In summary, we have shown that GSK-3β function is required for the NF-κB-mediated anti-apoptotic response to TNF-α. Our data also show that GSK-3α and -β have distinct biological roles, as the former is unable to compensate for the loss of the latter. □

Methods

Cytoplasmic and nuclear lysates were prepared as described[16]. Immunoblotting was carried out with I-κB-α (polyclonal, New England Biolabs), p65 NF-κB (polyclonal, Santa Cruz Biotechnology) and GSK-3 (mouse monoclonal, Upstate Biotechnology) antibodies.

Apoptosis assays

Where indicated, TNF-α-treated cells (10 ng ml^{-1} h) were collected, mixed with 4 μg ml^{-1} acridine orange (final concentration) and assessed by fluorescence microscopy. Cell survival following TNF-α treatment was determined by negative staining with trypan blue and expressed normalized to untreated controls. All experiments were repeated at least three times, and the data are shown as the mean ± standard error. *In situ* apoptosis was detected by TUNEL assay according to the manufacturer's instructions (Boehringer Mannheim), or by another fragmented DNA end-labelling protocol[30]. For β-galactosidase viability assays, two days after transfection with pCMV-β-galactosidase plus either pCDNA3-HA-GSK-3β or control plasmid, cells were treated as indicated and analysed.

EMSA

The κB-binding activities of embryonic fibroblasts incubated with lithium or potassium (30 mM, 4 h) and murine TNF-α (100 ng ml^{-1}, 30 min), as indicated, were compared by EMSA. Nuclear lysates were prepared and EMSAs were performed as described[17]. For oligonucleotide competition assays, equivalent amounts of nuclear extract protein (3 μg) were preincubated for 5 min with a 200-fold excess of either NF-κB-specific oligonucleotide probe containing two tandem NF-κB-binding sites (5′-ATCAGGGACTTTCCGC TGGGGACTTTCCG-3′ and 5′-CGGAAAGTCCCCAGCGGAAAGTCCCTGAT-3′) or mutant NF-κB oligonucleotides (5′-GATCACTCACTTTCCGCTTGCTCACTTTCCAG-3′ and 5′-CTGGAAAGTGAGCAAGCGCAAAGTGAGTGATC-3′) before addition of the radiolabelled NF-κB using the oligonucleotides 5′-TTCTAGTGATTTGCATTCGACA-3′ and 5′-TGTCGAATGCAAATCACTAGAA-3′.

Luciferase assay

Embryonic fibroblast cells were transfected with plasmids expressing ELAM-luciferase and β-galactosidase. Transfected cells were incubated in the presence of 30 ng ml^{-1} TNF-α or IL-1β for 6 h. Luciferase assays were carried out using the Promega assay kit and a Berthold luminometer. Activity was normalized to β-galactosidase activity and plotted as the mean ± standard deviation of triplicates from a representative experiment. To examine the effect of lithium treatment on NF-κB-mediated gene transcription, HEK293 epithelial cells were preincubated overnight with 30 mM lithium or potassium before being stimulated with 10 or 20 ng ml^{-1} TNF-α. Luciferase values were normalized to the unstimulated potassium and lithium controls.

