume: 15 μl $H_2O$). (iv) PCR Amplification: eluted DNA, 1× Phusion Master Mix (NEB M0531), 2U Phusion Enzyme (NEB M0530), 200 μM dNTP's, 1 μM each library amplification primer. The libraries were amplified between 18–21 cycles (annealing temperature, 65°C for 30 s.; polymerization time, 30 s). (v) The amplified products were purified using 0.8× volume of Agencourt Ampure beads. Eluted DNA was size-selected using 2% agarose gel electrophoresis followed by cutting out the 250–300 or 300–350 bp region. The DNA was purified from the gel using QIAquick Gel Extraction kit (Qiagen). Eluted DNA was quantitated on Qubit (Invitrogen) and size range was checked using Bioanalyzer 2100. The libraries were subsequently sequenced using Illumina MiSeq.

### ChIP-seq data analysis

Analysis was conducted from two independently grown and prepared samples for each transcription factor ChIP-seq as well as the untagged control ChIP-seq. The sequencing reads of the two biological experiments were inspected for consistency and were subsequently combined into one FASTQ file to provide more robust data analysis. The FASTQ files were mapped to the *S. pombe* genome (version: Schizosaccharomyces_pombe.ASM294v1.16) using Bowtie2_2.1.0 (46). Peak detection was conducted by HOMER tools using default settings (47). The untagged ChIP-seq experiment was used as the control dataset for finding peaks. We wrote a script to determine the sequence within the peaks to conduct motif analysis by MEME as well as one that determined the targets up to 1 kb downstream of the center of the ChIP enriched peaks. Gene Ontology (GO) analysis was conducted using GOTermFinder at go.princeton.edu (48) at 0.1 *P*-value cutoff. Statistics for peaks obtained and the IP experiment by HOMER are given in ChIP-seq Peak files in the GEO dataset (GSE60712).

### Selective spore germination assays

Heterozygous diploid strains were grown in YES medium until early log phase (200 ml, $OD_{600}$ of 0.2). The cells were washed twice with water and re-suspended in liquid ME medium (equal volume) and were shaken at room temperature. Spores were collected. The spore germination was conducted by inoculating 5 million spores per ml from each of the heterozygous, congenic diploids into pre-warmed EMM-LAH media at 32°C at 0 h. A part of this culture was treated with 15 mM hydroxyurea (HU). The cells were harvested for RNA preparation or fixed for flow cytometry at 6, 8, 10 and 12 h. The HU treated sample was collected at 10 h.

### RNA preparation, microarrays and clustering

Samples were harvested by adding ice directly to the culture and washing once with cold water. Harvested cells were snap frozen in liquid nitrogen and saved for RNA preparation. Total RNA was isolated using the RiboPure$^{TM}$-Yeast kit (Ambion). Microarray labeling and hybridization were performed using Quick Amp Labeling Kit (two color) or Low Input Quick Amp Labeling Kit (two color) (Agilent Technologies). Agilent Gene Expression Array 8 × 15 K custom design 020613 (49) was used for all experiments. All experimental samples were labeled with Cy3 and hybridized against a common (F108) wild-type (WT) asynchronous log phase control Cy5 labeled sample (GEO# GSE60718). Cross-reference was possible as labeled control sample was the same for all experiments. For all genes, the average gene expression value for all probes of a gene was used for further analysis. Microarray data from all synchronous spore germination experiments, *sak1–891-ts* strain and upstream ChIP-seq peaks (if present $Log_2$ value = 4) were clustered along with the top 750 genes from Oliva *et al.* using the hierarchical clustering method (Complete Linkage) (50) and visualized using Java Treeview (51,52) (data shown in Supplementary Material, Dataset S1). The control for the *fkh2Δ S.G.* (Spore germination), *sak1Δ S.G.* and *sep1Δ S.G.* was the WT S.G. experiment at each of the time points respectively. The control for *sak1–891-ts* strain was a WT (F14) strain kept at that temperature for an equal time. Each experiment from this study was given a weight of 1 and each of the experiment from Oliva *et al.* (29) was given a weight of 0.058 for clustering. Only experiments from this study are shown after clustering.

## RESULTS

### Targets of Fkh2

ChIP-seq was used to identify direct targets of Fkh2. Fkh2 was tagged at its C-terminus with the TAP-tag (53) and this tagged gene was found to be fully functional. qPCR was used to assay the enrichment of two known targets of Fkh2, *cdc15* and *spo12* (26,30), in the anti-TAP chromatin immunoprecipitates. There was good enrichment of *cdc15* and *spo12* in the tagged Fkh2 precipitates, when compared to either *cdc15* or *spo12* precipitated in untagged controls (∼200-fold enrichment), or when compared to *adh1* or *srp7* (control genes not regulated by Fkh2) precipitated by tagged Fkh2 (∼10-fold enrichment) (Figure 1A).

ChIP-seq (54) was performed and peaks of sequence reads were identified using HOMER (47) (e.g. Figure 1B). A candidate target gene was defined as a gene whose 5′ transcriptional start site is within 1 kb of an intergenic ChIP peak. Apparent intragenic ChIP peaks were removed from analysis, partly because heavily transcribed regions are artefactually precipitated in ChIP experiments (55,56).

Fkh2 peaks were observed upstream of 148 protein-coding genes (Supplementary Table S1). Of these, 72 were cell-cycle regulated (29,57). The majority of these 72 cell cycle genes are expressed in mitosis (Figure 1C) (58). Many of these genes contain upstream consensus sites for forkhead binding (29). An analysis of enriched GO terms from these 72 genes showed cytokinesis and M-phase regulation as the significant terms (Supplementary Table S2). Cell cycle independent targets had GO term enrichment for carbohydrate metabolism genes (Supplementary Table S2). These data are consistent with the prevailing notion that Fkh2 is predominantly and directly involved in regulating mitotic genes.

The upstream sequences of the 148 genes were analyzed for motifs using MEME (59). The classic forkhead consensus GTAAACAAA (Figure 1D) was identified, with an *E*-value of $10^{-81}$. 68/72 cell-cycle regulated genes contained this motif (motif *P*-value $< 10^{-3}$). But surprisingly, a second, distinct motif, GTTGnCntggcAAC, was also identified, with an *E*-value of $10^{-33}$. Less distinct but probably related motifs were previously found associated with *S. pombe* mitotic genes in at least five previous studies (25,29,31,33,60). Comparison of this second motif to known databases using TOMTOM (61) suggested that this second motif could define the binding site for an RFX transcription factor (Figure 1D) (62,63). In *S. pombe*, the only known RFX transcription factor is Sak1. Sak1 is essential and was obtained as a high-copy suppressor of the mating defect caused by high cAMP-dependent kinase (64). Thus, precipitation of genes binding the forkhead transcription factor Fkh2 enriched not only for Fkh2 binding motifs, but also for presumptive Sak1 binding motifs.

