Saccharomyces cerevisiae 14-3-3 proteins Bmh1 and Bmh2 participate in the process of catabolite inactivation of maltose permease

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Abstract In this study we show that Reg1, the regulatory subunit of the Reg1/Glc7 protein phosphatase (PP1) complex, interacts physically with the two yeast members of the 14-3-3 protein family, Bmh1 and Bmh2. By using different fragments of the Reg1 protein we mapped the interaction domain at the N-terminal part of the protein. We also show that Reg1 and yeast 14-3-3 proteins participate actively in the regulation of the glucose-induced degradation of maltose permease (Mal61).

Key words: Two-hybrid analysis; Reg1; Glc7; 14-3-3 protein; Catabolite inactivation; Maltose permease

1. Introduction

The yeast *Saccharomyces cerevisiae* regulates metabolism, gene expression and growth in response to carbon source availability. In the presence of glucose, the most preferred carbon source, yeast induces transcriptionally the expression of several genes such as those encoding glycolytic enzymes, ribosomal proteins and some glucose transporters (see [1] for review). At the same time, glucose represses at the level of transcription the expression of a large number of genes, including those involved in the utilization of alternative carbon sources, gluconeogenesis and respiration by a process known as glucose repression (see [2,3] for review). Glucose is also able to induce a rapid loss of activity of the enzymes of the gluconeogenic pathway (e.g. fructose 1,6-bisphosphatase) and of several sugar transporters (the high-affinity glucose transporter, galactose permease and maltose permease) and stimulate an increase in the rate of degradation of these proteins by a process known as catabolite inactivation [4–10].

In general, the regulation of the different nutrient signaling pathways is achieved by the modulation of the activity of specific protein kinases and phosphatases. For example, *GLC7*, an essential gene that encodes the catalytic subunit of the yeast protein phosphatase type 1 (PP1) [11], is involved in the regulation of glucose repression, glycogen metabolism and translation initiation, among other processes (see [12] for review), and participates in the regulation of these pathways by binding to specific regulatory subunits that target the phosphatase to the corresponding substrates. Reg1 (Hex2) is one of these regulatory subunits and targets Glc7 to substrates involved in the glucose repression pathway [13,14]. In fact, it is known that the Reg1/Glc7 phosphatase complex is involved in the regulation of the activity of the Snf1 kinase complex, a crucial player in the glucose repression pathway [15,16]. In addition, the Reg1/Glc7 phosphatase complex is also involved in the transduction of the glucose signal in the process of catabolite inactivation [17].

In order to identify additional components that could help Reg1 in performing its functions, we carried out a two-hybrid screening using LexA-Reg1 as bait. As a result of this screening we identified Bmh1 and Bmh2, the *S. cerevisiae* members of the 14-3-3 protein family. In this yeast, 14-3-3 proteins participate in the regulation of different crucial physiological processes such as exocytosis and vesicle transport, RAS/MAPK (mitogen-activated protein kinase) signalling cascade during pseudohyphal development, regulation of the subcellular localization of Msn2/4 [transcriptional activators of STRE (stress response element)-regulated genes], regulation of rapamycin-sensitive signalling (through the TOR kinase pathway) and proteasome-regulated protein degradation (see [18,19], for review).

In this study we also demonstrate that yeast 14-3-3 proteins participate, in addition, in the process of catabolite inactivation of maltose permease. We suggest that 14-3-3 proteins affect this pathway because they bind to the Reg1/Glc7 phosphatase complex, an already known regulator of this process [17].

2. Materials and methods

2.1. Strains and genetic methods

S. cerevisiae strain FY250 (*MATα his3Δ200 leu2Δ1 trp1Δ63 ura3-52*) was a gift from Dr. F. Winston (Harvard Medical School, Boston, MA, USA). Strain FY250 *reg1Δ::URA3* was described in [16]. Strains Σ1278 (*MATα his3 leu2 trp1 ura3-52*) and Σ1278 *bmh1Δbmh2Δ* were from Dr. G. Fink (Whitehead Institute for Medical Research, MIT, Cambridge, MA, USA) [20]. Strains ENY.WA-1A (*MATα ura3-52 leu2-3,112 trp1-289 his3-Δ1*) and ENY.hex2-3A (*reg1*) (*MATa leu2-3,112 ura3-52 hex2-3*) were from Dr. Karl-Dieter Entian (Institut für Mikrobiologie, Johann Wolfgang Goethe-Universität, Frankfurt, Germany) [21]. Strains CTY10-5d (*MATa ade2 his3 leu2 trp1 gal4 gal80 URA3::lexAop-lacZ*) and TAT-7 (*MATa ade2 his3 leu2 trp1 gal4 gal80 LYS2::lexAop-HIS3 URA3::lexAop-lacZ*) were a gift

