# Glucose-dependent and -independent signalling functions of the yeast glucose sensor Snf3

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**Abstract The yeast Snf3 protein has been described to function as a sensor for low concentrations of extracellular glucose. We have found that Snf3 is able to transduce a signal in the complete absence of extracellular glucose. High basal activity of the *HXT7* promoter during growth on ethanol required Snf3 as well as other components of the signalling pathway activated by Snf3. Moreover, the C-terminal domain of Snf3 was sufficient to complement the role of Snf3 in this regulation. As the C-terminal tail of Snf3 interacted with other components at the plasma membrane independent of the carbon source, our data suggest that Snf3 is involved in signalling complexes which can be activated by other signals than extracellular glucose.** 

*Key words:* Snf3; Rgt2; Glucose transport; Sensing; Hxt7; *Saccharomyces cerevisiae*

## 1. Introduction

There is emerging evidence that some members of solute transporter families have a regulatory instead of a transport function, i.e. instead of transporting solutes they are mainly involved in control of synthesis and/or activity of other transport proteins [1–4]. In the yeast *Saccharomyces cerevisiae*, two of these proteins, Snf3 and Rgt2, belong to the sugar transporter family and are involved in transcriptional induction by glucose of *HXT* genes encoding glucose transporters [5,6]. Snf3 appears to be a sensor of low levels of glucose whereas Rgt2 is a sensor of high glucose concentrations. The sensors resemble glucose transporters but additionally possess large cytoplasmic signalling domains at their C-terminus. The molecular mechanism through which Snf3 and Rgt2 activate their downstream signalling pathways in response to extracellular glucose remains unknown. The proteins have been suggested to act as glucose receptors, i.e. undergo intracellular modification upon binding of glucose to extracellular domains [5]. However, neither binding of glucose nor activation by glucose has ever been demonstrated. Here, we provide evidence for an additional function of Snf3 which is not dependent on the presence of extracellular glucose.

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## 2. Materials and methods

### 2.1. Yeast strains and growth conditions

The yeast strains described in this work were derived from strains CEN.PK2-1C (*MATa leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8$^c$ SUC2*) or MC971A (*MATa ura3-52 his3-11,15 MAL2 SUC2 GAL MEL*). Rich media were based on 1% yeast extract and 2% peptone (YP), minimal media consisted of 0.67% Difco yeast nitrogen base without amino acids (YNB), supplemented for auxotrophic requirements and with various carbon sources. Yeast cells were grown aerobically at 30°C on a rotary shaker or on agar plates.

### 2.2. Construction of yeast strains

Strains lacking the genes *SNF3*, *GRR1* and *RGT1* were constructed with the 'short flanking homology PCR' technology using either the *kanMX* module of Wach et al. [7] or the *loxP::kanMX::loxP*/Cre recombinase system [8]. The correct replacements were confirmed by PCR. For the construction of strain RWY28 (CEN.PK2-1C *HXT7promoter::lacZ-kanMX*) the *E. coli lacZ* gene was fused genomically to the promoter and the first 48 nucleotides of the truncated *HXT7* open reading frame (ORF). This was done by replacing parts of the *HXT7* ORF with a *lacZ-kanMX* reporter cassette as described by Boles et al. [9]. The same was done in strain SKY1 [10] after removal of the *kanMX* module, resulting in strain RWY29 (CEN.PK2-1C *snf3::loxP HXT7promoter::lacZ-kanMX*). For the construction of strain YFS707, a YIp356-*HXT7promoter::lacZ* plasmid was integrated into strain MC971A [11]. The *SNF3*, *GRR1* and *RGT1* genes were deleted in this strain as described [8,11]. For the construction of strain SDY02 (CEN.PK2-1C *snf3::loxP HXT7promoter::lacZ-kanMX Hxt6ΔC/C-Snf3*) the *SNF3* C-terminal tail was fused in a fusion PCR with six histidine codons, a stop codon and the *loxP::kanMX::loxP* module using the primers 'FUS-S-K' (5′-GATTTGAAACATCACCATCACCATCACTAATTCGTACGCTGCAGGTCGAC-3′), '2H6CSNF3' (5′-GCACAAATTAGAGCGTGATCATGAATTAATAAAAATGTTCGCAAAGCATAGGCCACTAGTGGATCTG-3′), and '1H6CSNF3' (5′-GGCTGTTTGGTCTTCATGTTCTTCTATGTTTTGTTAGTTGTTCCAGAAACGAAGGGTTTGACATTAG-3′), and plasmids pUG6 and pS3c11 (see below) as the templates. As the primer '1H6SNF3' contains at its 5′ end sequences homologous to the end of the membrane spanning domain of *HXT6* and primer '2H6SNF3' contains at its 5′ end sequences homologous to the *HXT6* termination region, the final PCR product was used to replace the short *HXT6* C-terminal domain with the long *SNF3* C-terminal tail after transformation into strain RWY29. The *kanMX* marker was removed afterwards.

