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Elm1p Is One of Three Upstream Kinases for the *Saccharomyces cerevisiae* SNF1 Complex

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Summary

Background: The yeast SNF1 protein kinase and the mammalian AMP-activated protein kinase are highly conserved heterotrimeric complexes that are "metabolic master switches" involved in the switch from fermentative/anaerobic to oxidative metabolism. They are activated by cellular stresses that deplete cellular ATP, and SNF1 is essential in the response to glucose starvation. In both cases, activation requires phosphorylation at a conserved threonine residue within the activation loop of the kinase domain, but identifying the upstream kinase(s) responsible for this has been a challenging, unsolved problem.

Results: Using a library of strains that express 119 yeast protein kinases as GST fusions, we identified Elm1p as the sole kinase that could activate the kinase domain of AMP-activated protein kinase in vitro. Elm1p also activated the purified SNF1 complex, and this correlated with phosphorylation of Thr210 in the activation loop. Removal of the C-terminal domain increased the Elm1p kinase activity, indicating that it is auto-inhibitory. Expression of activated, truncated Elm1p from its own promoter gave a constitutive pseudohyphal growth phenotype that was rescued by deletion of *SNF1*, showing that Snf1p was acting downstream of Elm1p. Deletion of *ELM1* does not give an *snf*− phenotype. However, Elm1p is closely related to Pak1p and Tos3p, and a *pak1*Δ *tos3*Δ *elm1*Δ triple mutant had an *snf1*− phenotype, i.e., it would not grow on raffinose and did not display hyperphosphorylation of the SNF1 target, Mig1p, in response to glucose starvation.

Conclusions: Elm1p, Pak1p, and Tos3p are upstream kinases for the SNF1 complex that have partially redundant functions.

Introduction

The yeast SNF1 complex and its mammalian homolog, AMP-activated protein kinase (AMPK), have many common features. Both are heterotrimeric complexes comprising related α, β, and γ subunits [1, 2]. Genes encoding the catalytic α subunit (*SNF1*) [3], the γ subunit (*SNF4*) [4], and at least one β subunit (*SIP1*, *SIP2*, or *GAL83*) are essential for function in yeast [5], while coexpression of all three subunits is required to obtain active kinase in mammalian cells [6]. The SNF1 complex is required for the switch from glucose to other carbon sources, as well as for the switch from fermentative to oxidative metabolism [7]. AMPK is a sensor of cellular energy status that has a wide range of functions [2]. However, an interesting parallel with SNF1 is that, in muscle, it is involved in the switch from the anaerobic metabolism of endogenous fuels (e.g., glycogen) to the oxidative metabolism of exogenous glucose and fatty acids [2, 8]. Both SNF1 and AMPK are activated by glucose deprivation [9, 10], while AMPK is activated by a variety of stresses that deplete ATP [2], including exercise in skeletal muscle [11]. In both cases, activation requires phosphorylation by upstream kinase(s) at a conserved threonine residue in the activation loop of the α subunit kinase domain (Thr210 in yeast; Thr172 in mammals) [9, 12–14]. Identifying these upstream kinases, which have not yet emerged either from genetic screens in yeast or from biochemical studies in mammals, has been a key, unsolved problem in both systems.

Using a library of protein fusions containing 119 protein kinases predicted from the yeast genome [15], we have identified Elm1p as the sole kinase that activated the bacterially expressed kinase domain of AMPK in vitro. Elm1p also activated the SNF1 complex and phosphorylated Thr210 in vitro. Although we provide evidence that Elm1p can act upstream of SNF1 in vivo, *elm1*Δ strains do not have an *snf*− phenotype, which suggested that there must be other kinases upstream of Snf1p in vivo. Elm1p is part of a small subfamily of yeast protein kinases with Pak1p and Tos3p [16]. We also report that a triple *elm1*Δ *pak1*Δ *tos3*Δ mutant does indeed have an *snf*− phenotype, showing that these three kinases act upstream of SNF1 in vivo in a partially redundant manner.

Results

Elm1p Activates the Bacterially Expressed AMPK Kinase Domain

From each of the strains expressing a single glutathione-S-transferase (GST)-protein kinase fusion [15], we purified the fusion protein and assayed its ability to activate a GST fusion of the bacterially expressed kinase domain of the α1 subunit of rat AMPK. We have previously shown that this substrate can be activated by mammalian AMPKK, and that this is abolished by mutation of Thr172 [17]. We reasoned that it was valid to use mammalian AMPK as substrate, because the sequences around Thr210 (Snf1p) and Thr172 (AMPK-α) are highly conserved, and because a mammalian upstream kinase can activate the yeast SNF1 complex in vitro [9]. Of the 119

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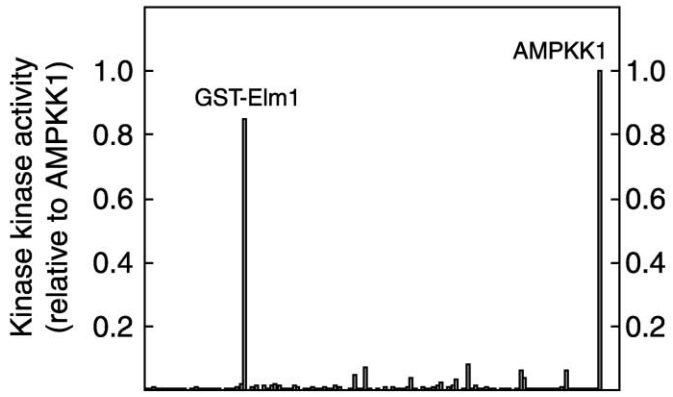
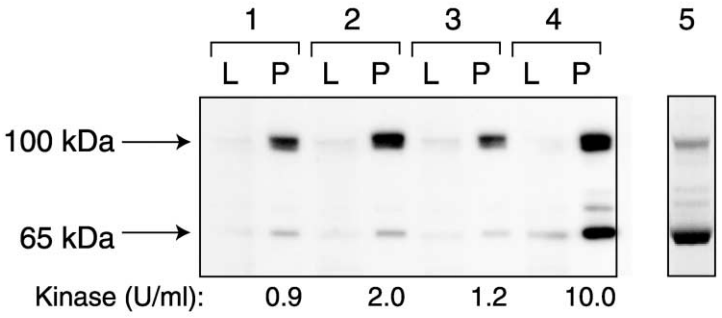
A Kinase kinase activity of yeast protein kinase-GST fusions



