The Snf1 kinase controls glucose repression in yeast by modulating interactions between the Mig1 repressor and the Cyc8–Tup1 co-repressor

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Among lower eukaryotes, glucose repression is a conserved, widely spread mechanism regulating carbon catabolism. The yeast Snf1 kinase, the Mig1 DNA-binding repressor and the Mig1-interacting co-repressor complex Cyc8(Ssn6)–Tup1 are central components of this pathway. Previous experiments suggested that cytoplasmic translocation of Mig1, upon its phosphorylation by Snf1 in the nucleus, is the key regulatory step for releasing glucose repression. In this report we re-evaluate this model. We establish the coordinated repressive action of Mig1 and Cyc8–Tup1 on *GAL1* transcription, but we find that Cyc8–Tup1 is not tethered by Mig1 to the promoter DNA. We demonstrate that both negative regulators occupy *GAL1* continuously under either repression or activation conditions, although the majority of the Mig1 is redistributed to the cytoplasm upon activation. We show that Snf1-dependent phosphorylation of Mig1 abolishes interaction with Cyc8–Tup1, and we propose that regulation of this interaction, not the Mig1 cytoplasmic localization, is the molecular switch that controls transcriptional repression/de-repression.

Keywords: Mig1; Cyc8–Tup1; repression

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INTRODUCTION

The regulation of both nuclear import and nuclear export has emerged as one of the most efficient mechanisms to adapt gene expression to the cell environment by restricting the access of transcriptional regulators to their target genes. The yeast Mig1 repressor, a Cys₂–His₂ zinc-finger DNA-binding protein, shuttles between the nucleus and cytoplasm in response to glucose (Nehlin & Ronne, 1990; De Vit *et al*, 1997; Ostling & Ronne,

1998). It is imported into the nucleus within minutes after the addition of glucose to the growth medium and is just as rapidly transported back to the cytoplasm when glucose is removed. This subcellular relocalization of Mig1 is regulated by the Snf1 protein kinase and is mediated by the nuclear transport receptor Msn5 (DeVit & Johnston, 1999).

Snf1 is a central component of the glucose signalling pathway in yeast. It is a heterotrimeric enzyme complex that contains the catalytic α -subunit Snf1, the γ -subunit Snf4 and one of the three related β -subunits Gal83, Sip1 or Sip2, which regulate the subcellular localization of the kinase complex. Gal83 directs Snf1 to the nucleus in response to glucose limitation (Vincent *et al*, 2001). *In vitro*, Mig1 is readily phosphorylated by Snf1, and in yeast mutants lacking *SNF1*, Mig1 is unphosphorylated and remains nuclear upon glucose depletion while transcription of glucose-regulated promoters is constitutively repressed (Treitel *et al*, 1998; Carlson, 1999; Smith *et al*, 1999). Msn5, a member of the β -importin family of nuclear transport receptors, mediates the nuclear export of Mig1 after its phosphorylation in the nucleus. *MSN5* gene deletion allows normal phosphorylation of the Mig1 protein but abolishes nuclear export (DeVit & Johnston, 1999).

Mig1 binds to glucose-regulated promoters such as that of the *GAL1* gene, and it is believed that it inhibits transcription by recruiting the general co-repressor complex Cyc8(Ssn6)–Tup1 (Nehlin *et al*, 1991; Treitel & Carlson, 1995; Tzamarias & Struhl, 1995). It has also been proposed that the relocalization of Mig1 to the cytoplasm results in dissociation of the Cyc8–Tup1 co-repressor complex from the target promoter, thus alleviating repression (Smith & Johnson, 2000). This hypothesis has not been proven up to now, and more importantly it is not consistent with recently reported data. First, Cyc8 and Tup1 proteins were shown to be continuously tethered at *GAL1* under both repression and activation conditions (Papamichos-Chronakis *et al*, 2002). Second, yeast mutants lacking Msn5, in which Mig1 remains nuclear, induce normal *GAL1* transcription (DeVit & Johnston, 1999), suggesting that Mig1 translocation may not be the key regulatory step.

In this report, we re-evaluate the previously proposed mechanism underlying glucose repression and de-repression by

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performing experiments on the *GAL1* promoter. We show that Mig1 is not required for tethering Cyc8–Tup1 to the promoter DNA, although both regulators act in concert to inhibit *GAL1* transcription. More importantly, we show that under inducing conditions Mig1 remains bound to the *GAL1* promoter, exactly as we have shown before for Cyc8–Tup1 (Papamichos-Chronakis *et al*, 2002). We further demonstrate that the phosphorylation of Mig1 by the Snf1 kinase alleviates its interaction with Cyc8–Tup1. We propose that it is this interaction, and not the Mig1 nuclear export, that functions as the key molecular switch controlling transcriptional repression/de-repression mechanism.

