

The nuclear exportin Msn5 is required for nuclear export of the Mig1 glucose repressor of *Saccharomyces cerevisiae*

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Background: Mig1 is a transcriptional repressor responsible for glucose repression of many genes in the budding yeast *Saccharomyces cerevisiae*. Glucose regulates Mig1 function by affecting its phosphorylation, which is catalyzed by the Snf1 protein kinase. Phosphorylation alters the subcellular localization of Mig1, causing it to be nuclear when glucose is present, and cytoplasmic when glucose is absent.

Results: Here, we report that Msn5, a member of the importin β family of nuclear transport receptors, is required to export Mig1 from the nucleus when glucose is removed. Mig1 and Msn5 interacted in a yeast two-hybrid assay. Within the portion of Mig1 that regulates its nuclear transport, we found a region that directed its nuclear export. Within this region, two leucine-rich sequences similar to known nuclear export signals were not required for Mig1 export. The corresponding domain of the yeast *Kluyveromyces lactis* Mig1 conferred glucose-regulated Msn5-dependent protein export from the nucleus in *S. cerevisiae*. Sequence comparison with *S. cerevisiae* Mig1 revealed short patches of homology in *K. lactis* and *K. marxianus* Mig1 that might be Msn5-interaction domains. These regions overlapped with the serine residues predicted to be Snf1 phosphorylation sites, suggesting that Msn5 and Snf1 recognize similar sequences in Mig1. Altering these serines abolished glucose-dependent phosphorylation of Mig1 and caused it to be a constitutive repressor that was retained in the nucleus.

Conclusions: Mig1 contains a new nuclear export signal that is phosphorylated by Snf1 upon glucose removal, causing it to be recognized by the nuclear exportin Msn5 and carried out of the nucleus into the cytoplasm where it contributes to derepression of glucose-repressed genes.

Background

Glucose represses the expression of many genes in the budding yeast *Saccharomyces cerevisiae* (for recent reviews, see [1,2]). A key component of the glucose repression mechanism is the Mig1 transcriptional repressor [3,4], which binds to sequences in the promoters of many glucose-repressed genes and recruits the general transcriptional repressors Ssn6 and Tup1 [5–7]. The subcellular localization of Mig1 is regulated by glucose: it is quickly transported into the nucleus upon glucose addition, and it rapidly leaves the nucleus after removal of glucose from the medium [8]. Export of Mig1 from the nucleus seems to be regulated by its phosphorylation, probably catalyzed by the Snf1 protein kinase [8–10]. Dephosphorylation of Mig1 might be the signal that initiates its nuclear import, leading to transcriptional repression [8,11,12].

Proteins enter and exit the nucleus through the nuclear pore, a large protein complex that regulates access to the nucleus (for recent reviews, see [13–15]). Proteins larger than about 50 kDa are carried through the pore by protein

receptors. A family of nuclear transport receptors in yeast has been identified from their sequence homology to importin β , the first characterized nuclear transport receptor [16]. Each receptor is thought to mediate transport of a unique set of proteins by binding to specific sequences in the cargo molecules (for reviews, see [17,18]). Transport receptors involved in nuclear import (importins) bind to nuclear localization signals (NLSs), the best characterized of which is rich in basic amino acids and is recognized by the importin α - β heterodimer. Receptors mediating nuclear export (exportins) bind to nuclear export signals (NESs). The first exportin discovered was Xpo1 (Crm1), which mediates export of proteins that contain short, leucine-rich NESs [19–22].

Proteins that function in the nucleus are commonly controlled by regulating their nuclear transport (for reviews, see [23–25]). Many proteins are transported from the cytoplasm to the nucleus as a consequence of a specific activating signal; some are transported back to the cytoplasm when the signal is removed. This probably occurs through

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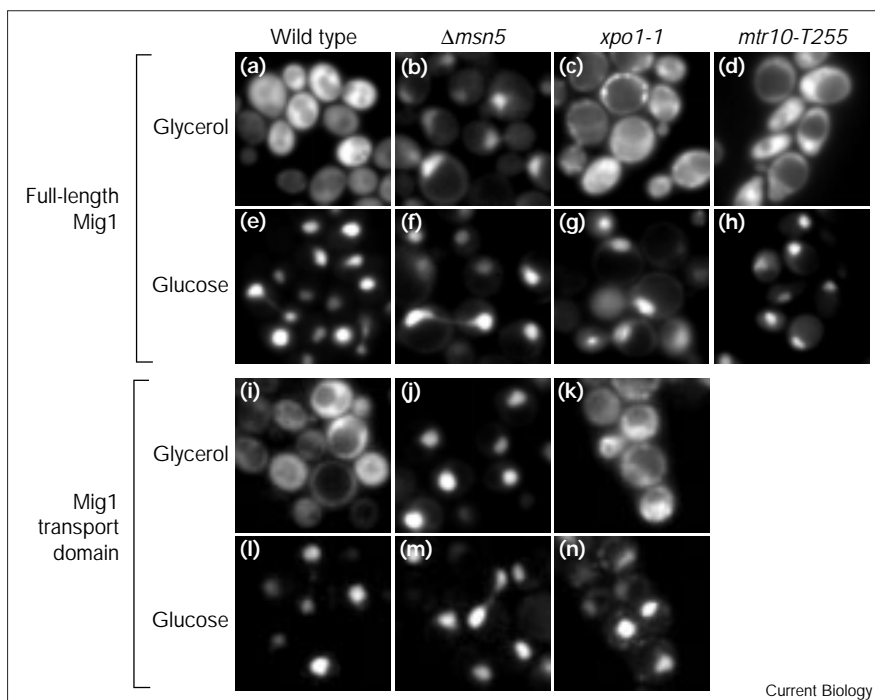
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Figure 1



Msn5 is required for export of Mig1 from the nucleus. (a–h) Wild-type (YM4342) and $\Delta msn5$ (FM414), $xpo1-1$ (FM416) and $mtr10-T255$ (FM419) mutant strains expressing a GFP–Mig1 (full-length) fusion protein (encoded by plasmid pBM3315) were (e–h) grown overnight on YM-uracil (minimal medium lacking uracil) + 2% glucose then (a–d) transferred to YM-uracil + glycerol for 20 min to induce export before imaging. The $xpo1-1$ and $mtr10-T255$ strains were incubated at 35°C for 1 and 3 h, respectively, before shifting to glycerol. (i–n) Sequences encoding the transport domain (amino acids 217–400) of Mig1 were fused to sequences encoding a GFP– β -galactosidase reporter (GFP– β -gal; pBM3495) and expression of the fusion protein analyzed as described above.

regulation of the interaction of the protein with its transport receptors, as has been shown for Pho4 [26,27], of which phosphate-regulated nuclear localization is mediated by the Pse1 importin [27] and the Msn5 exportin [26]. The exportin Xpo1 mediates regulated export of NF-AT [28], Yap1 [29] and cyclin B [30].

Msn5 is an importin β homolog implicated in glucose repression. *MSN5* was initially identified as a high-copy suppressor of a *snf1* mutation [31,32]. A *snf1* mutant is unable to grow on sucrose because it is unable to phosphorylate Mig1, causing it always to be in the nucleus, repressing *SUC2* expression [8]. High levels of the Mig1 export receptor would be expected to restore growth by exporting enough Mig1 to relieve repression. Here, we report that Msn5 does indeed mediate the export of Mig1 in response to glucose removal. Our results suggest that Msn5 recognizes a unique NES that requires phosphorylation by the Snf1 protein kinase to initiate export from the nucleus.