B Anti-GST blot/stained gel of five GST-Elm1p preparations



C Elm1 sequence with peptides identified by mass spectrometry

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MSPRQLIPTL IPEWAPLSQQ SCIREDELDS PPITPTSQTS SFGSSFSQQK 51
PTYSTIIGEN IHTILDEIRP YVKKITVSDQ DKKTINQYTL GVSAGSGQFG 101
YVRKAYSSTL GKVVAVKIIP KKPWNAQQYS VNQVMRQIQL WKSKGKITTN 151
MSGNEAMRLM NIEKCRWEIF AASRLRNNVH IVRLIECLDS PFSESIWIVT 201
NWCSLGELQW KRDDDEDILP QWKKIVISNC SVSTFAKKIL EDMTKGLEYL 251
HSQGCIHRDI KPSNILLDEE EKVAKLSDFG SCIFTPQSLP FSDANFEDCF 301
QRELNKIVGT PAFIAPELCH LGNSKRDFVT DGFKLDIWSL GVTLYCLLYN 351
ELPFFGENEF ETYHKIIEVS LSSKINGNTL NDLVIKRLLE KDVTLRISIQ 401
DLVKVLSRDQ PIDSRNHSQI SSSSVNPVRN EGPVRRFFGR LLTKKGKKKT 451
SGKGKDKVLV SATSKVTPSI HIDEEPDKEC FSTTVLRSSP DSSDYCSSLG 501
EEAIQVTDFL DTFCRSNESL PNLTVNNDKQ NSDMKTDRSE SSSHSSLKIP 551
TPIKAMIRLK SSPKENGNRT HINCSQDKPS SPLMDRTVGK RTVNNSGARK 601
LAHSSNILNF KAYINSEDSD IRETVEDVKT YLNFADNGQI
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Figure 1. Elm1p Activates the Kinase Domain of Mammalian AMPK in Cell-Free Assays and Is Activated by Proteolytic Removal of the C Terminus

(A) Activation of the α1 kinase domain by fusions between GST and yeast protein kinases; mammalian AMPKK1 is a positive control. A bar chart showing the activation of the bacterially expressed AMPK α1 kinase domain by 119 yeast protein kinase-GST fusions and by AMPKK1. The results are expressed relative to the activation obtained with AMPKK1. Only the kinases giving a positive signal (GST-Elm1p and AMPKK1) are labeled.

(B) A Western (anti-GST) blot of four GST-Elm1p preparations (lanes 1-4), and a Colloidal Blue-stained gel of large-scale preparation (lane 5). L, crude cell lysates; P, glutathione-Sepharose precipitates. The kinase kinase activities in the P fractions are given below each lane.

(C) The predicted amino acid sequence of Elm1p. The large shaded box enclosed in dashed lines represents the kinase domain, based on alignments with other kinases. Tryptic peptides identified in the fingerprinting analysis of the 65 kDa fragment are highlighted by using smaller boxes with continuous lines.

purified GST kinases tested, only Elm1p gave a signal that was well above background and was similar to the signal obtained with mammalian AMPKK1 [18] (Figure 1A).

The C-Terminal Region of Elm1p Is an Auto-Inhibitory Domain

To confirm these findings, we made several preparations of GST-Elm1p, but their activity was variable. When examined by Western blotting with anti-GST antibodies, we observed a 65 kDa polypeptide as well as the full-length 100 kDa GST-Elm1p (Figure 1B). Interestingly, the highest activity was found in the preparation that contained the highest proportion of 65 kDa polypeptide. The Elm1p sequence contains a central kinase domain (≈141–420), followed by a large C-terminal domain (CTD, ≈421–640). Since the GST detected by the antibody was fused at the N terminus, the 65 kDa polypeptide appeared to be truncated at the C terminus. To confirm this, we made a larger scale preparation (lane 5, Figure 1B), excised the 65 kDa fragment from the gel, digested it with trypsin, and analyzed the masses of the peptide mixture obtained by MALDI-TOF mass spectrometry. We could identify 14 peptides from the kinase domain, corresponding to 43% of the total sequence (Figure 1C). The peptides identified covered the region from residues 84 to 404, including almost the entire kinase domain. Tryptic peptides derived from GST were also detected, but we did not detect any peptides from the CTD. These results suggested that the CTD was an auto-inhibitory domain, and, to confirm this, we expressed DNA encoding full-length Elm1p, or Elm1p with the CTD removed (residues 1–420; Elm1p-ΔC), from a

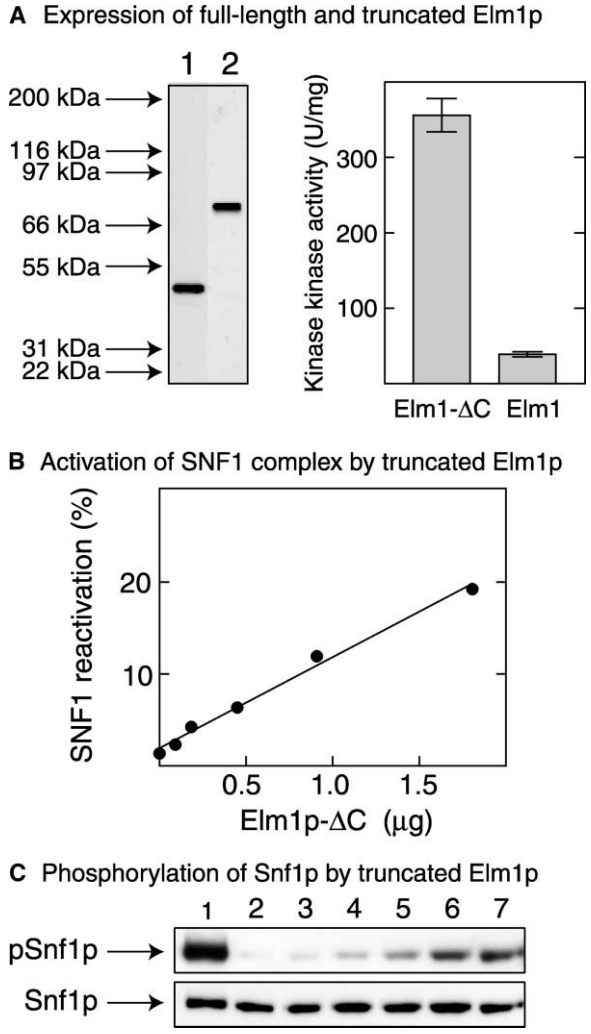

Figure 2. Elm1p Contains an Auto-Inhibitory Domain at the C Terminus and Activates the SNF1 Complex by Phosphorylation of Thr210

(A) Expression (left) and kinase kinase activity (right) of Elm1p-ΔC compared with full-length Elm1p. The protein kinases were expressed from a *GAL1* promoter, purified via hexahistidine tags, and analyzed by Western blotting (left) with an anti-Elm1p antibody (yN-19, Santa Cruz) or via kinase kinase assays (right).