RESULTS AND DISCUSSION

Mig1 is not required for Cyc8–Tup1 recruitment to *GAL1*

To test the requirement of Mig1 for Cyc8–Tup1 tethering to *GAL1*, we performed chromatin immunoprecipitation (IP) experiments to monitor Cyc8–Tup1 occupancy of *GAL1* in wild-type (WT) and *mig1*Δ mutant strains. As we have previously shown (Papamichos-Chronakis *et al*, 2002), haemagglutinin (HA)–Cyc8 and HA–Tup1 were present on the *GAL1* promoter in WT cells grown in glucose, in glycerol (no glucose) or in galactose medium (Fig 1). Interestingly, the same occupancy was observed in the *mig1*Δ yeast mutant strain grown under the same conditions (Fig 1); therefore, in contrast to what has been previously proposed, this result indicates that it is not the DNA-bound Mig1 that drives Cyc8–Tup1 to the *GAL1* promoter. As Cyc8–Tup1 does not bind to DNA directly (Komachi *et al*, 1994; Smith *et al*, 1995), it should be tethered, probably by interacting with additional protein factors. Mig2, a Mig1 homologue shown to bind *in vitro* DNA sites similar to those bound by Mig1, is unlikely to have such a role; Mig2 is not required for repression of the *GAL* genes (Lutfiyya *et al*, 1998), and additional *MIG2* disruption in a *mig1* background did not further alleviate glucose repression (Klein *et al*, 1999). Alternatively, physical interactions between Cyc8–Tup1 and specific HMG non-histone proteins, which have been previously reported (Laser *et al*, 2000), might contribute in holding Cyc8–Tup1 to specific chromosomal sites.

Mig1 and Cyc8–Tup1 repress *GAL1*

The data presented above raise an issue about the function of Mig1 in the *GAL1* repression process. Glucose repression of this

Fig 1 | Cyc8 and Tup1 occupancy of the *GAL1* in WT and *mig1*Δ mutants. Chromatin IP was performed in indicated strains expressing (A) HA–Cyc8 or (B) HA–Tup1 under repressive (3% glucose), de-repressive (5% glycerol) or inducing (2% galactose) conditions, using HA-specific antibody and primers for the *GAL1* promoter region. Results are the fold enrichment of the *GAL1* URS over a region of *GAL1* ORF after correction for the ratios of amplification achieved using input DNA.

Fig 2 | Mig1/Cyc8–Tup1 repression of Gal4-activated transcription. (A) Gal4 occupancy of *GAL1*. Chromatin IPs were performed in the indicated strains and growth conditions as in Fig 1, using anti-Gal4 antiserum and primers specific for UAS_G. Values represent the fold enrichment of the *GAL1* UAS over a region of *PHO5* ORF after correction for the ratios of amplification achieved using input DNA. (B) Northern blot analysis of *GAL1* mRNA isolated from the indicated strains and growth conditions. Values were quantified by PhosphorImager analysis and normalized to the *ACT1* transcript. Normalized *GAL1* transcript in WT cells grown in glucose was arbitrarily rated as 100.

promoter is a relatively complicated process, as Gal4, the transcriptional activator of *GAL1*, is also negatively regulated by glucose. The *GAL4* promoter contains binding sites for the Mig1 repressor, which is responsible for decreased Gal4 protein levels in cells grown in glucose (Nehlin *et al*, 1991; Johnston *et al*, 1994) while under these conditions the Gal4 activation domain is masked by the negative regulator Gal80 (Bhat & Murthy, 2001). To dissect the glucose repression effect of Mig1 and Cyc8–Tup1 solely on *GAL1* transcription, we used the *ADH1* promoter to overexpress the Gal4 activator in a yeast strain deleted for both the endogenous *GAL4* and *GAL80* genes (*gal4*Δ, *gal80*Δ strain). Although the *ADH*-driven Gal4 was expressed at lower levels in cells growing in glycerol (data not shown), its binding to the *GAL1* promoter (Fig 2A) was evident irrespective of the growth conditions and it was not affected by the *mig1*Δ and *cyc8*Δ mutations. Also, owing to the absence of Gal80, Gal4 could activate *GAL1* gene's transcription even in WT cells grown in glucose (Fig 2B). However, when glucose was removed, significantly higher levels of *GAL1* transcription were observed (Fig 2B), indicating that even when Gal4 is constitutively active, *GAL1* transcription is still subjected to glucose repression. Similarly high levels of *GAL1* mRNA were also obtained in *mig1*Δ and *cyc8*Δ strains grown in glucose media, and no further increase was observed in a *mig1*Δ, *cyc8*Δ double-mutant strain grown either in the presence or in the absence of glucose (Fig 2B), indicating that Mig1 and Cyc8–Tup1 act together to inhibit *GAL1* transcription. Thus, although Mig1 is dispensable for tethering Cyc8–Tup1 to the *GAL1* promoter, it is important for Cyc8–Tup1-mediated repression function in response to glucose.