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Abbreviations: GAD, Gal4 activating domain; GST, glutathione *S*-transferase; HA, hemagglutinin epitope; PCR, polymerase chain reaction; SC, synthetic complete medium; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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from Dr. R. Sternglanz (State University of New York, Stony Brook, NY, USA).

Standard methods for genetic analysis and transformation were used. Yeast cultures were grown in synthetic complete (SC) medium lacking appropriate supplements to maintain selection for plasmids [22], supplemented with different carbon sources.

2.2. Oligonucleotides

The oligonucleotides used were BMH2-1 (−18)CCGGGATCCC AAAAAAAAATGTCCCAAACTC, BMH2-2 (+843) CCCCTCGAG TTTGTCGACGCTCTTATTTGGTTGG, REG1-1 (−9)GCCGAAT TCATGTCAACAAATCTAGCAAATTACTTC, REG1-198 (+1503)-CCATGGGATCCATGAGGCATTTTCTAAC, REG1-120 (+1260)-CGTCATCTAGATCATCATCTGAAGAATTG- CTT. The number in parentheses corresponds to the first base of the oligonucleotide with respect to (+1)ATG. New restriction sites are underlined.

2.3. Plasmids

To construct pBTM-Reg1 (LexA-Reg1; *TRP1* as selection marker), an *Eco*RI/*Sal*I fragment from plasmid pRJ65 (LexA-Reg1, *HIS3* [23]) was subcloned into pBTM116 [24]. To construct pLexA-Reg1(1–313), an *Eco*RI/*Bam*HI fragment from plasmid pSB27 (LexA-Reg1(1–443) [16]) was subcloned into pEG202 [25]. To construct pLexA-Reg1-(1–198), oligonucleotides REG1-1 and REG1-198 (see above) were used to amplify by polymerase chain reaction (PCR) a fragment of the *REG1* gene using pSB27 as template; the amplified fragment was digested with *Eco*RI and *Bam*HI and subcloned in pEG202. To construct pLexA-Reg1(1-120), oligonucleotides REG1-1 and REG1-120 (see above) were used as before; the amplified fragment was digested with *Eco*RI and *Xba*I and subcloned in pUC18; then, an *Eco*RI/*Sal*I fragment was subcloned in pEG202.

To construct pEG-Bmh2 (LexA-Bmh2), oligonucleotides BMH2-1 and BMH2-2 (see above) were used to amplify by PCR the *BMH2* gene from genomic DNA of FY250. The amplified fragment was digested with *Bam*HI and *Xho*I and subcloned in pEG202. The same fragment was introduced in pACTII [26] and pGST [27], to obtain pACTII-Bmh2 (Gal4 activating domain [GAD]-Bmh2) and pGST-Bmh2 (glutathione *S*-transferase [GST]-Bmh2), respectively.

To construct YEpACT-hemagglutinin epitope (HA)-Glc7, a fragment from pHA-Glc7 [23] was introduced in the YEpACT vector [28].

Other plasmids used in this study were pRJ55 (LexA-Snf1), pRJ57 (LexA-Snf4) and pRJ65 (LexA-Reg1) [29]; pSB55 [GAD-Reg1(3–1014)], pSB52 [GAD-Reg1(3–760)], pSB32 [GAD-Reg1(3–485)], pSB31 [GAD-Reg1(3–447)], pSB27 [LexA-Reg1(1–443)], pBF414 [GAD-Reg1(424–1014)] and pSB17 (pSK-Reg1, HA-Reg1) [16]. Plasmid pSO74 (ADH1p-HXT1) was described in [30]; plasmids YCp50-MAL63 and pRS315-MAL61-HA were described in [17].