(B and C) (B) Activation and (C) phosphorylation at Thr210 of the dephosphorylated SNF1 complex by Elm1p-ΔC. (B) Activation of the dephosphorylated, purified SNF1 complex by increasing amounts of the hexahistidine-tagged Elm1p-ΔC; results are expressed as a percentage of the activity of the SNF1 complex prior to dephosphorylation. (C) Upper panel: phosphorylation of Thr210 assessed by probing blots with a phosphospecific antibody against the Thr172 site on mammalian AMPK [19]. Key to lanes: (1) purified SNF1 complex without phosphatase treatment; (2–7) phosphatase-treated SNF1 complex treated with 0, 0.09, 0.18, 0.45, 0.9, and 1.8 μg Elm1p-ΔC, as in (B). Lower panel: the same blot was stripped and reprobed with anti-Snf1p antibody (yK-16, Santa Cruz) to ensure uniform loading.

plasmid with a *GAL1* promoter. The products were purified via hexahistidine tags and were the expected size when probed with an anti-Elm1p antibody (Figure 2A, left panel). Although both protein products were active, the truncated product was 9-fold more active than the full-length protein (Figure 2A, right panel).

Elm1p Activates the SNF1 Complex and Phosphorylates Thr210 In Vitro

Elm1p-ΔC also activated the dephosphorylated SNF1 complex purified from yeast in a dose-dependent manner (Figure 2B). To test whether this was due to phosphorylation of Thr210, we utilized a phosphospecific antibody [19] against Thr172 on mammalian AMPK (apart from a conservative [Arg→Lys] replacement at the third residue, the sequence of the peptide antigen is perfectly conserved in Snf1p). Figure 2C (upper panel, lanes 1 and 2) shows that treatment with protein phosphatase-2A removed almost all of the phosphate from Thr210, while Elm1p-ΔC caused a dose-dependent increase in phosphorylation of Thr210 in the presence of MgATP (lanes 3–7). These findings correlate with the activation shown in Figure 2B. The lower panel shows reprobing of the same blot with a nonphosphospecific Snf1p antibody to confirm equal loading.

Expression of Activated Elm1p In Vivo Gives an Snf⁻-Dependent Phenotype

To examine the effect of the truncated, activated Elm1p in vivo, we compared a strain expressing the normal full-length *ELM1* with a strain in which the DNA encoding the CTD (residues 421–640) had been deleted from the endogenous gene. Using anti-Elm1p antibody, the expression of full-length and truncated Elm1p was identical (not shown). Cells were grown in medium containing 1 M sorbitol, since it was found that some of the strains described below were osmotically sensitive. Kinase kinase assays in anti-Elm1p immunoprecipitates confirmed that the Elm1p-ΔC strain had an activity that was 6-fold greater than that of the strain expressing full-length Elm1p. Figure 3 shows the appearance of colonies of these strains and others derived from them. The strain expressing full-length Elm1p (Figure 3A) and an *snf1*Δ strain (Figure 3B) gave normal, round colonies with a smooth margin. By contrast, cells expressing Elm1p-ΔC gave colonies with a ragged, irregular margin (Figure 3C). Deletion of the *SNF1* gene in cells expressing Elm1p-ΔC caused reversion to smooth colonies (Figure 3D), suggesting that Snf1p acted downstream of Elm1p to produce this phenotype. Consistent with this, the ragged colonies were regained by expressing from a plasmid wild-type Snf1p (Figure 3E), but not a Thr210Ala mutant (Figure 3F). The latter would be incapable of being phosphorylated and activated by the upstream kinase.

These strains and others derived from them were also studied by differential interference contrast microscopy (Figure 4). Cells expressing full-length Elm1p (Figure 4A) and an *snf1*Δ strain (Figure 4B) had a normal, rounded appearance with single buds. By contrast, cells expressing Elm1p-ΔC were misshapen, had elongated morphology, and had a pseudohyphal growth pattern that caused the mother and daughter cells to fail to separate (Figure 4C). When we deleted *SNF1* in the strain expressing Elm1p-ΔC, the morphology reverted to normal (Figure 4D). Western blotting confirmed that the expression of Elm1p-ΔC was not affected by the *SNF1* deletion (not shown). The elongated cells/pseudohyphal growth phenotype was restored to the Elm1p-ΔC *snf1*Δ strain

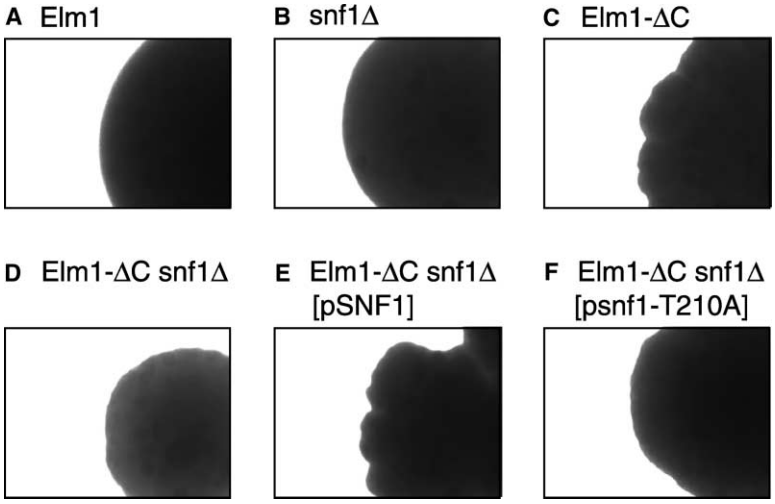

Figure 3. Appearance under the Dissecting Microscope of Colonies of Different Strains