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1. Welsh, G. I., Wilson, C. & Proud, C. G. GSK-3: a SHAGGY frog story. *Trends Cell Biol.* **6,** 274–279 (1996).
2. Dale, T. C. Signal transduction by the Wnt family of ligands. *Biochem. J.* **239,** 209–223 (1998).
3. Siegfried, E., Perkins, L. A., Capaci, T. M. & Perrimon, N. Putative protein kinase product of the *Drosophila* segment-polarity gene zeste-white3. *Nature* **345,** 825–829 (1990).
4. Ruel, L., Bourouis, M., Heitzler, P., Pantesco, V. & Simpson, P. *Drosophila* shaggy kinase and rat glycogen synthase kinase-3 have conserved activities and act downstream of Notch. *Nature* **362,** 557–560 (1993).
5. Harwood, A. J., Plyte, S. E., Woodgett, J., Strutt, H. & Kay, R. R. Glycogen synthase kinase 3 regulates cell fate in *Dictyostelium*. *Cell* **80,** 139–148 (1995).
6. Puziss, J. W., Hardy, T. A., Johnson, R. B., Roach, P. J. & Hieter, P. MDS1, a dosage suppressor of an mck1 mutant, encodes a putative yeast homolog of glycogen synthase kinase 3. *Mol. Cell. Biol.* **14,** 831–839 (1994).
7. Plyte, S. E., Feoktistova, A., Burke, J. D., Woodgett, J. R. & Gould, K. L. *Schizosaccharomyces pombe* skp1+ encodes a protein kinase related to mammalian glycogen synthase kinase 3 and complements a cdc14 cytokinesis mutant. *Mol. Cell. Biol.* **16,** 179–191 (1996).
8. Klein, P. S. & Melton, D. A. A molecular mechanism for the effect of lithium on development. *Proc. Natl Acad. Sci. USA* **93,** 8455–8459 (1996).
9. Stambolic, V., Ruel, L. & Woodgett, J. R. Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. *Curr. Biol.* **6,** 1664–1668 (1996).
10. He, X., Saint-Jeannet, J. P., Woodgett, J. R., Varmus, H. E. & Dawid, I. B. Glycogen synthase kinase-3 and dorsoventral patterning in *Xenopus* embryos. *Nature* **374,** 617–622 (1995).
11. Dominguez, I., Itoh, K. & Sokol, S. Y. Role of glycogen synthase kinase 3 beta as a negative regulator of dorsoventral axis formation in *Xenopus* embryos. *Proc. Natl Acad. Sci. USA* **92,** 8498–8502 (1995).
12. Nasevicius, A. *et al.* Evidence for a frizzled-mediated wnt pathway required for zebrafish dorsal mesoderm formation. *Development* **125,** 4293–4992 (1998).
13. Li, Q., Van Antwerp, D., Mercurio, F., Lee, K. F. & Verma, I. M. Severe liver degeneration in mice lacking the IκB kinase 2 gene. *Science* **284,** 321–325 (1999).
14. Li, Z. W. *et al.* The IKKβ subunit of IκB kinase (IKK) is essential for nuclear factor κB activation and prevention of apoptosis. *J. Exp. Med.* **189,** 1839–1845 (1999).
15. Beg, A. A., Sha, W. C., Bronson, R. T., Ghosh, S. & Baltimore, D. Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-κB. *Nature* **376,** 167–170 (1995).
16. Yeh, W. C. *et al.* Early lethality, functional NF-κB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. *Immunity* **7,** 715–725 (1997).
17. Beyaert, R., Vanhaesebroeck, B., Suffys, P., Van Roy, F. & Fiers, W. Lithium chloride potentiates tumor necrosis factor-mediated cytotoxicity *in vitro* and *in vivo*. *Proc. Natl Acad. Sci. USA* **86,** 9494–9498 (1989).
18. Beg, A. A. & Baltimore, D. An essential role for NF-κB in preventing TNF-α-induced cell death. *Science* **274,** 782–784 (1996).
19. Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R. & Verma, I. M. Suppression of TNF-α-induced apoptosis by NF-κB. *Science* **274,** 787–789 (1996).
20. Wang, C. Y., Mayo, M. W. & Baldwin, A. S. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB. *Science* **274,** 784–787 (1996).
21. Liu, Z. G., Hsu, H., Goeddel, D. V. & Karin, M. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. *Cell* **87,** 565–576 (1996).
22. Baueuerle, P. A. & Baltimore, D. IκB: a specific inhibitor of the NF-κB transcription factor. *Science* **242,** 540–546 (1988).
23. Oliver, F. J. *et al.* Resistance to endotoxic shock as a consequence of defective NF-κB activation in poly (ADP-ribose) polymerase-1 deficient mice. *EMBO J.* **18,** 4446–4454 (1999).
24. Beraud, C., Henzel, W. J. & Baeuerle, P. A. Involvement of regulatory and catalytic subunits of phosphoinositide 3-kinase in NF-κB activation. *Proc. Natl Acad. Sci. USA* **96,** 429–434 (1999).
25. Kane, L. P., Shapiro, V. S., Stokoe, E. & Weiss, A. Induction of NF-κB by the Akt/PKB kinase. *Curr. Biol.* **9,** 601–604 (1999).
26. Romashkova, J. A. & Makarov, S. S. NF-κB is a target of AKT in anti-apoptotic PDGF signalling. *Nature* **401,** 86–90 (1999).
27. Ozes, O. N. *et al.* NF-κB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* **401,** 82–85 (1999).
28. Sizemore, N., Leung, S. & Stark, G. R. Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-κB p65/RelA subunit. *Mol. Cell. Biol.* **19,** 4798–4805 (1999).
29. Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M. & Hemmings, B. A. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378,** 785–789 (1995).
30. Wijsman, J. H. *et al.* A new method to detect apoptosis in paraffin sections: *in situ* end-labeling of fragmented DNA. *J. Histochem. Cytochem.* **41,** 7–12 (1993).

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Two yeast forkhead genes regulate the cell cycle and pseudohyphal growth

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There are about 800 genes in *Saccharomyces cerevisiae* whose transcription is cell-cycle regulated[1,2]. Some of these form clusters of co-regulated genes[1]. The 'CLB2' cluster contains 33 genes whose transcription peaks early in mitosis, including *CLB1*, *CLB2*, *SWI5*, *ACE2*, *CDC5*, *CDC20* and other genes important for mitosis[1]. Here we find that the genes in this cluster lose their cell cycle regulation in a mutant that lacks two forkhead transcription factors, Fkh1 and Fkh2. Fkh2 protein is associated with the promoters of *CLB2*,

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***SWI5* and other genes of the cluster. These results indicate that Fkh proteins are transcription factors for the CLB2 cluster. The *fkh1 fkh2* mutant also displays aberrant regulation of the 'SIC1' cluster[1], whose member genes are expressed in the M–G1 interval and are involved in mitotic exit. This aberrant regulation may be due to aberrant expression of the transcription factors Swi5 and Ace2, which are members of the CLB2 cluster and controllers of the SIC1 cluster. Thus, a cascade of transcription factors operates late in the cell cycle. Finally, the *fkh1 fkh2* mutant displays a constitutive pseudohyphal morphology, indicating that Fkh1 and Fkh2 may help control the switch to this mode of growth.**

We determined the binding site for Fkh1 protein (Fig. 1). This site was similar to a motif found in front of the genes of the CLB2 cluster[1] (Fig. 1). This motif is the binding site for a transcription factor called 'SFF' (SWI five factor)[3–5], whose components have not been identified. Furthermore, transcription of *FKH1* and *FKH2* is regulated according to the cell cycle, with peak transcription during S phase[1], consistent with the idea that Fkh1 and Fkh2 might be involved in cell-cycle regulation.