### 2.3. Construction of plasmids

In most cases, genes were cloned into plasmids by homologous recombination in yeast cells, as described in Wieczorke et al. [12]. For the construction of plasmid YEpSNHX7 by fusion PCR, the membrane spanning domain of *SNF3* was amplified with GENEPAIRS primer 'YDL194W-forward' and 'Snf3Fus' (5′-TTCCAAAGTCAAACCCTTAGTTTCATAAACGGT-3′), and the C-terminal tail of *HXT7* was amplified with GENEPAIRS primer 'YDR342C-reverse' and 'HXT7Fus' (5′-GAAACTAAGGGTTTGACTTTGGAAGAAGTC-3′). The PCR products were fused using primers T1-ORFs and T2-ORFs [12], and transformed into yeast cells together with the linearized p426MET25 vector [13]. For the construction of plasmid


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Table 1
Transcriptional regulation of the *HXT7* gene in *snf3* mutants and mutants of the glucose induction pathway

| Strains (with YIp356-*HXT7promoter::lacZ*) | Genotype | 2% raffinose | 3% ethanol |
|---|---|---|---|
| YFS707 | WT | 1.7 | 1.0 |
| RWY81 | *snf3*Δ | 0.1 | 0.2 |
| YFS807 | *rgt1*Δ | 2.0 | 5.3 |
| RWY71-K | *grr1*Δ | 0.1 | 0.1 |
| RWY82 | *snf3*Δ *rgt1*Δ | 1.3 | 5.9 |
| RWY72-K | *grr1*Δ *rgt1*Δ | 2.1 | 3.5 |

All the strains are derived from MC971A. Cells were grown for about 15 h in YP medium with either 2% raffinose or 3% ethanol to a density of $1–2\times10^7$ cells/ml. Mean values of data from at least two independent experiments are given. β-Galactosidase activity is expressed in μmol/min/mg of protein.

pSnf3c-C-ADNS the C-terminal domain of *SNF3* was amplified with primers 'TOPSnf3' (5′-GATCCCAAGCTTACCATGGAGATCTCAACGAAGGGTTTGACATTAGAAGAG-3′) and 'BOTSnf3' (5′-GAGGATCCTCACCCGGGTTTCAAATCATTATTTTCATTTACAGG-3′). The PCR product was digested with *Hin*dIII and *Xma*I and cloned into the vector pADNS-p110-5′Sos [14]. pS3c11: the C-terminal domain of *SNF3* was amplified with primer pairs 'SNFC-T1/T1-ORFs' (5′-GTAATACAGGGTCGTCAGATACATAGATACAATTCTATTACCCCCATCCATACGGAATTCCAGCTGACCACCATGGAAACGAAGGGTTTGACATTAG-3′) and 'SNFC-2/SNFC-T2' (5′-TGAATGTAAGCGTGACATAACTAATTACATGACTCGAGGTTAGTGATGGTGATGGTGATGTTTCAAATCATTATTTTCATTTACAGG-3′) and cloned into p426MET25 via homologous recombination in yeast. pSH34: the C-terminal domain of *SNF3* was amplified with primers 'topSnf3cF' (5′-CGGGATCCACGAAGGGTTTGACATTAGAAGAG-3′) and 'botSnf3cF' (5′-CCGCTCGAGTTAACTTATAATACAACAGCCACCCGATCCTTTCAAATCATTATTTTCATTTACAGG-3′), digested with *Bam*HI and *Xho*I and cloned into the vector pUG34 (kindly provided by J. Hegemann and U. Güldener, Düsseldorf, Germany).

### 2.4. Enzymatic measurements and Western blot analysis

β-Galactosidase activity was measured as described previously [15]. Crude extracts were prepared using glass beads for breaking the cells according to published procedures [16]. Protein was determined according to the microbiuret method [17], with bovine serum albumin as a standard. Preparation of total protein extracts and Western blot analysis were performed as described previously [10]. Monoclonal anti-$His_6$ antibodies from mouse (Roche) were used to detect the $His_6$-tagged Snf3 C-terminal tail with anti-mouse peroxidase conjugate as secondary antibody. The C-terminal tail was visualized using Super Signal West Dura Extended Duration Substrate (Pierce).