We also separately analyzed the upstream sequences of the 76 Fkh ChIP-seq target genes that were not classified as cell cycle genes. Analysis by MEME again found the Fkh motif, but in this case no Sak1 motif, or any other second motif, was found. Thus, the Sak1 motif is specifically associated with the cell-cycle forkhead genes.

### Targets of Sak1

To see if indeed Sak1 binds the cell-cycle forkhead genes, we tagged Sak1 at its C-terminus with TAP and did ChIP-seq to identify its targets. We found 55 coding targets and 45 of these were genes we had already identified as cell cycle targets of Fkh2 (Supplementary Table S1). Since the filters for defining a peak were quite stringent, 45/55 may underestimate the true overlap. Motif analysis of the promoter regions identified by Sak1 ChIP-seq yielded both the Sak1 motif (*E*-value of $10^{-51}$) (Figure 1D) and the forkhead motif (*E*-value $10^{-59}$).

These results are reminiscent of *S. cerevisiae*, where the mitotic genes are regulated from a combined motif consisting of a motif for a forkhead transcription factor adjacent to a motif for the transcription factor Mcm1 (5), a MADS-box transcription factor (65). However, in *S. cerevisiae*, the Fkh and Mcm1 motifs are immediately adjacent to one another in a stereotypical arrangement with little variation from gene to gene (5). In contrast, the Fkh and Sak1 sites of *S. pombe* have varied arrangements in terms of spacing, order and strandedness (Figure 2). However, a Sak1 site is very commonly quite close to an Fkh2 site, typically within 40 bp (Figures 1E and 2).

Inspection of these promoters revealed shorter motifs that may be Sak1 half-sites. The RFX transcription factors usually bind as a dimer to a pair of half-sites which form the full palindromic motif (63,66), but some studies suggest that a single half-site might be functional (66–68). We note that one Sak1 half-site in the *cdc15* promoter appears to correspond to the particular PCB ('Pombe Cell cycle Box'–(60)) studied as a possible binding site for 'PCB Binding Factor', Mbx1 and polo kinase and thought to be involved in mitotic regulation (25,60).

### Targets of Sep1

Sep1 is another forkhead transcription factor (32) implicated in regulating mitotic genes. The division of labor between Fkh2 and Sep1 is not known. Therefore, we also attempted to define direct targets of Sep1 using ChIP-seq.

Consistent with previous results (69), our western analysis showed that the TAP-tagged Sep1 protein is sharply cell cycle regulated with peak abundance coinciding with mitosis (not shown). We attempted to do ChIP-seq experiments using synchronized *cdc25–22 sep1-TAP* cells and applying formaldehyde fixation at mitosis, but were unsuccessful, possibly due to the low levels of Sep1.

It has been predicted that Sep1 has signals for proteasomal degradation (70,71). To try to stabilize Sep1, we treated cells with bortezomib, a known proteasomal inhibitor in *S. pombe* (72). We observed a rapid increase in Sep1 abundance after addition of the drug, with peak accumulation after about 45 min (Figure 3A). Therefore, we treated asynchronous cells with bortezomib, and after 45 min fixed the cells with formaldehyde and did ChIP-seq analysis. Under these conditions, we succeeded in finding Sep1 ChIP-seq targets. There were significant peaks of Sep1 ChIP-seq reads passing all our filters for just nine coding genes (Figure 3D), with manual inspection showing reads above background for an additional three genes, *plo1*, *slp1* and