To see whether Fkh1 and Fkh2 regulate genes of the CLB2 cluster, we constructed *fkh1* and *fkh2* single and double mutants. Neither single mutant had an obvious phenotype, but the double mutant had unusual cell morphology (see below). We examined expression of cell-cycle regulated genes in the *fkh1 fkh2* mutant. *CLN2*, whose expression is independent of SFF, displayed its normal late-G1 peak in these cells (Fig. 2). However, *SWI5*, an SFF-dependent gene[4] and a member of the CLB2 cluster, failed to oscillate, but instead was constitutively expressed in moderate amounts (Figs 2 and 3). Interestingly, during α-factor arrest, *SWI5* was expressed in the *fkh1 fkh2* mutant but not in wild-type cells (not shown), indicating that Fkh1 and Fkh2 can repress as well as activate transcription.

We used microarrays for a more comprehensive analysis (Fig. 3). To examine cell cycle regulation, we compared synchronous Δ*fkh1* Δ*fkh2* cells to asynchronous Δ*fkh1* Δ*fkh2* cells. Although most genes were regulated normally in the *fkh1 fkh2* mutant after release from an α-factor block, the genes of the CLB2 cluster were an exception, and largely lost their cell cycle regulation. Of the 33 genes in the CLB2 cluster, 20 showed little or no oscillation in the *fkh1 fkh2* mutant (*ACE2*, *ALK1*, *BUD3*, *BUD4*, *CDC5*, *CLB1*, *CLB2*, *HST3*, *KIP2*, *IQG1*, *MOB1*, *MYO1*, *SWI5*, *YCL012w*, *YIL158w*, *YLR190w*, *YML033w*, *YML034w*, *YNL058c*, *YPL141c*), though they clearly oscillated in the wild type. Seven genes (*APC1*, *BUD8*, *NUM1*, *TEM1*, *YCL063w*, *YLR057w* and *YLR084c*) had little or no oscillation in the *fkh1 fkh2* mutant, but also had less than 2.5-fold oscillation in wild-type cells after release from an α-factor block, so their regulation by Fkh1 and Fkh2 is difficult to ascertain. The remaining six genes (*CDC20*, *CHS2*, *HOF1*, *YJL051w*, *YML119w* and *YPR156c*) retained a residual oscillation in the *fkh1 fkh2* mutant. Although the *fkh1 fkh2* mutations eliminated oscillation of the transcripts of the CLB2 cluster, moderate, constitutive expression remained for each gene (Fig. 3). Consistent with this, moderate, constitutive *CLB2* expression is seen when the SFF sites are removed from the *CLB2* promoter[5,6]. The constitutive expression of the genes in the CLB2 cluster explains why the *fkh1 fkh2* mutant is viable.

Misregulation of genes in the CLB2 cluster might result in secondary effects. In particular, the CLB2 cluster encodes the cell cycle transcription factors Swi5 and Ace2. These related factors[7,8] are responsible for the M–G1 phase transcription of genes in the downstream 'SIC1' cluster[1,8–10]. Indeed, genes in the SIC1 cluster were also misregulated in the *fkh1 fkh2* mutant (Fig. 3). Some genes had reduced expression (for example, *EGT2*, *CTS1*, *PCL9*); some genes had reduced oscillation (for example, *SIC1*, *YDL117w* and *PRY3*); and some genes had alterations in both time and amount of expression (for example, *YGL028c*). In the mutant, *SIC1* was expressed at the α-factor block, perhaps because *SWI5* is now expressed at the α-factor block. The diversity of responses may reflect different degrees of dependence on the amount of Swi5/Ace2.

Outside the CLB2 and SIC2 clusters, there were only a few genes whose regulation during the cell cycle was affected by the *fkh1 fkh2* mutation (for example, *BUD9*, *CLN3*, *YMR215w*, *KIN3* and *YOR315w)*. Several genes had an overall expression that increased (for example, *YGP1*) or decreased (for example, *TAO3*, *YGL028c*, *YHR143w*, *SPS4*, *SUN4*) in asynchronous *fkh1 fkh2* cells compared with wild-type cells (that is, these quantitative changes did not necessarily involve altered cell-cycle periodicity). Some of the downregulated genes are involved in cell-wall metabolism or cell separation, and this may help explain the cell separation defect of *fkh1 fkh2* cells (see below). The genes showing the largest quantitative effects in asynchronous cells did not include any genes from the CLB2 cluster (see Supplementary Information and our web site: genome-www.stanford.edu/fkh), suggesting that these quantitative effects were indirect.

To distinguish direct and indirect effects, and show that Fkh proteins regulate the CLB2 cluster directly, we performed formaldehyde crosslinking immunoprecipitation[11]. After immunoprecipitation of Fkh2 and associated chromatin, polymerase chain reaction (PCR) was used to test for various DNA fragments. Promoter fragments containing the SFF motifs from four genes of the CLB2 cluster (*SWI5*, *CLB2*, *YJL051w* and *HST3*) were assayed. All four were specifically present in the Fkh2 immunoprecipitate (Fig. 4a–c).

