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Role of Protein Phosphatase Reg2-Glc7 in the Regulation of the Yeast Stress Response Kinase, Snf1

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ROLE OF PROTEIN PHOSPHATASE REG2-GLC7 IN THE REGULATION OF THE YEAST
STRESS RESPONSE KINASE, SNF1

by

Marcin Maziarz

A Dissertation Submitted in
Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
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ABSTRACT

ROLE OF PROTEIN PHOSPHATASE REG2-GLC7 IN THE REGULATION OF THE YEAST STRESS RESPONSE KINASE, SNF1

by

Marcin Maziarz

The University of Wisconsin-Milwaukee, 2016
Under the Supervision of Professor Sergei Kuchin

Kinases of the AMP-activated protein kinase (AMPK) family are conserved in eukaryotes and play central roles in responses to reduced energy availability. AMPK, nicknamed the “fuel gauge” of the cell, monitors cellular energy status via the ratio of AMP to ATP nucleotides. AMPK restores energy homeostasis by reducing energy “spending” and increasing energy “income”. Correspondingly, defects in AMPK signaling have been implicated in diseases including type II diabetes, obesity, and cancer.

In yeast, the AMPK homolog is Snf1 protein kinase. Glucose is the preferred carbon/energy source of yeast, and thus limitation for glucose similarly activates Snf1. Snf1 activation requires phosphorylation of its T-loop threonine (Thr210) by upstream kinases. When glucose is abundant, Snf1 is inhibited by Thr210 dephosphorylation. The latter involves the function of type 1 protein phosphatase Glc7, which is targeted to Snf1 by a regulatory subunit, Reg1. The *reg1* mutation causes increased Snf1 activity and mimics various aspects of glucose limitation, including slower growth. Reg2 is another Glc7 regulatory subunit encoded by a paralogous gene, *REG2*. The goal of our study was to determine if Reg2 has a role in Snf1 regulation. Indeed, we have found that Reg2 contributes to Snf1 Thr210 dephosphorylation. Consistent with this role, Reg2 interacts with wild-type Snf1 but not with non-phosphorylatable Snf1-T210A.

Additionally, the ability of Reg2 to regulate Snf1 depends on the Reg2-Glc7 interaction. Reg2 accumulation increases in a Snf1-dependent manner during prolonged glucose deprivation, and glucose-starved cells lacking Reg2 exhibit delayed Snf1 Thr210 dephosphorylation and slower growth recovery upon glucose replenishment. Accordingly, cells lacking Reg2 are outcompeted by wild-type cells in the course of several glucose starvation/replenishment cycles. Collectively, our results support a model in which Reg2-Glc7 contributes to the negative control of Snf1 in response to glucose re-feeding after prolonged starvation. The competitive growth advantage provided by Reg2 underscores the evolutionary significance of this paralog for *S. cerevisiae*.

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I dedicate this dissertation
to my lovely fiancée,
Justyna Szulc.

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LIST OF ABBREVIATIONS

α -CTD	alpha carboxy-terminal domain
AID	Auto-inhibitory domain
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
AMPKK	AMP-activated protein kinase kinase
ATP	adenosine triphosphate
β -CBM	beta carbohydrate binding module
β -CTD	beta carboxy-terminal domain
CBS	cystathione- β -synthase
CaMKK	Ca ²⁺ /Calmodulin-activated kinase kinase
GAD	Gal4 Activation Domain
GBD	Glycogen binding domain
Glc7	Glycogen 7 (yeast type 1 protein phosphatase)
Lkb1	Liver kinase B1
PKA	Protein kinase A
PP1	Type 1 protein phosphatase
Snf1	Sucrose non-fermenting 1 (catalytic subunit)
SNF1	Sucrose non-fermenting 1 (heterotrimeric protein complex)
SUMO	Small ubiquitin-like modifier protein
TOR	Target of rapamycin

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Chapter 1: Introduction

Overview

The ability to sense and properly react to changes in energy levels is critical for all living organisms. When we are starving, it makes more sense to eat than to run a marathon. The same logic applies to eukaryotic cells under conditions of energy stress. Glucose is the preferred source of energy for many organisms, but is not always available. Fortunately, cells are well-equipped to deal with energy stress such as glucose limitation, with the help of metabolic pathways that react appropriately to falling energy levels. Likewise, once the stress is gone, the ability to quickly turn off this stress response is critical, especially for microorganisms in competitive environments. In eukaryotic cells, one of the major components of the energy stress response is AMP-activated protein kinase.

AMP-activated protein kinase

AMP-activated protein kinase (AMPK) is an energy sensing protein in eukaryotes that is often described as the “fuel gauge” of the cell (Hardie, Carling, & Carlson, 1998). Organisms ranging from simple yeast to multicellular humans utilize AMPK as a way to monitor cellular energy reserves, which can become depleted as a result of glucose starvation, exercise, or other energy stressors [reviewed in (Hardie, Ross, & Hawley, 2012; Hardie & Sakamoto, 2006)]. One way to monitor energy status is to sense levels of adenine nucleotides. As one of the main energy “currencies” in the cell, ATP (adenosine triphosphate) is used for a large number of enzymatic reactions. As this currency is spent, “change” is returned in the form of AMP and ADP. AMPK senses the relative ratio between AMP and ATP to determine if cells are experiencing energy stress [reviewed in (Hardie, 2011; Hardie & Hawley, 2001)].

Under stress conditions, AMPK becomes activated and springs into action to restore energy homeostasis and balance the energy “budget” (Fig. 1). This is accomplished by reducing energy “spending” and increasing energy “income”. AMPK reduces spending by reducing anabolic pathways (for instance, by inhibiting cell growth) and upregulating catabolic pathways (for instance, by increasing glucose uptake) (Hardie, 2007; Hardie et al., 2012). More specifically, AMPK uses its kinase activity to phosphorylate metabolic enzymes and to control gene expression by phosphorylating transcription factors and histone proteins (Hardie, 2011; Hardie et al., 2012)

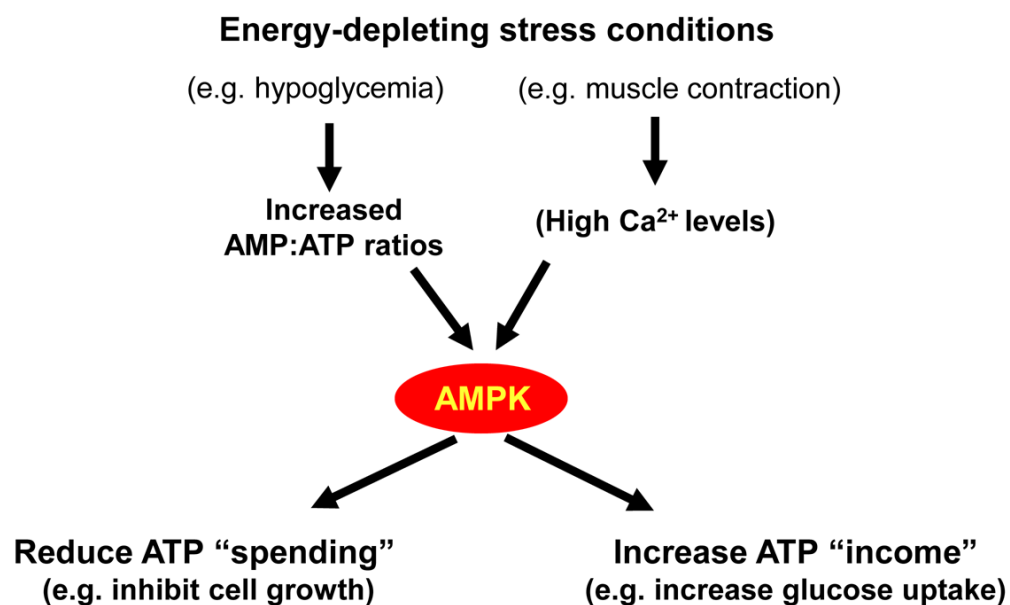


Fig. 1. General role of AMP-activated protein kinase (AMPK). AMPK is a stress response protein in eukaryotes, often nicknamed the “fuel gauge” of the cell. Energy-depleting conditions such as muscle contraction (which coincides with high Ca^{2+} levels) or glucose limitation promote high AMP to ATP ratios, which activate AMPK. AMPK functions to restore energy homeostasis by reducing anabolic/biosynthetic pathways, and increasing catabolic ATP-generating pathways. Adapted from Barrett 2011.

Heterotrimeric structure of AMPK

AMPK is a heterotrimeric protein complex composed of a catalytic α subunit, and regulatory β and γ subunits (Hardie, 2007; Hardie et al., 2012; Hardie, Schaffer, & Brunet, 2016) (Fig. 2). Mammalian genomes code for two α , two β , and three γ isoforms, resulting in 12 possible heterotrimeric complexes. The N-terminus of the α -subunit contains a conserved kinase domain, which provides AMPK with the enzymatic activity to phosphorylate serine or threonine residues. The kinase domain contains an important threonine residue (e.g. Thr172 in the rat protein) whose phosphorylation is required for maximal catalytic activity (Hawley et al., 1996; Suter et al., 2006). The α subunit also contains an auto-inhibitory domain (AID) which inhibits the activity of the kinase, since truncated constructs lacking the AID exhibit much higher activity than those with the AID still present (Crute, Seefeld, Gamble, Kemp, & Witters, 1998; Pang et al., 2007). A linker region joins the kinase domain to the C-terminal domain and tethers the α subunit to the gamma subunit (Hardie et al., 2012). The carboxy-terminal domain of the α subunit (α -CTD) interacts with the β subunit.

The β subunit contains a carbohydrate binding module (β -CBM) near the N-terminus of the protein. The β -CBM binds glycogen (Hudson et al., 2003; Polekhina et al., 2003), which inhibits AMPK (McBride, Ghilagaber, Nikolaev, & Hardie, 2009). The C-terminal domain of the β subunit (β -CTD) interacts with the other subunits of the AMPK complex.

The regulatory γ subunit acts as the energy-sensing subunit and regulates the activity of the complex [reviewed in (Hardie et al., 2016)]. It contains four consecutive regions (numbered 1-4) called cystathione- β -synthase (CBS) repeats, which function as binding sites for adenine nucleotides (Xiao et al., 2007). Binding of the low-energy nucleotides AMP or ADP to sites 1 and 3 can trigger increased kinase activity by multiple mechanisms including allosteric activation

(by AMP) or by blocking phosphatases from access to Thr172 (by AMP or ADP) (Xiao et al., 2011).

Much of the knowledge of AMPK structure and function has been derived from numerous crystallographic structures. However, genetic and mutational studies have also provided significant information about the regulation of AMPK.

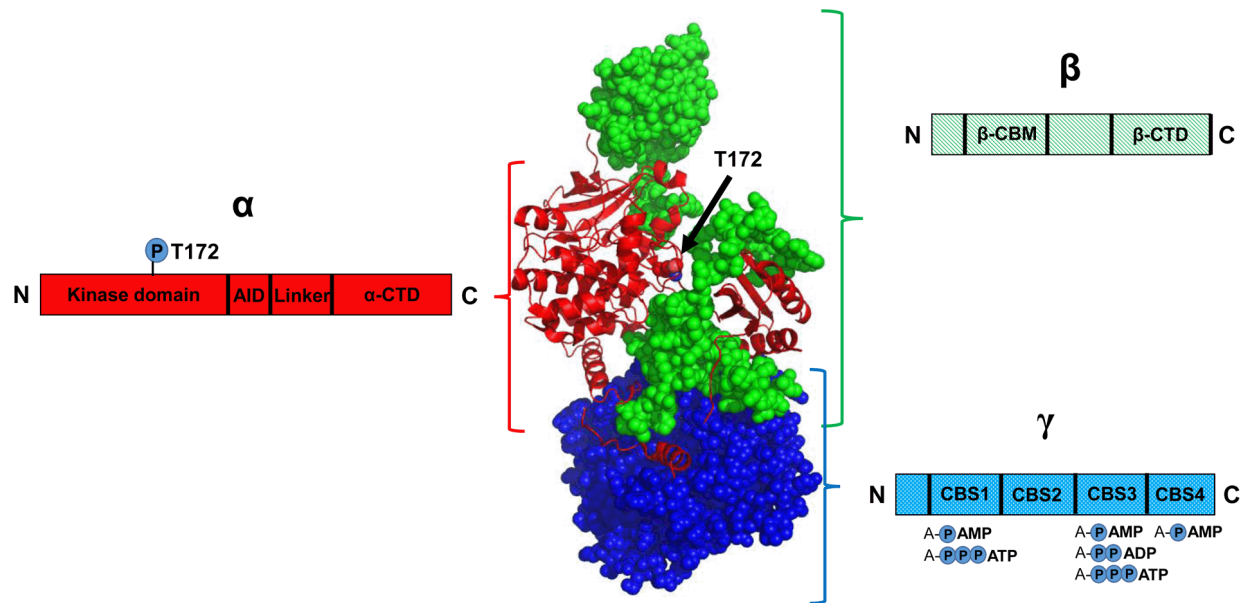


Fig. 2. Domain organization in AMPK subunits. AMPK is a heterotrimer. The α subunit contains the catalytic kinase domain, including the activation loop threonine 172 whose phosphorylation increases the activity of the kinase. The auto-inhibitory domain (AID) interacts with and inhibits the kinase domain. The linker domain wraps around the γ subunit to tether it to the α subunit. In the β subunit, the carbohydrate-binding module (β -CBM) binds glycogen and the C-terminal domain (β -CTD) interacts with the α and γ subunits. The γ subunit is the energy-sensing subunit. It contains four consecutive cystathione- β -synthase (CBS) domains which bind adenine nucleotides. Site 2 is unoccupied, site 4 is permanently bound to AMP. The other sites can be occupied by either AMP, ATP (sites 1 and 3), or ADP (site 3), which affects AMPK kinase activity. AMPK model was rendered in Pymol from PDB file 4RER. α subunit is in “cartoon” view. β and γ subunits are in “sphere” view. Adapted from Hardie *et al.* 2012 and Hardie *et al.* 2016.