(A) A strain expressing (from its own promoter) full-length Elm1p with a protein A tag.
(B) A strain deleted for *SNF1* in the same background.
(C) The same strain as in (A), but expressing C-terminally truncated (1–420) Elm1p (Elm1p-ΔC).
(D) The same strain as in (C), but with *SNF1* also deleted.
(E) The same strain as in (D), but expressing plasmid pRJ79 encoding a Snf1p-VP16 fusion.
(F) The same strain as in (D), but expressing plasmid pRJ81 encoding a Snf1p(T210A)-VP16 fusion.
All pictures were taken at 16× magnification.

by expression of wild-type Snf1p (Figure 4E), but not a Thr210Ala mutant (Figure 4F). As expected [20], deletion of endogenous *ELM1* yielded elongated cells with a pseudohyphal growth pattern (Figure 4G). However, unlike the Elm1p-ΔC strain, the morphology of this strain was not normalized by deletion of *SNF1* (Figure 4H). The phenotype of the double disruptant, which involved large cells that flattened under the coverslip, cells that did not detach from each other, and cells that were very prone to rupture, even in 1 M sorbitol, was more severe than either of the single disruptions.

A *pak1*Δ *tos3*Δ *elm1*Δ Triple Mutant Has an *snf*− Phenotype

While these studies with Elm1p were being carried out by one of our two groups, the other group [21] reported that the protein kinase Pak1p could also phosphorylate Thr210 on Snf1p in vitro and provided genetic evidence

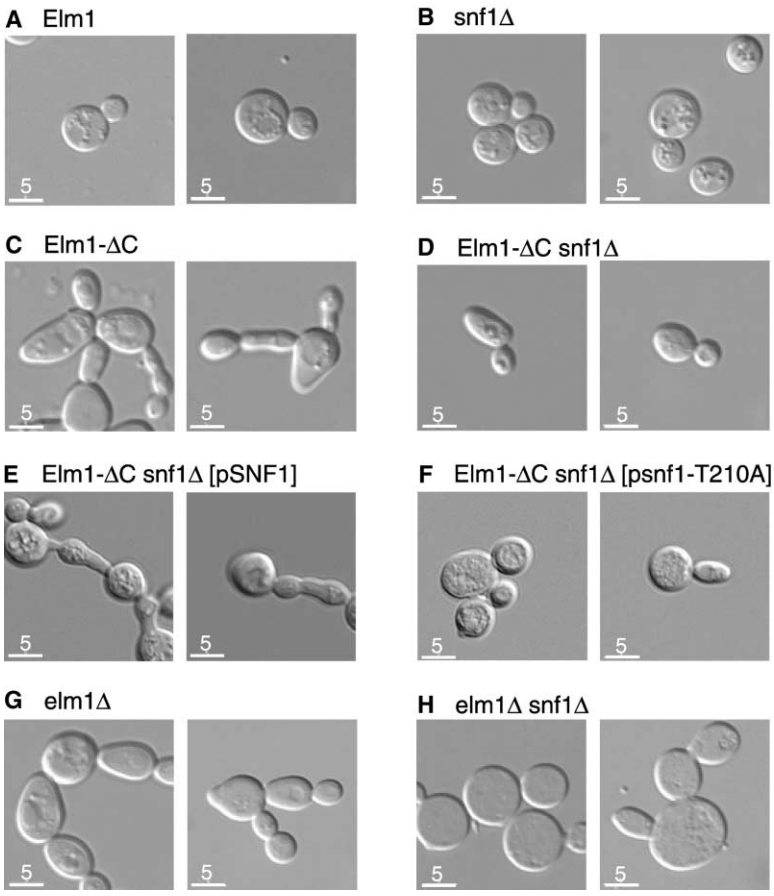

Figure 4. Appearance by Differential Interference Contrast Microscopy of Cells of Different Strains

(A) A strain expressing (from its own promoter) full-length protein A-tagged Elm1p.
(B) An *snf1*Δ deletion in the same genetic background.
(C) The same strain as in (A), but expressing C-terminally truncated (1–420), rather than full-length, Elm1p (Elm1p-ΔC).
(D) The same strain as in (C), but with an additional *snf1*Δ deletion.
(E) The same strain as in (D), but also expressing plasmid pRJ79 encoding a Snf1p-VP16 fusion.
(F) The same strain as in (D), but also expressing plasmid pRJ81 encoding a Snf1p(T210A)-VP16 fusion.
(G) An *elm1*Δ deletion in the same background.
(H) An *ELM1 SNF1* double disruptant (*elm1*Δ *snf1*Δ).
The scale bars are 5 μm.