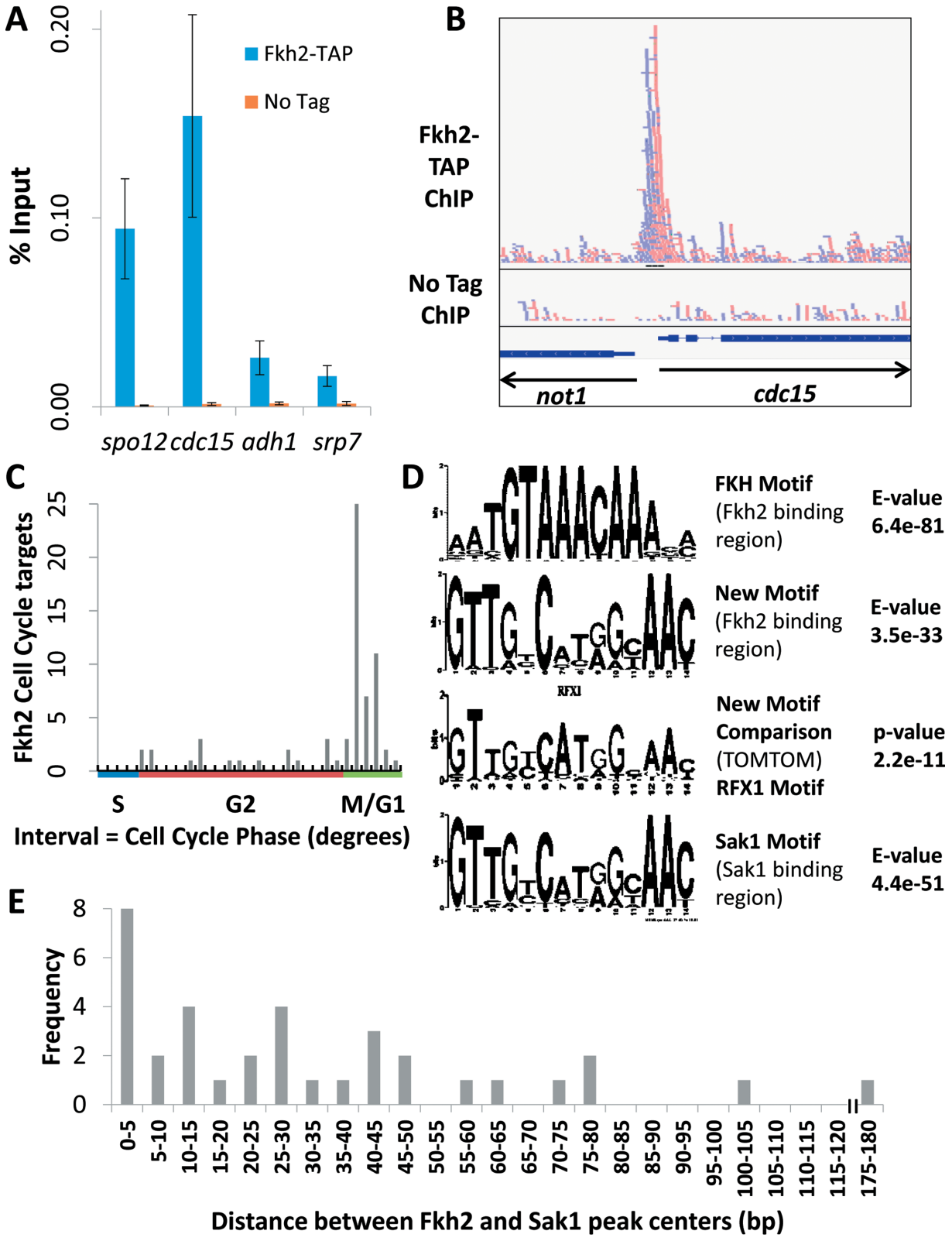


**Figure 1.** Fkh2 ChIP enrichment and analysis of ChIP-seq targets. (**A**) ChIP analysis by qPCR of asynchronous cultures of No Tag cells (F108) and cells expressing Fkh2-TAP tag (JLP1586). qPCR was done for two positive controls, *cdc15* and *spo12*, and two negative controls, *adh1* and *srp7*. Data show the mean ± S.E.M, ($N = 7$). *P*-value < 0.021. (**B**) Sequencing reads aligned to the *Schizosaccharomyces pombe* genome comparing Fkh2-TAP ChIP to No Tag ChIP upstream of *cdc15* transcript. Blue and red indicate sequencing reads aligning to opposite strands (Also see Supplementary Figure S1). (**C**) Cell cycle time of peak expression of Fkh2 cell cycle ChIP-seq targets. Each target is assigned a cell cycle phase at peak expression, in degrees (58). (**D**) Motif analysis from the sequence of Fkh2 and Sak1 peaks. Motif comparison of New Motif (Fkh2 ChIP binding region) to the known motifs (all yeast) was by TOMTOM. (**E**) Frequency distribution of the distances (bp) between the centers of Fkh2 and Sak1 ChIP Seq peaks.

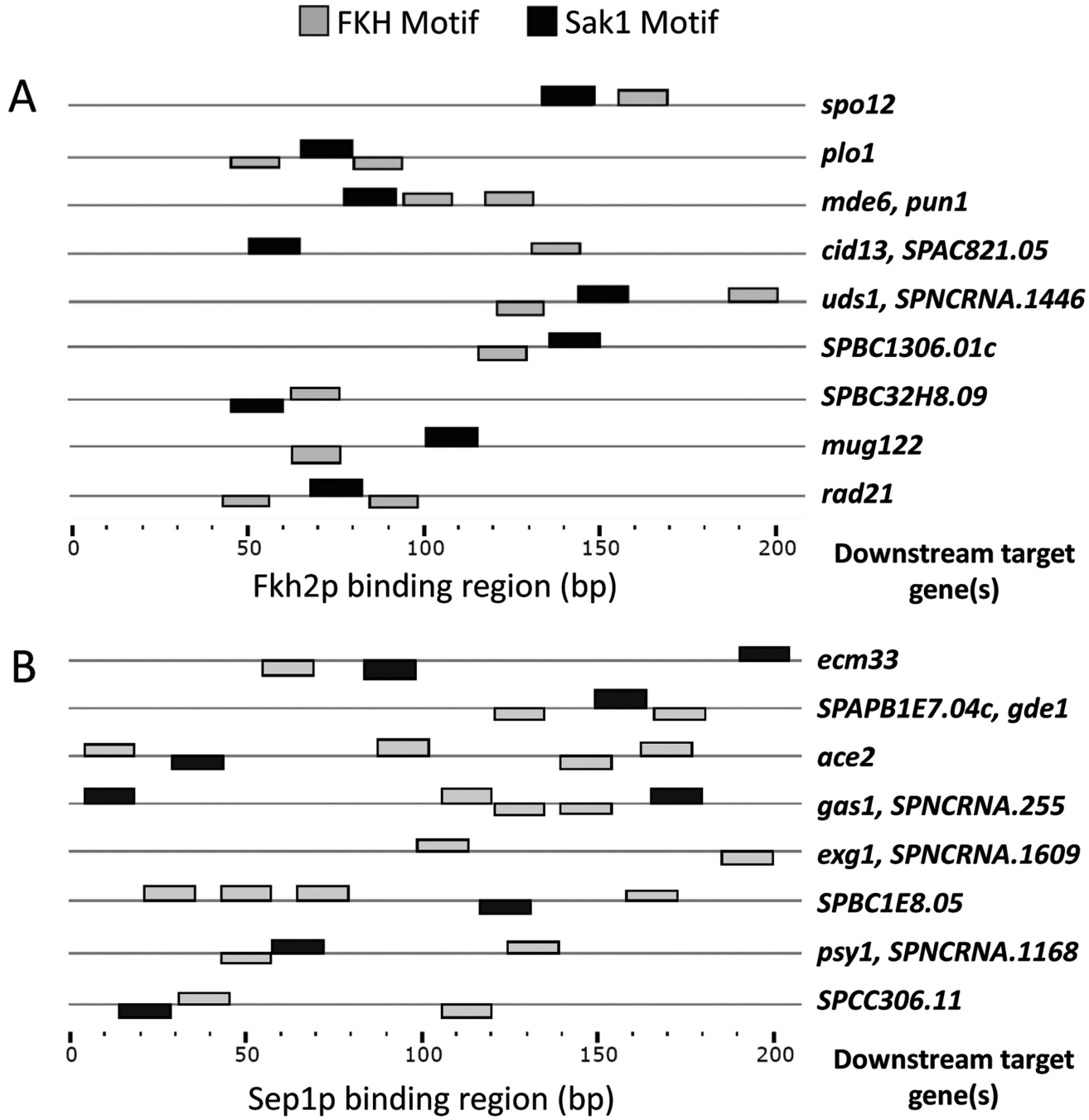


**Figure 2.** Organization of Sak1 and Fkh motifs. Organization of the Sak1 (black) and Fkh (gray) motifs of (**A**) Fkh2 and (**B**) Sep1 targets. Motifs are displayed above or below the line depending on strandedness. The height of a motif is proportional to its quality (i.e. -log(*P*-value)) (max height; *P*-value $= 10^{-10}$). Eight regions are shown for nine Sep1 targets because one pair of genes (SPAPB1E7.04c and *gde1*) is divergently transcribed.