Regulation of AMPK

Although AMPK is a protein kinase, it is itself phosphorylated by upstream kinases to promote its catalytic activation. AMPK is phosphorylated on a highly conserved residue in the kinase domain known as the T-loop threonine (Thr172) (Hawley et al., 1996) in response to an increase in the intracellular AMP to ATP ratio (Hardie, 2011; Hardie & Hawley, 2001), which corresponds to low energy status. Following typical eukaryotic nomenclature, this phosphorylation is performed by AMPKK (AMPK kinase). In most cell types, AMPKK is a protein complex composed of the liver kinase B1 (LKB1) bound to the proteins STRAD and MO25 (Hawley et al., 2003). Mutations in *LKB1* are associated with a rare disease known as Peutz-Jeghers syndrome (Hemminki et al., 1998; Mehenni et al., 1998).

AMPK can also be activated by Ca²⁺/Calmodulin-activated kinase kinase (CaMKK) in response to elevated calcium inside the cell (Hawley et al., 2005; Hurley et al., 2005; Woods et al., 2005). Since high cytosolic calcium levels are often associated with ATP-consuming processes, it is believed that this could be a wise anticipatory stress adaptation (Hardie, 2007).

Although a great deal of information is known about the upstream activating kinases, the identity of the phosphatase(s) that inactivate AMPK is still unclear. Some studies have found evidence that protein phosphatase 2C (PP2C) and protein phosphatase 1 (PP1) are responsible (Davies et al., 1995; Garcia-Haro et al., 2010; Sanders, Grondin, Hegarty, Snowden, & Carling, 2007).

Other than falling energy levels or increasing calcium levels, there is also a growing body of evidence that AMPK activity is regulated by “non-canonical mechanisms”. As a result of high reactive oxygen species (ROS) concentrations, AMPK also becomes activated, although the

exact nature of this mechanism is unclear (Hardie et al., 2012). DNA damage may also trigger activation of AMPK by the ATM kinase (Sanli et al., 2010; Zhou et al., 2011).

Although research on AMPK has been continuing for almost 30 years, much is still unknown. More knowledge about AMPK structure and regulation can be gleaned by studying AMPK homologs in other species. For instance, an assembled (partial) heterotrimeric complex was first crystallized from the yeast *S. pombe* (Townley & Shapiro, 2007). Even more has been determined using the budding yeast, *S. cerevisiae*; the identity of the upstream AMPKKs was discovered via homology to the yeast upstream kinases (Hawley et al., 2003; Hong, Momcilovic, & Carlson, 2005; Hurley et al., 2005).

SNF1 protein kinase

In the yeast *Saccharomyces cerevisiae*, the AMPK α homolog is called Snf1. As the name suggests, Snf1 (sucrose non-fermenting 1) was discovered in 1981 in a screen for yeast mutants that were unable to utilize sucrose (Carlson, Osmond, & Botstein, 1981). In many microorganisms including yeast, glucose is the preferred carbon and energy source, and cells must respond to glucose limitation stress by rewiring gene expression to import and break down alternative sugars such as sucrose (see reference (Conrad et al., 2014) for a review).

Comparisons

Yeast SNF1 is an excellent model for studying AMPK due to the numerous structural, functional, and regulatory similarities between them. Like AMPK, SNF1 is also a heterotrimeric complex (Jiang & Carlson, 1997; Woods et al., 1996). It is composed of the catalytic α kinase subunit, Snf1, which associates with one of three alternative targeting/regulatory β subunits:

Gal83, Sip1, or Sip2 (Erickson & Johnston, 1993; Jiang & Carlson, 1997; Yang, Hubbard, Carlson, Celenza, & Eng, 1992; Yang, Jiang, & Carlson, 1994), and with a regulatory γ subunit, Snf4 (Celenza & Carlson, 1989; Celenza, Eng, & Carlson, 1989). The kinase domain of the Snf1 subunit is 61% identical to its mammalian counterpart, and two out of the three alternate β subunits have a glycogen binding domain (GBD) that strongly resembles the β -CBM found in AMPK β (Hedbacker & Carlson, 2008).

There is also a great deal of conservation between AMPK and SNF1 regulation. Snf1 is similarly activated by phosphorylation on a conserved T-loop in its kinase domain (Thr210 in Snf1) (McCartney & Schmidt, 2001). Experiments have shown that the upstream AMPK kinases (LKB1 and CaMKK) are able to phosphorylate and activate Snf1 in yeast (Hong et al., 2005; Woods et al., 2005). Likewise, some of the yeast upstream kinases (see below) are able to activate AMPK *in vitro* (Hong, Leiper, Woods, Carling, & Carlson, 2003; Sutherland et al., 2003). Further demonstration of the functional conservation between SNF1 and AMPK is that the activity and phosphorylation of an AMPK complex still remains glucose regulated even when expressed in yeast cells (Ye et al., 2014). Unlike mammalian AMPK, yeast SNF1 is not believed to be regulated by AMP. However, consistent with the role of SNF1 as an energy-sensing regulator, there is evidence that ADP may help activate this kinase (Mayer et al., 2011).

In the following sections, the upper-case “SNF1” will be used when specifically referring to the heterotrimeric complex. The term “Snf1” will be used to refer to the catalytic alpha subunit (the kinase) and its activity.

Stresses that activate Snf1

As a central stress response kinase, Snf1 is known to respond to a multitude of environmental stimuli. Most notably, glucose limitation activates Snf1 (McCartney & Schmidt, 2001; W. A. Wilson, Hawley, & Hardie, 1996; Woods et al., 1994). Yeast cells prefer glucose as their carbon and energy source (2% is considered abundant glucose), and therefore glucose limitation is a stress condition that triggers Snf1 activation. Nitrogen limitation activates Snf1, and this may involve negative regulation by the nutrient-sensing TOR kinase (Orlova, Kanter, Krakovich, & Kuchin, 2006). High pH activates Snf1 (Casamayor et al., 2012; Hong & Carlson, 2007), and it has been proposed that this is because glucose utilization is poor in alkaline pH environments (Casamayor et al., 2012). Sodium stress activates Snf1 (Hong & Carlson, 2007). Recent evidence points to Snf1 as being involved in the DNA damage response as well (Simpson-Lavy, Bronstein, Kupiec, & Johnston, 2015). Even artificial stresses such as rapid centrifugation are known to activate Snf1, complicating laboratory analysis of live cells and cell extracts (W. A. Wilson et al., 1996).

Function of Snf1

Snf1 is able to maintain energy homeostasis by turning on (de-repressing) glucose-repressed genes, turning on genes involved in the utilization of alternative carbon sources, promoting respiration, and by regulating the activity or localization of metabolic enzymes (Fig. 3) (Hedbacker & Carlson, 2008).

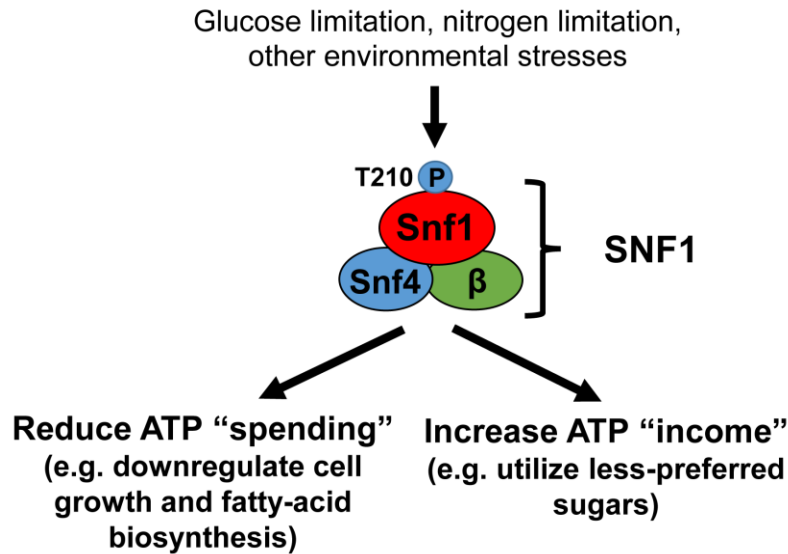


Fig. 3. Role of Snf1. During glucose limitation and other environmental stresses, the catalytic Snf1 subunit becomes phosphorylated and activated. Once activated, Snf1 enforces energy homeostasis by downregulating energy-expensive biosynthetic pathways and upregulating ATP-generating pathways. The regulatory β subunit can be one of three alternate subunits: Sip1, Sip2, or Gal83. Adapted from Barrett et al., 2011.

Snf1 regulates gene expression by phosphorylating transcriptional activators and repressors and by affecting chromatin structure (Lin, Manchester, & Gordon, 2003; Lo et al., 2001; Lo et al., 2005; Young, Kacherovsky, & Van Riper, 2002). Once activated, Snf1 migrates to the nucleus (Hedbacker, Hong, & Carlson, 2004; Vincent, Townley, Kuchin, & Carlson, 2001) where it acts as a transcriptional regulator by phosphorylating activators and repressors (De Wever, Reiter, Ballarini, Ammerer, & Brocard, 2005; Ostling & Ronne, 1998; Treitel, Kuchin, & Carlson, 1998).

Snf1 most famously regulates the Mig1 transcriptional repressor. Snf1 activates the expression of glucose-repressed genes by phosphorylating the Mig1 repressor at four sites, which makes Mig1 lose its interaction with the Ssn6-Tup1 corepressor complex, effectively removing it from promoters of glucose-repressed genes (Papamichos-Chronakis, Gligoris, & Tzamarias, 2004; Smith, Davies, Wilson, Carling, & Hardie, 1999; Treitel et al., 1998), as well as promotes

Mig1 nuclear exclusion (DeVit & Johnston, 1999). One such gene is *SUC2* (Nehlin & Ronne, 1990; Vallier & Carlson, 1994), whose product, invertase, cleaves sucrose into glucose and fructose. Snf1 also phosphorylates and regulates other transcriptional regulators such as Msn2 (De Wever et al., 2005), Cat8 (Randez-Gil, Bojunga, Proft, & Entian, 1997), and Sip4 (Vincent & Carlson, 1998). For instance, Snf1 regulates Msn2 by phosphorylating it and promoting its cytoplasmic localization (De Wever et al., 2005; Mayordomo, Estruch, & Sanz, 2002). Snf1 is even able to directly interact with and activate the transcriptional machinery (RNA Polymerase II holoenzyme), which can be utilized as a readout of Snf1 activity and localization (Kuchin, Treich, & Carlson, 2000).

Snf1 also phosphorylates other proteins that are not transcriptional regulators. For example, Snf1 phosphorylates and inactivates Acc1, acetyl-acid carboxylase, to downregulate energetically-expensive fatty-acid biosynthesis during energy limitation (Woods et al., 1994; Zhang, Galdieri, & Vancura, 2013).

Structure of the SNF1 complex

Like AMPK, SNF1 is a heterotrimeric complex composed of one each of an α , β , and γ subunit (Fig. 4). The α subunit, simply called Snf1, is the catalytic subunit. Its N-terminal kinase domain includes the activation loop threonine 210 (orthologous to threonine 172 in AMPK) whose phosphorylation is required for Snf1 activity. The C-terminal domain of Snf1 contains an AID region which bends back and interacts with the kinase domain to inhibit Snf1 activity (Celenza & Carlson, 1989; Jiang & Carlson, 1996), as well as a region known to interact with the β subunits and with Snf4.

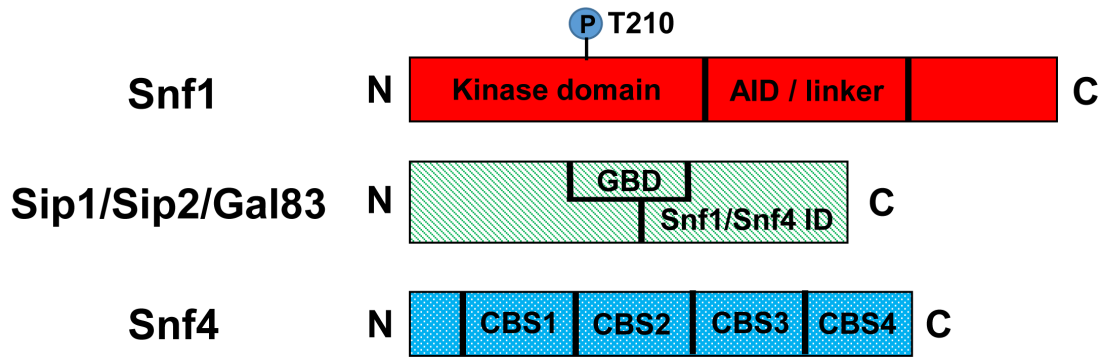


Fig. 4. Domain organization of SNF1 subunits. SNF1 is a heterotrimer. The Snf1 α subunit contains the catalytic kinase domain, including the activation loop threonine 210 whose phosphorylation increases the activity of the kinase. The auto-inhibitory domain/linker (AID/linker) interacts with and inhibits the kinase domain and is also required for Snf4 interaction; this domain shares limited homology with the AMPK linker region and has not been crystallographically resolved. The C-terminus of Snf1 interacts with the regulatory β subunits. Sip1, Sip2, and Gal83 are alternate β subunits, with the latter two containing a verified glycogen-binding domain (GBD). The GBD partially overlaps with the Snf1 and Snf4 interaction domain (Snf1/Snf4 ID). Snf4 is the energy-sensing γ subunit. It contains four consecutive cystathione- β -synthase (CBS) domains, which bind adenine nucleotides. Binding of ADP protects SNF1 from dephosphorylation. Adapted from Hedbacker et al 2008 and Chandrashekarappa et al 2011.

The β subunits are responsible for targeting the SNF1 complex to the appropriate subcellular compartments (Hedbacker, Hong, et al., 2004; Hedbacker, Townley, & Carlson, 2004; Vincent et al., 2001). Studies with mutant strains expressing only one β subunit have shown that Gal83 contributes the most to SNF1 kinase activity (Hedbacker, Hong, et al., 2004). Gal83 also seems to be the most abundant SNF1 β subunit during growth on glucose, and it is also responsible for targeting Snf1 to the nucleus during glucose limitation. The Sip1 and Sip2-containing isoforms are targeted to the vacuole and the cytoplasm, respectively (Vincent et al., 2001).