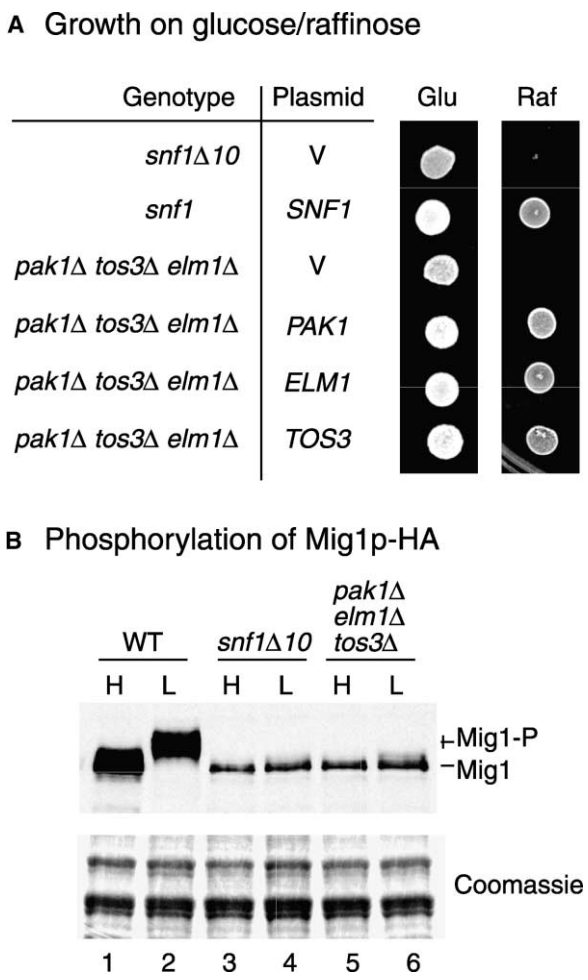

Figure 5. The Pak1p, Elm1p, and Tos3p Kinases Are Required for Snf1p Function

(A) Comparison of the growth properties of yeast cells lacking either Snf1p or the Snf1p-activating kinases. Equivalent numbers of cells were spotted onto synthetic complete media lacking leucine and containing either glucose (Glu) or raffinose (Raf) as the carbon source. The yeast strains used (FY1193 (*snf1Δ10*) and MSY858 (*pak1Δ elm1Δ tos3Δ*)) were transformed with either empty vector (V) or plasmids containing the indicated gene.

(B) Snf1-dependent phosphorylation of Mig1 is blocked in *pak1Δ elm1Δ tos3Δ* cells. Cells were transformed with a plasmid expressing Mig1 protein tagged with the HA epitope. Protein extracts prepared from cells grown in high (H, 2%; lanes 1, 3, and 5) or low (L, 0.05%; lanes 2, 4, and 6) glucose were analyzed by Western blotting. The yeast strains used in this study were MSY182 (WT; lanes 1 and 2), FY1193 (*snf1Δ10*; lanes 3 and 4), and MSY858 (*pak1Δ elm1Δ tos3Δ*; lanes 5 and 6). The mobility of the Mig1p protein and phosphorylated Mig1p (Mig1-P) is indicated. The lower panel shows a Coomassie blue-stained gel run in duplicate to show that the six extracts contained equivalent quantities of total protein.

that it acted upstream of the SNF1 complex in vivo. However, *pak1* deletion did not cause an *snf−* phenotype, suggesting that Snf1 may be activated in vivo by other upstream kinases in addition to Pak1p. Intriguingly, the kinase domains of Elm1p and Pak1p are closely related to each other and to that of Tos3p [16]. Figure 5A shows that an *snf1Δ10* strain would grow on glucose (Glu), but not on raffinose (Raf) as expected. Growth on raffinose was restored by providing *SNF1* on a plasmid instead. Figure 5A shows that a *pak1Δ tos3Δ elm1Δ* triple mutant also had an *snf−* phenotype in that it would grow on glucose but not on raffinose. Provision of either *PAK1*, *ELM1*, or *TOS3* function on a plasmid allowed growth on raffinose, providing strong evidence that any one of these three kinases can activate the SNF1 complex in vivo.

Growth on raffinose requires derepression of the invertase gene (*SUC2*) via multiple phosphorylation of the repressor protein, Mig1p, by the SNF1 complex [22, 23]. Figure 5B shows that multiple phosphorylation of HA-tagged Mig1p on transfer of a wild-type strain to low glucose was evident as reduced mobility on a Western blot. The hyperphosphorylation of Mig1p in response to low glucose was completely abolished in an *snf1Δ10* strain and in a *pak1Δ tos3Δ elm1Δ* triple mutant.

Discussion

Our approach using the library of yeast strains expressing GST-protein kinase fusions [15] detected Elm1p, but not Pak1p or Tos3p, as an upstream kinase for the SNF1 complex. There are several reasons why this approach might fail to find all protein kinases acting upstream of SNF1. For example, the GST domain might interfere with enzymatic function, or the kinase might require additional inputs, such as covalent modifications or interaction with regulatory subunits. Indeed, we might have missed Elm1p if there had not been some fortuitous degradation of the protein to generate an active fragment (Figure 1B). Our results support the idea that the CTD of Elm1p (residues 421–640) is an auto-inhibitory domain that represses the kinase activity. Koehler and Myers [24] previously reported that Elm1p lacking the CTD was functional in vivo and displayed some kinase activity against artificial substrates, but they did not address whether the truncation caused a quantitative effect on Elm1p function. We are not proposing that proteolysis is a physiological mode of regulation of Elm1p, but instead that there is some natural stimulus (perhaps during cytokinesis, see below) that relieves inhibition by the CTD via an allosteric transition or a reversible covalent modification.

The results in Figure 2 show that Elm1p activates the SNF1 complex by phosphorylation of Thr210 on Snf1p, while results supporting the idea that this can occur in vivo are presented in Figures 3 and 4. When compared with cells expressing full-length Elm1p, expression from the endogenous promoter of an activated Elm1p lacking the inhibitory CTD (Elm1p-ΔC) produced colonies with a ragged margin, elongated cell morphology, and pseudohyphal growth, the latter presumably being responsible for the unusual colony shape. This was dependent on the function of Snf1p, because the cell and colony shape reverted to normal if the *SNF1* gene was deleted in the strain expressing the activated Elm1p-ΔC. Moreover, the ability of Snf1p to mediate the effect of activated Elm1p required an intact phosphorylation site, i.e., Thr210, because the phenotype reverted upon expression of wild-type Snf1p, but not a Thr210Ala mutant. This suggests that the unusual cell growth pattern requires

phosphorylation of Thr210 by Elm1p-ΔC, and that the latter is acting as an upstream kinase for Snf1p.

ELM1 was originally defined via mutations that caused an elongated cell morphology (*ELM*), constitutive, pseudohyphal growth, and invasion of agar [20]. Pseudohyphal, invasive growth normally only occurs upon nutrient starvation, and it is viewed as a mechanism to allow yeast to forage for nutrients. Intriguingly, starvation for glucose and other fermentable sugars causes invasive filamentous growth of haploid yeast, and this response requires *SNF1* [25]. Surprisingly, none of the strains studied by us in Dundee (even *elm1*Δ) invaded agar significantly, but this may be a function of genetic background [26] Additional insights into the role(s) of Elm1p came from findings of genetic interactions with the cell cycle controller, Cdc28p [27]. Expression of *ELM1* mRNA [28] and protein [29] is maximal at the time of cytokinesis. In *elm1*Δ mutants, the septin Cdc11p, a cytoskeletal protein that (with other septins) forms the 10 nm filament in the bud neck, is mislocalized and is found throughout the bud, and cytokinesis is delayed [27]. Expression of an Elm1p-GFP fusion from the *ELM1* promoter suggested that Elm1p also localizes to the bud neck [27, 29]. Interestingly, overexpression *or* deletion of *ELM1* result in elongated cell morphology, and it was suggested that overexpression might cause mislocalization, because an Elm1p-GFP fusion failed to localize to the bud neck when overexpressed [29]. This may be related to our observations that the phenotype of a strain expressing an activated Elm1p (Elm1p-ΔC) was superficially similar to that of an *elm1*Δ strain. However, the two strains differed in the effect of the additional deletion of *SNF1* (Figure 4), and this difference rules out the possibility that Elm1p-ΔC was simply acting as a dominant-negative mutant.