2.4. Two-hybrid screening

A two-hybrid screening [31] for proteins that interacted with LexA-Reg1 (pBTM-Reg1, see above) was carried out in strain TAT-7 (see above). The strain was transformed with a library of *S. cerevisiae* cDNAs fused to GAD [32]. Transformants were selected in SC+2% glucose plates and were subsequently screened for β-galactosidase activity using a filter lift assay [33].

2.5. Invertase and β-galactosidase assays

Invertase activity was assayed in whole cells as previously described [29]. β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units as in [15].

2.6. Preparation of cell extracts by the fast boiling method

Cells corresponding to 1 U A_{600} were collected by rapid centrifugation (14 000 rpm, 1 min), resuspended in 100 μl of Laemmli sample buffer [34] and boiled for 3 min. Glass beads (0.3 g, 450 μm diameter) were added to the suspension and then cells were vortexed at full speed for 30 s. The suspension was boiled again for 3 min and centrifuged at 14 000 rpm for 1 min. 20 μl of the supernatant was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting.

2.7. Pull-down assay

Preparation of protein extracts and pull-down assays were essentially as described previously [16].

2.8. Immunoblot analysis

Protein samples were separated by SDS–PAGE and analyzed by immunoblotting using the corresponding antibodies: anti-GST (Amersham), anti-Hxk2 polyclonal antibodies [21] or anti-HA (Boehringer Mannheim) monoclonal antibodies. Antibodies were detected by enhanced chemiluminescence with ECL or ECL Plus reagents (Amersham).

3. Results and discussion

3.1. Reg1 interacts with members of the yeast 14-3-3 protein family, Bmh1 and Bmh2

Reg1 (a regulatory subunit of the PP1 protein phosphatase

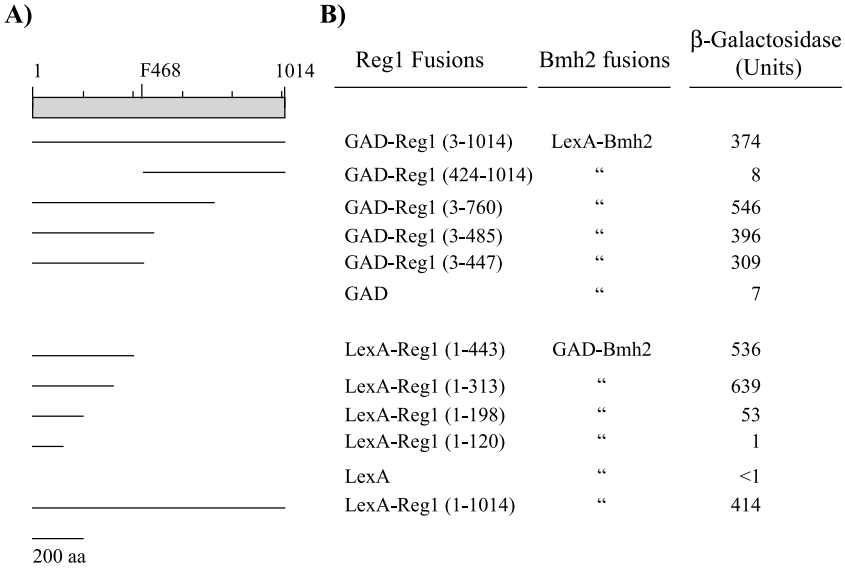

Fig. 1. Bmh2 interacts with the N-terminal domain of Reg1. A: Reg1 sequences fused to either GAD or LexA that were used in the two-hybrid analysis. None of these fusions showed detectable self-activating activity. B: CTY10-5d transformants expressing the indicated pair of proteins were grown exponentially in 4% glucose medium; all the GAD fusions were based on plasmid pACTII except for GAD-Reg1(424–1014) that was based on pACT; all the LexA fusions were based on plasmid pEG202. Values are average β-galactosidase activities of four to six transformants, with standard deviation lower than 15% in all cases. Protein levels of interacting proteins were similar in all cases (not shown).