Snf4 is the γ regulatory subunit of the SNF1 complex. During glucose limitation, Snf4 interacts with the AID of the α catalytic subunit. By doing so, this releases Snf1 from its auto-inhibited state and, together with Snf1 Thr210 phosphorylation, promotes the activation of the

kinase (Leech, Nath, McCartney, & Schmidt, 2003). Much work has been done to determine the identity of the energy-carrying nucleotide(s) that bind to the SNF1 complex to regulate its activity. Due to the difficulty of accurately measuring intracellular binding of specific adenine nucleotides to a specific protein, much of this work has been performed *in vitro*. The current consensus is that the γ subunit of the Snf1 complex is able to bind to all three variants of adenine nucleotides (AMP, ADP, and ATP) with varying affinity (Mayer et al., 2011). However, of these three, ADP is the nucleotide that protects Snf1 from dephosphorylation, thus shifting the balance toward the Thr210-phosphorylated form of Snf1 (Chandrashekarappa, McCartney, & Schmidt, 2011; Mayer et al., 2011).

Regulation of Snf1

Snf1 activity is regulated post-translationally, most notably via phosphorylation of its activation loop Thr210 residue (Tabba, Mangat, McCartney, & Schmidt, 2010), by ubiquitination (M. A. Wilson et al., 2011) and SUMOylation (Simpson-Lavy et al., 2015; Simpson-Lavy & Johnston, 2013), as well as by ADP binding to the γ subunit Snf4 (Fig. 5). In addition, Thr210 phosphorylation depends on the presence of the β subunits (Schmidt & McCartney, 2000). Snf1 does not seem to be regulated at the level of *SNF1* gene transcription (Celenza & Carlson, 1984), although *GAL83* gene transcription is regulated by glucose (Santangelo, 2006).

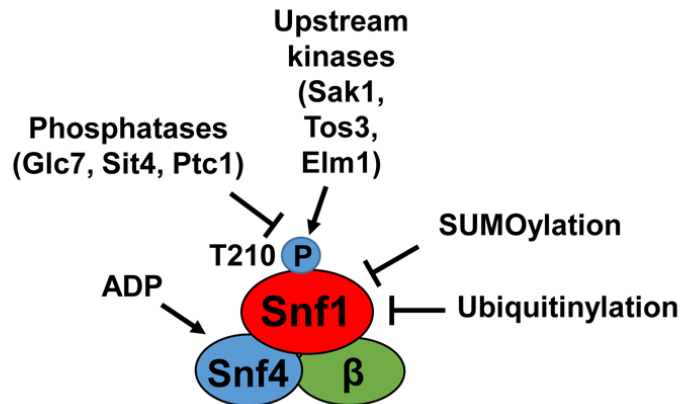


Fig. 5. Summary of regulatory mechanisms controlling SNF1 activity. During glucose limitation and other environmental stresses, the catalytic Snf1 subunit is phosphorylated on its Thr210 residue by upstream kinases. The low energy nucleotide, ADP, binds to Snf4 and protects Snf1 from dephosphorylation. During growth on abundant glucose, the phospho-Thr210 residue on Snf1 becomes dephosphorylated by phosphatases, including the Reg1-Glc7 type 1 protein phosphatase. Snf1 activity and stability are decreased by SUMOylation and ubiquitinylation.

Regulation of Snf1 by SUMOylation and ubiquitination

Although Snf1 Thr210 phosphorylation is considered to be the classical way of Snf1 regulation that affects catalytic activity and localization of the kinase (see below), studies have pointed to other post-translational modifications of Snf1, which affect its regulation and function.

It was recently shown that Snf1 is SUMOylated. SUMOylation is the attachment of a small ubiquitin-like modifier protein (SUMO) to a lysine of a protein with the help of an E3 ligase. Generally, SUMOylation has a few functions: (i) it marks proteins for ubiquitination and degradation (Uzunova et al., 2007), (ii) it promotes new interactions between the SUMOylated protein and target proteins (Song, Durrin, Wilkinson, Krontiris, & Chen, 2004). Snf1 is SUMOylated on Lys549 by the E3 ligase Mms21 (Simpson-Lavy & Johnston, 2013). Mutation of this lysine to an arginine results in a non-SUMOylatable Snf1 which is more stable and which

exhibits higher kinase activity in glucose. This suggests that SUMOylation of Snf1 functions to promote Snf1 degradation and inhibit its activity (Simpson-Lavy & Johnston, 2013). One benefit for such glucose-induced inhibition of Snf1 by SUMO is that it could allow for expression of *HXT* genes that encode hexose transporters.

Snf1 is also ubiquitinated. Although the identity of the ubiquitin ligase is not known, Snf1 is deubiquitinated by Ubp8 (M. A. Wilson et al., 2011). As one would expect if Snf1 is truly ubiquitinated – a mechanism that reduces protein stability - deletion of the antagonistic deubiquitinating enzyme Ubp8 tips the scales towards ubiquitination and results in lower Snf1 protein stability (M. A. Wilson et al., 2011). Ubiquitination also affects Snf1 Thr210 phosphorylation and kinase activity (M. A. Wilson et al., 2011).

Regulation of Snf1 by Thr210 phosphorylation/dephosphorylation

Although SUMOylation and ubiquitinylation have recently emerged as processes that regulate Snf1, phosphorylation and dephosphorylation of its activation loop threonine is the most recognized method by which Snf1 is regulated. Snf1 catalytic activity increases substantially by phosphorylation of its T-loop threonine (Thr210). In 2003, three different groups published studies describing that Snf1 is phosphorylated by three upstream kinases: Sak1, Tos3, and Elm1 (Hong et al., 2003; Nath, McCartney, & Schmidt, 2003; Sutherland et al., 2003). The three kinases are partially redundant, since the presence of any one of them is sufficient to activate Snf1 (Hong et al., 2003). Cells are unable to grow on alternative carbon sources only when all three upstream kinases are missing (Hong et al., 2003), providing further evidence of their redundancy. Despite this overlap, Sak1 seems to play the largest role in Snf1 catalytic activity. Of the three single upstream kinase deletion strains, cells lacking Sak1 exhibit the lowest Snf1

kinase activity (Hedbacker, Hong, et al., 2004; Hong et al., 2003). Moreover, Sak1 is required not only for catalytic activity of Snf1-Gal83, but also for its nuclear localization (Hedbacker, Hong, et al., 2004).

Snf1 is inactivated by dephosphorylation. The main Snf1 phosphatase is Glc7, the yeast homolog of the highly conserved type 1 protein phosphatase (PP1). Whereas protein kinases tend to be fairly specific in their substrate recognition, serine/threonine protein phosphatases lack specificity on their own. To remedy this, eukaryotes have numerous regulatory subunits that bind to phosphatases and regulate their activity and/or substrate specificity (Ceulemans & Bollen, 2004). Reg1-Glc7 is considered the “main” phosphatase complex that dephosphorylates Snf1. Reg1 was first identified as a gene involved in glucose signaling, since cells lacking Reg1 fail to undergo glucose repression (Matsumoto, Yoshimatsu, & Oshima, 1983). Reg1 was soon identified as a regulatory subunit of Glc7 (Tu & Carlson, 1995), and was shown to interact with Snf1 and regulate the conformation of the kinase complex (Ludin, Jiang, & Carlson, 1998; Sanz, Alms, Haystead, & Carlson, 2000). Cells lacking Reg1 exhibit constitutive Snf1 Thr210 phosphorylation and Snf1 activity even in glucose (McCartney & Schmidt, 2001), when Snf1 is normally turned off. Cells lacking Reg1 also grow slowly (Tu & Carlson, 1995), which is a Snf1-dependent phenotype (Frederick & Tatchell, 1996), suggesting that Snf1 activation results in slow growth, which is consistent with its role in enforcing energy conservation.

Interestingly, although *reg1* cells show constitutive Snf1 phosphorylation during growth in high glucose (McCartney & Schmidt, 2001), Snf1 phosphorylation is still higher in low glucose, raising the possibility that other PP1 complexes or even entirely different phosphatases could be involved in Snf1 inactivation. Sit4, a type 2A-like protein phosphatase and Ptc1, a type 2C protein phosphatase have been more recently identified as Snf1 phosphatases (Ruiz, Xu, &

Carlson, 2011, 2013). However, identifying the full set of Snf1 phosphatases has been a challenge since combinatorial mutants such as *reg1 sit4* are lethal due to hyperactivation of Snf1. In this regard, it is also interesting that Reg2 was identified as another PP1 regulatory subunit with homology to Reg1. *reg1 reg2* double mutants grow extremely slowly, which can be remedied by deleting Snf1 (Frederick & Tatchell, 1996). Since previous work had shown that mutations that affect the slow growth of the *reg1* mutant could be potential components of the Snf1 pathway (Barrett, Orlova, Maziarz, & Kuchin, 2012), we set out to examine if Reg2 could be involved in Snf1 regulation.

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Chapter 2:

Physical and functional interactions between Reg2, a subunit of type 1 protein phosphatase, and the Snf1 protein kinase

Abstract

Snf1 protein kinase of *Saccharomyces cerevisiae* regulates responses to glucose/energy limitation. Under glucose-limiting conditions, Snf1 is activated by phosphorylation of its T-loop threonine (Thr210) by upstream kinases. Thr210 dephosphorylation under glucose-rich conditions involves the function of type 1 protein phosphatase Glc7, which is targeted to Snf1 by a regulatory subunit, Reg1. Reg2 is another regulatory subunit of Glc7, and previous genetic evidence suggested a link between Reg2 and Snf1. Here, we have explored this link in more detail. Yeast two-hybrid and coimmunoprecipitation assays indicate that Reg2 interacts with wild-type Snf1 but not with non-phosphorylatable Snf1-T210A. Experiments with overexpression and deletion of *REG2* provide evidence that Reg2 contributes to Snf1 Thr210 dephosphorylation. Collectively, our results suggest that Reg2 interacts with phospho-Thr210-Snf1 and makes a fine-tuning contribution to its inactivation in response to abundant glucose.

Introduction

Kinases of the AMP-activated protein kinase (AMPK) family are conserved in eukaryotes from yeast to humans and play central roles in responses to reduced energy availability. In mammalian cells, AMPK is activated by conditions that lead to an increase in the ratio of low-energy AMP nucleotides to ATP nucleotides, such as hypoglycemia [reviewed in references (Hardie, 2007b; Hardie, Ross, & Hawley, 2012)]. AMPK restores the balance between energy consumption and

energy generation by regulating gene expression and metabolic pathway activity (Fogarty & Hardie, 2009). AMPK also coordinates cell growth and proliferation with energy availability (Jones et al., 2005), such as by turning off biosynthetic pathways during energy starvation. Correspondingly, defects in AMPK signaling have been implicated in diseases including type II diabetes, obesity, and cancer (Fogarty & Hardie, 2009; Hardie, 2007a).

In the yeast *Saccharomyces cerevisiae*, the AMPK homolog Snf1 regulates responses to limitation for glucose, which is the most preferred carbon/energy source [reviewed in (Hedbacker & Carlson, 2008)]. Snf1 is largely inactive when glucose is abundant, but becomes activated during glucose limitation. Once activated, Snf1 “harvests” energy by promoting the utilization of alternate carbon sources, such as sucrose. Three protein kinases (Sak1, Tos3, and Elm1) function in a partially redundant manner to activate Snf1 by phosphorylation of its T-loop threonine residue (Thr210); dephosphorylation and inactivation of Snf1 involves PP2A-like protein phosphatase Sit4 and type 1 protein phosphatase Glc7 (Hedbacker & Carlson, 2008; Ruiz, Liu, Xu, & Carlson, 2012; Ruiz, Xu, & Carlson, 2011).

Type 1 protein phosphatase Glc7 associates with various regulatory subunits that target it to specific substrates. Collectively, these phosphatase complexes have a broad repertoire of cellular functions, including the regulation of metabolism (Ceulemans & Bollen, 2004). One of these regulatory subunits, Reg1 (Tu & Carlson, 1995), is known to play a major role in facilitating Glc7’s ability to recognize and dephosphorylate Thr210 of Snf1 (Ludin, Jiang, & Carlson, 1998; McCartney & Schmidt, 2001; Zhang, McCartney, Chandrashekarappa, Mangat, & Schmidt, 2011). Cells lacking Reg1 display increased levels of phospho-Thr210-Snf1 and Snf1 kinase activity, as well as constitutive (glucose-insensitive) expression of Snf1-dependent genes required for alternate carbon source utilization (Hong, Momcilovic, & Carlson, 2005;

McCartney & Schmidt, 2001; Tu & Carlson, 1995). In addition, mutation of *REG1* confers a Snf1-dependent slow growth phenotype (Barrett, Orlova, Maziarz, & Kuchin, 2012; Frederick & Tatchell, 1996; Ruiz et al., 2011). This slow growth, which is observed even in the presence of abundant glucose, likely reflects hyperactivation of an energy-saving function of Snf1 and represents an interesting phenotype in terms of its possible relationship to the anti-cancer roles of the mammalian AMPK pathway. Genetic interactions of the *reg1* mutation with respect to the slow growth phenotype could identify additional regulators in the Snf1 pathway.

Reg2 is another regulatory subunit of Glc7 and shares homology with Reg1 (Frederick & Tatchell, 1996). Although the *reg2* mutation alone does not cause any phenotypic defects comparable to those caused by *reg1*, the *reg2* mutation exacerbates the slow growth phenotype of the *reg1* mutant; importantly, the slow growth of the *reg1 reg2* double mutant depends on Snf1, suggesting that, like Reg1, Reg2 might be functionally related to Snf1 (Frederick & Tatchell, 1996).

Here, we have addressed the possible role of Reg2 in Snf1 regulation. Yeast two-hybrid and co-immunoprecipitation assays provide evidence that Reg2 physically interacts with phospho-Thr210-Snf1. In addition, growth assays as well as immunoblot analysis of Snf1 Thr210 phosphorylation provide initial evidence that Reg2 makes a fine-tuning contribution to the dephosphorylation of Snf1 in response to abundant glucose.