These results suggested that the activated Elm1p could phosphorylate and activate the SNF1 complex in vivo, but because *elm1*Δ strains do not have an *snf*− phenotype, it remained formally possible that this was a function that was not performed by normal, full-length Elm1p. However, an alternative explanation for the lack of an *snf*− phenotype in *elm1*Δ strains was that there were multiple kinases acting upstream of the SNF1 complex that are partially redundant. While the studies of Elm1p were being performed by one of our two groups, the other group reported that Pak1p could phosphorylate Snf1p at Thr210 in vitro, and they also provided genetic evidence that Pak1p activates the SNF1 complex in vivo [21]. Intriguingly, Pak1p, Tos3p, and Elm1p form a small protein kinase subfamily in yeast; the kinase domains of the first two are particularly closely related [16]. We therefore constructed a *pak1*Δ *tos3*Δ *elm1*Δ triple mutant. Like an *snf1*Δ strain, the triple mutant would grow on glucose but not on raffinose. Metabolism of raffinose requires the derepression of the *SUC2* gene, encoding invertase. Derepression of *SUC2* is achieved, at least in part, via phosphorylation of Mig1p by the SNF1 complex at four serine residues within the central R1 domain [22]. This in turn promotes binding of Mig1p to the nuclear export protein, Msn5p, and the consequent translocation of the repressor protein to the cytoplasm [23]. As shown in Figure 5A, the growth defect of the *pak1*Δ *tos3*Δ *elm1*Δ triple mutant on raffinose could be restored by provision of any one of the three kinases on a plasmid. In addition, the hyperphosphorylation of Mig1p that occurred in response to glucose deprivation of a wild-type strain was abolished in an *snf1*Δ*10* strain and in the *pak1*Δ *tos3*Δ *elm1*Δ triple mutant. Thus, while any one of these three kinases can activate the SNF1 complex in vivo, the presence of at least one of them is essential for SNF1 function.

One of our original aims was to use the yeast system to identify upstream kinases that might enable us to identify homologs in mammals. Unfortunately, searches of predicted mammalian proteins reveal no clear homolog of Elm1p, Pak1p, or Tos3p. The best matches are to the two isoforms of calmodulin-dependent protein kinase kinase (CaMKK), which is the upstream kinase that activates calmodulin-dependent protein kinases I and IV via phosphorylation of a threonine residue in the activation loop equivalent to Thr172 in AMPK-α and Thr210 in Snf1p [30]. Although the sequence similarity was confined to the kinase domain, this was intriguing because one of our groups had already shown that CaMKK can activate AMPK in cell-free assays [31]. We do not believe that the upstream kinases previously purified from rat liver [13, 18] are CaMKKs because: (i) they were not stimulated by Ca^{2+} and calmodulin; (ii) relative to CaMKK, they were more efficient at activating AMPK and less efficient at activating CaMKI; and (iii) they are not immunoprecipitated by anti-CaMKK antibodies (Jennifer Reid, personal communication). However, it seems likely that the true AMPKKs may correspond to one or more of the predicted mammalian protein kinases with kinase domains related to those of the CaMKKs, many of which still have unknown functions.

Conclusions

We have identified Elm1p as an upstream kinase that can phosphorylate and activate the SNF1 complex in vitro and in vivo. Elm1p contains a regulatory C-terminal domain whose influence can be removed by proteolysis or truncation, but which may be regulated physiologically by some other unknown mechanism. Our results and those published previously [21] identify Elm1p, Pak1p, and Tos3p as a subfamily of yeast kinases that can phosphorylate Thr210 on Snf1p, and at least one of these is required for activation of the SNF1 complex in vivo. Although these kinases are partially redundant with respect to this function, deletion of the individual kinases causes different phenotypes, so they may phosphorylate the SNF1 complex under different conditions, or at different times or subcellular locations, in vivo. Furthermore, phenotypic variation may be explained if Pak1, Tos3, and Elm1 kinases were to activate additional and distinct sets of downstream kinases [32]. Further work is required to delineate their differing physiological roles, but their overlapping function in activation of the SNF1 complex explains why these kinases have not emerged from previous genetic screens that identified genes that interact with *SNF1*. Although Elm1p, Pak1p, and Tos3p do not have direct homologs in mammals, our results should assist in the identification of the elusive upstream kinases in the mammalian AMPK system.

Supplemental Data

Supplemental Data including the Experimental Procedures for this paper are available at http://www.current-biology.com/cgi/content/full/13/15/1299/DC1/.

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Supplemental Data

Elm1p Is One of Three Upstream Kinases for the *Saccharomyces cerevisiae* SNF1 Complex

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Supplemental Experimental Procedures

Yeast Strains and General Methods, Plasmids, and Strain Construction

The *S. cerevisiae* strains used for most of this study are listed in Table S1 and are isogenic with W303. Strains used for the results in Figure 5 were MSY182 (*MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200*), FY1193 (*MATα ura3-52 leu2Δ1 his3Δ200 trp1Δ63 snf1Δ10*), and MSY858 (*MATα ura3Δ0 leu2Δ0 his3Δ1 pak1Δ::KAN elm1Δ::KAN tos3::KAN*). Oligonucleotides used are shown in Table S2, and plasmids are shown in Table S3. Standard yeast methods and growth media were used [S1], and all strains were grown at 30°C. Medium containing raffinose as the carbon source was supplemented with 1 μg/ml actimycin A to block respiration. Yeast was transformed as described by Agatep et al. [S2]. *Escherichia coli* strain TOP10F′ (InVitrogen) was used for plasmid manipulations. Standard molecular biology and microbiology techniques were carried out as described by Ausubel et al. [S3].