Results

Msn5 is required for the export of Mig1 from the nucleus

In the absence of glucose, a fusion protein between the green fluorescent protein (GFP) and Mig1 (GFP–Mig1) was localized in the cytoplasm in a wild-type *S. cerevisiae* strain (Figure 1a), but in the nucleus in an *msn5* mutant (Figure 1b). Two other nuclear transport receptors appeared to play little if any role in Mig1 export: incubation of an *xpo1* or *mtr10* mutant at the restrictive temperature for

1–3 hours inhibited mRNA export (data not shown) without affecting the rate of Mig1 export (Figure 1c,d). This was not because of an inability of Snf1 to phosphorylate Mig1 in *msn5* mutants (an event required for nuclear export of Mig1 and inhibition of its function [8–10]): Mig1 was phosphorylated within 5 minutes of glucose removal in an *msn5* mutant (data not shown). Thus, Msn5 functions after phosphorylation of Mig1 occurs. Msn5 is not involved in import of Mig1 into the nucleus because this was normal in a $\Delta msn5$ mutant (Figure 1f), and addition of glucose to $\Delta msn5$ cells grown in glycerol induced import of the small portion of Mig1 that remains in the cytoplasm (data not shown). The similarity of Msn5 to *bona fide* nuclear import receptors, and its requirement for normal export of Mig1 from the nucleus suggests that Msn5 is an exportin for Mig1.

Mig1 interacts with Msn5

Msn5 and amino acids 1–400 of Mig1 interacted in the yeast two-hybrid assay (Figure 2). The interaction was strongest when Msn5 was fused to the Gal4 DNA-binding domain (BD–Msn5) and Mig1 to the Gal4 transcriptional-activation domain (AD–Mig1). No interaction was detectable between BD–Mig1 and AD–Xpo1 (Figure 2).

Identification of the nuclear transport domain

Amino acids 261–400 of Mig1 are sufficient for glucose-dependent nuclear transport [8], but export mediated by this region is somewhat slower than for the full-length protein. Addition of amino-acid residues 217–260 restored

export to a rate identical to that of the full-length protein (Figure 3a–c). Thus, amino acids 217–400 contain all the sequences required for glucose-regulated nuclear transport and will be referred to as the transport domain. In a strain lacking *msn5*, a Mig1 (amino acids 217–400)–GFP– β -gal fusion was completely nuclear in the absence of glucose (Figure 1j), indicating that this region mediates Msn5-dependent export.

The leucine-rich regions in Mig1 are not required for export

The Mig1 transport domain contains two leucine-rich regions similar to nuclear export signals known to be required for export of some proteins from the nucleus [33,34]. A mutant version of Mig1 with all eight of these leucines and one valine changed to alanine was exported normally, demonstrating that these leucine-rich regions of Mig1 are not NESs (Figure 3d–f). This is also consistent with our observation that Xpo1, a nuclear exportin for proteins that contain the leucine-rich NES, is not the Mig1 exportin.

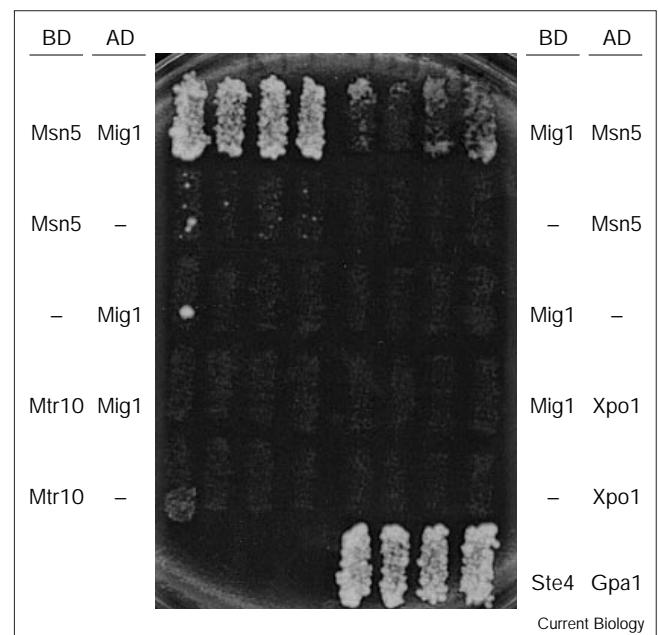
A 96 amino-acid region within Mig1 is required for export

To identify the sequences required for export of Mig1 from the nucleus, we fused various portions of the transport domain to the GFP– β -gal reporter (Figure 4). In the absence of any added sequence, GFP– β -gal was localized in both the cytoplasm and the nucleus (the β -galactosidase protein apparently contains a weak NLS), regardless of the carbon source (Figure 4, line 25). This made it useful for identifying polypeptides that direct import or export, because addition of an NLS would result in an exclusively nuclear localization; addition of an NES would result in an exclusively cytoplasmic localization. We assessed the ability of each Mig1–GFP– β -gal fusion protein to be exported from the nucleus rapidly when glucose was removed and to be imported into the nucleus rapidly upon addition of glucose, as well as localization after overnight growth on glucose or glycerol.

In the carboxy-terminal portion of the transport domain, a stretch of basic amino acids (residues 364–368) that is similar to known NLSs is likely to be the Mig1 NLS because amino acids 359–373 caused the GFP– β -gal reporter to be exclusively localized to the nucleus, with or without glucose (Figure 4, line 7). Any fusion protein lacking this putative NLS could not enter the nucleus (though we acknowledge that other sequences that could contribute to NLS function might be missing from the fusion protein); any containing it had the ability to be imported.

The exclusively cytoplasmic localization directed by amino acids 217–351 (Figure 4, line 13) suggests that the NES is within this region. The sequences directing export were further defined using a series of deletions from each side of this region. Removal of amino acids up to residue 244 had

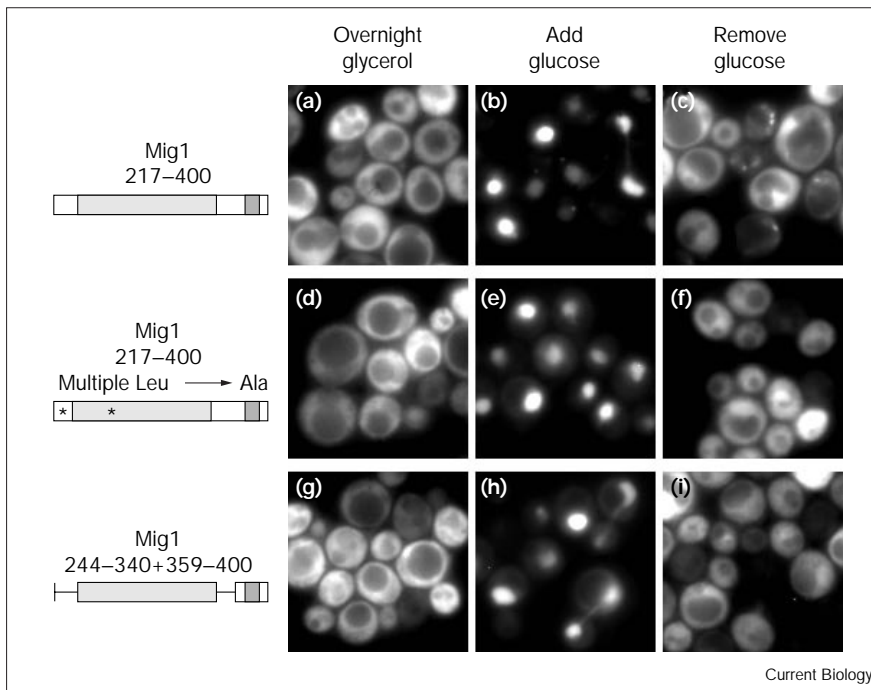
Figure 2



Mig1 interacts with Msn5. Yeast strain FM425 expressing proteins fused to either the Gal4 DNA-binding domain (BD) or Gal4 transcriptional-activation domain (AD) were grown on YM + 2% glucose plates lacking leucine, tryptophan and adenine to assay the ability of the fusion proteins to induce expression of the *GAL2–ADE2* reporter. All patches grew when plated on YM + 2% glucose plates lacking leucine and tryptophan but containing adenine (data not shown). Four separate transformants for each interaction were plated. A dash indicates empty vector. The strains expressing BD–Mig1 and AD–Msn5 grew only slightly slower than strains expressing BD–Msn5 and AD–Mig1, but the former strains did not image as well because they turned a reddish color. Identical results were obtained when the yeast were grown on media lacking leucine, tryptophan, histidine and containing 10 mM aminotriazole to assay the ability of the fusion proteins to induce expression of the *GAL7–HIS3* reporter, except that the strain containing BD–Mtr10 and AD–Mig1 also grew (data not shown). The Mig1 fusions lack the Mig1 transcriptional-repression domain located in the carboxy-terminal 25 amino acids [53] because these sequences interfere with transcriptional activation: no interaction could be detected with full-length Mig1 (data not shown). Units of β -galactosidase (expressed from the *GAL7–lacZ* reporter) were 6.8 ± 0.34 in the strain containing BD–Msn5 and AD–Mig1; 1.5 ± 0.17 in the strain containing only BD–Msn5; and 0.72 ± 0.19 in the strain containing only AD–Mig1.