Materials and Methods

Strains and growth conditions. The *S. cerevisiae* strains used in this study are listed in Table 1. Except for strain CTY10-5d (R. Sternglanz, SUNY, Stony Brook, NY) used for initial yeast two-hybrid assays, all strains were in the Σ 1278b genetic background and were descendants of strains

MY1384 (*MATa*, prototroph), MY1401 (*MAT α* *ura3 Δ* *leu2 Δ* *his3 Δ*), and MY1402 (*MATa* *ura3 Δ* *leu2 Δ* *trp1 Δ*) of the isogenic Sigma2000 series (Microbia, Cambridge, MA). To generate Σ 1278b derivatives with *reg2 Δ ::KanMX6*, the *KanMX6* marker sequence was amplified by PCR with primers flanking the *REG2* open reading frame (ORF). The mutant allele was first introduced into a wild-type diploid (MKY324 X MKY341) by transformation; all yeast transformations were performed using the standard lithium acetate method (Rose, Winston, & Hieter, 1990). The genotype of the heterozygous *REG2/reg2 Δ ::KanMX6* diploid was confirmed by PCR analysis of genomic DNA, and haploid *reg2 Δ ::KanMX6* segregants were recovered from the heterozygous diploid by tetrad analysis. Construction of the *reg1 Δ ::URA3* and *snf1 Δ ::KanMX6* derivatives was described previously (Kuchin, Vyas, & Carlson, 2002; Orlova, Kanter, Krakovich, & Kuchin, 2006). The *reg1 Δ reg2 Δ* double mutant was constructed by crossing MMY9 (*reg2 Δ ::KanMX6*) and KBY247 (*reg1 Δ ::URA3*), followed by tetrad analysis.

Rich medium was yeast extract-peptone (YEP) supplemented with extra tryptophan (40 mg/liter) and adenine (20 mg/liter); synthetic complete (SC) medium lacking appropriate supplements was used to select for plasmids (Rose et al., 1990). Unless indicated otherwise, the media contained 2% glucose, and yeast cells were grown at 30°C.

Yeast two-hybrid assays. Plasmids pIT469 (Kuchin, Treich, & Carlson, 2000) and pRJ79 (Jiang & Carlson, 1996) express LexA-Snf1 and VP16-Snf1 from vectors pEG202 (Golemis, Serebriiskii, Gjuris, & Brent, 1997) and pVP16 (Vojtek, Hollenberg, & Cooper, 1993), respectively. Plasmids pRJ215 and pRJ217 express LexA-Snf1-K84R and LexA-Snf1-T210A, respectively, from vector pEG202 (Kuchin, Vyas, Kanter, Hong, & Carlson, 2003; Treitel, Kuchin, & Carlson, 1998). To construct in-frame Reg2 fusion proteins, a PCR fragment

encompassing the *REG2* ORF was inserted at the BamHI site of pEG202 and pACTII (Legrain, Dokhelar, & Transy, 1994), yielding pLexA-Reg2 and pGAD-Reg2, respectively. The two-hybrid reporter strains were CTY10-5d carrying an integrated *lexAop-lacZ* reporter, or MKY343 carrying a *lexAop-lacZ* reporter plasmid pSH18-18, a derivative of pLR1Δ1 (West, Yocum, & Ptashne, 1984). The reporter strains were co-transformed with pairs of plasmids expressing the protein pairs being tested. Transformants were grown to mid-log phase with plasmid selection in SC lacking the appropriate supplements, and containing 2% glucose, and then shifted for 3 h to an otherwise identical medium containing 0.05% glucose. β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units, as described previously (Vyas, Kuchin, & Carlson, 2001). Briefly, cells are resuspended in buffer, permeabilized by the addition of SDS and chloroform, and the colorimetric ONPG substrate is added. Once yellow color is observed, the reaction is stopped by the addition of sodium carbonate, and Miller units are calculated based on the reaction time and OD₄₂₀ of the sample.

Expression of the fusion proteins was confirmed by immunoblotting, as follows. Representative transformants were grown under conditions identical to those used to measure β-galactosidase activity. Cell extracts were prepared using the boiling/alkaline lysis method (Orlova, Barrett, & Kuchin, 2008). Briefly, cultures were boiled for five minutes and centrifuged at 4,000 rpm to pellet cells. The collected cells were treated with 0.1 N NaOH and boiled in the presence of SDS-PAGE loading buffer. Proteins were separated by SDS-PAGE and analyzed by immunoblotting. LexA fusion proteins were detected with anti-LexA antibody (Millipore). The GAD-Reg2 fusion protein contains a hemagglutinin (HA) epitope tag between the Gal4 activation domain (GAD) and Reg2, and was detected with anti-HA antibody HA-7 (Sigma-Aldrich). VP16-Snf1 was detected using anti-polyhistidine antibody H1029 (Sigma-Aldrich),

which strongly recognizes Snf1 due to the presence of a natural tract of 13 consecutive histidines (Orlova et al., 2008). Thr210-phosphorylated Snf1 proteins were detected with anti-phospho-Thr172-AMPK (Cell Signaling Technology) as described previously (Orlova et al., 2008), which also recognizes the analogous phospho-Thr210 residue in Snf1. Signals were detected by enhanced chemiluminescence using Pierce ECL2 or HyGlo (Denville Scientific).

Coimmunoprecipitation assays. Plasmid pHA-Reg2 expresses N-terminal triple HA epitope-tagged Reg2 (HA-Reg2). It was constructed by inserting the *REG2* ORF into the BamHI site of vector pSK134HA (Orlova et al., 2006), which allows for the expression of proteins from the strong *ADHI* promoter. Plasmids pSM14 and pMO16 (Orlova, Ozcetin, Barrett, & Kuchin, 2010) express untagged wild-type Snf1 and Snf1-T210A, respectively, from vector pRS316 (Sikorski & Hieter, 1989). To ensure that only the desired version of Snf1 is present in immunoprecipitates, *snf1* Δ cells (strain MKY362) were transformed with plasmid pSM14 expressing Snf1 or with plasmid pMO16 expressing Snf1-T210A. This strain was also co-transformed with plasmid pHA-Reg2. Cells were grown to mid-log phase with plasmid selection in SC lacking leucine and uracil and containing 2% glucose and then shifted to an otherwise identical medium containing 0.05% glucose for 15 min, which is sufficient for Snf1 activation (Ruiz et al., 2011).

Protein extracts were prepared and immunoprecipitations (from 200 μ g of protein per reaction) were performed essentially as described previously (Treitel et al., 1998). Briefly, protein extracts were incubated at 4°C in the presence of protein A beads (Repligen) in a buffer containing 0.1% Triton X-100 and 100 mM NaCl; the antibody used for immunoprecipitation of Snf1 and Snf1-T210A was anti-polyhistidine antibody H1029 (Sigma-Aldrich), which

recognizes the natural polyhistidine tract in Snf1 (Orlova et al., 2008). This was followed by three washes in the presence of 250 mM NaCl. Proteins were eluted from the beads by boiling in the presence of SDS-PAGE loading buffer. The immunoprecipitates were examined for the presence of HA-Reg2 by immunoblotting with anti-HA-peroxidase (Roche). The total levels of immunoprecipitated Snf1 and its Thr210 phosphorylation state were determined using anti-polyhistidine and anti-phospho-Thr172-AMPK antibodies, respectively (Orlova et al., 2008). Signals were detected by enhanced chemiluminescence using Pierce ECL2 or HyGlo (Denville Scientific). The extracts were analyzed by immunoblotting similarly (10 µg protein per lane).

Results

Reg2 interacts with Snf1 in the two-hybrid system.

In light of previous evidence that Reg2 is a component of the Snf1 pathway (Frederick & Tatchell, 1996), we first tested whether, like Reg1, Reg2 physically interacts with Snf1. To address this possibility, we first used the yeast two-hybrid system. The yeast two-hybrid system is a genetic method of detecting protein-protein interactions (Fig. 6). It involves fusing one of the proteins being tested to the DNA-binding domain of a transcriptional activator, fusing the other protein to the activation domain, and co-expressing both hybrid proteins in a reporter strain. A physical interaction between the proteins of interest results in the reconstitution of the transcriptional activator, and in the expression of a reporter gene such as *lacZ* (Chien, Bartel, Sternglanz, & Fields, 1991)

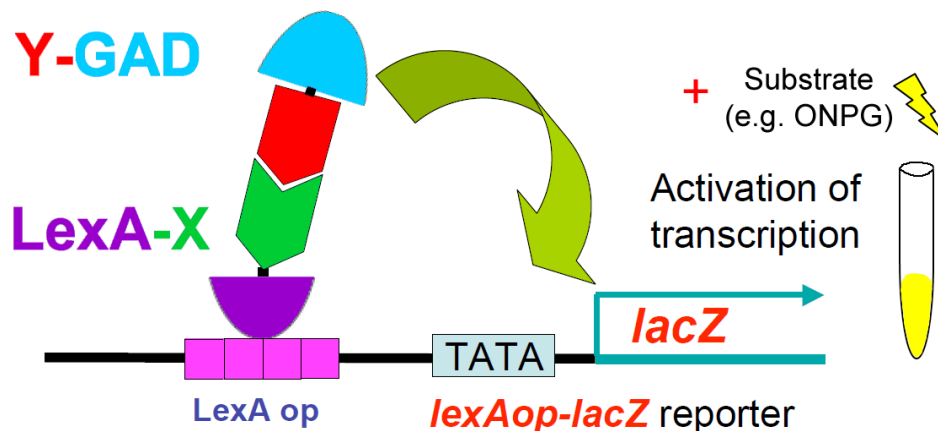


Fig. 6. Overview of the yeast two-hybrid system. The yeast two-hybrid system allows for the *in vivo* detection of protein-protein interactions. One protein of interest is fused to the DNA-binding domain of a transcription factor (e.g. LexA), and the other is fused to an activation domain (Gal4 activation domain, GAD). Both fusion proteins are co-expressed in a reporter strain, such as CTY10-5d, which has an integrated *lexAop-lacZ* reporter. If the two proteins of interest interact, the transcription factor is reconstituted, RNA Pol II is recruited, and the reporter gene is expressed. Expression of the reporter can be determined by a simple colorimetric assay.

We constructed a fusion protein in which the Gal4 transcription activation domain (GAD) is fused at the amino terminus of Reg2 (GAD-Reg2). We tested the ability of GAD-Reg2 to activate transcription of an integrated *lexAop-lacZ* reporter gene when co-expressed with a LexA-Snf1 fusion protein (Kuchin et al., 2000) in reporter strain CTY10-5d. Under high glucose conditions (2% glucose), reporter activation in cells expressing LexA-Snf1 and GAD-Reg2 was not substantially higher relative to the vector controls, but under glucose-limiting conditions (0.05% glucose), LexA-Snf1 and GAD-Reg2 interacted strongly (Fig. 7A), and the increased interaction could not be attributed to increased fusion protein levels (Fig. 7B).

To further confirm the two-hybrid interaction, We also constructed the reciprocal two-hybrid fusion protein (Reg2 fused to a DNA-binding domain: LexA-Reg2) and examined its ability to interact with a VP16-Snf1 fusion (Jiang & Carlson, 1996). Similarly, the interaction

was stronger under glucose-limiting conditions (Fig. 7A), without an increase in the levels of the interaction partners (Fig. 7C).

Thus, these experiments indicated that Reg2 and Snf1 interact in the two-hybrid system in a glucose-regulated manner.

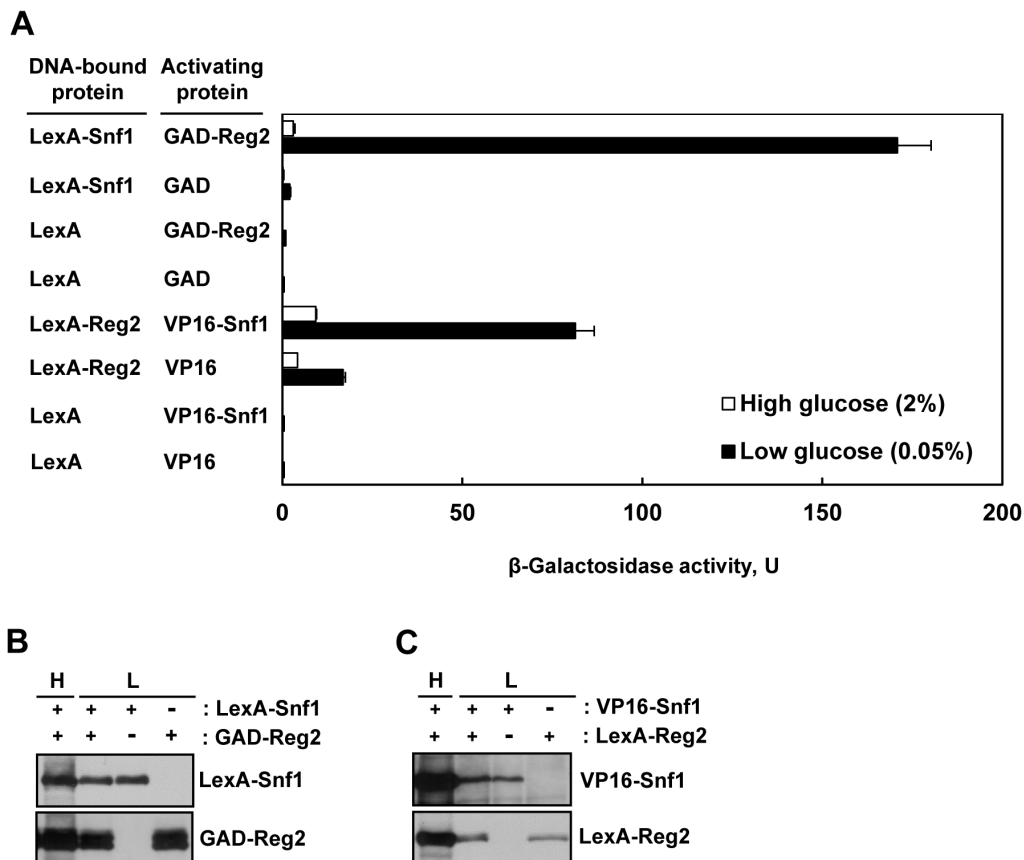


Fig. 7. Reg2 and Snf1 interact in the two-hybrid system. (A) Transformants of reporter strain CTY10-5d with plasmids expressing the indicated protein pairs were grown to mid-log phase with plasmid selection in the presence of 2% glucose, and then shifted for 3 h to an otherwise identical medium containing 0.05% glucose. β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units. Values are averages for 4-8 transformants. The error bars indicate standard errors. (B, C) Expression of the fusion proteins during growth in 2% glucose (H, high glucose), and after shift to 0.05% glucose (L, low glucose). Transformants expressing the indicated proteins (+) or carrying the corresponding vectors (-) were grown as for panel A, and expression of the fusion proteins was analyzed by immunoblotting.