complex) is involved in both the regulation of the glucose repression pathway and the transduction of the glucose signal that regulates the process of catabolite inactivation. In order to identify additional components that could help Reg1 in performing its functions, we carried out a two-hybrid screening using LexA-Reg1 as bait (see Section 2). After screening 10^6 transformants, we recovered 12 positive transformants that grew in the absence of histidine and were blue in the β-galactosidase filter assay. Inserts contained in these 12 plasmids were sequenced and the identity of the corresponding genes determined by BLAST analysis [35]. Three inserts corresponded to in frame C-terminal fragments of the *SSB1* and *SSB2* genes, members of the heat shock protein HSP70 family [36]. Eight inserts corresponded to in frame cDNAs of the *BMH1* gene that started at different positions either at the 5′ untranslated region or in the coding region. Finally, one insert corresponded to an in frame fusion to 53 nucleotides upstream of the starting ATG of the *BMH2* gene. Since the clones containing *BMH1* and *BMH2* were the most abundant, we focused our attention on their interaction with Reg1. Our results were in agreement with those recently reported in which the interaction between Reg1 and Bmh1 was confirmed by affinity precipitation [37].

Bmh1 and Bmh2 are the yeast members of the 14-3-3 protein family (see [18] for review). Reg1 was able to interact with both Bmh1 and Bmh2 proteins, although the interaction was better with Bmh2. As is shown in Table 1, pACT-Bmh1-(clone16) (an in frame fusion of GAD to five nucleotides upstream of the starting ATG of *BMH1*) showed lower interaction values than pACT-Bmh2(clone10) (an in frame fusion of GAD to 53 nucleotides upstream of the starting ATG of *BMH2*). Since the interaction between LexA-Reg1 and Bmh2 was stronger than in the case of Bmh1 (see Table 1), and since it has been described that Bmh2, although being produced in lower amounts than Bmh1, was more active [38], we focused our attention on the Bmh2 protein. Using synthetic oligonucleotides we amplified by PCR the coding region of *BMH2*, which was sequenced to confirm that no modifications had been introduced by the Taq polymerase. This fragment was used to construct LexA-Bmh2 and GAD-Bmh2 derivatives that were used to study the domains of the Reg1 protein involved in their interaction (Fig. 1). We observed that Bmh2 interacted only with the N-terminal part of Reg1 (residues 1–313) (Western blot analysis indicated that the interacting proteins were produced at similar levels in all cases; data not shown). This Bmh2 interaction domain was independent of the motif of interaction with Glc7 ($RHIHF_{468}ND$), located more towards the middle of the protein [16].

Table 1
Reg1 interacts with Bmh1 and Bmh2

		β-Galactosidase (U)
LexA-Reg1	pACT-Bmh1(16)	217
LexA-Reg1	pACT-Bmh2(10)	314
LexA-Reg1	pACT	< 1
LexA	pACT-Bmh2(10)	< 1

Two-hybrid interaction between pBTM-Reg1 (LexA-Reg1) and different clones recovered after the two-hybrid screening was measured in TAT-7 cells. Values for cells growing exponentially in 4% glucose are average β-galactosidase activities of four to six transformants, with standard deviation lower than 15% in all cases.