A	**6**	0	**19**	**19**	**19**	0	**19**	**12**	**12**	
T	0	**15**	0	0	0	1	0	**5**	**7**	
C	1	4	0	0	0	**17**	0	2	0	
G	**12**	0	0	0	0	1	0	0	0	
	R	Y	A	A	A	C	A	W	W	Consensus FKH1
	G	T	A	A	A	Y	A	A		Published SFF
	R	W	A	A	A	Y	A	W		SFF/Fkh site

Figure 1 The Fkh1 binding site. The Fkh1 site was determined using a GST–Fkh1 fusion and a modified oligonucleotide selection and amplification binding protocol (SAAB)[25]. Nineteen oligonucleotides from the fifth cycle of SAAB were sequenced. The number of occurrences of each nucleotide at each position is shown. Published SFF is from ref. 1. SFF/FKH site is our best current estimate of the site. R, A or G; Y, T or C; W, A or T.

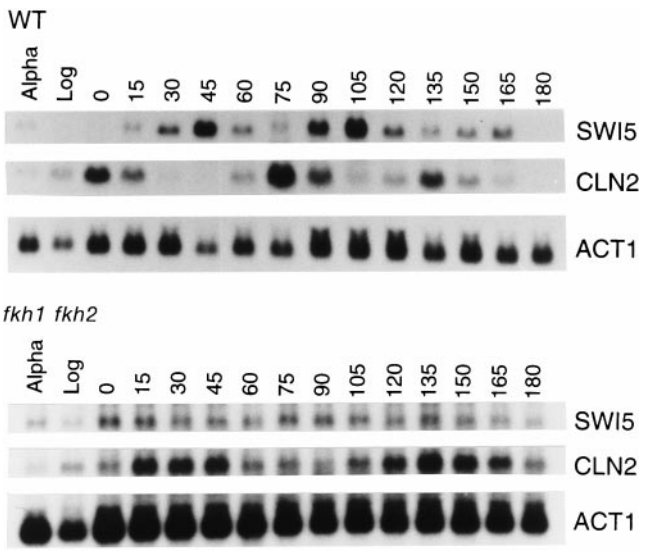

Figure 2 Regulation of *SWI5* during the cell cycle is lost in an *fkh1 fkh2* mutant. Wild-type (WT) or mutant cells were synchronized in G1 using α-factor, then released. *SWI5*, *CLN2*, and *ACT1* (loading control) messenger RNAs were assayed using northern blots. Time after release is shown (0—180 min). Alpha, α-factor arrested cells; Log, asynchronous cells.

In addition, we examined full-length promoters from three genes of the SIC1 cluster, *EGT2*, *SIC1* and *PCL9*. No fragment from the intergenic region upstream of these genes was specifically present in the immunoprecipitate (Fig. 4c–g, and data not shown for *PCL9*). Thus, Fkh2 directly regulates *SWI5*, *CLB2*, *YJL051w* and *HST3*, but only indirectly regulates *EGT2*, *SIC1* and *PCL9*.