Construction of *S. cerevisiae* Deletion Strains

Yeast genes were deleted by using a one-step polymerase chain reaction (PCR)-mediated method as described by Longtine et al. [S4]. To replace *ELM1* with *Schizosaccharomyces pombe his5*$^+$ (producing strain CSY100), a deletion cassette was amplified from the pFA6a-His3MX6 vector by using the oligonucleotides ELM1DF and ELM1DR. This cassette was then transformed into *S. cerevisiae* AY925, and transformants were selected on synthetic drop-out (SDO)-His medium. Integration of the cassette into the *ELM1* gene was confirmed by PCR. The *SNF1* gene was deleted in exactly the same way, by using oligonucleotides SNF1DF and SNF1DR3, to produce CSY101.

Purification of GST Kinases and Analysis by Mass Spectrometry

Starter cultures (1 ml) were grown in a 96-well plate format from glycerol and were used to inoculate 50 ml synthetic drop-out (SDO)-Ura medium with 2% raffinose. The expression of the GST kinases was induced for 4 hr by the addition of galactose to 4% when cultures reached an OD_{600} of 0.8–1.0. Cells were harvested by centrifugation and were washed with ice-cold phosphate-buffered saline (PBS). Cells were lysed by vortexing for 3 min with acid-washed glass beads (425–600 μm diameter) and 1 ml RIPA buffer (50 mM HEPES [pH 7.4], 1% Triton X-100, 1% Na deoxycholate, 0.1% SDS, 150 mM NaCl, 0.1 mM phenylmethane sulphonyl fluoride, 80 μM glycerophosphate, 50 μM NaF, 100 μM Na_3VO_4, plus complete protease inhibitor cocktail, EDTA-free [Roche]). Cell debris and glass beads were removed by centrifugation, and the lysates were removed and snap frozen. GST kinases were purified in batch mode from the lysates. Glutathione-Sepharose (50 μl) (Pharmacia) was added, and the suspensions were incubated at 4°C for 30 min and were then washed 4 times with 1 ml PBS. The GST kinases were eluted with glutathione elution buffer (50 mM Tris-HCl [pH 8.5], 20 mM glutathione). Purification of the GST kinases was confirmed by Western blotting with an anti-GST antibody conjugated to horseradish peroxidase (Sigma). The identity of the Elm1p degradation products was confirmed by tryptic peptide mass fingerprinting as described previously [S5].

Kinase Kinase Assays

Kinase kinase assays were performed by using a GST fusion with the kinase domain of the α1 subunit of AMPK as substrate as described previously [S6]. For assays of Elm1p-PrA and Elm1p-ΔC-PrA, the proteins were precipitated from yeast lysates as described below. After the final wash with HEPES buffer, the Protein G (PrG)-Sepharose (Pharmacia) was resuspended in this same buffer and split, such that each assay would receive 5 μl Elm1p bound beads. The buffer was aspirated off, and the kinase kinase assays were performed as described above.

Expression and Purification of His-Tagged Elm1p

DNAs encoding full-length Elm1p and the C-terminally truncated Elm1p (residues 1–420) were amplified from AY925 genomic DNA by using the oligonucleotides Elm1V5F/Elm1V5R and Elm1V5F/Elm1ΔCV5R, respectively. The PCR products were cloned into the pYES2.1/V5-His-TOPO vector (InVitrogen) following manufacturers'

Table S1. Yeast Strains Used in This Study

Strain	Genotype[a]
AY925	*MATa* W303
CSY100	*MATa* W303 *elm1::* Sp*his5*
CSY101	*MATa* W303 *snf1::* Sp*his5*
CSY102	*MATa* W303 *elm1::ELM1::PrA::* Sp*his5*
CSY103	*MATa W303 elm1::ELM1(1-1260)::PrA::* Sp*his5*$^+$
CSY104	*MATa W303 elm1::* Sp*his5 snf1::TRP1*
CSY105	*MATa* W303 *elm1::ELM1(1-1260)::PrA::* Sp*his5 snf1::TRP1*

[a] W303 background: *ade2-1 his3-11,15 leu2-3 trp1-1 ura3-1 can1-100 ssd1-d2 FUS3 KSS1* gal+.

Table S2. Oligonucleotides Used in This Study

Name	Sequence (5′-3′)
ELM1DF	GCCAGGTTAACAATAATTACTTAGCATGAAATGT CACCTCCGGATCCCCGGGTTAATTAA
ELM1DR	CCGACAGATATCATCCTGTAGTTTCATCTATATTT GACCAGAATTCGAGCTCGTTTAAAC
SNF1DF	ACAAGTTTTGCTACACTCCCTTAATAAAGTCAACA TGAGCAGTAACAACAACCGGATCCCCGGGT TAATTAA
SNF1DR3	CGTTACGATAAAAAAAAGGGAACTTCCATATCAT TCTTTTACGTTCCACCATCGAATTCGAGCTCG TTTAAAC
Elm1ΔCHISF	TCGCGTGACCAGCCCATAGATTCTAGGAATCACA GTCAAATTTGAGCGCCACTTCTAAA
Elm1ΔC HISR	ATTATCAGCTAACCCAATCCGACAGATATCATCC TGTAGGAATTCGAGCTCGTTTAAAC
Elm1V5F	ACTTAGCATGAAATGTCA
Elm1V5R	TATTTGACCATTATCTGC
Elm1ΔCV5R	AATTTGACTGTGATTCCT
Elm1ZZF	GAAGATGTAAAAACGTATCTGAACTTTGCAGATA ATGGTCAAATAGGAGCAGGGGCGGGTGC
Elm1ZZR	ATTATCAGCTAACCCAATCCGACAGATATCATCC TGTAGTTTCATGAGGTCGACGGTATCGATAAG
Elm1ZZΔCF	TCGCGTGACCAGCCCATAGATTCTAGGAATCACA GTCAAATTGGAGCAGCAGGGGCGGGTGC