Mutation of the Snf1 activation loop threonine abolishes interaction with Reg2.

To determine if the Reg2-Snf1 interaction depends on the Thr210 phosphorylation state or catalytic activity of Snf1, we used yeast two-hybrid assays to compare the ability of GAD-Reg2 to interact with LexA-Snf1 and two mutant derivatives: non-phosphorylatable LexA-Snf1-T210A and kinase-dead LexA-Snf1-K84R, with a mutation in the ATP-binding site (Estruch, Treitel, Yang, & Carlson, 1992). These two mutant derivatives could allow us to distinguish between three possibilities: (i) Reg2 interacts with Snf1 regardless of the Thr210 phosphorylation state, (ii) Reg2 requires that Snf1 be in a catalytically active conformation for its interaction, or (iii) Reg2 requires the physical presence of a phospho-Thr210 residue, but not necessarily catalytic activation, for its interaction with Snf1.

Under high glucose conditions, the mean β -galactosidase activities were low for all combinations examined (2.5 U or lower); under glucose-limiting conditions, GAD-Reg2 strongly interacted with LexA-Snf1 (170 U) and with kinase-dead LexA-Snf1-K84R (86 U), but there was no significant interaction with non-phosphorylatable LexA-Snf1-T210A (<3 U) (Fig. 8A). The observed interaction defect between GAD-Reg2 and LexA-Snf1-T210A could not be attributed to reduced expression of the fusion proteins, as determined by immunoblot analysis (Fig. 8B). Furthermore, this defect could not be attributed to the lack of catalytic activity, since GAD-Reg2 still interacted quite strongly with the kinase-dead LexA-Snf1-K84R, which supports hypothesis (iii) above.

We further examined the interaction between Reg2 and non-phosphorylatable Snf1-T210A by co-immunoprecipitation. We co-expressed HA-Reg2 and either Snf1 or Snf1-T210A in a *snf1 Δ* strain. Cells were cultured in limiting glucose (to promote Thr210 phosphorylation), protein extracts were prepared, and the Snf1 and Snf1-T210A proteins were immunoprecipitated

with anti-polyhistidine antibody. The convenient natural stretch of thirteen histidines in Snf1 allow us to use the anti-polyhistidine antibody to pull-down Snf1 without the need for an epitope tag. The immunoprecipitates were then examined for the presence of HA-Reg2 by immunoblotting with anti-HA-peroxidase antibody. In support of our previous two-hybrid results, HA-Reg2 co-immunoprecipitated with Snf1 but not with Snf1-T210A (Fig. 8C).

Collectively, these experiments indicated that Thr210 of Snf1 is critical for its interaction with Reg2, suggesting that like Reg1 (Ludin et al., 1998), Reg2 interacts with the phosphorylated form of Snf1.

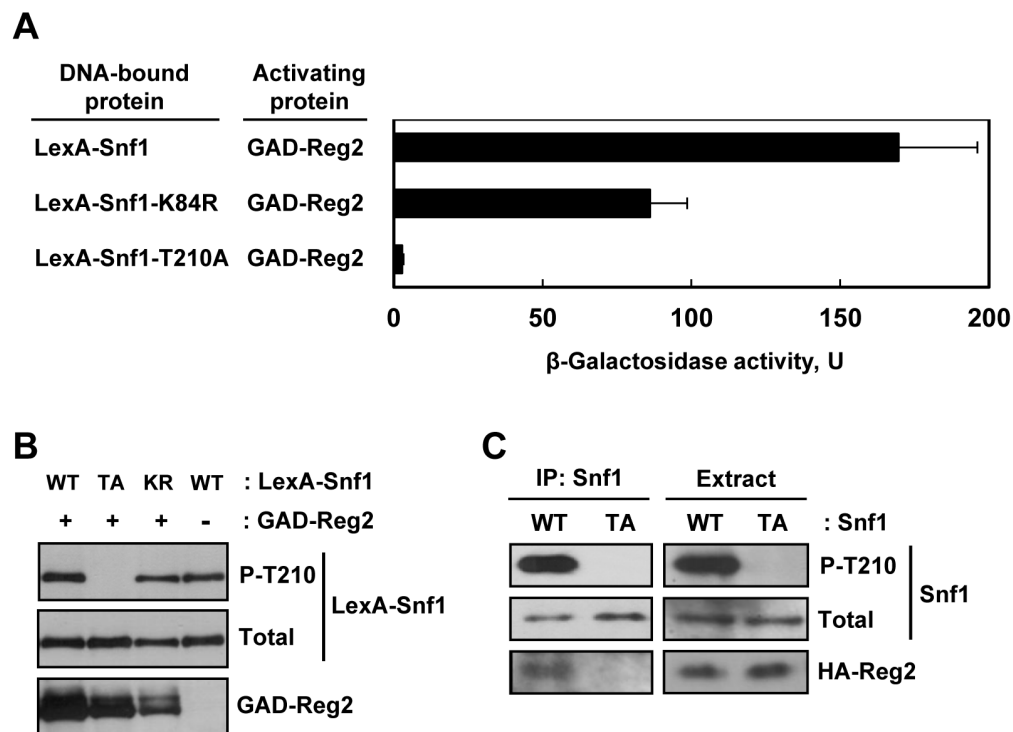


Fig. 8. Mutation of the Snf1 activation loop threonine abolishes interaction with Reg2. (A) Wild-type strain MKY343 carrying a *lexAop-lacZ* reporter and expressing the indicated protein pairs was grown to mid-log phase with plasmid selection in the presence of abundant (2%) glucose, and then shifted for 3 h to an otherwise identical medium containing limiting (0.05%) glucose. β -Galactosidase activity was assayed in permeabilized cells and expressed in Miller units. Shown are the results for limiting glucose; values are averages for five transformants; the error bars indicate standard errors. In glucose-rich conditions, the values were 2.5 U or lower. (B) Representative

transformants were cultured under glucose-limiting conditions as for panel A, and the levels of LexA-Snf1 phosphorylated at Thr210 (P-T210), total LexA-Snf1 (Total), and GAD-Reg2 were confirmed by immunoblotting. WT, LexA-Snf1; TA, LexA-Snf1-T210A; KR, LexA-Snf1-K84R. Cells also expressed GAD-Reg2 (GAD-Reg2, +) or carried the corresponding vector pACTII (GAD-Reg2, -). (C) Coimmunoprecipitation assays. Cells of strain MKY362 (*snf1* Δ) transformed with combinations of plasmids expressing HA-Reg2 and wild-type Snf1 (Snf1, WT) or non-phosphorylatable Snf1-T210A (Snf1, TA) were grown to mid-log phase with plasmid selection in abundant (2%) glucose, and then shifted to an otherwise identical medium containing limiting (0.05%) glucose for 15 min. Following the shift to limiting glucose, protein extracts were prepared, and Snf1 was immunoprecipitated with anti-polyhistidine antibody. The immunoprecipitates (IP: Snf1) and extracts (Extract) were examined by immunoblotting for the presence of phospho-Thr210-Snf1 (P-T210), total Snf1 protein (Total), and HA-Reg2.

The *reg2* Δ mutation does not result in constitutive Thr210 phosphorylation of Snf1 during exponential growth on high glucose.

After determining that a physical interaction between Reg2 and Snf1 exists, we next investigated the role of Reg2 in Snf1 regulation. To do this, we tested for possible effects of the *reg2* Δ mutation on the Thr210 phosphorylation status of Snf1. Since the *reg1* Δ mutation results in constitutive Snf1 phosphorylation during growth in high glucose - when Snf1 is normally inactive (McCartney & Schmidt, 2001) - it seemed possible that *reg2* Δ cells could exhibit a similar phenotype. Wild-type and mutant cells were grown to mid-log phase in the presence of abundant (2%) glucose, shifted to limiting (0.05%) glucose for 1 h, and the Thr210 phosphorylation status of Snf1 was determined by immunoblotting. The levels of phospho-Thr210-Snf1 in the *reg2* Δ mutant were comparable to those in the wild type under both conditions (Fig. 9A). Thus, unlike the *reg1* Δ mutation (McCartney & Schmidt, 2001, and see Fig. 7), the *reg2* Δ mutation did not lead to any discernible difference in the Thr210 phosphorylation status of Snf1 during exponential growth in high glucose.

It remained possible that the *reg2Δ* mutant differs from the wild type with respect to rapid Snf1 dephosphorylation in response to glucose replenishment. To test this possibility, wild-type and *reg2Δ* cells were grown in the presence of abundant glucose, shifted to limiting glucose for 1 h, and then replenished with glucose for 1 min. However, no obvious dephosphorylation defect was observed in the mutant (Fig. 9B, C).

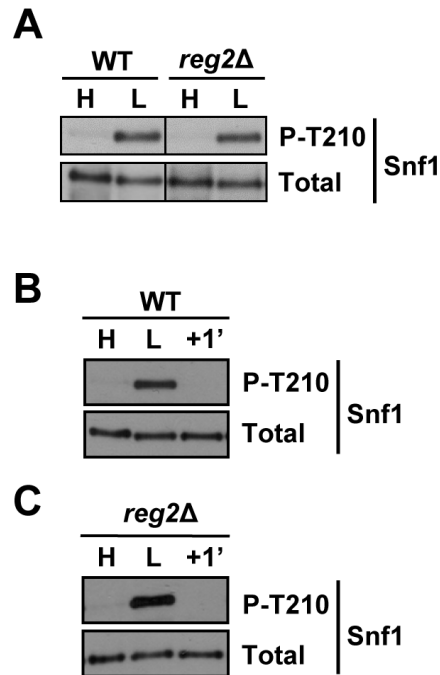


Fig. 9. Lack of Reg2 does not lead to constitutive accumulation of phospho-Thr210-Snf1. (A) Cells of the indicated genotypes were grown to mid-log phase in YEP containing 2% glucose (H, high glucose), followed by a shift for 1 h to YEP containing 0.05% glucose (L; low glucose). (B-C) Cells were grown as for panel A, and the glucose-limited cells were then replenished with abundant (2%) glucose for 1 min (+1'). The levels of phospho-Thr210-Snf1 (P-T210) and total Snf1 protein (Total) were analyzed by immunoblotting. The strains were MMY128 (WT) and MMY18 (*reg2Δ*).

Overexpression of Reg2 leads to reduced phospho-Thr210-Snf1 during growth in abundant glucose.

Although deletion of *REG2* did not result in constitutive Thr210 phosphorylation, it remained possible that Reg2 could have a role in Snf1 regulation that is not easily discernible by gene knockout. For instance, Reg2 could have the ability to dephosphorylate a subpopulation of phospho-Thr210-Snf1 proteins, or its contribution could otherwise be minor. As an alternative strategy, we next examined the effects of Reg2 overexpression. To this end, we used LexA-Reg2, which is expressed from the *ADHI* promoter of multicopy vector pEG202 (Golemis et al., 1997). Overexpression of LexA-Reg2 resulted in a significant reduction of the levels of phospho-Thr210-Snf1 both in the wild type and in the *reg1* Δ mutant during growth in abundant glucose (Fig. 10A). Consistent with previous results (Frederick & Tatchell, 1996), in control experiments we observed that overexpression of LexA-Reg2 improves the growth of the *reg1* Δ mutant (Fig. 10B), which supports the role of Reg2 in reducing Snf1 phosphorylation and provides evidence that Reg1 and Reg2 have some overlapping functions in the Snf1 pathway.

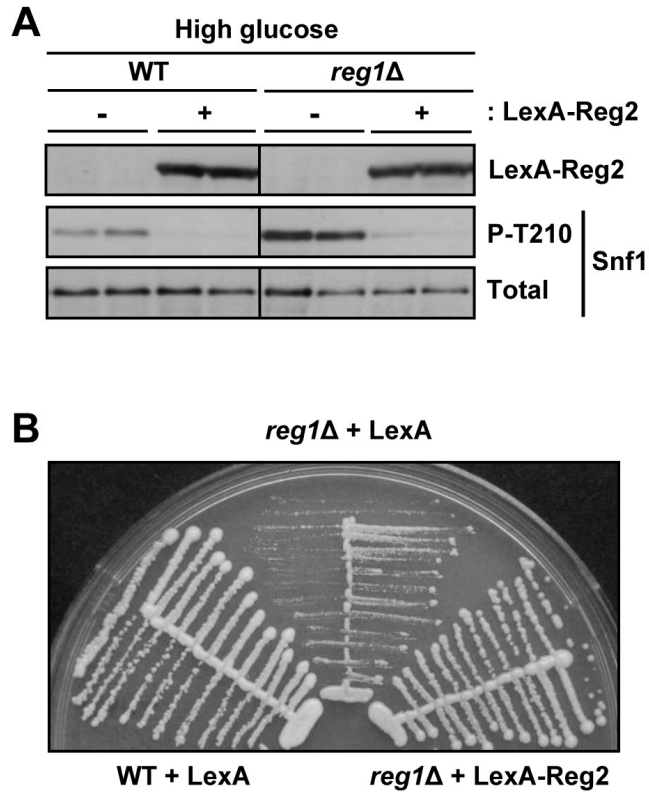


Fig. 10. Effects of Reg2 overexpression in glucose-rich conditions. (A) Overexpression of Reg2 during growth in abundant glucose reduces the phospho-Thr210-Snf1 levels. Strains of the indicated genotypes carrying plasmid pLexA-Reg2 (LexA-Reg2, +) or the corresponding vector pEG202 (LexA-Reg2, -) were grown to mid-log phase with plasmid selection in abundant (2%) glucose. The levels of LexA-Reg2, phospho-Thr210-Snf1 (P-T210) and total Snf1 protein (Total) were analyzed by immunoblotting. (B) Overexpression of LexA-Reg2 suppresses the slow growth caused by *reg1Δ*. Cells of the indicated genotypes carrying vector pEG202 (LexA) or plasmid pLexA-Reg2 (LexA-Reg2) were streaked on SC medium lacking histidine and containing 2% glucose, and grown for 3 days. The strains were MKY343 (WT) and KBY247 (*reg1Δ*).