Mig1 remains at *GAL1* even under induction conditions

We next questioned whether, upon glucose depletion, Mig1 dissociates from its target promoters, thus alleviating Cyc8–Tup1-mediated repression function. We tagged the endogenous *MIG1* gene with multiple Myc epitopes, thus generating a yeast strain expressing a Myc–Mig1 protein from the native *MIG1* promoter and chromosomal position. This Myc–Mig1 protein was fully functional in *GAL1* repression or de-repression (data not shown)

Fig 3 | Mig1 binding to actively transcribed GAL1. (A) Immunoblot analysis of the Myc–Mig1 protein expressed in WT and *snf1Δ* strains with Myc-specific antibody. Cells were grown in glucose or in glycerol for 2 h. Total proteins were separated by SDS–PAGE. The altered mobility of Myc–Mig1 indicates the differential phosphorylation (P–Mig1). (B) Mig1 (upper panel) and Cyc8 (middle panel) occupancy of GAL1 and Mig1 occupancy of SUC2 (lower panel) under repressive, de-repressive or activating conditions. Chromatin IP was performed in strains expressing HA–Cyc8 or Myc–Mig1 with HA- and Myc-specific antibodies and primers for GAL1 or SUC2 promoters and ORF sequences. Results are the fold enrichment of the indicated regions over a region of *PHO5* ORF after correction for the ratios of amplification achieved using input DNA. (C) Immunostaining of WT, *snf1Δ* and *msn5Δ* cells expressing Myc–Mig1 protein under repressive or de-repressive conditions, using Myc-specific antibody and an FITC-conjugated secondary antibody.

and, as shown in Fig 3A, it clearly showed a glucose-dependent shift in mobility, which was not observed in yeast mutants lacking the *SNF1* gene. Thus, our Myc–Mig1 was subject to Snf1-dependent phosphorylation upon glucose depletion, as has also been previously reported by Treitel & Carlson (1995).

Previous *in vivo* photofootprinting experiments failed to detect Mig1 binding to *GAL1* in steady-state glucose-grown cells (Frolova *et al*, 1999). However, the chromatin IP experiment of Fig 3B shows that Myc–Mig1 occupied the *GAL1* promoter under these conditions. Surprisingly, though, Myc–Mig1 remained bound to *GAL1* even in cells grown in glucose-depleted medium or in cells grown in galactose. Under these same conditions, HA–Cyc8 protein also occupied *GAL1* (Fig 3B, middle; Papamichos-Chronakis *et al*, 2002). Myc–Mig1 occupancy persisted after 3–4 h of culture in glycerol or galactose medium and it was specific for promoter sequences, as binding to the *GAL1* coding region was at background levels (Fig 3B). Identical results were obtained with a plasmid-borne HA-epitope-tagged Mig1 protein (data not shown). As shown in Fig 3B (lower panel), continuous Myc–Mig1 binding under normal and low-glucose conditions was also observed for *SUC2*, a distinct glucose-regulated promoter (Nehlin & Ronne,

1990). Notably, Mig1 occupancy of *SUC2* was considerably less efficient than that observed for *GAL1*, indicating that Mig1 DNA binding persists even at a lower-affinity binding site.

This intriguing observation prompted us to apply immunofluorescence microscopy to verify the subcellular localization of Myc–Mig1 under the various conditions. Myc–Mig1 was strictly localized to the nucleus of cells grown in glucose and it was dispersed to the cytoplasm either upon glucose depletion (glycerol; Fig 3C) or in galactose (data not shown). Moreover, Myc–Mig1 remained nuclear in the *snf1Δ* and *msn5Δ* mutant strains (Fig 3C), indicating that its subcellular localization was regulated by Snf1-dependent phosphorylation and was dependent on Msn5. However, our chromatin IP results, showing Mig1 permanently bound to glucose-repressible promoters, strongly suggested that shuttling to the cytoplasm is definitely not complete. It does not entirely exclude Mig1 from being nuclear and does not preclude Mig1 from DNA binding. Therefore, in contrast to the previously proposed model, we propose that de-repression of glucose-regulated genes is not a consequence of Mig1 cytoplasmic localization and thus dissociation from the relevant promoters.