no effect on export (Figure 4, line 2), but removal of the next nine amino acids caused an export defect (Figure 4, line 3). The amino-terminal side of the Mig1 NES is therefore between amino acids 244 and 253. A series of fusions were made lacking sequence between amino acid 320 and 359 (the Mig1 NLS — amino acids 359–400 — was included in this series of fusions to permit glucose-induced nuclear loading). Removal of amino acids 340–359 (Figure 4, lines 8 and 9) had no effect on export, but removal of 10 more amino acids (to residue 330; Figure 4, line 10) prevented normal export. The carboxy-terminal boundary of the NES is, therefore, between amino acids 330 and 340. Other internal deletions that remove any

Figure 3



The minimal NES of Mig1 is a 96 amino-acid region that does not depend on two leucine-rich regions. Mig1 sequences were fused to GFP-β-gal and expressed in a wild-type yeast strain (YM4342). The localization of each Mig1-GFP-β-gal fusion protein was imaged after (a,d,g) overnight growth on YM-uracil + 5% glycerol, and then (b,e,h) 10–15 min after adding glucose to a concentration of 2%. The ability of the Mig1 sequence to promote export from the nucleus was then determined by imaging the cells (c,f,i) 15–20 min after washing away the glucose. (a–c) Amino acids 217–400 (the transport domain; pBM3495) contained all the sequences necessary for glucose-regulated nuclear import and export. (d–f) Mutation of leucines 224, 226, 299, 312, 313, 315, 318, 319 and valine 320 to alanine (pBM3672) had no effect on export of the transport domain from the nucleus. (g–i) Amino acids 244–340 (pBM3681) were sufficient for nuclear export upon removal of glucose. The Mig1 NLS is included in residues 359–400 and was included in this construct to allow its nuclear entry. The light-shaded region represents the minimal Mig1 NES; the dark-shaded region represents the Mig1 NLS. The asterisks denote the two leucine-rich sequences in the Mig1 NES.

sequence between amino acids 244 to 340 also prevented export (Figure 4, lines 14–17). The minimal sequence required for export therefore includes amino acids 244–340 (Figure 3i and Figure 4, line 12). Although this 96 amino-acid region was required for normal glucose-regulated export, smaller portions exhibited some export activity that could be detected when the Mig1 NLS was absent (leaving only the weak β-galactosidase NLS); for example, some regions of the transport domain promoted an exclusively cytoplasmic localization (Figure 4, lines 18–20).

Only the Snf1 kinase sites are conserved in three Mig1 orthologs

To identify other amino acids important for export and import we took advantage of the fact that Mig1 orthologs in two *Kluyveromyces* species, *K. lactis* and *K. marxianus*, are functional in *S. cerevisiae* and regulated by glucose in a Snf1-dependent manner [35,36]. Outside of the DNA-binding domain, only a few scattered patches of sequence are conserved between the three proteins (Figure 5) [36]. Comparison of these sequences led us to predict which sequences comprise the transport domains of *K. lactis* Mig1 (KIMig1) and *K. marxianus* Mig1 (KmMig1). Indeed, a portion of this region of KIMig1 (amino acids 261–390) conferred on the GFP-β-gal reporter nuclear transport properties indistinguishable from the *S. cerevisiae* Mig1 (ScMig1) transport domain (Figure 6a–c). The rate of transport following removal or addition of glucose was

identical to that obtained with the ScMig1 transport domain (data not shown). Export of the KIMig1 transport domain also depended on Msn5 (Figure 6d). The localization of the KmMig1 transport domain was not determined but, as *KmMIG1* can complement a *mig1Δ* mutation in *S. cerevisiae* [36], its localization is also likely to be glucose-regulated and require Msn5 for export.

The known or likely transport domains of these three proteins are similar in only three regions (Figure 5). Both KIMig1 and KmMig1 have a stretch of basic amino acids in the carboxy-terminal portion that in ScMig1 was shown to direct nuclear import and is likely, therefore, to be the NLS in each homolog. Within the region required for export in ScMig1, there were only two patches of similarity with KIMig1 and KmMig1, which, interestingly, included two of the four predicted Snf1 phosphorylation sites (serines 278 and 311). The phosphorylated serines (Figure 5, circled Ps) and the amino acids thought to be required for recognition by the Snf1 kinase (Figure 5, asterisks) are conserved in all three proteins.

Predicted Snf1 sites are required for the export of Mig1 upon glucose removal

Four serines in Mig1 (positions 222, 278, 311 and 381) are predicted to be Snf1 kinase phosphorylation sites [10,37], and this appears to be the case because changing these residues to alanine abolished the Mig1 phosphorylation

Figure 4

Domains required for Mig1 nuclear export. The indicated Mig1 sequences were expressed as fusion proteins with GFP-β-gal (pBM3098) in a wild-type yeast strain (YM4342) and assayed for the ability to promote glucose-regulated export or import; +, most of the fusion protein was exported to the cytoplasm or imported into the nucleus 15–30 min after changing the carbon source; –, no significant change in localization was apparent 15–30 min after changing the carbon source. Subcellular localization was also determined after overnight growth on YM-uracil + 2% glucose or YM-uracil + 5% glycerol; N, predominantly nuclear; C, predominantly cytoplasmic; N/C, distributed equally between nucleus and cytoplasm (this is the default state of the GFP-β-gal reporter when no transport activity is provided by the added sequence); C/N, predominantly cytoplasmic but partly nuclear; N/A, transport activity could not be ascertained because the fusion protein was either always in the nucleus so it was not possible to determine whether glucose induces nuclear import, or always in the cytoplasm so it was not possible to determine whether it could be exported from the nucleus when glucose was removed. The transport domain is schematically illustrated at the top; dark grey box (amino acids 244–340), the minimal export sequence; light grey box (amino acids 359–370), the nuclear import signal; asterisks, Snf1 phosphorylation sites. The portion of the transport domain included in each fusion protein is represented by a solid bar and the numbers above the bar indicate the exact ends of the Mig1 sequence in each fusion protein.

Line	NES	NLS	Glucose regulated		Localization on	
			Export	Import	Glucose	Glycerol
1	217	400	+	+	N	C
2	244		+	+	C/N	C
3	253		–	+	N	C
4	276		–	+	N	C
5	290		–	+	N	C/N
6	335		–	N/A	N	N
7	359	373	–	N/A	N	N
8	350		+	+	N	C
9	340		+	+	N	C
10	330		–	N/A	N	N
11	320		–	N/A	N	N
12	244	340	+	+	N	C
13		351	N/A	–	C	C
14	237	305	–	+	N	N/C
15	237	335	–	+	N	N/C
16	237	359	–	N/A	N	N
17	301	335	–	+	N/C	C
18	275		N/A	–	C	C
19	261	351	N/A	–	C	C
20	290	351	N/A	–	C	C
21		321	–	–	N/C	N/C
22	290	324	–	–	N/C	N/C
23	261	301	–	–	N/C	N/C
24	237		–	–	N/C	N/C
25	GFP-β-gal (no Mig1 sequence added)		–	–	N/C	N/C

that occurs in the absence of glucose, and impaired derepression of *SUC2* [10] and *GALI* (Figure 7e). Changing all four serines to alanine caused both full-length Mig1 and a protein containing only its transport domain to remain in the nucleus in the absence of glucose (Figure 7a,c). Phosphorylation of Mig1 appears to promote its export, possibly by directing its interaction with Msn5. A small fraction of the full-length Mig1 in which the four Snf1 phosphorylation sites were mutated was still cytoplasmic in the absence of glucose, and entered the nucleus on addition of glucose (data not shown), suggesting that there are other phosphorylation sites in Mig1. These additional sites must lie

outside amino acids 217–400 because the transport domain lacking the four Snf1 phosphorylation sites appeared to be exclusively nuclear in the absence of glucose.