## 3. Results and discussion

### 3.1. Snf3 is able to transduce a signal in the absence of glucose

To analyze the function of the glucose sensor Snf3 in glucose-induced signal transduction, in previous studies the promoters of the genes *HXT1–4* have been used as reporters [1,5,6]. Expression of all these genes is completely dependent on the presence of glucose. In contrast, the *HXT7* promoter exhibits a basal activity on alternative carbon sources like ethanol [11]. We integrated a *HXT7promoter::lacZ* fusion gene into the genome of a wild-type and of a *snf3* deletion strain. The *HXT7* promoter was induced by the low concentrations of hexoses derived from the extracellular hydrolysis of raffinose and exhibited a lower basal activity during growth of the yeast cells on ethanol (Table 1). Surprisingly, in a *snf3* mutant the activity of the *HXT7* promoter was not only abolished under inducing conditions but also on ethanol (Table 1), indicating that Snf3 is able to transduce a signal even in the absence of extracellular glucose.

To test whether the glucose-independent signal generated by Snf3 is transduced via the same signalling pathway as the glucose-dependent signal, regulation of a *HXT7promoter::lacZ* gene was analyzed in different mutants affected in the Snf3 signal transduction pathway. Indeed, all the factors involved in the glucose-dependent regulation (Rgt1, Grr1) were also involved in the independent regulation (Table 1). Rgt1 is a zinc cluster protein that binds directly to promoters of the *HXT* genes and acts as a repressor in the absence of glucose [18]. Grr1 is an essential component of the $SCF^{Grr1}$ ubiquitin protein ligase complex that mediates the signal generated by Snf3 to inhibit Rgt1 in response to glucose [19]. In *grr1* mutants like in the *snf3* mutants the *HXT7* promoter was not active neither during growth on raffinose nor on ethanol (Table 1). Deletion of *RGT1* could overcome repression and led to a strong induction of the *HXT7* promoter in *snf3* and in *grr1* mutants, in the presence and absence of an inducing carbon source (Table 1).

### 3.2. The C-terminal tail of Snf3 constitutively induces a transcriptional signal

To analyze the function of Snf3 in glucose-dependent and -independent regulation of *HXT7* promoter activity in more detail, the membrane spanning part of Snf3 was separated from the long cytoplasmic C-terminal tail. The Snf3 membrane spanning part without the C-terminal tail was fused to the short C-terminal tail of the high-affinity glucose trans-

Table 2
Transcriptional regulation of the *HXT7* gene by Snf3 and variants of Snf3

| Strains (with *HXT7promoter::lacZ-kanMX*) | Plasmid | 2% raffinose (inducing) | 3% ethanol |
|---|---|---|---|
| RWY28 (WT) | p426MET25 (without insert) | 2.8 | 1.2 |
| RWY29 (*snf3*Δ) | p426MET25 (without insert) | 0.3 | 0.4 |
| RWY29 (*snf3*Δ) | YEpSNHX7 (Snf3ΔC/C-Hxt7) | 0.4 | 0.6 |
| RWY29 (*snf3*Δ) | pS3c11 (C-Snf3/6His) | 1.8 | 0.9 |
| RWY29 (*snf3*Δ) | pSH34 (GFP/C-Snf3/Farn) | 3.3 | 5.5 |
| RWY29 (*snf3*Δ) | pSH34+YEpSNHX7 | 2.1 | 3.1 |
| SDY02 (*snf3*Δ *Hxt6*Δ*C/C-Snf3*) | – | 1.5 | 1.4 |

All the strains are derived from CEN.PK2-1C. Cells were grown for about 15 h in selective YNB medium with either 2% raffinose or 3% ethanol to a density of $1–2\times10^7$ cells/ml. Mean values of data from at least two independent experiments are given. β-Galactosidase activity is expressed in μmol/min/mg of protein.

porter Hxt7 (YEpSNHX7), and the sequence encompassing the C-terminal tail of Snf3 was cloned into the vector p426MET25 [13] (pS3c11). The impact of both parts of Snf3 on *HXT7* expression was studied after transformation into a *snf3* deletion strain containing a genomically integrated *HXT7promoter::lacZ* fusion gene. Again, in the *snf3* mutant strain the activity of the *HXT7* promoter was not only abolished under inducing conditions but also on ethanol (Table 2). The membrane spanning domain of Snf3 alone could not induce the *HXT7* promoter. However, the C-terminal tail of Snf3 partly restored the induced as well as the basal activity of the *HXT7* promoter (Table 2).