In contrast to cells grown in high glucose, wild-type cells with LexA-Reg2 exhibited no discernible reduction in the phospho-Thr210 level in limiting glucose (Fig. 11A). It should be noted that – as we often observe with proteins expressed from vector pEG202 – the LexA-Reg2 protein level was somewhat reduced in limiting glucose relative to abundant glucose (Fig. 11B),

but it seems unlikely that this reduction alone could be responsible for the lack of any apparent effect on Snf1. We conclude that Reg2 overexpression negatively affects Snf1, at least in the presence of abundant glucose.

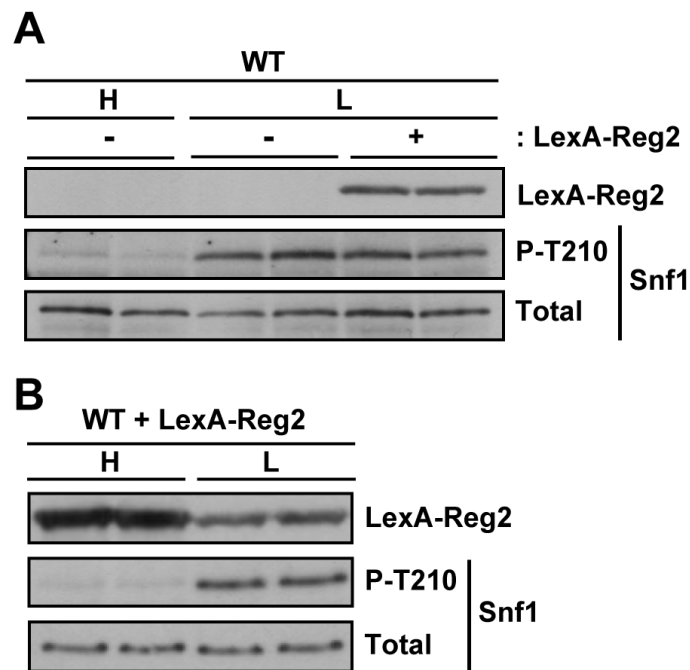


Fig. 11. Overexpression of Reg2 has no significant effect on the Thr210 phosphorylation status of Snf1 in low glucose. (A) Wild-type cells (MKY343) transformed with pLexA-Reg2 (LexA-Reg2, +) or with the corresponding vector pEG202 (LexA-Reg2, -) were grown to mid-log phase with plasmid selection in abundant (2%) glucose (H, high glucose) and then shifted to an otherwise identical medium containing 0.05% glucose (L, low glucose). The levels of LexA-Reg2, phospho-Thr210-Snf1 (P-T210) and total Snf1 protein (Total) were analyzed by immunoblotting. (B) Expression of LexA-Reg2 in 2% glucose (H, high glucose) and in 0.05% glucose (L, low glucose). Cells of MKY343 (WT) transformed with pLexA-Reg2 were grown as for panel A.

Combined effects of *reg1*Δ and *reg2*Δ. The above overexpression and interaction experiments provided evidence that Reg2 is involved in the negative control of Snf1. Since the *reg2*Δ single mutation did not affect the Thr210 phosphorylation status of Snf1 in our earlier experiments (Fig. 9), we next examined whether *reg2*Δ could affect Snf1 when combined with *reg1*Δ.

We constructed *reg1Δ reg2Δ* double mutant cells by genetic crossing and used immunoblotting to monitor the levels of phospho-Thr210-Snf1. Cells were grown in the presence of abundant glucose, and then shifted to limiting glucose. In this experimental setting, however, the levels of phospho-Thr210-Snf1 in the *reg1Δ reg2Δ* double mutant were similar to those in the *reg1Δ* single mutant (Fig. 12A).

In the course of our experiments with the *reg1Δ* single mutant, we noted that glucose re-addition to glucose-limited cells results in a rapid (1 min) reduction of phospho-Thr210-Snf1 to a level that is below that observed during exponential growth in abundant glucose (Fig. 12B; compare lanes 5 and 7). The effect was transient, as the level of phospho-Thr210-Snf1 increased again 5 min after glucose re-addition (Fig. 12B; compare lanes 7 and 8). One potential explanation is that this rapid/transient “dip” in phospho-Thr210-Snf1 upon glucose replenishment might involve Reg2. Therefore, we compared the Thr210 phosphorylation status of Snf1 in the *reg1Δ* single, *reg2Δ* single, and *reg1Δ reg2Δ* double mutants in response to re-addition of glucose. Unlike with the *reg1Δ* single mutant, there was no such extra “dip” in the *reg1Δ reg2Δ* double mutant at 1 min after glucose addition (Fig. 12C; compare lanes 7 and 9), suggesting that Reg2 might contribute to the rapid dephosphorylation of Snf1 upon glucose replenishment.

We note that these results still do not provide a conclusive explanation for the slow growth of the *reg1Δ reg2Δ* double mutant [(Frederick & Tatchell, 1996), and Fig. 12D]. It is known that this phenotype is Snf1-dependent (Frederick & Tatchell, 1996), but - as described above - there was no obvious increase in phospho-Thr210-Snf1 in the *reg1Δ reg2Δ* double mutant cells relative to *reg1Δ* single mutant cells during exponential growth in abundant glucose. It is possible that the slow growth of the double mutant is not directly related to Reg2’s role in

the negative control of Snf1 itself; instead, it could be related to a role in dephosphorylation of a Snf1 target involved in growth control, as was proposed previously (Frederick & Tatchell, 1996). However, we cannot exclude that immunoblot analysis simply does not have sufficient resolution to capture potentially relevant effects of *reg2Δ* on Snf1 that are either transient, or small by comparison to the effects of *reg1Δ*, yet sufficient to translate into larger phenotypic effects.

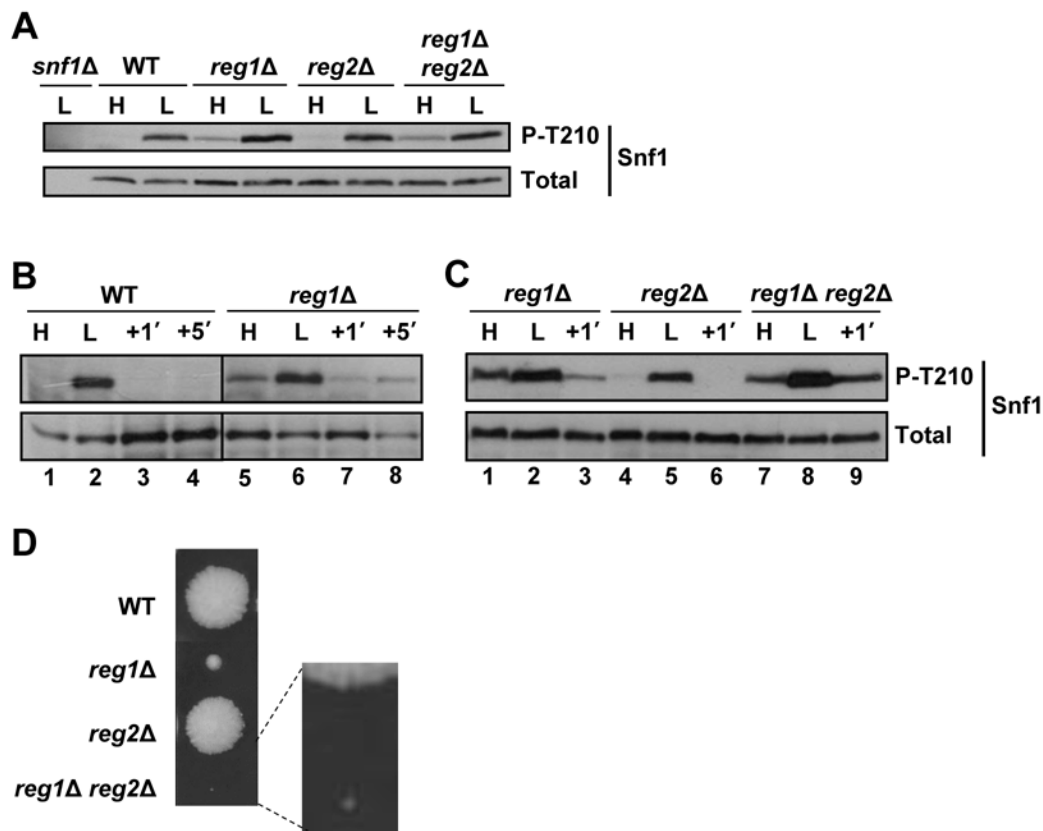


Fig. 12. Combined effects of *reg1Δ* and *reg2Δ*. (A-C) Cells of the indicated genotypes were grown to mid-log phase in YEP containing 2% glucose (H, high glucose) and shifted for 1 h to YEP containing 0.05% glucose (L, low glucose); where indicated, the glucose-limited cells were then replenished with abundant (2%) glucose for 1 min (+1') or 5 min (+5'). The levels of phosphorylated Thr210 (P-T210) and total Snf1 protein (Total) were analyzed by immunoblotting. The strains were MMY128 (WT), MMY40 (*reg1Δ*), MMY41 (*reg2Δ*), MMY42 (*reg1Δ reg2Δ*), and MKY362 (*snf1Δ*). (D) Strains MMY9 (*reg2Δ*) and KBY247 (*reg1Δ*) were crossed, and the resulting diploid was grown on sporulation medium to induce meiosis. Segregants were micromanipulated onto rich YEP medium containing 2% glucose and grown for 4 days. A representative tetratype tetrad (yielding four haploids with four different genotypes) is shown here. The centers of the colonies are ~5 mm apart.

Discussion

Previous evidence suggested that Reg2 could have a role in the negative control of the Snf1 pathway. First, like Reg1, Reg2 is a regulatory subunit of the type 1 protein phosphatase Glc7 that plays a prominent role in Snf1 inactivation. Second, Reg2 shares homology with Reg1. Third, the *reg1Δ reg2Δ* double mutant grows slower than either single mutant, and this phenotype can be suppressed by elimination of Snf1. However, it remained unclear whether Reg2 can participate in the regulation of Snf1 itself.

Here, we present evidence that Reg2 indeed contributes to the negative control of this kinase. First, like Reg1, Reg2 interacts with Snf1; moreover, our results strongly suggest that this interaction requires that Snf1 is phosphorylated at Thr210, as would be expected if Snf1 is a dephosphorylation target for Reg2-Glc7. One piece of evidence in support of an interaction between Reg2 and the phosphorylated form of Snf1, is that the two-hybrid interaction is significantly stronger during growth in low glucose - a condition in which Snf1 is phosphorylated. Even more convincing is that Reg2 is unable to interact with a Snf1 protein in which the threonine 210 residue has been replaced with the non-phosphorylatable alanine (Fig. 8A,C).

Second, Reg2 overexpression rescues the Snf1-dependent slow growth phenotype of the *reg1Δ* mutant, and this effect is accompanied by a detectable reduction in phospho-Thr210-Snf1. Third, Reg2 contributes to rapid Snf1 dephosphorylation upon glucose replenishment; interestingly, this observation correlates with the recent evidence that Reg2 contributes to a transient spike in total Glc7 protein phosphatase activity that occurs during the first few minutes of glucose addition to glucose-deprived cells (Castermans et al., 2012).

Previous results indicate that the *REG2* gene is expressed at a significantly higher level in low glucose than in abundant glucose (Lutfiyya et al., 1998; Young, Dombek, Tachibana, & Ideker, 2003). These observations suggested that Reg2 plays a more important cellular role when glucose is limiting. Our results from the overexpression of Reg2 (Fig. 10), however, show that Reg2 is also capable of further reducing levels of phospho-Thr210-Snf1 during growth in abundant glucose. One possible interpretation is that Reg2 protein levels could naturally be low in abundant glucose, and therefore boosting Reg2 levels via overexpression results in further Snf1 dephosphorylation and improved growth of the *reg1* Δ mutant.

Based on these results, we propose the following model. During steady-state growth in abundant glucose, Reg2 has little or no role in Snf1 inactivation, and the most important role is played by Reg1-Glc7 and Sit4 (Ruiz et al., 2012; Ruiz et al., 2011). During conditions that have yet to be determined, but can be mimicked by deletion of *REG1*, *REG2* gene expression increases, as does the level of phospho-Thr210-Snf1. Reg2 then interacts with a portion of the phospho-Thr210-Snf1 pool, thus preparing for its rapid dephosphorylation should glucose become abundant.

In the case of Snf1 regulation by glucose, Reg2 appears to play only a fine-tuning role. Further experiments will be required to determine when *REG2* expression increases, as well as to comprehensively determine how Reg2 differs from Reg1 in the regulation of the Snf1 protein kinase.

Table 1. *S. cerevisiae* strains

Strain	Genotype	Source
CTY10-5d	<i>MATa gal4 gal80 URA3::lexAop-lacZ his3 leu2 ade2 trp1</i>	R. Sternglanz
MKY343	<i>MATa ura3Δ leu2Δ his3Δ trp1Δ</i>	This laboratory
MKY362	<i>MATa ura3Δ leu2Δ his3Δ trp1Δ snf1Δ::KanMX6</i>	This laboratory
KBY247	<i>MATa ura3Δ leu2Δ his3Δ trp1Δ reg1Δ::URA3</i>	This study
MMY9	<i>MATa ura3Δ reg2Δ::KanMX6</i>	This study
MMY18	<i>MATa ura3Δ his3Δ trp1Δ reg2Δ::KanMX6</i>	This study
MMY40	<i>MATa leu2Δ trp1Δ reg1Δ::URA3</i>	This study
MMY41	<i>MATa ura3Δ his3Δ reg2Δ::KanMX6</i>	This study
MMY42	<i>MATa leu2Δ trp1Δ reg1Δ::URA3 reg2Δ::KanMX6</i>	This study
MMY128	<i>MATa ura3Δ his3Δ</i>	This study

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Chapter 3:

Role of the RVxF motif of Reg2 in mediating its interactions and function

Abstract

In the yeast *Saccharomyces cerevisiae*, Snf1 protein kinase regulates responses to glucose/energy limitation. Under glucose-limiting conditions, Snf1 is activated by phosphorylation of its T-loop threonine (Thr210) by upstream kinases. Under glucose-rich conditions, Snf1 is inactivated by dephosphorylation of its Thr210 residue by phosphatases including type 1 protein phosphatase (PP1), known as Glc7 in yeast. PP1 is a highly conserved eukaryotic protein that requires regulatory subunits for substrate recognition. Many of these regulatory subunits, such as Reg1, require a small region known as the RVxF motif to bind to PP1. In the previous chapter, we provided evidence that a homolog of Reg1, Reg2, has a role in the negative regulation of Snf1. Here, we have examined the role of the putative RVxF motif present in Reg2. We show that mutation of the RVxF motif disrupts the Reg2-Glc7 interaction. We also show that unlike overexpression of wild-type Reg2, overexpression of the mutant Reg2 protein neither reduces the Snf1 T210 phosphorylation level nor suppresses the slow growth of *reg1* Δ cells, indicating that mutation of the RVxF motif affects the ability of Reg2 to regulate Snf1. Our results strongly suggest that Reg2 is a *bona fide* PP1 regulatory subunit, and that the Reg2-Glc7 interaction is required for the function of Reg2 in Snf1 regulation.