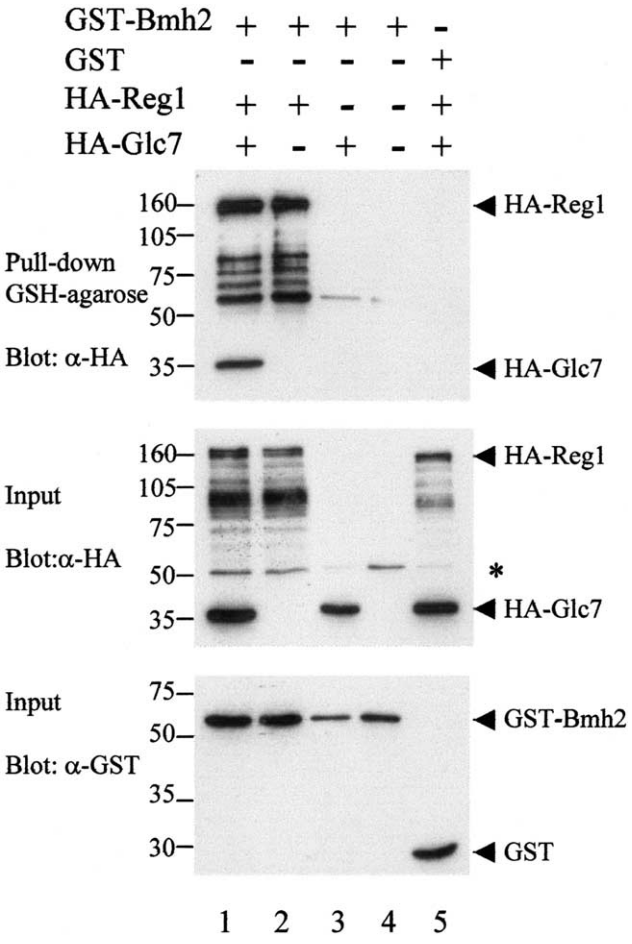

Fig. 2. Bmh2 interacts physically with Reg1 and Glc7. Protein extracts (250 μg) were prepared from FY250 *reg1*Δ::*URA3* glucose growing cells expressing GST-Bmh2 (pGST-Bmh2), HA-Reg1 (pSK-Reg1) and HA-Glc7 (YEpACT-HA-Glc7) or the corresponding empty vectors pGST, pSK93 and YEpACT. GST-Bmh2 fusion proteins were pulled down with glutathione-agarose. Pelleted proteins were analyzed by SDS–PAGE and immunodetected with anti-HA monoclonal antibodies (upper panel). Proteins in the input crude extracts (5 μg) were also immunodetected with either anti-HA (middle panel) or anti-GST (lower panel). Additional bands observed in lanes with HA-Reg1 were degradation products. The position of a protein in the crude extracts that cross-reacts non-specifically with anti-HA is indicated with an asterisk. Size standards are indicated in kDa.

The physical interaction between Bmh2 and Reg1 was confirmed by pull-down assays. Using a GST-Bmh2 fusion protein we observed a specific interaction between Bmh2 and Reg1 (Fig. 2, compare lanes 2, 4 and 5). If in the *reg1*Δ cells expressing GST-Bmh2 and HA-Reg1 we additionally overexpressed HA-Glc7 (the catalytic subunit of the Reg1/Glc7 phosphatase complex), we observed that GST-Bmh2 was also able to pull down HA-Glc7 (Fig. 2, lane 1), but this was dependent on the presence of Reg1 (Fig. 2, lane 3). These results indicate that GST-Bmh2 was able to interact physically with the Reg1/Glc7 phosphatase complex in glucose growing cells. We repeated the pull-down assay in cells that had been shifted to carbon starvation conditions for 10, 30 and 60 min and observed that the binding between GST-Bmh2 and the Reg1/Glc7 phosphatase complex remained after the shift (data not shown).

3.2. Yeast 14-3-3 proteins are not involved in the regulation of the glucose repression pathway

Since the Reg1/Glc7 protein phosphatase complex is involved in the regulation of the glucose repression pathway (see Section 1), we checked for possible interactions between Bmh2 and other components of this pathway. However, LexA fusions to Snf1, Snf4 (Snf1 activator subunit), and Hxk2 (Snf1 negative regulator) did not show any positive interaction with GAD-Bmh2, in either high or low glucose conditions (data not shown). To analyze the possible involvement of Bmh2 in the process of glucose repression we studied the regulation of *SUC2* expression, a gene encoding the enzyme invertase whose expression is repressed by glucose [2], in a double *bmh1Δbmh2Δ* mutant. It is known that a double *bmh1Δbmh2Δ* mutation is lethal in most yeast genetic backgrounds but it is still viable in the Σ1278b background [20]. Using mutants in this background we observed that regulation of *SUC2* expression was not affected in the absence of 14-3-3 proteins (data not shown). We also overexpressed Bmh2 and found that regulation of *SUC2* expression was not affected either (data not shown). Therefore, yeast 14-3-3 proteins were not involved in the regulation of the glucose repression pathway.

3.3. Yeast 14-3-3 proteins are involved in the regulation of the catabolite inactivation pathway of maltose permease (Mal61)