*uds1*. Consistent with the delayed cell separation phenotype of a *sep1Δ* strain, the targets showed GO term enrichment for cell wall polysaccharide metabolism and cell wall organization/biogenesis (Supplementary Table S2). All nine were genes we had previously identified as joint targets of Fkh2 and Sak1 (Figure 3B); that is, Sep1 binds to a subset of the Fkh2 and Sak1 targets.

An alternative interpretation might be that the number of Sep1 ChIP-seq peaks found was limited by the low abundance of the Sep1 protein, and that in reality, Sep1 might bind a larger set of genes, perhaps all Fkh2 targets. Or, oppositely, it could be argued that bortezomib artefactually increased Sep1 abundance and the apparent number of targets, and that in reality Sep1 binds to less than nine genes, and this possibility should be kept in mind. However, the phenotypes of *sep1* mutants, and the gene expression results shown below (Figure 4C), are consistent with the idea that

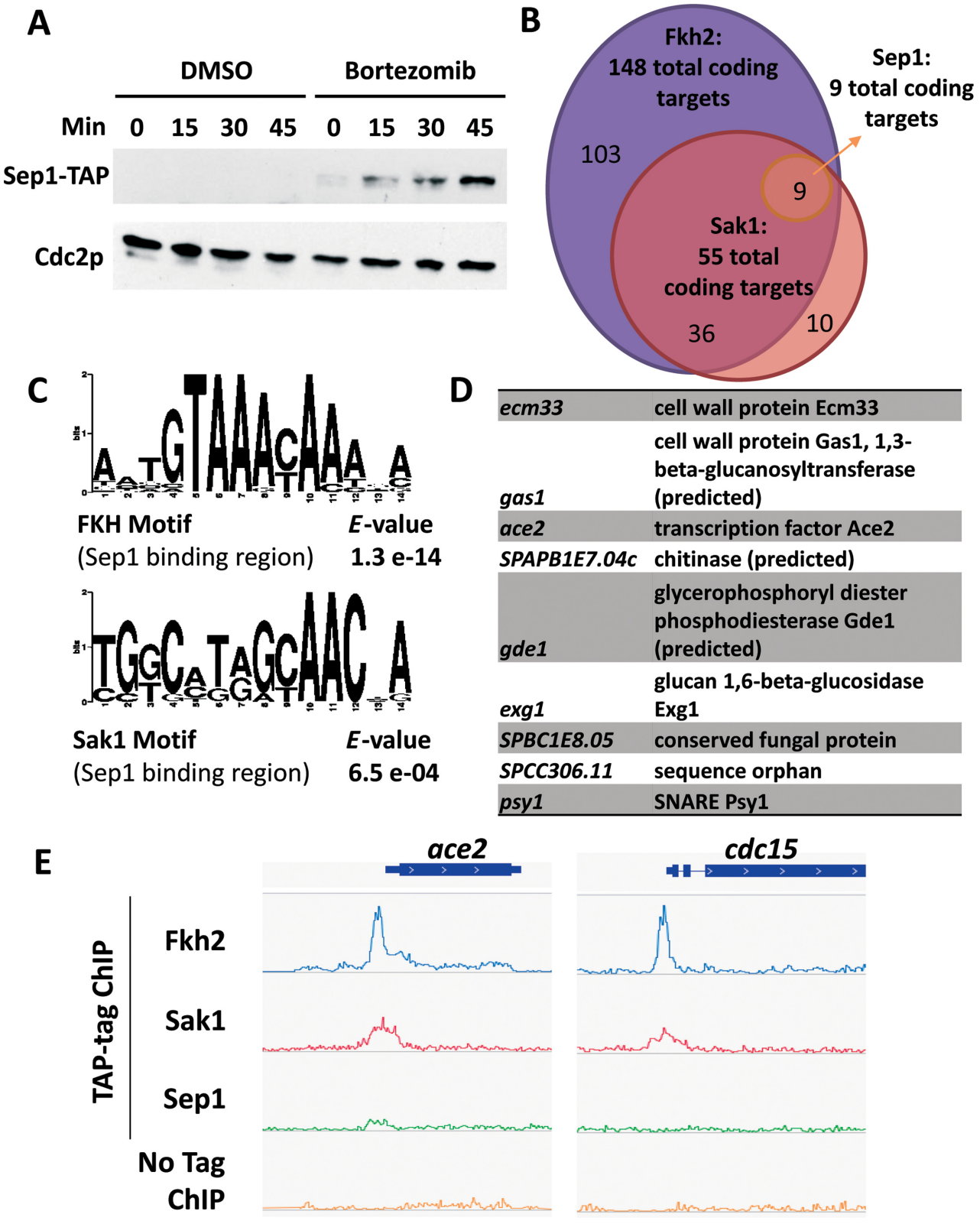


**Figure 3.** Sep1 targets. (**A**) Western analysis of Sep1-TAP tagged cells treated with Bortezomib or DMSO (solvent). (**B**) Venn diagram of the overlap between Fkh2, Sak1 and Sep1 coding targets. (**C**) Motif analysis of Sep1 binding regions by MEME. (**D**) A list of all Sep1p targets identified by ChIP-seq analysis. (**E**) Fkh2, Sep1 and Sak1 ChIP-seq coverage graphs upstream of *ace2* and *cdc15*. All graphs depict sequence tag counts per base (average: 25 bp window; normalization: count/million reads); scale: 60. They are visualized in the integrated genome viewer, IGV (Broad Institute).