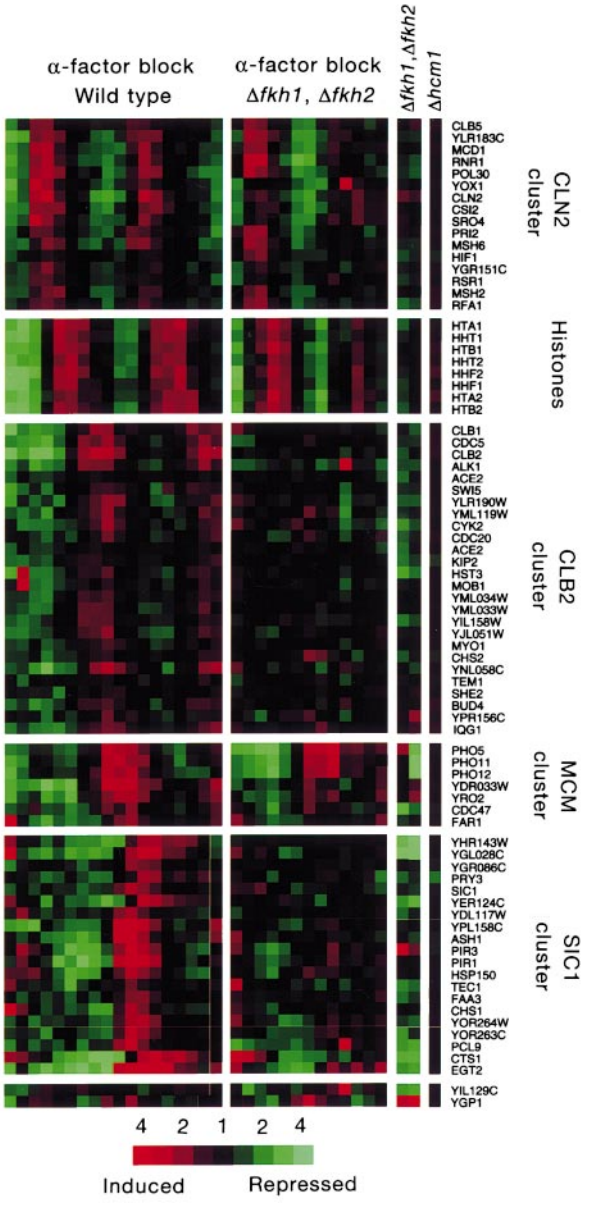

Figure 3 Microarray analysis of *fkh1 fkh2* mutants. Wild-type (left column) or isogenic *fkh1 fkh2* cells (GZ45-17a) (second column) were synchronized with α-factor, released, and sampled (left to right) through two cell cycles. Relative mRNA abundance was analysed by competitive microarray hybridization[1]. Red, gene induction compared to asynchronous cells; green, repression; dynamic range is 16-fold. Representative genes from five clusters are shown. For α-factor experiments, synchronous wild-type cells were compared to asynchronous wild-type cells, and synchronous mutant cells were compared to asynchronous mutant cells, thus showing the effect of the mutations on oscillations in gene expression over the cell cycle. The '*Δfkh1, Δfkh2*' column compares asynchronous *fkh1 fkh2* cells to asynchronous wild-type cells, and shows the effect of the mutations on overall expression. The '*Δhcm1*' column compares asynchronous *hcm1* (a third forkhead-related gene) mutant cells to asynchronous wild-type cells. The bottom row shows the most upregulated gene in *fkh1 fkh2* mutants, *YGP1* (up 12-fold), and the most downregulated gene, *YIL129C* (down 30-fold). See Supplementary Information and our Web site (genome-www.stanford.edu/fkh) for complete data.

To summarize, almost all of the genes in the CLB2 cluster mostly or completely cease to oscillate in the *fkh1 fkh2* mutant, though they continue to be expressed. Many genes in the SIC1 cluster lose their normal regulation qualitatively and/or quantitatively, presumably because the transcription factors controlling them are encoded in the CLB2 cluster. Relatively few other genes are affected. We conclude that Fkh1 and Fkh2 are responsible for the regulation of the CLB2 cluster and probably encode components of SFF, because the Fkh1 binding site matches the SFF site found in the promoters of the CLB2 cluster, because SFF-regulated genes fail to oscillate in the *fkh1 fkh2* mutant, and because Fkh2 is actually present at four of these promoters. Our best estimate of the site consensus is RWAAAYAW. The slight residual periodic expression of some CLB2 cluster genes in the *fkh1 fkh2* mutant may be due to two related forkhead transcription factors, Hcm1 and Fhl1 (ref. 12).

The *fkh1 fkh2* mutants had striking morphological phenotypes:

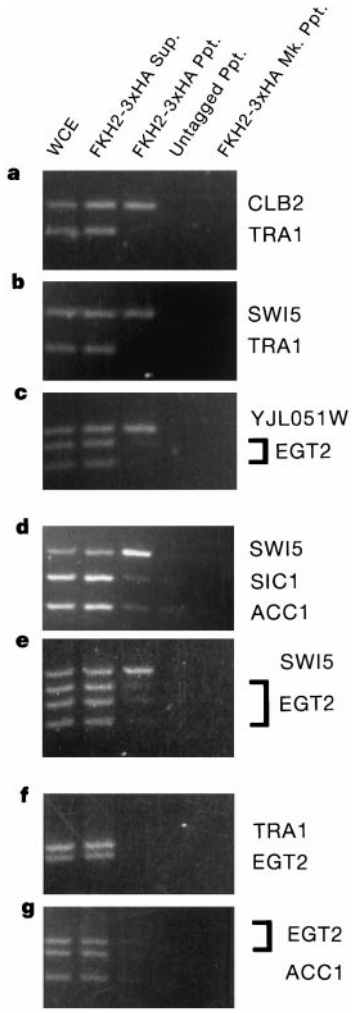

Figure 4 Fkh2 is at the promoters of *SWI5*, *CLB2* and *YJL051w*, but not at *EGT2* or *SIC1*. PCR fragments amplified from anti-Fkh2-3xHA immunoprecipitation or other chromatin fractions are shown after gel electrophoresis and ethidium bromide staining. WCE, whole cell extract; FKH2–3×HA Sup, supernatant from the immunoprecipitation of the haemagglutinin (HA)-tagged strain; FKH2–3×HA Ppt., material eluted from the immunoprecipitation of the HA-tagged strain; Untagged Ppt., material eluted from the immunoprecipitation of the untagged control strain; FKH2–3×HA Mk. Ppt., material eluted from the mock immunoprecipitation (no antibody) of the HA-tagged strain; CLB2, SWI5 and YJL051w, fragments from the promoters of CLB2 cluster genes; TRA1 and ACC1, negative control fragments; EGT2 (three different fragments) and SIC1, fragments encompassing the complete upstream regions of *EGT2* and *SIC1* genes of the SIC1 cluster.