The ability of Mig1 to be exported from the nucleus correlated with the number of Snf1 phosphorylation sites it possesses. Mutants with single, double and triple Ser→Ala substitutions in the Snf1 sites were all defective in nuclear export of Mig1, with the extent of the defect correlating

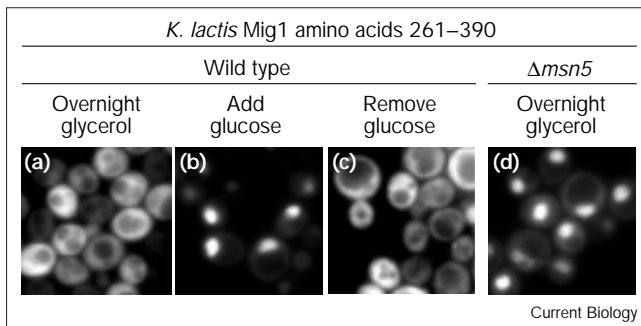
Figure 5

ScMig1 244	R T V F I D G P E Q K Q L Q Q Q Q N S L S P R Y S N T V I	L P R P R S L T	D F Q G L N N A N P N N N G S L R A Q T Q
KiMig1 271	S Q Q N L V H L H H P A P N R P L T E F V D N E Y I S N G	L P R T R S W T	N L S E Q Q S P S G F S - - - - -
KmMig1 342	P A A S L P H L Q Q V S S N - - - G F L D T N G H A A S	V A R N K S W T	N L S G V M P S P S E S G - - - - -
ScMig1 302	S S V Q L K R P S S V L S L N D L I V G Q - R N T	N E S D S D F T T G G E	D E E D G L K D P S N S S I D N L E Q D Y
KiMig1 320	S S A L N S R F S S S N S L N Q L I D Q H S R N S	S T V S I S T L L K Q E	T V I S Q D E D M S T E D A Y - - - - -
KmMig1 387	S S A L V S R F S S S A S L N K L M D P S S R T S	S A V S I A T L M N E D	K L Q S Q D D L S V V D E F - - - - -
ScMig1 359	L Q E Q S R K K S K T S T P	T T M L S R S	379
KiMig1 372	- - G R P L K K S K A I M P	I M R P S S T	390
KmMig1 438	- - G R S R K K S K T S T P	I R R P S S	456

Comparison of the transport domains in Mig1 orthologs from three yeast species identifies similar sequences in the regions required for nuclear import and export. Alignment of the sequences of the known transport domain of *S. cerevisiae* Mig1 (ScMig1) and the corresponding region in the Mig1 orthologs of *K. lactis* (KiMig1) and *K. marxianus* (KmMig1). Dark grey, identical amino acids; light grey,

conserved amino acids; circled P, a serine residue predicted to be phosphorylated by the Snf1 kinase; asterisks, amino acids thought to be important for recognition by the Snf1 kinase [29]. The alignments were made by the Clustal method with the Megalign protein alignment program and adjusted manually.

Figure 6



Localization directed by the *K. lactis* Mig1 transport domain. KIMig1 amino acids 261–390 were fused to GFP- β -gal (pBM3699) and expressed in (a–c) wild-type (YM4342) and (d) Δ *msn5* *S. cerevisiae* strains. The assays for nuclear import and export were carried out as described in Figure 4.

with the number of altered sites (Figure 8a–e). Mig1 became increasingly nuclear in glycerol-grown cells as more Snf1 kinase sites were removed. Likewise, the ability of Mig1 to repress transcription in the absence of glucose increased as more Snf1 kinase sites were removed. Thus, the four Snf1 kinase sites seem to contribute additively to the regulation of Mig1.

Msn5 is only partially responsible for the regulation of Mig1 function

Surprisingly, Mig1 function appeared to be regulated normally in the absence of Msn5: *GAL1* expression was derepressed normally in an *msn5* mutant (7229 ± 1868

units of β -galactosidase activity in glycerol-grown *msn5* mutant cells; 8437 ± 2100 units in the wild type) despite the fact that Mig1 was always in the nucleus. Mig1 function must, therefore, be regulated by another mechanism. This additional regulatory mechanism also responds to phosphorylation by Snf1, because Mig1 missing all four Snf1 consensus phosphorylation sites was a constitutive repressor (Figure 7e).

Discussion

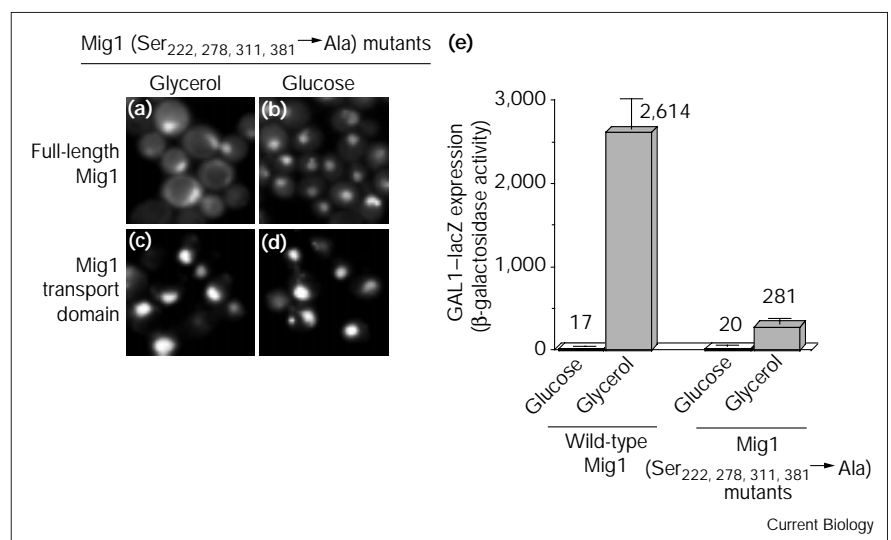
Msn5 appears to be a nuclear exportin for several regulatory proteins

Msn5, an importin β homolog that was isolated as a high-copy suppressor of a *snf1* mutation, is probably the export receptor for Mig1, because deletion of Msn5 abolished export of full-length Mig1 and of a protein containing the transport domain of Mig1, and Msn5 and Mig1 interacted *in vivo*. Although we have not yet been able to demonstrate a Mig1–Msn5 interaction *in vitro*, it seems probable that Msn5 acts directly on Mig1.

Our results explain the fact that Msn5 is a high-copy suppressor of mutations that reduce Snf1 function [31,32]. Because Snf1 is required for export of Mig1 from the nucleus, Mig1 is always in the nucleus repressing *SUC2* expression in a mutant with crippled Snf1, thereby preventing its growth on sucrose. High levels of Msn5 apparently result in enough export of Mig1 from the nucleus, even when Snf1 activity is severely reduced, to derepress *SUC2* and permit growth on sucrose. Indeed, we found that overexpression of Msn5 caused Mig1 to accumulate in the cytoplasm in the presence of glucose (data not shown).