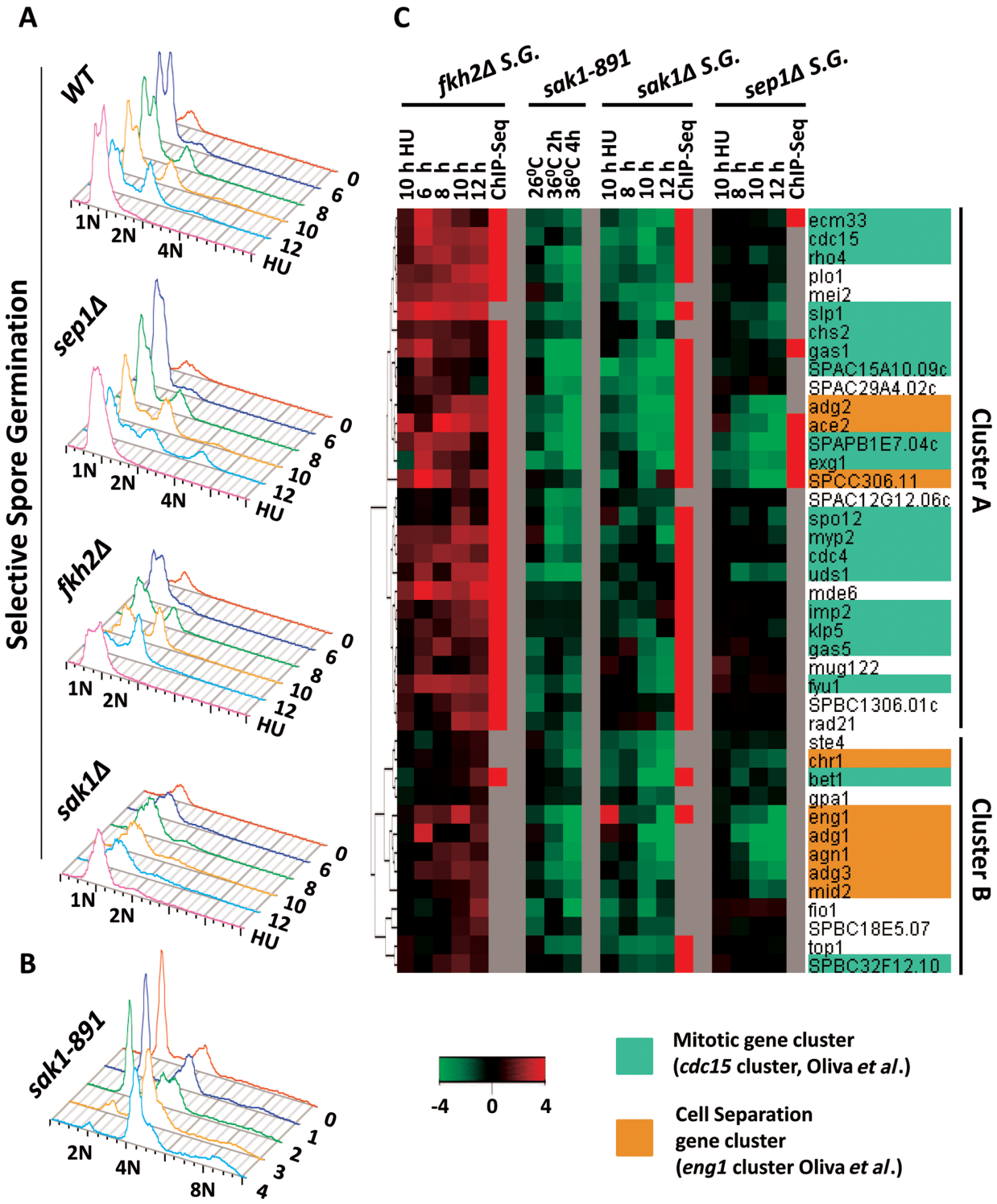


**Figure 4.** Selective spore germination assays for *fkh2Δ, sak1Δ* and *sep1Δ* cells. (**A**) Flow cytometry analysis of DNA content for the selective spore germination of *WT*, *fkh2Δ*, *sak1Δ* and *sep1Δ* mutants. (**B**) Flow cytometry analysis as in Figure 4A for *sak1–891* at restrictive temperature. 0 time: permissive temperature. Figure 4A, B: Y axis, cell number; Z axis, hours. (**C**) Gene expression analysis from the selective spore germination (S.G.) of the *fkh2Δ*, *sak1Δ* and *sep1Δ* deletion mutants and the *sak1–891-ts* mutant. The control for the *fkh2Δ S.G.*, *sak1Δ S.G.* and *sep1Δ S.G.* was the WT S.G. experiment at each of the time points respectively. The control for *sak1–891-ts* strain was a WT (F14) strain kept at that temperature for an equal duration of time. The cluster represents both direct and indirect roles of each transcription factor (whole-genome cluster analysis: Dataset S1). The clustering process is described in the methods section. Each "ChIP-Seq" column represents the results described above of the ChIP-Seq experiments with the relevant transcription factors (Fkh2, Sak1, or Sep1) for each gene in the cluster (e.g., *cdc15* bound to Fkh2 and Sak1 in the ChIP-Seq experiments, but not to Sep1). Every row represents a gene and every column represents a microarray or ChIP-Seq experiment. Red signifies upregulation (Experiment/Control > 1), green signifies downregulation (Experiment/Control < 1). Black signifies no change. Dynamic range is 16-fold from reddest to greenest (key: fold change). HU: Hydroxyurea.

Sep1 binds to the small subset of the Fkh2 targets implied by the Sep1 ChIP-seq data.

The sequences of the upstream regions of the Sep1 ChIP-seq targets were extracted and analyzed using MEME. Again, forkhead and Sak1 motifs were found (Figure 3C). It is perhaps noteworthy that there is little if any difference between the forkhead sequence motifs obtained by Fkh2 ChIP-seq and Sep1 ChIP-seq. One possibility for the different phenotypes of *fkh2* mutants and *sep1* mutants might have been that these two forkhead transcription factors have different binding motifs, but this does not seem to be the case. Other than, perhaps, a slight preference for an 'A' at the first position of the motif (compare Figures 1D and 3C), Sep1 seems to have the same consensus binding site as Fkh2.

Fkh2, Sak1 and Sep1 ChIP-seq profiles for one Sep1 target (*ace2*) and one non-target (*cdc15*) are compared in Figure 3E. In comparison to the genes that are Fkh2-but-not-Sep1 targets (Figure 2A), the Sep1 targets appear to have more Fkh consensus motifs (Figure 2B). The mean number of Fkh motifs for the Fkh2-only genes was 2.4, while the mean number of Fkh motifs for the Sep1 genes was 5.2 ($P < 0.03$).

A noteworthy Sep1 target is *ace2*. Ace2 is the transcription factor responsible for expression of genes needed for cell separation (29,73–75). The main phenotype of a *sep1* mutant is lack of cell separation and in principle this phenotype could be explained by lack of expression of *ace2*. Indeed, phenotypes of the *sep1* mutant, the *ace2* mutant and the *sep1 ace2* double mutant are very similar to each other (35).