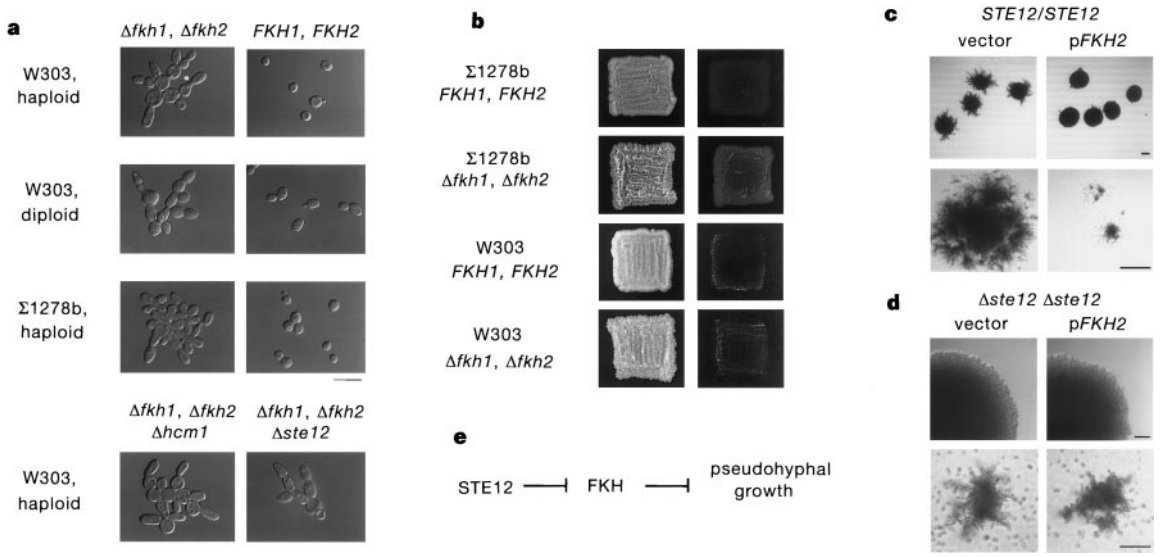

Figure 5 Phenotype of Δ*fkh1* Δ*fkh2* cells. **a**, Morphology. Yeast strains were grown in rich medium to mid-log phase, concentrated by centrifugation, sonicated and photographed. Scale bar: 10 μm. **b**, Invasiveness. Cells were patched onto rich medium and grown for two days (Σ1278b background) or three days (W303 background) at 30 °C. The first column shows the patches before washing, the second column shows the patches after washing with a stream of water. **c**, Extra copies of *FKH2* suppress formation of filaments and invasion into the agar. A diploid strain from the Σ1278b background (L5366) was transformed with a multicopy control plasmid (pGF29) or a multicopy plasmid containing *FKH2* (pGF53), and grown on SLAD plates[26] at 30 °C. Top, colonies before washing; bottom, colonies after washing. Scale bars: 50 μm. **d**, As in **c** except the diploid from the Σ1278b background carried a homozygous deletion Δ*ste12*/Δ*ste12*.

mother and daughter cells remained attached; mother and daughter cells budded synchronously (by time-lapse photography, not shown); cells were elongated (Fig. 5a); and cells were somewhat invasive on agar plates (Fig. 5b). These phenotypes occurred in W303 haploids and diploids in nutrient-rich solid or liquid media. The morphology is characteristic of pseudohyphal growth, which usually occurs after nitrogen starvation on solid medium and allows yeast to forage more efficiently[13–15]. However, pseudohyphal growth does not usually occur in strain W303. The phenotypes were not seen in *fkh1 hcm1* or *fkh2 hcm1* mutants, nor were they intensified in a *fkh1 fkh2 hcm1* triple mutant (Fig. 5a). It is consistent with our findings that *clb2* mutants themselves are weakly pseudohyphal[16], as transcription of the CLB2 cluster is activated by Clb2/Cdc28 kinase activity[17]. A *Schizosaccharomyces pombe* mutant defective in a forkhead transcription factor, *sep1*, also has a cell separation defect[18].

Unlike strain W303, strain Σ1278b undertakes robust pseudohyphal growth when starved for nitrogen on solid media[13]. The *fkh1 fkh2* mutations allowed pseudohyphal and invasive growth of Σ1278b even in rich media (Fig. 5a, b).

It is possible that the *fkh* mutants are only mimicking pseudohyphal growth (that is, they may be pseudo-pseudohyphal). However, overexpression of *FKH2* suppressed the normal ability of Σ1278b-related cells to become pseudohyphal upon nitrogen starvation (Fig. 5c), indicating that Fkh2 may be a part of the normal pathway for this adaptation.

Deletion of *STE12* dramatically reduces (but does not abolish) normal pseudohyphal growth[14]. In contrast, a *ste12 fkh1 fkh2* triple mutant is pseudohyphal, like the *fkh1 fkh2* double mutant (Fig. 5a). Thus, the Fkh proteins either act downstream of Ste12, or in a parallel pathway. However, overexpression of *FKH2* did not reduce the residual capacity of an *ste12* mutant for pseudohyphal growth (Fig. 5d), arguing that the Fkh proteins are not in a parallel pathway. In summary, Ste12 may control pseudohyphal growth in part by repressing Fkh expression or activity, which in turn may repress some aspects of pseudohyphal growth (Fig. 5e).

The pseudohyphal phenotypes can be explained in terms of genes in the CLB2 and SIC1 clusters. The CLB2 cluster contains *BUD3*, *4* and *8*, which affect budding pattern. The synchronous budding and elongated cells might be caused by a delay in mitosis, and many genes important for mitosis are in the CLB2 and SIC1 clusters. Finally, the lack of mother–daughter separation could be due to the decreased expression of the cell separation genes *EGT2* (ref. 9) and *CTS1* (ref. 19) of the SIC1 cluster, and perhaps also to the decreased expression of *YIL129c*, *YGL028c*, *YHR143w*, *SPS4*, *PRY3* and *SUN4*.