Figure 7

Mutation of the predicted Snf1 phosphorylation sites in Mig1 prevents nuclear export. (a–d) Full-length Mig1 fused to GFP (pBM3726), and the transport domain fused to GFP- β -gal (pBM3838), both with serines 222, 278, 311 and 381 mutated to alanine, were expressed in wild-type yeast (YM4342) and their localization determined after growth on (a,c) YM-uracil + 5% glycerol or (b,d) YM-uracil + 2% glucose (long exposure times, which were identical for samples grown on both carbon sources, were required to image the poorly expressed full-length mutant Mig1–GFP, resulting in the appearance of high cytoplasmic background fluorescence). (e) Wild-type Mig1 and the Mig1 (Ser^{222, 278, 311, 381} → Ala) mutant were expressed in a *mig1* Δ mutant containing a *GAL1-lacZ* reporter integrated in the genome (YM4374); β -galactosidase activity was measured after growth on YM-uracil + 4% glucose or YM-uracil + 5% glycerol. Assays were carried out on duplicate cultures of three independent



transformants grown to early logarithmic phase. We believe the small amount of glucose repression that remains in the

mutant is due to phosphorylation at a fifth consensus Snf1 site present in Mig1, possibly Ser108 [9].

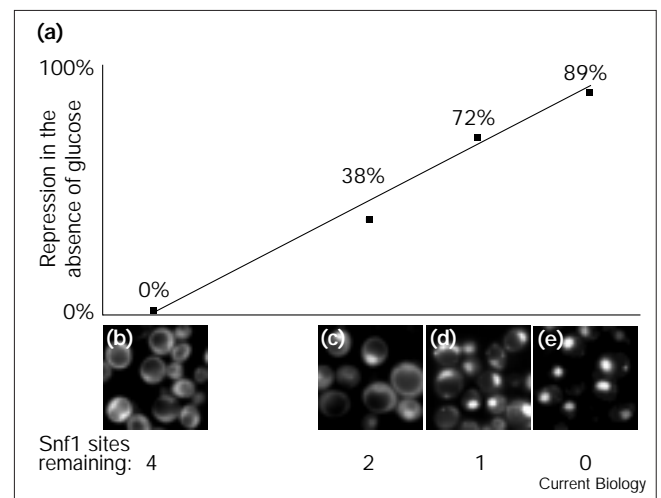
Msn5 is also the nuclear exportin for Pho4. Msn5 interacts only with the phosphorylated form of Pho4, which is generated by the Pho85–Pho80 cyclin-dependent kinase (CDK)–cyclin complex [26,38]. Because this protein kinase recognizes a target sequence that is different from that recognized by Snf1, Msn5 probably does not recognize a specific primary sequence, but rather perhaps a specific secondary structure. This would explain why such a large region of Mig1 (96 amino acids) is required for its nuclear export. Msn5 has also been shown to be responsible for the export of Msn2, an activator of stress-response genes (C. Schüller and H. Ruis, personal communication). Mutations in Msn5 also affect calcium-induced transcription [39,40] and cause defects in the mating pathway [41]. It seems probable, therefore, that Msn5 is responsible for the nuclear export of several transcription factors or signal transduction proteins that are regulated by environmental signals. Because the loss of Msn5 causes no obvious growth defect in yeast, and we observed no defect in mRNA export in an *msn5* mutant (our unpublished observations), it must transport a limited set of non-essential proteins.

Mig1 contains a new NES that is activated by phosphorylation

A 184 amino-acid transport domain within Mig1 is sufficient for its glucose-regulated nuclear import and export. Present in this region is an NLS, and two short sequence stretches rich in leucine that resemble many previously characterized NESs. These leucine-rich sequences were, however, not responsible for the export of Mig1 (Figure 3). Mig1 appears, therefore, to contain a new NES. The smallest portion of Mig1 that supported regulated nuclear export consisted of 96 amino acids. We cannot say at this time whether this region contains sequences for interaction with Msn5, or is required for phosphorylation of Mig1 by Snf1, or both. Such a large NES would be unusual: most that have been identified consist of 8–20 amino acids [33]. Several smaller non-overlapping portions of this region promoted export (Figure 4) that was dependent on Msn5 (data not shown), but only in the presence of a weak NLS (like the one that fortuitously exists in β -galactosidase), as if nuclear export activity was not due to a single strong NES, but instead was the sum of several weaker NESs scattered throughout the region.

Only two patches of sequence within the minimal export domain of *S. cerevisiae* were found to be conserved in the two *Kluyveromyces* Mig1 orthologs. It is significant that these patches of sequence included two putative Snf1 phosphorylation sites. The phosphorylated serines and the amino acids thought to be recognized by the Snf1 kinase, along with a few of the surrounding amino acids, were conserved in all three proteins. The conservation of these sequences in all three Mig1 proteins suggests they are important for export, and therefore are likely to be the sites of Msn5 interaction. The suggestion that these

Figure 8

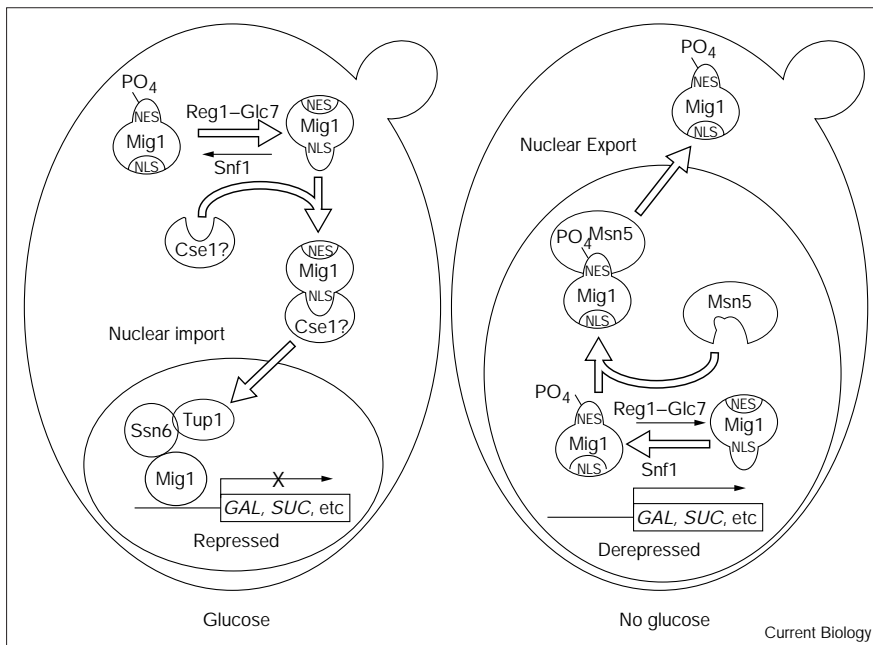


The amount of glucose repression and Mig1 in the nucleus correlates with the number of Snf1 sites present. (a) Full-length Mig1 possessing no Snf1 phosphorylation sites (Ser_{222,278,311,381}→Ala; pBM3726), one remaining site (Ser_{278,311,381}→Ala; pBM3582), two remaining sites (Ser_{278,381}→Ala; pBM3728), or all four sites intact (pBM2433) were introduced into a Δ *mig1* mutant containing a *GAL1-lacZ* reporter (YM4374) and grown in YM-uracil + 5% glycerol; β -galactosidase activity was measured in duplicate cultures of three independent transformants grown to early logarithmic phase. Repression is expressed as the percentage of expression of *GAL1* in the mutants growing on glycerol relative to wild type (fully induced, or 0% repression). Standard deviations were less than 25% for all samples. (b–e) Localization of fusion proteins between the Mig1 transport domain and GFP- β -gal in strain YM4374, determined after growth on YM-uracil + 5% glycerol. The fusion proteins differed in the number of intact Snf1 phosphorylation sites: all sites intact (pBM3495); two remaining sites (Ser_{278,381}→Ala, pBM3865); one remaining site (Ser_{278,311,381}→Ala, pBM3866); no remaining sites (Ser_{222,278,311,381}→Ala; pBM3867).

regions include sites likely to be phosphorylated by Snf1 implies that Snf1 plays a direct role in regulating nuclear export of Mig1. This idea is strongly supported by the observation that mutation of the serines at these sites (along with two other serines in Snf1 sites outside this region) abolished nuclear export of Mig1.