Table S3. Plasmids Used in This Study

Name	Description	Source/Reference
pFA6a-His3MX6	Plasmid template for PCR	[S4]
pFA6a-TRP1	Plasmid template for PCR	[S4]
pZZ-His5	pBluescript KS- carrying a ZZ tag and *S. pombe his5*$^+$	[S9]
pYES2.1/V5-His-TOPO	Linearized 2μ *URA3* yeast expression vector with *GAL1* promoter, V5 epitope and His_6 tag	InVitrogen
pYES-Elm1	pYES2.1/V5-His-TOPO containing full length *ELM1* gene	This study
pYES-$Elm1_{1–420}$	pYES2.1/V5-His-TOPO *ELM1* nucleotides 1–1260	This study
pRJ79	Expresses VP16-Snf1	[S12]
PRJ81	Expresses VP16-Snf1T210A	[S12]

instructions. The vectors were amplified by transformation into *E. coli* Topp10′ (InVitrogen), isolated from *E. coli*, and then transformed into *S. cerevisiae* AY925. Transformants were selected on SDO-Ura medium. Expression of the fusion proteins was induced by the addition of galactose (4%) to mid-log phase cultures grown in SDO-Ura medium with 2% raffinose. Cells were harvested by centrifugation and were washed with ice-cold PBS, and lysates were prepared with RIPA buffer and glass beads. Ni-NTA-agarose (Qiagen) was washed with native binding buffer (20 mM Na phosphate, 500 mM NaCl [pH 7.2–7.6]). The lysate was then bound to the Ni-NTA-agarose at 4°C. The agarose was washed with native wash buffer (20 mM Na phosphate, 500 mM NaCl [pH 6.0]), and the His-tagged protein was eluted with native wash buffer containing increasing concentrations of imidazole (50–500 mM). Purification of Elm1p-V5-His_6 and Elm1p-ΔC-V5-His_6 was confirmed by Western blotting analysis, and proteins were detected with either anti-Elm1p (y-640, Santa Cruz Biotechnology) or anti-V5-HRP (InVitrogen).

Purification and Phosphorylation of the Yeast SNF1 Complex by Elm1p-ΔC

The SNF1 complex was purified as described previously [S7]. The purified kinase (52 U/ml) was incubated with the catalytic subunit of bovine protein phosphatase-2A [S8] (110 mU/ml) for 10 min at 30°C, then okadaic acid was added to a 1 μM final concentration to inhibit the phosphatase. The reaction mixture was divided into aliquots and was incubated with ATP (200 μM), $MgCl_2$ (5 mM), and the indicated amounts of Elm1p-ΔC for 5 min at 30°C in a final volume of 20 μl. One aliquot (5 μl) was removed for kinase assays, and a second (15 μl) was removed for SDS-PAGE and Western blotting.

Expression and Immunoprecipitation of Protein A-Tagged Elm1 Constructs

Full-length Elm1p and the C-terminally truncated Elm1p (residues 1–420) were tagged at the C terminus with Protein A (Strains CSY102 and CSY103, respectively) by using homologous recombination with PCR products. The oligonucleotides, ELM1ZZF and ELM1ZZR (full-length Elm1p) or ELM1ΔCZZF and ELM1ZZR (Elm1p-ΔC), amplified a transformation cassette containing the *S. pombe HIS5* gene, the TEV protease cleavage site, and two Z domains from protein A [S9]. Cassettes were individually transformed into *S. cerevisiae* AY925, and transformants were selected on SDO-His medium. Integration of the cassettes into the *ELM1* gene was confirmed by PCR.

Elm1p-PrA and Elm1p-ΔC-PrA were immunoprecipitated from yeast lysates as follows (an anti-Elm1p antibody was used because precipitation via the protein A tag was not successful). Protein G (PrG)-Sepharose was washed with IP buffer (50 mM Tris [pH 7.4] at 4°C, 150 mM NaCl, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, complete protease inhibitor cocktail [Roche]). Anti-Elm1p (y-640, Santa Cruz Biotechnology, 6 μg) was bound to the PrG-Sepharose at 4°C for 1 hr, and the beads werewashed again and were resuspended in IP buffer. Anti-Elm1p-PrG-Sepharose was added to 350 μg yeast lysate, such that each IP received 1.5 μg antibody, and incubated on a roller mixer for 2 hr at 4°C. The Sepharose was then washed 3 times with 1 ml IP buffer, 3 times with 1 ml IP buffer containing 1 M NaCl, and two times with 1 ml HEPES buffer (50 mM Na HEPES [pH 7.4], 1 mM DTT, 0.02% Brij-35). To prepare the immunoprecipitated samples for gel analysis, the HEPES buffer was aspirated off the Sepharose beads, which were resuspended in 20 μl NuPAGE sample buffer (InVitrogen), then incubated at 100°C for 5 min. The PrG-Sepharose was removed by centrifugation, and the 20 μl sample was run on a NuPAGE 4%–12% Bis-Tris gel (InVitrogen) with MOPS running buffer. The separated proteins were transferred to nitrocellulose and were probed with anti-Elm1p (yN-19, Santa Cruz Biotechnology), then with PrG-HRP.

The *SNF1* gene was deleted in *S. cerevisiae* strains CSY102 and CSY103 by homologous recombination with a deletion cassette amplified as described for the construction of strain CSY101, except that the template was pFA6a-TRP1. This cassette was then transformed into *S. cerevisiae* strains CSY102 and CSY103, and transformants were selected on SDO-Trp medium. Integration of the cassettes into the *SNF1* gene was confirmed by PCR.

Microscopy

Colonies were examined with a Wild Heerbrugg M37 dissecting microscope with a digital camera mounted. Cells were prepared for differential interference contrast microscopy by resuspending a small portion of a 2 mm diameter colony in 50 μl YPD containing 1 M sorbitol. The suspension was briefly vortexed, 2 μl of the suspension was transferred to a microscope slide, and a coverslip was added. DIC microscopy was performed by using a DeltaVision Restoration Microscope (Applied Precision) built around a Nikon Inverted microscope fitted with a 100×/1.4 NA PlanApo lens.

Western Blotting of HA-Tagged Mig1p

Mig1 protein was tagged with three copies of the haemaglutinin epitope (HA) [S10]. The abundance and phosphorylation state of Mig1 was detected by Western blotting [S11] by using a monoclonal antibody against the HA epitope (Santa Cruz Biotechnology).

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