Snf1 modulates Mig1 interaction with Cyc8

Yeast two-hybrid data indicated that the C-terminal domain of Mig1 interacts with Cyc8, and mutations that weaken this interaction lower repression activity as well (Treitel & Carlson, 1995; Tzamarias & Struhl, 1995; Ostling *et al*, 1998). This observation and the data presented above prompted us to investigate whether Snf1-dependent phosphorylation of Mig1 abolishes Cyc8 interaction upon glucose depletion. We reasoned that Mig1 and Cyc8–Tup1, although continuously tethered to *GAL1*, might inhibit or release transcription in a manner dictated by their physical interaction.

As indicated in Fig 4A, His–Cyc8 interacted with Myc–Mig1, but not with Myc–His3 that served as a negative control (Fig 4D), when both proteins were expressed in WT cells grown in glucose. However, no interaction was observed in cells grown in glucose-depleted medium where Myc–Mig1 was phosphorylated (Fig 4B). On the contrary, in *snf1Δ* yeast mutant cells, where Mig1 remained in the non-phosphorylated form and target promoters were constitutively repressed, interaction between His–Cyc8 and Myc–Mig1 was observed even in cells grown in glucose-depleted medium (Fig 4B). Because the phosphorylated form of Mig1 was mostly localized to the cytoplasm of WT cells grown in glucose-depleted medium, its failure to interact with Cyc8 could be a consequence of the different compartmentalization of the two proteins. To exclude this possibility, we repeated the co-IP experiment in the *msn5Δ* yeast mutant strain in which Mig1 is continuously localized to the nucleus. As shown in Fig 4C, interaction with Cyc8 was again observed specifically in cells grown in normal glucose where Mig1 was not phosphorylated, but not in the glucose-depleted cells. The above experiments demonstrate that Cyc8 interacts specifically with the non-phosphorylated form of Mig1 and, upon glucose depletion, Snf1-dependent phosphorylation of Mig1 releases its interaction with Cyc8–Tup1.

Collectively, the data presented above suggest that regulation of Mig1 interaction with Cyc8–Tup1 through Snf1-mediated phosphorylation is a key molecular switch that controls *GAL1*

Fig 4 | Snf1 regulates Mig1–Cyc8 interaction. (A) Mig1–Cyc8 interaction. Proteins obtained from glucose-grown WT cells expressing Myc–Mig1 from its original chromosomal position and His-tagged Cyc8 by the ADH promoter cloned in a multicopy vector. Extracts were treated with preimmune serum (–anti-His) or anti-His antibody (+ anti-His) coupled to protein A–Sepharose beads. Input (Inp) and precipitated proteins (IP) were separated by SDS–PAGE and analysed using anti-Myc antibody. Input proteins were equal to 1:150th the total extract incubated with protein A–Sepharose. (B) Snf1-dependent loss of Mig1–Cyc8 interaction. Immunoblotting of total input (Inp) and immunoprecipitated proteins (IP) obtained from WT or *snf1*Δ strains coexpressing His–Cyc8 and Myc–Mig1 as in (A) and grown under de-repressing conditions, using anti-Myc antibody. (C) Mig1–Cyc8 interaction in *msn5*Δ cells. IP of extracts obtained from *msn5*Δ cells expressing His–Cyc8 and Myc–Mig1 grown in glucose or glycerol using preimmune serum (–anti-His) or anti-His antibody (+ anti-His). Total input (Inp) and precipitated proteins (IP) were analysed by immunoblotting using anti-Myc antibody. (D) Immunoblotting of total Inp and IP proteins as in (A) with cells expressing Myc–His3 from its original chromosomal position and His-tagged Cyc8.

repression. In glucose-grown cells, *GAL1* is occupied by the non-phosphorylated Mig1 form, which interacts with Cyc8–Tup1. This interaction is not responsible for tethering Cyc8–Tup1 to the promoter DNA, but is important for transcriptional repression. Possibly, conformational changes induced by this interaction, at this promoter context, potentiate the Cyc8–Tup1 repression function. Under low-glucose conditions, the Snf1 kinase complex enters the nucleus, where Mig1 is phosphorylated in an Snf1-dependent manner (Treitel *et al*, 1998; Vincent *et al*, 2001). Phosphorylation is a prerequisite for nuclear export but not sufficient to exclude Mig1 from occupying target promoters. We suggest that under these growth conditions, Mig1 binds to *GAL1* in its phosphorylated form that precludes interaction with

Cyc8–Tup1, which may lead to nonfunctional conformations of the co-repressor complex.