It has recently been described that the Reg1/Glc7 phosphatase complex plays an additional role in the transduction of the glucose signal in the process of catabolite inactivation [10,17]). In the absence of Reg1, there was a significant reduction in the rate of glucose-induced proteolysis of maltose permease (see [17] and Fig. 3A). To analyze if the yeast 14-3-3 proteins also affected this pathway, we checked the glucose-induced degradation of maltose permease in double *bmh1Δbmh2Δ* mutants and found a significant reduction in the rate of degradation of maltose permease, similar to that found in *reg1Δ* mutants (Fig. 3B). The only difference was that the amount of HA-Mal61 in the *reg1Δ* mutant appeared to increase 3 h after the addition of glucose, but it decreased significantly in the *bmh1Δbmh2Δ* mutants, although this may indicate that in the *reg1Δ* mutant, new synthesis of HA-Mal61 may proceed in the presence of a mixture of glucose and maltose, since *reg1Δ* is also a glucose repression mutant, while this process is blocked in the *bmh1Δbmh2Δ* mutant. To rule out the possibility that the reduction in the degradation rate of Mal61 was due to a deficient glucose uptake in the double mutant, we transformed these cells with a plasmid containing the *HXT1* low affinity glucose transporter gene under the control of the *ADH1* gene promoter (plasmid pS074 [30]). The process of glucose-induced degradation of maltose permease was still significantly reduced in these cells (data not shown). In addition, and as we have already mentioned before, the regulation of *SUC2* expression was as in wild type, indicating that glucose uptake was not severely affected in the double mutants. Therefore, we suggest that yeast 14-3-3 proteins Bmh1 and Bmh2 participate actively in the transduction of the glucose signal in the process of catabolite inactivation of maltose permease, and that they might do so by binding to the Reg1/Glc7 phosphatase complex, an already known regulator of this process. Since 14-3-3 proteins affect a great variety of physiological pathways [18], we cannot rule out the possibility that 14-3-3 proteins affect, in addition, other steps in the regulation of glucose-induced degradation of maltose permease, independent of Reg1/Glc7.

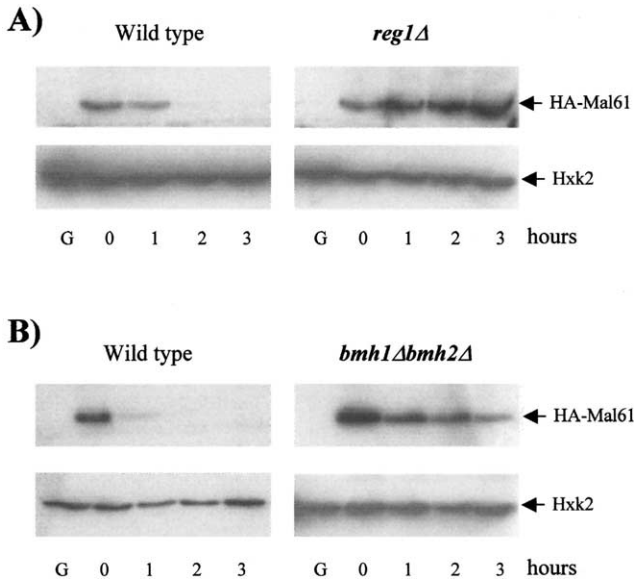

Fig. 3. Glucose-induced degradation of maltose permease is prevented in *reg1Δ* and double *bmh1Δbmh2Δ* mutants. A,B: The indicated mutants (Σ1278 *bmh1Δbmh2Δ* and ENY.hex2-3A [*reg1*]) and their corresponding wild types (Σ1278 and ENY.WA-1A), transformed with plasmids YCp50-MAL63 and pRS315-MAL61-HA [17], were grown overnight in SC−2% glucose medium. When they reached the exponential phase (G), cells were washed twice with sterile water and resuspended in SC−2% maltose medium. Cells were then incubated at 30°C for 5 h. After this time, a pulse of 2% glucose (final concentration) was added to the cultures and aliquots were taken at time 0 and after 1, 2 and 3 h of incubation at 30°C. Crude extracts were obtained by the fast boiling method (Section 2). The presence of maltose permease (HA-Mal61) in the crude extracts (20 μl) was analyzed by 6% SDS–PAGE and western blotting, using anti-HA monoclonal antibodies. The same blot was re-probed with anti-Hxk2 polyclonal antibodies as loading control.

We tried to extend our results to the regulation of the glucose-induced degradation of fructose 1,6-bisphosphatase (Fbp1) and galactose permease (Gal2). However, for unknown reasons, we were unable to detect these proteins in the double *bmh1Δbmh2Δ* mutants growing in glucose-containing media and shifted for to up to 16 h to media containing either 2% glycerol or 2% galactose. The lack of expression of galactose permease could be the reason why these double *bmh1Δbmh2Δ* mutants were unable to grow in galactose as the only carbon source [20].

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