Although only two of the four putative Snf1 phosphorylation sites in Mig1 occur within the region identified as the minimal export domain, the other sites are involved in regulation of nuclear export because mutation of all four sites in Mig1 was required to almost completely abolish Mig1 nuclear export (Figure 8). Conversion of any two or three sites to alanine caused only a partial defect in Mig1 nuclear export (data not shown). It also appeared that at least one more site outside of the transport domain (possibly Ser108 [9]) is required for complete regulation of full-length Mig1, because a small fraction of full-length protein lacking the four serines of the Snf1 sites was still exported from the nucleus. The two Snf1 phosphorylation sites within the minimal NES are, therefore, sufficient to

Figure 9



Proposed mechanism for glucose control of Mig1 nuclear localization and its role in regulating glucose repression. PO₄, phosphate groups. See text for details. Our preliminary evidence suggests that Cse1 might be the nuclear importin for Mig1.

promote export of this region, independent of the rest of the molecule. Perhaps the other sites contribute additional Msn5-binding sites.

Is Mig1 nuclear import or export or both glucose-regulated?

Our model implies that both import into and export from the nucleus of Mig1 are regulated by carbon source. We have provided evidence that nuclear export is regulated through Snf1: export of Mig1 occurred only when glucose removal promoted the Snf1-dependent phosphorylation of Mig1 that makes it a target for Msn5. Evidence for glucose-regulated import came from analysis of deletions of the transport domain. Sequences within amino acids 253–335 lacked export activity, but prevented import until glucose was added. If this region was removed, Mig1 import became glucose independent. Although these results suggest that a region in Mig1 confers glucose regulation of nuclear import independent of nuclear export activity, further work is needed to solidify this hypothesis. Pho4 is regulated similarly: high phosphate activates its nuclear export and low phosphate activates the import pathway [26,27].

Mig1 is regulated at two levels

We were surprised to find that glucose still regulated the ability of Mig1 to repress gene expression in an *msn5* mutant. This indicates that glucose regulates two functions of Mig1: its nuclear localization (through Msn5), and its ability to repress transcription (possibly by regulating its ability to work with the general repressors Ssn6 and Tup1). Both of these functions are regulated by Snf1-catalyzed

phosphorylation of Mig1, because Mig1 lacking all four Snf1 phosphorylation sites was always in the nucleus (Figure 7a–d) and was a constitutive repressor (Figure 7e). The same situation occurs for other proteins whose nuclear localization is regulated by Msn5: regulation of Pho4 function by phosphate levels occurs normally in an *msn5* mutant [42]; Msn2 is regulated by cellular stress in an *msn5* mutant (C. Shüller and H. Ruis, personal communication). Yeast cells appear to have evolved multiple mechanisms for stringent regulation of transcription factor function.

Conclusions

Our results suggest the following mechanism for regulation of Mig1-mediated glucose repression (Figure 9). When glucose is added to cells, Snf1 is inhibited, and a phosphatase (possibly the Reg1–Glc7 complex) dephosphorylates Mig1. This promotes an interaction of Mig1 with its importin and/or weakens its interaction with Msn5, resulting in transport of Mig1 into the nucleus where it binds target genes and recruits Ssn6 and Tup1 to repress transcription. Upon glucose removal, Snf1 is activated, phosphorylates Mig1 at four (possibly five) sites, which promotes interaction of Mig1 with Msn5 and results in Mig1 transport to the cytoplasm. The removal of Mig1 from the nucleus relieves transcriptional repression, leading to expression of the target genes.

Materials and methods

Yeast strains and growth

Yeast strains were derived from strain S288C (Table 1). Standard methods were used for genetic crosses, sporulation and tetrad dissection

Table 1

Yeast strains used in this study.		
Yeast strain	Genotype	Source
FM414	<i>MATα ura3-52 his3 lys2 msn5Δ::HIS3</i>	F. Estruch
FM416	<i>MATα ura3-1 his3-11,15 ade2-1 trp1-1 leu2-3,112 xpo1::Leu2 [xpo1-1 on pRS313]</i>	[22]
FM419	<i>MATα ura3-52 lys2-801 mtr10-T255</i>	[41]
FM423	<i>MATα Klyveromyces lactis wild type strain (K1282)</i>	S. Johnston
FM425	<i>MATα ura3-52 his3Δ200 trp1-901 leu2-3,112 gal4Δ gal80Δ GAL2-ADE2 Lys2::GAL1-HIS3 met2::GAL7-lacZ</i>	[50]
YM4342	<i>MATα ura3Δ::LEU2 his3Δ200 trp1-903 lys2-801</i>	
YM4374	<i>MATα ura3-52 his3Δ200 ade2-101 lys2-801 trp1-901 met gal80 mig1Δ::LEU2 LEU2::GAL1-lacZ</i>	
YM6153	<i>MATα ura3-52 his3 trp1::hisG lys2 msn5Δ::HIS3</i>	
YM6238	<i>MATα ura3-52 his3-200 ADE2 lys2-801 trp1-903 leu2-3,112 gal80? mtr10-T255</i>	

[43]. Yeast were grown at 30°C in rich (YP) or minimal (YM) medium containing the appropriate carbon source. Yeast transformations were carried out as described by Gietz *et al.* [44].

Plasmids. Standard procedures for the manipulation of plasmid DNA and transformation of bacteria were followed [45]. To ensure that no unintentional mutations were introduced into DNA fragments produced by the PCR for cloning, the relevant DNA segments were sequenced and/or three independently created clones were examined. All MIG1 sequences are derived from pBM2433 (carries the MIG1 XbaI-HindIII fragment from pMIG1 [4] inserted between the XbaI and HindIII sites of pRS316, a URA3-CEN plasmid [46]). The full-length Mig1-GFP fusion protein used to localize Mig1 (pBM3315) is functional, as it complements a mig1 mutation [8].

Site-directed mutagenesis to change leucines or serines in Mig1 to alanine

The various mutations that convert the Snf1 phosphorylation sites (serines 278, 311 and 381) to alanine were generated by site-directed mutagenesis using the PCR, as described in [47]. Briefly, each mutation was generated in two PCRs. First, *MIG1* sequence was amplified using one oligonucleotide primer that contains the mutations to change one of the sites. The second primer for this reaction was chosen to include sequence past a unique restriction enzyme site within *MIG1*. In a second PCR, the DNA fragment was extended in the opposite direction past another unique restriction enzyme site. The final PCR product was used to replace the wild-type sequence between the two restriction sites by recombination in yeast by cotransforming yeast with the

MIG1-containing plasmid (pBM2608) cut with the appropriate restriction enzymes, and the PCR product. Two of the Snf1 sites, serines 311 and 381, have adjacent phosphorylatable amino acids (Ser310 and Thr380) that were also changed to alanine. A DNA fragment containing a mutation that changes Ser222 to alanine was generously provided by M. Treitel and M. Carlson [10]. The various Snf1 site mutations were introduced into full-length Mig1 or the Mig1-GFP- β -gal fusion by recombination in yeast (as described in [47]). Mutations that change leucines 224, 226, 229, 312, 313, 315, 318, 319 and Val320 to alanine were generated by a similar method (described in detail in [47]). All mutations were verified by determining their DNA sequence changes. Oligonucleotides used to make the sequence changes are listed in Table 2.