### Transcriptional roles: Fkh2 is a repressor, while the related forkhead transcription factor Sep1 is an activator and Sak1 is an activator

To define the individual roles of Fkh2, Sak1 and Sep1, we wished to examine gene expression through the cell cycle in each mutant. *cdc25–22* block-release has traditionally been used to assess cell cycle events in mutants. However, *sak1*Δ mutants are nonviable (64) and *fkh2*Δ mutants are very sick, i.e. they have growth, morphological and cell cycle defects (25–26,34). Further, since Fkh2 binds upstream of the *cdc25* gene (Supplementary Table S1, data not shown) and *fkh2Δ cdc25–22* mutants show synthetic lethality (see below, Figure 5F), an alternative approach to assess gene expression in the *fkh2Δ* mutant was essential. Fortunately, a 'selective spore germination' experiment could be used. In this method, heterozygous diploids are constructed where the gene of interest (here, *fkh2*, *sep1* or *sak1*) is disrupted with *ura4*. After sporulation, spores are incubated in -ura medium and only the *ura4+* cells (i.e. cells bearing the disruption) germinate and grow. Germination is reasonably synchronous (Figure 4A; WT), so by sampling with time, gene expression can be assayed through the cell cycle. Since the transcripts are assessed in the first cell cycle of the mutant, secondary effects due to downstream targets are mitigated. Therefore, we did selective spore germination for heterozygous *sak1Δ*, *fkh2Δ* and *sep1Δ* mutants and measured transcripts by microarrays. In addition, we used a *sak1–891-ts* strain (76) to assess the effects of Sak1 inactivation by an independent method. Finally, we used hydroxyurea to arrest germinating cells in early S-phase to get an independent cell cycle block. By flow cytometry, the germinating WT, *fkh2Δ* and *sep1Δ* spores gave the expected synchronous progression from G1 through S phase to 2N DNA content; *sak1–891* (ts) also arrested at G2 (which, given that these cells started in exponential growth, is in this case a 4N DNA content). Surprisingly, the *sak1*Δ mutants arrested with 1N DNA content in the spore germination experiment; the significance of this G1 arrest is discussed below (Figure 4A and B).

Samples were taken from the synchronously germinating cells (WT, *fkh2Δ*, *sak1Δ* and *sep1Δ*) and from *sak1–891* ts cells shifted to 36°C and gene expression was analyzed using microarrays. There were several clear conclusions. First, deletion of *fkh2* allows early upregulation of all Fkh2 targets (Figure 4C) to roughly the same high levels found at peak expression in WT cells. Upregulation of some of these genes could be seen even in the hydroxyurea arrest (i.e. in S-phase). This supports the notion that Fkh2 is primarily a repressor of these genes.

In contrast to Fkh2, the forkhead transcription factor Sep1 behaves as an activator of a small subset of genes. Its ChIP-seq target genes (*ace2*, *exg1*, *SPCC306.11*, etc.) are strongly downregulated in *sep1*Δ (Figure 4C, cluster A), supporting the validity of the ChIP-seq results. Several other genes are also down-regulated in *sep1*Δ (e.g. Figure 4C, cluster B and *adg2*). However, most or perhaps all of these are activated by the transcription factor Ace2 (29,73–75) and *ace2* is a target of Sep1. Thus, the downregulation of the genes in cluster B can be understood as an indirect effect of downregulation of *ace2*.

Sak1 behaves as a general activator of the mitotic genes. All the Sak1 target genes, as well as the genes in cluster B, are downregulated in the *sak1–891* ts mutant at the G2 arrest at restrictive temperature (Figure 4C). Notably, the Sak1 target genes are downregulated in the ts mutant whether the genes require Sep1 (e.g. *ace2*) or not (e.g. *spo12*). Thus, the Sep1 targets seem to require both Sak1 and Sep1, two activators, for their expression. These results are most consistent with the idea that Sak1 is a transcriptional activator of all these genes.

Finally, the germinating *sak1*Δ cells appeared to arrest in G1 phase. Although growth and germination were relatively poor, forward light scattering and microscopy showed that many cells grew to large sizes, and yet there was no accumulation of 2N cells with time. This suggested an arrest at the G1/S boundary (a 'Start' arrest). One of the Sak1 ChIP-seq targets is *rep2*, a gene needed for the *MluI* Cell Cycle Box Binding Factor (MBF) transcription factor, which promotes 'Start'. Furthermore, all the targets of MBF were downregulated in the germinating *sak1*Δ cells (not shown). On the other hand, these same MBF targets were upregulated in the hydroxyurea-arrested *sak1*Δ cells, suggesting that these cells could activate MBF. Understanding whether the Start arrest is due to a specific transcriptional defect of the *sak1* cells or to a less specific defect in germination and growth will require further experiments.