FKH1 and *FKH2* encode proteins homologous to the forkhead transcription factor of *Drosophila*[20]. This family of transcription factors is highly conserved, with at least 20 homologues in humans[21,22]. Here, we have shown that a pair of forkhead transcription factors is responsible for the M-phase transcription of a cluster of genes. As transcription of vertebrate B-type cyclins is also regulated during the cell cycle[23,24], it will be interesting to see whether any aspects of the regulatory system have been conserved. Our study of the *FKH* genes also helps understanding of the yeast cell cycle—the Fkh transcription factors are expressed in S phase to help to induce a set of genes in mitosis; this mitotic cluster includes the Swi5 and Ace2 transcription factors which then induce yet another cluster of genes during the M–G1 interval.

In developmental processes controlled by forkhead transcription factors in other species, it is not clear what the ultimate target genes may be; here, we have identified a number of direct and indirect targets. Similar microarray studies in other organisms may define the final target genes of other developmental pathways. □

Methods

Determination of the FKH1 binding site

The FKH1 site was determined using a modified selection and amplification binding protocol (SAAB)[25]. See Supplementary Information and our Web site (genome-www.stanford.edu/fkh).

Analysis of gene expression

Strain W303a (*MATa ade2 leu2 his3 trp1 ura3 ssd1-d can1-100*) and isogenic GZY45-17a *MATa bar1 fkh1 fkh2* cells were synchronized using α-factor. Samples were taken every 15 min after release from α-factor. RNA was analysed by northern blotting using probes for

SWI5, *CLN2* and *ACT1*. Microarray analysis was as described[1]. Microarray data for α-factor block-release of wild-type cells is from ref. 1. Strain GZY45-17a showed twofold increases in expression for many genes on chromosome 16, indicating that this strain could be disomic.

Crosslinking chromatin immunoprecipitations

Chromatin immunoprecipitations were carried out as described[11] with minor modifications. Exact methods and oligos are described in Supplementary Information and at our Web site (genome-www.stanford.edu/fkh).

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1. Spellman, P. T. *et al.* Comprehensive identification of cell cycle-regulated genes of the yeast *Saccharomyces cerevisiae* by microarray hybridization. *Mol. Biol. Cell* **9,** 3273–3297 (1998).
2. Cho, R. J. *et al.* A genome-wide transcriptional analysis of the mitotic cell cycle. *Mol. Cell* **2,** 65–73 (1998).
3. Althoefer, H., Schleiffer, A., Wassmann, K., Nordheim, A. & Ammerer, G. Mcm1 is required to coordinate G2-specific transcription in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **15,** 5917–5928 (1995).
4. Lydall, D., Ammerer, G. & Nasmyth, K. A new role for MCM1 in yeast: cell cycle regulation of SWI5 transcription. *Genes Dev.* **5,** 2405–2419 (1991).
5. Maher, M., Cong, F., Kindelberger, D., Nasmyth, K. & Dalton, S. Cell cycle-regulated transcription of the CLB2 gene is dependent on Mcm1 and a ternary complex factor. *Mol. Cell. Biol.* **15,** 3129–3137 (1995).
6. Seufert, W., Futcher, B. & Jentsch, S. Role of a ubiquitin-conjugating enzyme in degradation of S- and M-phase cyclins. *Nature* **373,** 78–81 (1995).
7. Moll, T., Tebb, G., Surana, U., Robitsch, H. & Nasmyth, K. The role of phosphorylation and the CDC28 protein kinase in cell cycle- regulated nuclear import of the *S. cerevisiae* transcription factor SWI5. *Cell* **66,** 743–758 (1991).
8. Dohrmann, P. R. *et al.* Parallel pathways of gene regulation: homologous regulators SWI5 and ACE2 differentially control transcription of HO and chitinase. *Genes Dev.* **6,** 93–104 (1992).
9. Kovacech, B., Nasmyth, K. & Schuster, T. EGT2 gene transcription is induced predominantly by Swi5 in early G1. *Mol. Cell. Biol.* **16,** 3264–3274 (1996).
10. Knapp, D., Bhoite, L., Stillman, D. J. & Nasmyth, K. The transcription factor Swi5 regulates expression of the cyclin kinase inhibitor p40SIC1. *Mol. Cell. Biol.* **16,** 5701–5707 (1996).
11. Hecht, A. & Grunstein, M. Mapping DNA interaction sites of chromosomal proteins using immunoprecipitation and polymerase chain reaction. *Methods Enzymol.* **304,** 399–314 (1999).
12. Zhu, G. & Davis, T. N. The fork head transcription factor Hcm1p participates in the regulation of SPC110, which encodes the calmodulin-binding protein in the yeast spindle pole body. *Biochim. Biophys. Acta* **1448,** 236–244 (1998).
13. Gimeno, C. J., Ljungdahl, P. O., Styles, C. A. & Fink, G. R. Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and RAS. *Cell* **68,** 1077–1090 (1992).
14. Liu, H., Styles, C. A. & Fink, G. R. Elements of the yeast pheromone response pathway required for filamentous growth of diploids. *Science* **262,** 1741–1744 (1993).
15. Kron, S. J., Styles, C. A. & Fink, G. R. Symmetric cell division in pseudohyphae of the yeast *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **5,** 1003–1022 (1994).
16. Edgington, N. P., Blacketer, M. J., Bierwagen, T. A. & Myers, A. M. Control of *Saccharomyces cerevisiae* filamentous growth by cyclin-dependent kinase Cdc28. *Mol. Cell. Biol.* **19,** 1369–1380 (1999).
17. Amon, A., Tyers, M., Futcher, B. & Nasmyth, K. Mechanisms that help the yeast cell cycle clock tick: G2 cyclins transcriptionally activate G2 cyclins and repress G1 cyclins. *Cell* **74,** 993–1007 (1993).
18. Grallert, A., Grallert, B., Ribar, B. & Sipiczki, M. Coordination of initiation of nuclear division and initiation of cell division in *Schizosaccharomyces pombe*: genetic interactions of mutations. *J. Bacteriol.* **180,** 892–900 (1998).
19. King, L. & Butler, G. Ace2p, a regulator of CTS1 (chitinase) expression, affects pseudohyphal production in *Saccharomyces cerevisiae*. *Curr. Genet.* **34,** 183–191 (1998).
20. Weigel, D., Jurgens, G., Kuttner, F., Seifert, E. & Jackle, H. The homeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of the *Drosophila* embryo. *Cell* **57,** 645–658 (1989).
21. Kaufmann, E. & Knochel, W. Five years on the wings of fork head. *Mech. Dev.* **57,** 3–20 (1996).
22. Lai, E., Clark, K. L., Burley, S. K. & Darnell, J. E. Hepatocyte nuclear factor 3/fork head or "winged helix" proteins: a family of transcription factors of diverse biologic function. *Proc. Natl Acad. Sci. USA* **90,** 10421–10423 (1993).
23. Cogswell, J. P., Godlevski, M. M., Bonham, M., Bisi, J. & Babiss, L. Upstream stimulatory factor regulates expression of the cell cycle-dependent cyclin B1 gene promoter. *Mol. Cell. Biol.* **15,** 2782–2790 (1995).
24. Hwang, A., Maity, A., McKenna, W. G. & Muschel, R. J. Cell cycle-dependent regulation of the cyclin B1 promoter. *J. Biol. Chem.* **270,** 28419–28424 (1995).
25. Overdier, D. G., Porcella, A. & Costa, R. H. The DNA-binding specificity of the hepatocyte nuclear factor 3/forkhead domain is influenced by amino-acid residues adjacent to the recognition helix. *Mol. Cell. Biol.* **14,** 2755–2766 (1994).
26. Gimeno, C. J. & Fink, G. R. Induction of pseudohyphal growth by overexpression of PHD1, a *Saccharomyces cerevisiae* gene related to transcriptional regulators of fungal development. *Mol. Cell. Biol.* **14,** 2100–2112 (1994).