Mig1-GFP- β -gal fusions

MIG1 coding sequence amplified from pBM2433 was inserted into the *Bam*HI site located between the *ADH1* promoter and the GFP coding sequence in plasmid pBM3098 (contains a fusion of the coding sequence of wild-type GFP upstream of the *Escherichia coli lacZ* gene in vector pVT103-U (2 μ , *URA3* selectable marker, [48])). The *MIG1* sequences were inserted by either of two methods. The first was standard restriction enzyme digestion and ligation: *MIG1* sequences were amplified by PCR with oligonucleotide primers that introduce a *Bgl*II site and an initiation codon before the *MIG1* sequence and a *Bam*HI site after the *MIG1* sequence. The DNA fragment was digested with *Bgl*II and *Bam*HI and then inserted into the *Bam*HI site of pBM3098 by ligation. The second method was homologous recombination in yeast: the *MIG1* sequences were amplified

Table 2

Oligonucleotides used to make site-directed mutations.		
Number	Sequence	Mutation
OM841	TTGAAGAGACCAGCAGCTGTTTAAAGTTTGAAC	Ser _{310,311} →Ala
OM977	CAAATTCGTACC GGCCGCGGATCTAC	Thr ₃₈₀ →Ala and Ser ₃₈₁ →Ala (non-coding strand)
OM1244	CCAAGTTCAGCTGCTAGcGCGAACGACGCGGCGGCTGGCCAAAGA	Leu _{312,313,315,318,319} →Ala and Val ₃₂₀ →Ala
OM1312	AGTGCCAGTAGAGCAAAGCAAACGCIAGCTCGTCCCTAC	Leu _{224,226,229} →Ala
OM1343	GAAAATCCGTTAAAGCTCGCGGCCTgGGTAATATAAC	Ser ₂₇₈ →Ala (non-coding strand)

The codons altered are italicized. Changes that add or remove a restriction enzyme site for diagnostic purposes are indicated in lower case letters.

with oligonucleotide primers that introduce sequence identical to that surrounding the *Bam*HI site in pBM3098 to each side of the *MIG1* sequence. The amplified DNA fragment was then cotransformed with *Bam*HI digested pBM3098 into yeast strain YM4342. Recombinant plasmids isolated from the Ura3⁺ yeast colonies were analyzed for the presence of the *MIG1* coding sequence in frame with the *GFP* coding sequence.

KIMig1 (amino acids 261–390)–GFP–β-gal fusion (pBM3699)

The *K. lactis* *MIG1* coding sequence was amplified from genomic DNA. This DNA fragment was inserted into the *Bam*HI site of pBM3098 by recombination in yeast as described above.

Gal4 fusions for the yeast two-hybrid interaction assay

All Gal4 fusions were made by recombination in yeast [49]. DNA fragments containing *MIG1*, *MSN5* or *MTR10* coding sequences for recombination were produced in two PCR steps. In the first PCR, DNA fragments containing the entire open reading frames of *MSN5* and *MTR10* were produced using Research Genetics Gene Pairs oligonucleotides for amplification from genomic DNA of YM701 (strain S288C) for *MTR10*, and from pBM3692 (*MSN5* in YE24, provided by F. Estruch) for *MSN5*. *MIG1* sequence was generated using pBM2433 and primers that amplify sequence coding for amino acids 1–400. In the second PCR, the DNA produced in the first reaction was further amplified to add sequence identical to that surrounding the cloning site in the vector and served to direct homologous recombination. The DNA fragments from the second PCR were cotransformed into yeast strain YM4342 with pOAD plasmid that had been cut with *Pvu*I and *Nco*I, for activation-domain fusions; or pOBD, for binding-domain fusions. Recombinant plasmids were isolated from Leu⁺ (for activation-domain fusions) or Trp⁺ (for binding-domain fusions) yeast colonies and analyzed for the presence of the proper hybrid sequences. The Gal4–*Msn5* fusion is functional because it complements the *Mig1* export defect of a Δ *msn5* yeast strain.

Yeast two-hybrid assay for Mig1–Msn5 interaction

Gal4 fusions to be tested for interaction were introduced into the two-hybrid strain PJ69-4A (FM425) [50]. Patches of four transformants for each pair were first grown on rich YPD plates then replica-plated to minimal media lacking tryptophan, leucine and adenine; and minimal media lacking tryptophan, leucine, histidine, and containing various concentrations of aminotriazole, to test for interaction. Assays of β-galactosidase activity were performed using a kit from Pierce (Rockford), according to the manufacturers instructions. Miller units (1000 × OD₄₂₀ / OD₆₀₀ × volume of cells assayed (ml) × time of assay (min)) are reported.

Imaging of GFP fluorescence

GFP fluorescence was imaged in living cells as described previously [10]. Briefly, 1–3 ml cultures of yeast were grown to an OD₆₀₀ of 0.4–0.8 in minimal media containing 2% glucose or 5% glycerol, centrifuged, washed in a nonfluorescent media (described in [51]) containing 2% glucose or 5% glycerol. Finally, the cells were resuspended in approximately 20–30 μl of the nonfluorescent media, and 1 μl of this was placed on an agar pad for microscopy.

Import/export assay

Cultures (1 ml) of yeast strains carrying GFP fusions were grown overnight on media containing 5% glycerol. Cells were concentrated by centrifugation and a 1 μl sample was taken for imaging ('overnight glycerol' sample). The cells were then resuspended in media containing 2% glucose for 10–20 min, concentrated by centrifugation and another 1 μl sample removed for imaging ('add glucose' sample). Finally the cells were washed two times with media containing 5% glycerol to remove glucose, concentrated by centrifugation and a 1 μl sample was imaged ('remove glucose' sample). Strains with temperature-sensitive mutations were grown at the permissive temperature (25°C) to early log phase, then incubated at a restrictive temperature (35°C) for appropriate times before, and during, carbon source shifts.

Measurement of glucose repression

Repression by *Mig1* was determined by measuring β-galactosidase expressed from a *GAL1–lacZ* fusion (pRY181) integrated at the *LEU2* locus as described previously [52], except cell densities (OD₆₀₀) and product formation (OD₄₂₀) were quantified in microtiter plates on a Molecular Devices plate reader. Yeast were grown in minimal medium lacking uracil and containing 2% glucose or 5% glycerol to mid-log phase (OD₆₀₀ of about 1.0). Duplicate yeast cultures from at least three independent clones were assayed for each plasmid construction.

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References

- Carlson M: Regulation of glucose utilization in yeast. *Curr Opin Genet Dev* 1998, 8:560-564.
- Gancedo JM: Yeast carbon catabolite repression. *Microbiol Mol Biol Rev* 1998, 62:334-361.
- Nehlin JO, Carlberg M, Ronne H: Control of yeast *GAL* genes by *MIG1* repressor: a transcriptional cascade in the glucose response. *EMBO J* 1991, 10:3373-3377.
- Nehlin JO, Ronne H: Yeast *MIG1* repressor is related to the mammalian early growth response and Wilms' tumour finger proteins. *EMBO J* 1990, 9:2891-2898.
- Treitel MA, Carlson M: Repression by *SSN6-TUP1* is directed by *MIG1*, a repressor/activator protein. *Proc Natl Acad Sci USA* 1995, 92:3132-3136.
- Tzamaras D, Struhl K: Distinct TPR motifs of *Cyc8* are involved in recruiting the *Cyc8-Tup1* corepressor complex to differentially regulated promoters. *Genes Dev* 1995, 9:821-831.
- Tzamaras D, Struhl K: Functional dissection of the yeast *Cyc8-Tup1* transcriptional co-repressor complex. *Nature* 1994, 369:758-761.
- DeVit MJ, Waddle JA, Johnston M: Regulated nuclear translocation of the *mig1* glucose repressor. *Mol Biol Cell* 1997, 8:1603-1618.
- Ostling J, Ronne H: Negative control of the *mig1p* repressor by *snf1p*-dependent phosphorylation in the absence of glucose. *Eur J Biochem* 1998, 252:162-168.
- Treitel MA, Kuchin S, Carlson M: *Snf1* protein kinase regulates phosphorylation of the *Mig1* repressor in *Saccharomyces cerevisiae*. *Mol Cell Biol* 1998, 18:6273-6280.
- Tu J, Carlson M: The *GLC7* type 1 protein phosphatase is required for glucose repression in *Saccharomyces cerevisiae*. *Mol Cell Biol* 1994, 14:6789-6796.
- Tu J, Carlson M: *REG1* binds to protein phosphatase type 1 and regulates glucose repression in *Saccharomyces cerevisiae*. *EMBO J* 1995, 14:5939-5946.
- Cole CN, Hammell CM: Nucleocytoplasmic transport: driving and directing transport. *Curr Biol* 1998, 8:R368-R372.
- Corbett AH, Silver PA: Nucleocytoplasmic transport of macromolecules. *Microbiol Mol Biol Rev* 1997, 61:193-211.
- Doye V, Hurt E: From nucleoporins to nuclear pore complexes. *Curr Opin Cell Biol* 1997, 9:401-411.
- Gorlich D, Dabrowski M, Bischoff FR, Kutay U, Bork P, Hartmann E, et al.: A novel class of RanGTP binding proteins. *J Cell Biol* 1997, 138:65-80.
- Pemberton LF, Blobel G, Rosenblum JS: Transport routes through the nuclear pore complex. *Curr Opin Cell Biol* 1998, 10:392-399.
- Wozniak RW, Rout MP, Aitchison JD: Karyopherins and kissing cousins. *Trends Cell Biol* 1998, 8:184-188.
- Fornerod M, Ohno M, Yoshida M, Mattaj JW: CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* 1997, 90:1051-1060.
- Fukuda M, Asano S, Nakamura T, Adachi M, Yoshida M, Yanagida M, et al.: CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature* 1997, 390:308-311.
- Ossarehazari B, Bachelier F, Dargemont C: Evidence for a role of *crm1* in signal-mediated nuclear protein export. *Science* 1997, 278:141-144.