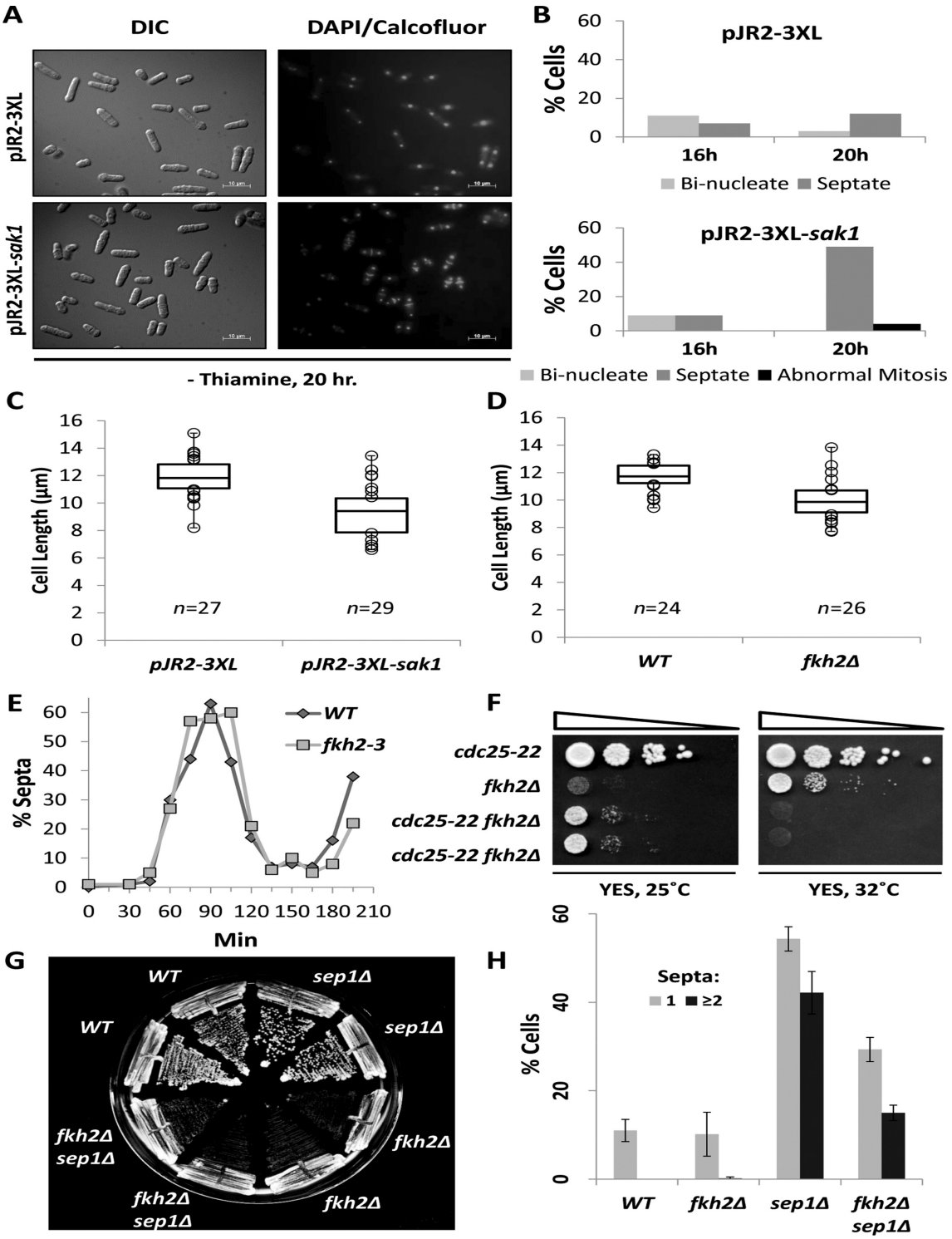


**Figure 5.** Genetic phenotypes and interactions. (**A**) Sak1 overexpression. DIC (Differential Interference Contrast) microscopy and fluorescence images (DAPI/Calcofluor) of WT (JLP988) cells either containing an empty vector (pJR-3XL) or a plasmid overexpressing Sak1 from the *nmt1* promoter (pJR-3XL-*sak1*) under derepressed (-thiamine) conditions at 20 h. Scale bar: 10 μm. (**B**) Percentage of cells from the experiment in Figure 5A showing 2 nuclei (bi-nucleate), 2 nuclei with septa in between (septate) or showing an aberrant phenotype (e.g. multiple septa). Hundred cells were counted for each experiment at the given times. (**C**) The lengths of septated control cells and septated Sak1-overexpressing cells from the experiment of Figure 5A (*t*-test, *P*-value $< 3.51 \times 10^{-7}$). (**D**) The lengths of septated control cells and septated *fkh2Δ* cells at 12 h into the selective spore germination experiment (*t*-test, *P*-value $< 1.5 \times 10^{-5}$). (**E**) *cdc25–22* block release of WT (*cdc25–22*, JLP1679) and *fkh2–3 cdc25–22* (JLP1770) cells. Septation is shown as a function of time. (**F**) Serial dilutions of *cdc25–22* (JLP1635), *fkh2Δ* (JLP1501) and *cdc25–22 fkh2Δ* (JLP1741, JLP1745) were grown on YES plates at the indicated temperatures. (**G**) Growth assays of two biological duplicates each of WT, *fkh2Δ*, *sep1Δ* and *fkh2Δ sep1Δ* mutants obtained from a cross of *fkh2::ura4*$^+$ (JLP1501) and *sep1::LEU2* (JLP1823) on a YES plate. (**H**) Percentage of cells bearing 1 septum, or 2 or more septa, in WT, *fkh2Δ*, *sep1Δ*, or *fkh2Δ sep1Δ* mutants. Two hundred cells from each of three biological replicates grown in liquid YES medium were counted and are represented by the mean ± S.E.M.

### Mitosis is advanced by overexpression of *sak1* or deletion of *fkh2*

If Sak1 is an activator of the mitotic genes, then its over-expression might advance mitosis. To test this, we cloned *sak1* behind the thiamine-repressible *nmt1* promoter and used this construct to over-express *sak1* in transformants. Cells overexpressing *sak1* had a 'Wee' phenotype. They entered mitosis early and cultures contained a large fraction of septated cells (Figure 5A and B). Measurements of cell length showed that cells overexpressing *sak1* formed septa at shorter cell lengths (i.e. earlier) than controls (Figure 5C).

Similarly, cells deleted for *fkh2* formed septa at shorter lengths than control cells in the spore germination experiment (Figure 5D), suggesting that deletion of *fkh2* also advanced mitosis, again consistent with the idea that Fkh2 is a repressor of the mitotic genes. In established cultures of *fkh2*Δ cells, which are very sick, mean cell lengths are longer than in WT ((25) and data not shown). We attribute this to the generally sick state of the cells and also to the fact that the mean length is greatly affected by the longest cells, which may be non-dividing.

If Fkh2 represses the mitotic genes, then overexpression of Fkh2 might delay cells in mitosis. Over-expression of Fkh2 is lethal (Supplementary Figure S2A) (25), with cells accumulating a variety of aberrant mitotic phenotypes (Supplementary Figure S2B).

Fkh2 has an FHA domain, a domain that binds phosphopeptides (77–79). In *S. cerevisiae*, the Fkh2 FHA domain is critical; it binds the phosphorylated co-activator Ndd1 to form the ultimate activator (12,14–15). We wanted to see whether loss of the *S. pombe* Fkh2 FHA domain might reveal some new phenotype of *fkh2*. We constructed allele *fkh2–3*, which bears R100A, S128A and N151A mutations, which are predicted to eliminate phosphopeptide binding (80,81). However, *fkh2–3*, despite the probable loss of a functional FHA domain, had nearly WT growth and in a *cdc25–22* block release experiment, gave a septation profile similar to WT (Figure 5E). (We note, however, that a deletion of the entire FHA domain gave a strong phenotype similar to that of the *fkh2*Δ; this might be due to a general effect on protein folding and function.) In contrast, a *cdc25–22 fkh2*Δ mutant is synthetically lethal at a semi-permissive temperature (32°C) for *cdc25–22* (Figure 5F). It has been shown that Fkh2 interacts with the Clr6 histone deacetylase complex (82). It is possible that the Fkh2 FHA domain contributes to the recruitment of this complex independent of its phospho-peptide binding ability much like the *S. cerevisiae* Fkh2 (11).

In addition, we tested the requirement of Fkh2 for Sak1 binding and found that Sak1 can bind upstream of its targets in the absence of *fkh2* (Supplementary Figure S3A). We also observed that even though Sak1 protein levels remain unchanged during the cell cycle (Supplementary Figure S3B), it is predominantly recruited to its target gene promoters during M phase (Supplementary Figure S3C and D). We also tried to check the genetic interactions between *fkh2* and *sak1* by generating an *fkh2Δ sak1Δ* mutant. These mutants germinated very poorly with 5 microcolonies/million spores observed. These 'surviving' microcolonies had very long and branched cells and failed to grow (data not shown). This suggests that deletion of *fkh2* is not sufficient to suppress the lethality of *sak1Δ* and that Sak1 plays an active part in the activation of the mitotic genes.