The model proposed above provides alternative explanations to several previously reported observations. The fact that the C-terminal deletion derivatives of Mig1, which fail to interact with Cyc8–Tup1, do not repress transcription (Ostling *et al*, 1998) can be attributed to their failure to potentiate the Cyc8–Tup1 repression activity, and not to their inability to recruit Cyc8–Tup1 physically. In addition, Mig1 mutants lacking phosphorylation sites behave as constitutive repressors (DeVit & Johnston, 1999) because they may interact with Cyc8–Tup1 even under activation conditions. Finally, the observation that *msn5*Δ mutants normally derepress *GAL1* transcription, although Mig1 remains nuclear (DeVit & Johnston, 1999), is now explained by the above model, which proposes that Mig1 phosphorylation and lack of Cyc8 interaction, rather than Mig1 nuclear export, is the important regulatory step that releases transcription from glucose repression. However, an important issue that remains unresolved concerns the physiological relevance of Mig1 cytoplasmic redistribution upon glucose depletion.

METHODS

Yeast media, strains and plasmids. Standard media were used: YPD (3% glucose), YPG (2% galactose), YPGly (5% glycerol) and CS supplemented with the required amino acids. For *GAL1* de-repression or induction, overnight cultures were diluted and cultured in fresh YPD for 3 h (OD₆₀₀ 0.6) before transfer to YPGly or YPG, respectively. WT FT5 strain and the derivatives *mig1*Δ and *cyc8*Δ have been described by Tzamarias & Struhl (1992). PJ694, *MATα trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3GAL2-ADE2 met2::GAL7-lacZ* was provided by D. Alexandraki. *SNF1* and *MSN5* gene disruptions were generated as described by Knop *et al* (1999) and confirmed by Southern analysis. *MIG1* and *CYC8* disruptions in the PJ694 strain were generated as described by Tzamarias & Struhl (1995). Mig1 was tagged at the C-terminus with 9-Myc at their original chromosomal loci according to Knop *et al* (1999). HA–Cyc8 has been described by Tzamarias & Struhl (1995). The Gal4 activation domain (amino acids 768–881) was fused to the Gal4-binding domain of the pGBT9 plasmid (Clontech) expressed under the *ADH1* promoter. 6-His-tagged Cyc8 protein was expressed by the *ADH* promoter cloned in the 2μ vector pVTU263.

Gene expression analysis, co-IP and immunofluorescence assays.

Total RNA was isolated from cells grown to OD₅₅₀ ~0.4. by the acid phenol method. In all, 5 μg of total RNA was loaded on 1.5% agarose formaldehyde gel and hybridized following standard protocols. Quantification was performed by Phosphor-Imager analysis (Molecular Dynamics) and normalized to *ACT1* transcript.

In vivo interaction assays were performed as in Kadosh & Struhl (1997). The bound fraction was eluted by SDS sample buffer, and proteins were separated by 10% polyacrylamide gel electrophoresis (PAGE) and analysed by immunoblotting using monoclonal Myc-specific antibody (Santa Cruz).

Immunostaining of yeast cells was performed according to the protocol available at www.bioprotocol.com. A monoclonal antibody against the Myc epitope tag (Santa Cruz) was used followed by an FITC-conjugated anti-mouse IgG secondary antibody (Sigma). Cells were viewed with appropriate filters in a Bio-Rad Radiance 2000 microscope.

Chromatin immunoprecipitation. Chromatin was immunoprecipitated as described by Kuo & Allis (1999) using polyclonal antibody against Myc or HA epitope tag (Santa Cruz), and polyclonal antibody specific for the Gal4 DNA-binding domain (Santa Cruz). The recovered DNA was subjected to quantitative real-time PCR and PCR products were visualized by gel electrophoresis to ensure the correct size of the DNA product. Primers amplified the following DNA fragments (coordinates relative to the ATG (+1): *GAL1* UAS/URS (–169 to –370); *GAL1* open reading frame (ORF) (+1342 to +1558); *SUC2* URS (–544 to –393); *SUC2* ORF (+544 to +710)). Quantification reflected the fold enrichment of the tested promoter DNA relative to an ORF region after correction for the ratios of amplification achieved using input DNA. Each chromatin IP was repeated at least three times and the variation between experiments was $\pm 15\%$.

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