We wanted to test whether a tight localization at the plasma membrane might influence the signalling properties of the Snf3 C-terminal domain. Therefore, a farnesylation/palmitoylation sequence was fused at its C-terminus by PCR and it was cloned behind and in frame with the green fluorescence protein (GFP) sequence of the vector pUG34, resulting in plasmid pSH34. After transformation of the plasmid pSH34 into the *snf3* deletion strain RWY29, fluorescence microscopy revealed cell surface localization of the farnesylated Snf3 C-terminal domain–GFP fusion protein (not shown). Moreover, the membrane-attached C-terminal domain induced the *HXT7* promoter much stronger than the soluble C-terminal domain, not only on raffinose but even more on ethanol (Table 2). Coexpression of the farnesylated C-terminal domain together with the membrane spanning domain of Snf3 did not restore normal glucose regulation of Snf3 (Table 2), indicating that the C-terminal domain must be tightly linked to the membrane spanning parts of Snf3 in order to become regulated by glucose.

### 3.3. The C-terminal tail of Snf3 does not convert Hxt6 into a glucose sensor

To test whether glucose regulation of the Snf3 C-terminal tail can be restored if it is fused to a glucose transporter, the short C-terminal domain of the Hxt6 high-affinity glucose transporter was replaced with the Snf3 C-terminal domain. This was done with the genomic allele of *HXT6* (see Materials and methods), and regulation of a genomically integrated *HXT7promoter::lacZ* fusion gene was analyzed. However, the Hxt6ΔC/C-Snf3 fusion protein behaved similar to the C-terminal domain of Snf3 alone (Table 2). It did not restore glucose regulation of the *HXT7* promoter, but rather produced a glucose-independent constitutive signal. This is in contrast to other findings with Hxt1ΔC/C-Snf3 and Hxt2ΔC/C-Snf3 fusion proteins [5] and shows that specific residues in the membrane spanning domain might be important for optimal function of a glucose sensor.

### 3.4. The C-terminal tail of Snf3 interacts with the plasma membrane and is phosphorylated

As a plasma membrane-attached C-terminal domain of Snf3 produced a much stronger signal than a soluble C-terminal domain, we wanted to analyze its intracellular localization with a genetic system. Previously, it has been shown that targeting of the human nucleotide exchange factor SOS to the plasma membrane in the vicinity of Ras is sufficient for activating the Ras signalling pathway of *S. cerevisiae* [14,20]. Only plasma membrane-targeted human SOS could replace the essential function of the yeast Cdc25 protein, thus enabling *cdc25*$^{ts}$ mutants to grow at the restrictive temperature.

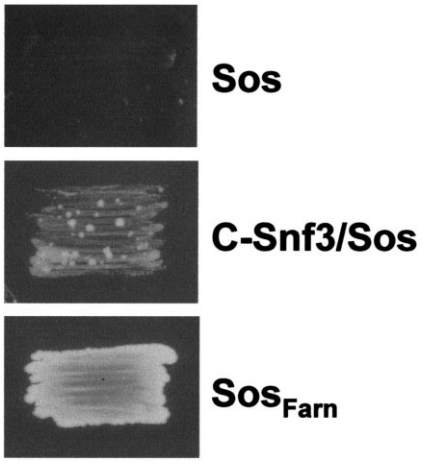


Fig. 1. The Snf3 C-terminal domain interacts with components at the plasma membrane. A *cdc25-2*$^{ts}$ strain [14] was transformed with plasmids expressing the human Sos GEF protein (hSos) or various fusion proteins, and incubated on selective YNB 2% glucose medium at 37°C for 4 days. hSos was expressed either unmodified (Sos, plasmid pADNS-p110-5′Sos), fused with a farnesylation sequence (Sos$_{Farn}$, plasmid pADNS-5′SosF) [14], or the soluble C-terminal domain of Snf3 was fused at its carboxy-terminus to hSos (C-Snf3/Sos, plasmid pSnf3c-C-ADNS). hSos can complement the *cdc25* mutation only if targeted to the plasma membrane.

To test whether the soluble C-terminal tail of Snf3 interacts with other components at the plasma membrane, it was fused in front of a highly active truncated version of the SOS coding region, resulting in plasmid pSnf3c-C-ADNS, and transformed into the *cdc25*$^{ts}$ mutant strain [14]. In contrast to a plasmid expressing the human SOS gene alone, the pSnf3c-C-ADNS plasmid supported slow growth at the restrictive temperature of 37°C (Fig. 1), indicating that the isolated C-terminal domain of Snf3 is partially localized at the plasma membrane. As growth could be observed on media with either inducing (glucose) or non-inducing (galactose) carbon sources (not shown), plasma membrane interactions of the Snf3 tail are not regulated by the carbon source, consistent with the constitutive signalling function of the isolated C-terminal tail.