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Forkhead-like transcription factors recruit Ndd1 to the chromatin of G2/M-specific promoters

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Many cell-cycle-specific events are supported by stage-specific gene expression. In budding yeast, at least three different nuclear factors seem to cooperate in the periodic activation of G2/M-specific genes[1–3]. Here we show, by using chromatin immunoprecipitation polymerase chain reaction assays, that a positive regulator, Ndd1, becomes associated with G2/M promoter regions in manner that depends on the stage in cell cycle. Its recruitment depends on a permanent protein–DNA complex consisting of the MADS box protein, Mcm1, and a recently identified partner Fkh2, a forkhead/winged helix related transcription factor[4,5]. The lethality of Ndd1 depletion is suppressed by *fkh2* null mutations, which indicates that Fkh2 may also have a negative regulatory role in the transcription of G2/M-induced RNAs. We conclude that Ndd1–Fkh2 interactions may be the transcriptionally important process targeted by Cdk activity.

From the initiation of S phase to the completion of anaphase, yeast cells need to maintain a high level of B-type cyclin (Clb)-mediated Cdk1 activity. If Clb kinase activity decreases too much during this interval, then re-replication may occur before chromosome separation and cytokinesis[6]. One mechanism that ensures continuous production of the main mitotic cyclin, Clb2, is based on a positive feedback loop between the Clb2/Cdk1 kinase and the transcriptional activation system of *CLB2* (ref. 7). In G1 and early S phase, the messenger RNA level of this gene is low because of the lack of promoter activity[2,3]. Re-accumulation of the mRNA during later stages of the cell cycle depends largely on Clb-dependent kinase activity. The machinery for *CLB2* activation

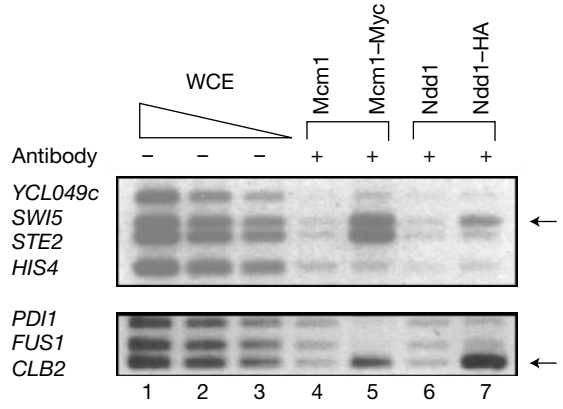

Figure 1 Ndd1 binds specifically to Mcm1-dependent G2/M-specific promoters. Chromatin immunoprecipitation PCR (ChIP) assays with *STE2*, *SWI5* and *CLB2* using tagged versions of Mcm1 and Ndd1. Lanes 1–3, dilution series of the whole-cell extract control (WCE); lanes 4 and 5, immunoprecipitation with anti-Myc antibody; lanes 6 and 7, immunoprecipitation with HA-specific antibody. Arrows emphasize the signals created by the *SWI5* and *CLB2* primer pairs. Gene names specify other promoter regions amplified by the control primer pairs. Top and bottom panels show results of independent experiments.