Introduction

The Snf1/AMP-activated protein kinase (AMPK) family is conserved from simple eukaryotes like yeast to complex eukaryotes including mammals. AMPK is a stress responder

that monitors cellular energy levels and maintains energy homeostasis (Hardie, 2007b). In low energy conditions, such as those caused by hypoglycemia and exercise, AMPK becomes activated and functions to reduce energy-consuming processes and to increase ATP-generating processes (Hardie, Ross, & Hawley, 2012). Defects in AMPK signaling are associated with metabolic diseases including type II diabetes and obesity (Fogarty & Hardie, 2009; Hardie, 2007a).

In the yeast *Saccharomyces cerevisiae*, the AMPK homolog, Snf1, similarly responds to energy stresses such as glucose limitation. During growth in low glucose, Snf1 becomes activated by upstream kinases (Hong, Leiper, Woods, Carling, & Carlson, 2003; Nath, McCartney, & Schmidt, 2003; Sutherland et al., 2003), which phosphorylate it on Thr210 (McCartney & Schmidt, 2001). When glucose is abundant, Snf1 is inactivated by Thr210 dephosphorylation by the Reg1-Glc7 phosphatase (Ludin, Jiang, & Carlson, 1998; McCartney & Schmidt, 2001; Tu & Carlson, 1995).

Glc7 is the catalytic subunit of type 1 protein phosphatase (PP1), another highly conserved eukaryotic protein. PP1 is known to associate with numerous regulatory subunits, such as Reg1, which modulate the activity or substrate specificity of the phosphatase (Ceulemans & Bollen, 2004; Heroes et al., 2013). Many of these subunits have a small PP1-interacting region known as the “RVxF” motif (Ceulemans & Bollen, 2004). Previous work has shown that the RVxF-mutant Reg1 fails to interact with PP1/Glc7 (Sanz, Alms, Haystead, & Carlson, 2000; Tabb, Mangat, McCartney, & Schmidt, 2010), and exhibits defects related to its role in Snf1 regulation. For instance, the mutant Reg1 protein cannot complement the slow growth or the constitutive expression of invertase exhibited by *reg1Δ* cells (Dombek, Voronkova, Raney, & Young, 1999; Tabb et al., 2010).

In the previous chapter, we showed that Reg2, a homolog of Reg1, has a role in the negative regulation of Snf1. However, it remained unclear if Reg2 requires its RVxF motif for Glc7 interaction, or whether the function of Reg2 in Snf1 regulation is even related to this motif. In this study, we mutated the RVxF motif and examined the physical interaction between mutant Reg2 and Glc7, and between mutant Reg2 and Snf1. We show that the Reg2 RVxF motif is required for Reg2-Glc7 interaction. Additionally, we found that the Reg2 RVxF motif is required for the ability of overexpressed Reg2 to dephosphorylate Snf1 and to functionally complement the slow growth of *reg1Δ* cells. Together, these results indicate that the RVxF motif is important for the Reg2-Glc7 interaction and for the negative regulation of Snf1 by Reg2.

Materials and Methods

Strains and growth conditions. The *S. cerevisiae* strains used in this study are listed in Table 2. The reporter strain, CTY10-5d (R. Sternglanz, SUNY, Stony Brook, NY) carries an integrated *lexAop-lacZ* reporter and was used for yeast two-hybrid assays. Strain KBY247 is in the Σ1278b genetic background and a descendant of strains MY1384 (*MATa*, prototroph), MY1401 (*MATα ura3Δ leu2Δ his3Δ*), and MY1402 (*MATa ura3Δ leu2Δ trp1Δ*) of the isogenic Sigma2000 series (Microbia, Cambridge, MA). Construction of the Σ1278b *reg1Δ::URA3* allele was described previously (Tu & Carlson, 1995).

Synthetic complete (SC) medium lacking appropriate supplements was used to select for plasmids (Rose, Winston, & Hieter, 1990). Unless indicated otherwise, the media contained 2% glucose, and yeast cells were grown at 30°C.

Yeast two-hybrid assays. All plasmids expressing fusion proteins for the yeast two-hybrid experiments were based on vectors pEG202 or pACTII, which provide expression from the *ADHI* promoter (Golemis, Serebriiskii, Gjuris, & Brent, 1997; Legrain, Dokhlar, & Transy, 1994; Vojtek, Hollenberg, & Cooper, 1993). Plasmid pIT469 (Kuchin, Treich, & Carlson, 2000) expresses LexA-Snf1 from vector pEG202. Plasmids pRJ215 and pRJ217 express LexA-Snf1-K84R and LexA-Snf1-T210A, respectively, from vector pEG202 (Kuchin, Vyas, Kanter, Hong, & Carlson, 2003; Treitel, Kuchin, & Carlson, 1998). To construct pLexA-Glc7, a PCR fragment encompassing the *GLC7* open reading frame (ORF) was inserted at the BamHI site of pEG202. To construct pLexA-Reg2 and pGAD-Reg2, a PCR fragment encompassing the *REG2* ORF was inserted at the BamHI site of pEG202 and pACTII, respectively. Plasmids pLexA-Reg2-IF and pGAD-Reg2-IF are identical to plasmids pLexA-Reg2 and pGAD-Reg2, respectively, except that they carry mutations that change the Ile169 and Phe171 codons of Reg2 to Met and Ala codons, respectively; the mutations were introduced essentially as described previously (Tabba et al., 2010) using a mutagenic primer that also creates silent diagnostic restriction sites overlapping the Met and Ala codons (NdeI and HaeIII, respectively).

The reporter strain was CTY10-5d carrying an integrated *lexAop-lacZ* reporter. This strain was co-transformed with pairs of plasmids expressing the protein pairs being tested. Transformants were grown to mid-log phase with plasmid selection in SC lacking the appropriate supplements, and containing 2% glucose, and then shifted for 3 h to an otherwise identical medium containing 0.05% glucose. β -galactosidase activity was assayed in permeabilized cells and expressed in Miller units, as described previously (Vyas, Kuchin, & Carlson, 2001).

Immunoblotting. Expression of the fusion proteins was confirmed by immunoblotting, as follows. Representative transformants were grown under conditions identical to those used to measure β -galactosidase activity. Cell extracts were prepared using the boiling/alkaline lysis method (Orlova, Barrett, & Kuchin, 2008). Briefly, cultures were boiled for five minutes and centrifuged at 4,000 rpm to pellet cells. The collected cells were treated with 0.1 N NaOH and boiled in the presence of SDS-PAGE loading buffer. Proteins were separated by SDS-PAGE and analyzed by immunoblotting. LexA fusion proteins were detected with anti-LexA antibody (Millipore). The GAD-Reg2 and GAD-Reg2-IF fusion proteins contain a hemagglutinin (HA) epitope tag between the Gal4 activation domain (GAD) and Reg2 (or Reg2-IF), and were detected with anti-HA antibody HA-7 (Sigma-Aldrich). Thr210-phosphorylated Snf1 proteins were detected with anti-phospho-Thr172-AMPK (Cell Signaling Technology) as described previously (Orlova et al., 2008). Signals were detected by enhanced chemiluminescence using Pierce ECL2 or HyGlo (Denville Scientific).

Results

The RVxF motif is required for Reg2-Glc7 interaction

Reg1 and Reg2 both possess variants of the RVxF motif found in many eukaryotic type 1 protein phosphatase-interacting proteins (RHIHF and RHIKF, respectively) (Fig. 13). Despite the four-letter abbreviation for the motif, it can actually be composed of five amino acids (as in Reg1 and Reg2), and a more accurate consensus sequence is [RK]-X₀₋₁-[VI]-X-[FW] (Wakula, Beullens, Ceulemans, Stalmans, & Bollen, 2003).

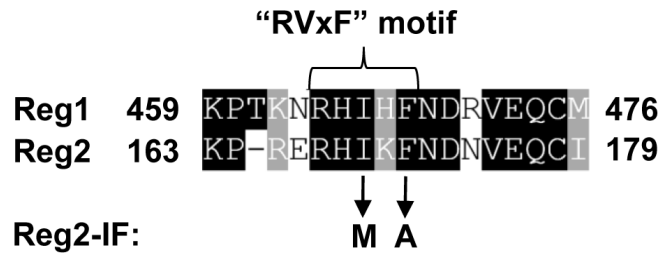


Fig. 13. Alignment of the RVxF motifs of Reg1 and Reg2. The amino acid identities and similarities are shaded black and gray, respectively. Arrows indicate the introduced changes in the Reg2-IF mutant.

It was previously shown that the RVxF motif of Reg1 is required for its interaction with Glc7 (Alms, Sanz, Carlson, & Haystead, 1999; Dombek et al., 1999; Sanz et al., 2000; Tabbā et al., 2010). To determine the role of the apparent RVxF motif of Reg2, we mutated the two critical hydrophobic residues, isoleucine (Ile169) and phenylalanine (Phe171), to methionine and alanine, respectively, by analogy to prior studies with Reg1 (Dombek et al., 1999; Tabbā et al., 2010). These two hydrophobic residues have been shown to interact with a hydrophobic groove in PP1 (Terrak, Kerff, Langsetmo, Tao, & Dominguez, 2004). Since the Reg1 double mutant (I466M, F468A) has been previously called Reg1-IF (Tabbā et al., 2010), we will similarly refer to the equivalent Reg2 double mutant (I169M, F171A) as “Reg2-IF”.

Reg2 was first identified in 1996 in a yeast two-hybrid screen for proteins that interact with Glc7 (Frederick & Tatchell, 1996). We have constructed similar plasmids expressing LexA-Glc7, GAD-Reg2, as well as the mutant derivative, GAD-Reg2-IF, and expressed them in cells with an integrated *lexAop-lacZ* reporter, allowing for the determination of interactions between these proteins using the yeast two-hybrid system. Consistent with previous results (Frederick & Tatchell, 1996), wild-type Reg2 interacted strongly with Glc7 in the yeast two-hybrid assays (Fig. 14A). This interaction could be detected both in high- and low-glucose conditions, although it was stronger in low glucose (~75 U) than in high glucose (~15 U). By contrast, the Reg2-IF

mutant exhibited a severe Glc7 interaction defect, as there was no detectable interaction in either of the tested conditions. To confirm that the Reg2-IF interaction defect was not due to decreased levels of the fusion proteins, we performed immunoblot analysis with cells expressing these two-hybrid protein pairs. Immunoblot analysis showed similar levels of the LexA-Glc7, GAD-Reg2, and GAD-Reg2-IF fusion proteins in both cases (Fig. 14B). These results strongly suggest that the RVxF motif of Reg2 is required for its interaction with Glc7, and that Reg2 is a *bona fide* PP1 regulatory subunit in the sense that the canonical RVxF motif is required for its PP1 interaction.

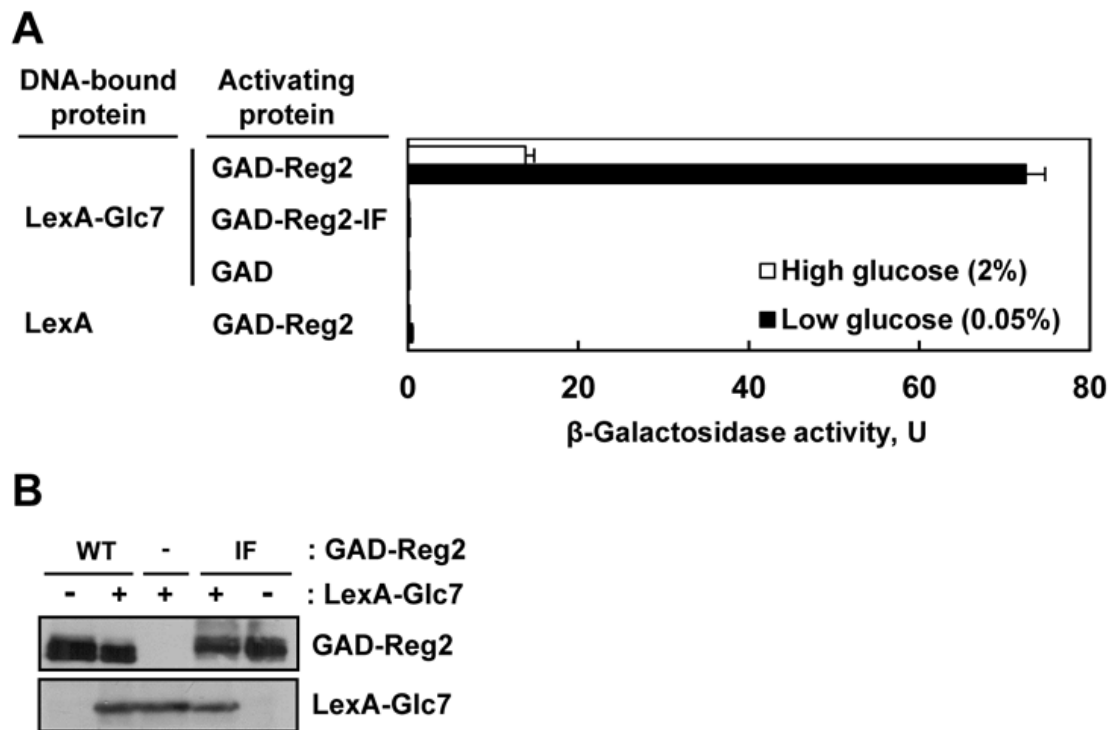


Fig. 14. Reg2-IF does not interact with Glc7 in the yeast two-hybrid system. (A) Transformants of the reporter strain CTY10-5d with plasmids expressing the indicated protein pairs were grown to mid-log phase with plasmid selection in the presence of 2% glucose, and then shifted for 3 h to an otherwise identical medium containing 0.05% glucose. β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units. Values are averages for 4-8 transformants. The error bars indicate standard errors. (B) Representative transformants of CTY10-5d expressing

GAD-Reg2 (GAD-Reg2, WT), GAD-Reg2-IF (GAD-Reg2, IF), or carrying the corresponding vector (GAD-Reg2,-) and simultaneously expressing LexA-Glc7 (LexA-Glc7, +) or carrying the corresponding vector (LexA-Glc7, -) were grown as for panel A, and expression of the fusion proteins was confirmed by immunoblotting.