22. Stade K, Ford CS, Guthrie C, Weis K: **Exportin 1 (crm1p) is an essential nuclear export factor.** *Cell* 1997, **90**:1041-1050.
23. Boulikas T: **Nuclear import of protein kinases and cyclins.** *J Cell Biochem* 1996, **60**:61-82.
24. Jans DA, Hubner S: **Regulation of protein transport to the nucleus: central role of phosphorylation.** *Physiol Rev* 1996, **76**:651-685.
25. Vandromme M, Gauthier RC, Lamb N, Fernandez A: **Regulation of transcription factor localization: fine-tuning of gene expression.** *Trends Biochem Sci* 1996, **21**:59-64.
26. Kaffman A, Rank NM, O'Neill EM, Huang LS, O'Shea EK: **The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus.** *Nature* 1998, **396**:482-486.
27. Kaffman A, Rank NM, O'Shea EK: **Phosphorylation regulates association of the transcription factor Pho4 with its import receptor Pse1/Kap121.** *Genes Dev* 1998, **12**:2673-2683.
28. Zhu J, McKeon F: **NF-AT activation requires suppression of Crm1-dependent export by calcineurin.** *Nature* 1999, **398**:256-260.
29. Yan C, Lee LH, Davis LI: **Crn1p mediates regulated nuclear export of a yeast AP-1-like transcription factor.** *EMBO J* 1998, **17**:7416-7429.
30. Yang J, Bardes ES, Moore JD, Brennan J, Powers MA, Kornbluth S: **Control of cyclin B1 localization through regulated binding of the nuclear export factor CRM1.** *Genes Dev* 1998, **12**:2131-2143.
31. Estruch F, Carlson M: **Increased dosage of the *MSN1* gene restores invertase expression in yeast mutants defective in the SNF1 protein kinase.** *Nucleic Acids Res* 1990, **18**:6959-6964.
32. Estruch F, Carlson M: **Two homologous zinc finger genes identified by multicopy suppression in a SNF1 protein kinase mutant of *Saccharomyces cerevisiae*.** *Mol Cell Biol* 1993, **13**:3872-3881.
33. Nakielny S, Dreyfuss G: **Nuclear export of proteins and RNAs.** *Curr Opin Cell Biol* 1997, **9**:420-429.
34. Wen W, Meinkoth JL, Tsien RY, Taylor SS: **Identification of a signal for rapid export of proteins from the nucleus.** *Cell* 1995, **82**:463-473.
35. Cassart JP, Georis J, Ostling J, Ronne H, Vandenhaute J: **The MIG1 repressor from *Kluyveromyces lactis*: cloning, sequencing and functional analysis in *Saccharomyces cerevisiae*.** *FEBS Lett* 1995, **371**:191-194.
36. Cassart JP, Ostling J, Ronne H, Vandenhaute J: **Comparative analysis in three fungi reveals structurally and functionally conserved regions in the mig1 repressor.** *Mol Gen Genet* 1997, **255**:9-18.
37. Dale S, Wilson WA, Edelman AM, Hardie DG: **Similar substrate recognition motifs for mammalian AMP-activated protein kinase, higher plant HMG-CoA reductase kinase-A, yeast SNF1, and mammalian calmodulin-dependent protein kinase I.** *FEBS Lett* 1995, **361**:191-195.
38. Kaffman A, Herskowitz I, Tjian R, O'Shea EK: **Phosphorylation of the transcription factor PHO4 by a cyclin-CDK complex, PHO80-PHO85.** *Science* 1994, **263**:1153-1156.
39. Matheos DP, Kingsbury TJ, Ahsan US, Cunningham KW: **Tcn1p/Crz1p, a calcineurin-dependent transcription factor that differentially regulates gene expression in *Saccharomyces cerevisiae*.** *Genes Dev* 1997, **11**:3445-3458.
40. Stathopoulos AM, Cyert MS: **Calcineurin acts through the crz1/tcn1-encoded transcription factor to regulate gene expression in yeast.** *Genes Dev* 1997, **11**:3432-3444.
41. Akada R, Kallal L, Johnson DI, Kurjan J: **Genetic relationships between the G protein beta gamma complex, Ste5p, Ste20p and Cdc42p: investigation of effector roles in the yeast pheromone response pathway.** *Genetics* 1996, **143**:103-117.
42. Komeili A, O'Shea EK: **Roles of phosphorylation sites in regulating activity of the transcription factor Pho4.** *Science* 1999, **284**:977-980.
43. Rose MD, Winston F, Heiter P. *Methods In Yeast Genetics: A Laboratory Manual.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 1990.
44. Gietz D, St Jean A, Woods RA, Schiestl RH: **Improved method for high efficiency transformation of intact yeast cells.** *Nucleic Acids Res* 1992, **20**:1425.
45. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: A Laboratory Manual.* Plainview, New York: Cold Spring Harbor Laboratory Press; 1989.
46. Sikorski RS, Hieter P: **A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*.** *Genetics* 1989, **122**:19-27.
47. DeVit M: **Glucose regulation of the Mig1 transcriptional repressor of the yeast *Saccharomyces cerevisiae*.** Washington University, Saint Louis, MO; 1999.
48. Vernet T, Dignard D, Thomas DY: **A family of yeast expression vectors containing the phage f1 intergenic region.** *Gene* 1987, **52**:225-233.
49. Hudson JJ, Dawson EP, Rushing KL, Jackson CH, Lockshon D, Conover D, *et al.*: **The complete set of predicted genes from *Saccharomyces cerevisiae* in a readily usable form.** *Genome Res* 1997, **7**:1169-1173.
50. James P, Halladay J, Craig EA: **Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast.** *Genetics* 1996, **144**:1425-1436.
51. Waddle JA, Karpova TS, Waterston RH, Cooper JA: **Movement of cortical actin patches in yeast.** *J Cell Biol* 1996, **132**:861-870.
52. Yocum RR, Hanley S, West R Jr, Ptashne M: **Use of *lacZ* fusions to delimit regulatory elements of the inducible divergent GAL1-GAL10 promoter in *Saccharomyces cerevisiae*.** *Mol Cell Biol* 1984, **4**:1985-1998.
53. Ostling J, Carlberg M, Ronne H: **Functional domains in the Mig1 repressor.** *Mol Cell Biol* 1996, **16**:753-761.

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