### Genetic interactions between *fkh2* and *sep1*

If Sep1 were a general activator of the mitotic genes, it ought to have a severe growth defect. However, the *sep1*Δ grows almost as well as WT (Figure 5G), except that it fails to separate daughter cells after mitosis, like an *ace2* mutant (35). Furthermore, if Sep1 were a general activator, then loss of this activator might be suppressed by loss of the opposing repressor, Fkh2. On the contrary, the double mutant is about as sick as *fkh2*Δ (Figure 5G). However, deletion of *fkh2* does partly suppress the septation defect of *sep1Δ* (Figure 5H). This is consistent with the idea that Fkh2 and Sep1 oppose each other specifically for septation. Expression of *ace2* in these cells lacking both the Sep1 activator and the Fkh2 repressor could now be due to activation from Sak1.

## DISCUSSION

Sak1, an RFX transcription factor, appears to be a new, central factor activating transcription of the mitotic genes of *S. pombe*. Evidence for this is that: ChIP-seq finds Sak1 at 45 Fkh2-regulated mitotic genes; motif searches find an RFX-consensus motif in front of the mitotic genes with a very small *E*-value ($10^{-51}$); *sak1* is an essential gene; the expression of the mitotic genes is strongly downregulated at restrictive temperature in a *sak1-ts* mutant; and overexpression of *sak1* advances mitosis.

Forkhead transcription factors are involved in mitotic gene control from yeasts to humans (5,18). The mechanisms of this control are partly understood in *S. cerevisiae*, but not elsewhere. In mammals, targeting of the forkhead transcription factors to mitotic genes involves protein–protein interactions with other sequence specific DNA binding proteins, including the DREAM complex, Myb and NF-Y (17,22–23). Interestingly, the NF-Y motif is, in other contexts, associated with regulation via RFX transcription factors (83–86).

The RFX transcription factors are an ancient, well-conserved family (63). Structurally, like the forkhead transcription factors, they have a 'winged helix', a variation of the helix-turn-helix, but with a unique method of sequence-specific DNA recognition involving the 'wings' and the major groove (87,88). RFX transcription factors have been found collaborating with forkhead transcription factors in the development of cilia in *Drosophila* (89,90), for cell separation, and possibly other mitotic processes, in *Acremonium chrysogenum* and *Penicillium marneffei* (91,92), and, recently, in front of many mouse genes, including the homeobox transcription factor Cdx2 (93). In *S. cerevisiae*, the RFX homolog *RFX1* is involved in response to DNA damage (94). DNA damage in G2 typically causes a cell cycle arrest in G2; in *S. cerevisiae*, this is implemented, in part, via Ndd1 (95,96), the forkhead-associated co-activator of mitotic gene expression. Thus, Sak1 would be well-placed to carry out an analogous role in preventing mitotic gene expression in response to DNA damage. Indeed, inspection

of the protein sequence shows that Sak1 has nine consensus sites for phosphorylation by the Cds1 damage-responsive checkpoint protein kinase.

In *S. pombe*, it has been thought for some time that mitotic gene expression involves the two forkhead factors Fkh2 and Sep1, with Fkh2 thought to be a general repressor (25,26,30) and Sep1 thought to be a general activator (25,30,33). However, a general role for Sep1 in activation is difficult to reconcile with the weak phenotype of *sep1*Δ and furthermore it has been unclear how the switch from repression to activation and back again would occur. Here, we resolve at least one of these issues by showing that Sak1, not Sep1, is the most likely general activator. But several acute issues remain. First, are there still additional proteins involved? In view of the number of proteins used for mitotic gene control in *S. cerevisiae* and mammals, it would not be surprising if there are additional components of the *S. pombe* system.

Second, why do the genes targeted by Sep1 apparently need two activators, Sep1 and Sak1? These genes are downregulated when either of the two activators is missing (Figure 4). Possibly Sak1 alone is capable of partial activation of these Sep1 genes when Fkh2 is missing (Figure 5H).

Third, does Fkh2, though on its own a repressor, play some role in the formation or assembly of the ultimate activator? The forkhead transcription factors can effectively compete with nucleosomes to find their binding sites and therefore can act as 'pioneer' factors to displace nucleosomes from regulatory regions (97,98). It is possible that Fkh2 is acting as a pioneer factor to aid the eventual assembly of an activating transcription complex. Our results show that gene activation can occur in the *fkh2* deletion, but we cannot be certain that the levels of mitotic expression achieved in the *fkh2* deletion are as high or as sharp as in the WT.

Fourth and most important, how is the transition from repression to activation achieved and how is this regulated by CDK activity? Sak1 is predominantly recruited to its target gene promoters during M phase (Supplementary Figure S3C and D) and Fkh2 is apparently phosphorylated by CDK (25–26,99). One very simple model is that this phosphorylation reduces the affinity of Fkh2 for DNA (Alves-Rodrigues *et al.*, personal communication), allowing it to fall off the DNA (30). This could unmask the Sak1 motif allowing Sak1 to get recruited during mitosis and also allow the Sep1 activator to replace Fkh2 at some sites. An alternative model is the cooperative binding of Sak1 with phosphorylated Fkh2 which would allow activation. Even though Sak1 can bind in the absence of *fkh2* (Supplementary Figure S3A), we cannot be certain whether the strength of binding is equivalent to WT.

McInerny *etal*. have suggested the involvement of Mbx1, Mid1, Plo1 polo kinase and Clp1 phosphatase in the control of mitotic gene expression (25,30,60,100–101). It seems that Mid1 and Mbx1 act as adaptors for the efficient recruitment of Plo1 and subsequently Clp1 (in late M phase). Thus, it may be that when Sak1 binds upstream of its targets at M phase it is phosphorylated by Plo1 and this event activates transcription. The transcription may then subsequently be repressed by de-phosphorylation of Sak1 by Clp1.

In summary, in this study we have delineated the roles of the major transcription factors involved in the mitotic transcriptional circuitry in *S. pombe* and have discovered the role of Sak1 as the main activator for mitosis.

## ACCESSION NUMBERS

All high-throughput sequencing and microarray data are available at NCBI Gene Expression Omnibus. Accession numbers—All data: GSE60719; Sub-series: GSE60712 (ChIP-seq), GSE60718 (microarray).

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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