Plasmid pS3c11 carries the C-terminal tail of Snf3 followed by six histidine codons. The plasmid was transformed into the *snf3* deletion mutant SKY1 [10], and protein crude extracts were prepared. The His$_6$-tagged C-terminal tail of Snf3 was purified on nickel-agarose columns, separated by SDS–PAGE and detected with anti-His$_6$ antibodies. As can be seen in Fig. 2, the Snf3 C-terminal tail runs with a molecular weight around 56 kDa but showing two distinct bands. To test whether the upper band might result from phosphorylation, the crude extracts were incubated with 10 U alkaline phosphatase for 4 h at 37°C before gel electrophoresis. Indeed, in this case the upper of the two bands was nearly completely lost (Fig. 2), indicating that the C-terminal tail of Snf3 is phosphorylated. Again, phosphorylation of the isolated C-terminal domain was independent of the carbon source (data not shown).

### 3.5. The glucose sensors cannot transport glucose

Snf3 is not able to transport glucose [5,12]. It might be suggested that the large C-terminal cytosolic domain of Snf3 inhibits an intrinsic transport activity. To test this, plasmid YEpSNHX7, containing the Snf3 membrane spanning part

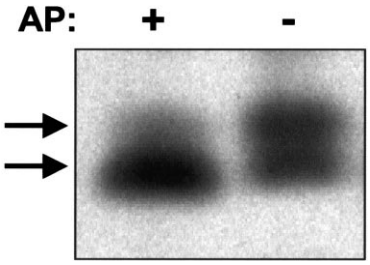


Fig. 2. The Snf3 C-terminal domain is phosphorylated. The separated C-terminal domain of *SNF3* was fused with six histidine codons (plasmid pS3c11), transformed into a *snf3* deletion strain (SKY1) and purified on nickel-agarose columns. It was separated by SDS–PAGE and detected with anti-$His_6$ antibodies. Two bands could be observed at about 56 kDa. After incubation with 10 U alkaline phosphatase (AP) for 4 h at 37°C (+) the upper of the two bands disappeared.

fused to the short C-terminal tail of the high-affinity glucose transporter Hxt7, was transformed into a hexose transporter-deleted ($hxt^-$) yeast strain, EBY.VW1000 [12], and plated first on a permissive maltose medium. After replica plating it could not restore growth of the $hxt^-$ strain on 0.1% or 2% glucose media, indicating that it is not able to transport glucose. Even after extensive selection for suppressor mutations using irradiation with UV light in sub-lethal doses or after random PCR mutagenesis of the *SNF3ΔC/C-HXT7* gene or the complete *SNF3* and *RGT2* genes, we did not obtain any mutant sensor genes able to restore growth of the $hxt^-$ strain on glucose (data not shown). These results indicate that despite their high similarities with the hexose transporters, Snf3 and Rgt2 do not have any intrinsic capacities to transport glucose.

### 3.6. Conclusions

We have found that Snf3 exhibits a signalling activity that is independent of the presence of extracellular glucose. However, Snf3 was further activated by low concentrations of glucose. Thus, it might be that Snf3 has a high basal signalling activity even in the absence of glucose which is transduced only to very sensitive promoters like *HXT7*. Alternatively, Snf3 activity during growth on non-fermentable carbon sources might be induced by the very low amounts of intracellular glucose which are produced by hydrolysis of storage carbohydrates or gluconeogenic glucose-6-phosphate. Binding of intracellular glucose to the glucose sensor would not be unexpected if one considers that also the glucose transporters act as symmetrical carriers (Maier, Völker, Boles and Fuhrmann, submitted). Signalling by free intracellular glucose is supported by the finding that an Snf3-dependent strong induction of invertase could be observed in an $hxt^-$ strain growing on ethanol [21]. An $hxt^-$ strain is supposed to be unable to secrete glucose leading to increased intracellular glucose concentrations.

A third possibility is that Snf3 is not only involved in glucose sensing. Indeed, Snf3 together with Rgt2 have recently been proposed to play dual roles as both glucose and cation sensors [22]. It was shown that mutations in the glucose sensors confer a growth advantage under conditions of $Na^+$ ions stress and a growth disadvantage under conditions of $K^+$ ion stress. These effects were completely independent of glucose concentration, which was kept at 2% in these studies. Thus, it seems possible that Snf3 is involved in various signalling complexes at the plasma membrane. Such a role would be consistent with the finding that the signal transducing part of Snf3 is its large cytoplasmic C-terminal tail which can function independent of the membrane spanning part. Attachment to the membrane spanning region of Snf3 is only required for glucose sensitivity while it might also act in other signalling complexes which act independent of glucose.

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