Reg2-IF is functionally defective in Snf1 regulation

After determining that the RVxF motif is required for the physical interaction between Reg2 and Glc7, we next addressed whether this interaction has a role in Snf1 regulation.

Previous work with Reg1 has shown that Reg1 constructs lacking the RVxF motif may not be functional in Snf1 regulation. For instance, these mutant proteins are unable to complement the slow growth of *reg1Δ* cells (which exhibit increased Snf1 kinase activity), or to restore normal glucose-regulated expression of the *SUC2* and *ADH2* genes (Dombek et al., 1999; Tabbà et al., 2010).

Consistent with a role as a regulatory/targeting subunit of Glc7, we hypothesized that Reg2, like Reg1, may also require the RVxF motif to function in Snf1 regulation. To this end, we overexpressed wild-type Reg2 as well as Reg2-IF in *reg1Δ* cells. As before (Fig. 10B), overexpression of wild-type Reg2 (as a LexA-Reg2 protein) improved the growth of *reg1Δ* cells. On the contrary, overexpression of Reg2-IF (as a LexA-Reg2-IF protein) was unable to restore the growth of the *reg1Δ* mutant to the wild-type level (Fig. 15A). Although growth is a convincing phenotypic readout, we also used a more direct approach to examine Snf1 Thr210 phosphorylation. As before, overexpression of LexA-Reg2 reduced the levels of phospho-Thr210-Snf1. However, overexpression of the mutant derivative was unable to reduce phospho-Thr210-Snf1 levels (Fig. 15B). These results suggest that the Reg2-Glc7 interaction, mediated by the RVxF motif, is important for the ability of Reg2 to dephosphorylate Snf1.

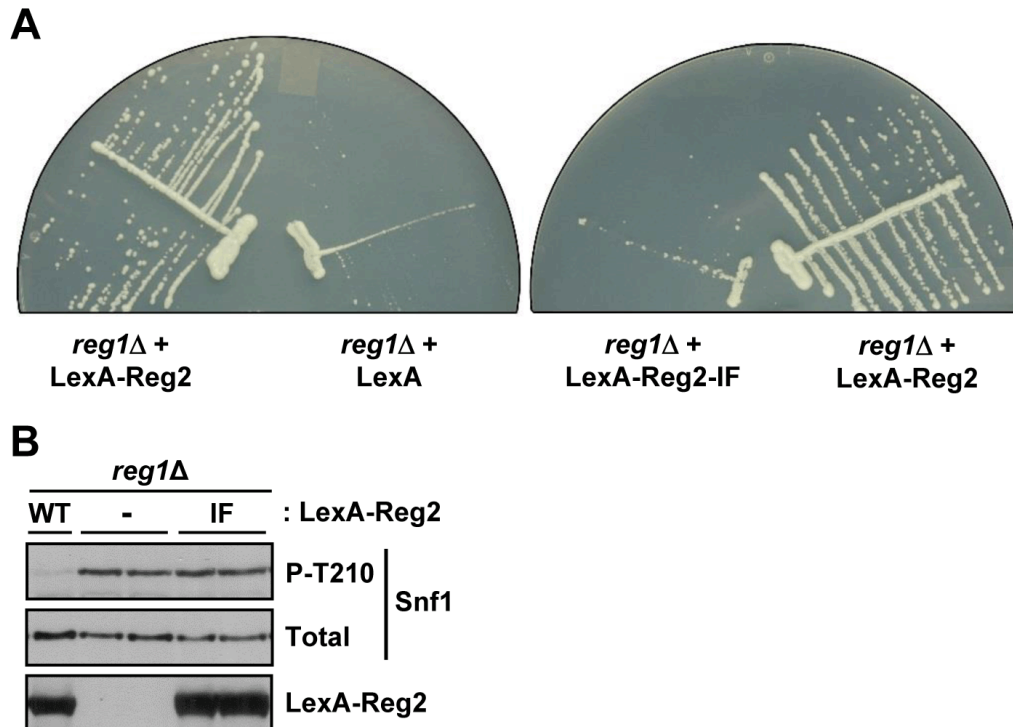


Fig. 15. Reg2-IF is defective in the regulation of Snf1. (A) Overexpression of wild-type Reg2, but not the Reg2-IF mutant improves growth of *reg1Δ* cells. *reg1Δ* cells of strain KBY247 carrying vector pEG202 (LexA), plasmid pLexA-Reg2 (LexA-Reg2), or plasmid pLexA-Reg2-IF (LexA-Reg2-IF) were streaked on SC medium lacking histidine and containing 2% glucose and grown for 3 days. (B) Overexpression of LexA-Reg2-IF during growth in abundant glucose does not reduce phospho-Thr210-Snf1 levels. *reg1Δ* cells of strain KBY247 carrying plasmid pLexA-Reg2 (LexA-Reg2, WT), pLexA-Reg2-IF (LexA-Reg2, IF), or the corresponding vector pEG202 (LexA-Reg2, -) were grown to mid-log phase in selective SC medium lacking histidine and containing 2% glucose. The levels of LexA-tagged proteins (LexA-Reg2), phospho-Thr210-Snf1 (P-T210) and total Snf1 protein (Total) were analyzed by immunoblotting.

The RVxF motif of Reg2 is not required for the Reg2-Snf1 interaction

Interestingly, it was previously reported that the RVxF motif of Reg1 is required for interaction not only with Glc7, but also with Snf1, since Reg1-IF was unable to interact with either (Tabba et al., 2010). A “competitive binding” model was proposed in which Glc7, the phosphatase, and Snf1, the substrate, compete with each other for binding to Reg1. We therefore also tested Reg2-IF for interaction with Snf1 to determine if Reg2 follows a similar competitive

binding model. We used the yeast two-hybrid system to address this possibility for Reg2. We co-expressed LexA-Snf1 (and LexA-Snf1-T210A) and GAD-Reg2 (or GAD-Reg2-IF) in the yeast two-hybrid reporter strain CTY10-5d and quantified the interactions between these protein pairs. As seen in our previous two-hybrid assays, LexA-Snf1 and the wild-type GAD-Reg2 interacted strongly in low glucose (Fig. 7A, and Fig.16). The observed interaction pattern was similar using the mutant GAD-Reg2-IF, as it also interacted strongly with LexA-Snf1 (Fig. 16). In control experiments, we also observed that Reg2-IF interacted with kinase-dead LexA-Snf1-K84R, but failed to interact with non-phosphorylatable LexA-Snf1-T210A, as expected if the Reg2 protein interacts with the phospho-Thr210 form of Snf1 (see Fig. 8A,C). Thus, these results provide no evidence that the RVxF motif of Reg2 is required for its interaction with Snf1.

Together, the overexpression and two-hybrid experiments suggest that Reg2 requires the RVxF motif to interact with Glc7 (but not Snf1), and to carry out its role in Snf1 Thr210 dephosphorylation.

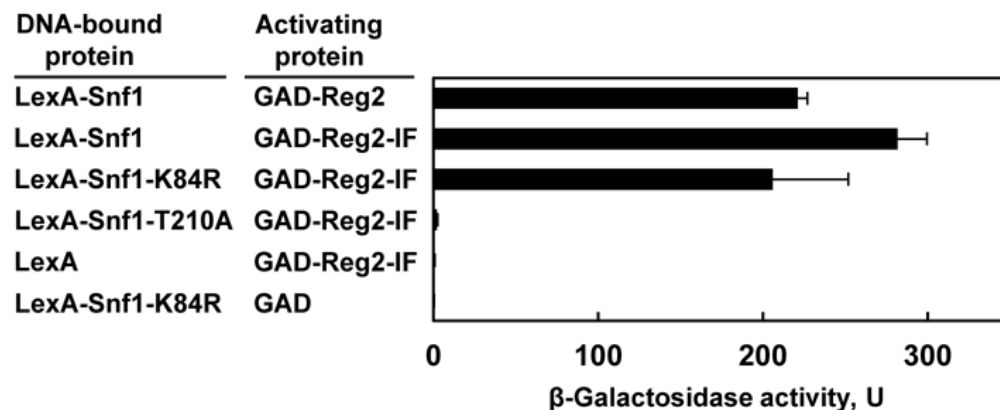


Fig. 16. Reg2-IF interacts with Snf1 in the yeast two-hybrid system. Transformants of the reporter strain CTY10-5d with plasmids expressing the indicated protein pairs were grown to mid-log phase with plasmid selection in the presence of 2% glucose, and then shifted for 3 h to an otherwise identical medium containing 0.05% glucose. β -Galactosidase activity was assayed in permeabilized cells and expressed in Miller units. Shown are the results for limiting glucose; values are averages for 4-8 transformants; the error bars indicate standard errors.

Discussion

Since protein phosphatases play an antagonistic role to protein kinases in numerous biochemical pathways, they are interesting drug targets for diseases caused by alterations in protein phosphorylation (Bollen, Peti, Ragusa, & Beullens, 2010), such as Alzheimer's disease and certain forms of cancer (den Hertog, 2003; Gong et al., 2000). PP1, for instance, is hijacked by the HIV-1 Tat protein to promote HIV-1 transcription (Ammosova et al., 2005).

Pharmaceutical compounds that inhibit phosphatases already exist, but many of these drugs can have off-target effects since the catalytic subunits of protein phosphatases lack specificity. For this reason, phosphatase regulatory subunits are even more intriguing, since they are known to modulate the activity or substrate specificity of phosphatases (Bollen et al., 2010; Ceulemans & Bollen, 2004).

PP1 is known to interact with hundreds of regulatory proteins in various eukaryotes. This interaction is often mediated by a small region in the regulatory proteins known as the "RVxF motif". Recent studies have found that small molecules or peptides that bind to the RVxF motif can inhibit the dephosphorylation of specific PP1 targets (Ammosova et al., 2012; Ammosova et al., 2011). For these reasons, understanding the physical and functional interactions between PP1, its regulatory subunits, and PP1 substrates can have broad medical relevance.

In this study, we examined the role of a putative PP1-binding RVxF motif found in Reg2. Since the same motif in Reg1 was previously shown to be critical for the ability of Reg1 to act on Snf1, we hypothesized that the Reg2 RVxF motif would also be important for Snf1 regulation. Using the yeast two-hybrid system, we examined the physical interactions between the mutant Reg2-IF protein and either Glc7 or Snf1. Like Reg1, Reg2 also requires the RVxF motif for interaction with Glc7. However, the Reg2 RVxF motif is not necessary for Snf1

binding, which makes a “competitive binding” model less likely to be true for Reg2. It remains possible that a similar binding competition could occur via an as yet unknown region of Reg2 other than its RVxF motif. However, it is also possible that the functional cycle of Reg2 is simpler than that of Reg1, and it does not require such competition.

Table 2. *S. cerevisiae* strains

<u>Strain</u>	<u>Genotype</u>	<u>Source</u>
CTY10-5d	<i>MATa gal4 gal80 URA3::lexAop-lacZ his3 leu2 ade2 trp1</i>	R. Sternglanz
KBY247	<i>MATa ura3Δ leu2Δ his3Δ trp1Δ reg1Δ::URA3</i>	This study

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Chapter 4:

Springing into action: Reg2 negatively regulates Snf1 protein kinase and facilitates recovery from prolonged glucose starvation

Abstract

The ability of microorganisms to respond to stress is essential for their survival. However, rapid recovery from stress could be equally crucial in competitive environments. Therefore, a wise stress response program should prepare the cells for quick recovery upon re-exposure to favorable conditions. Glucose is the preferred carbon source for the yeast *Saccharomyces cerevisiae*. Glucose depletion activates the stress-response protein kinase Snf1, which functions to limit energy-consuming processes such as growth. We show that prolonged glucose deprivation also leads to Snf1-dependent accumulation of Reg2, and that this protein helps inhibit Snf1 and accelerate growth recovery upon glucose replenishment. In addition, cells lacking Reg2 are readily outcompeted by wild-type cells during glucose depletion/replenishment cycles. Thus, while prolonged glucose deprivation might seem to put yeast cells “on their knees”, concomitant accumulation of Reg2 helps configure the cells into a “sprinter’s crouch start position” to spring into action once glucose becomes available.

Introduction

Glucose is a preferred source of carbon and energy for a wide variety of microorganisms. Although the yeast *Saccharomyces cerevisiae* can exist in many different environments, it is particularly known for its ability to flourish under glucose-rich conditions - a property exploited by humans for millennia to make wine [reviewed in references (Goddard & Greig, 2015;

Pretorius, 2000)]. Considering its prevalence in wine-making, a common assumption is that *S. cerevisiae* is abundant on grapes. However, *S. cerevisiae* begins its journey to the winery as a rare species, making up just a small portion of the microbial residents in vineyards. Eventually it dominates during fermentation, in large part due to a rapid response to the glucose-rich environment found on winery surfaces and grape must (Goddard & Greig, 2015; Pretorius, 2000). Consistent with the importance of glucose for its growth and metabolism, *S. cerevisiae* has evolved a complex regulatory network that controls responses to glucose availability, with a central role played by a protein kinase called Snf1 [reviewed in (Santangelo, 2006)].

Yeast Snf1 (sucrose non-fermenting 1) is a founding member of the highly conserved Snf1/AMP-activated protein kinase (AMPK) family [reviewed in (Hedbacker & Carlson, 2008)]. A unifying feature of Snf1/AMPK kinases is that they serve as the “fuel gauge” of the eukaryotic cell (Hardie & Carling, 1997): as energy levels decrease, these kinases are activated and function to balance the energy “budget” by reducing energy spending and by increasing energy “income”. In mammals, AMPK coordinates cell growth and proliferation with energy availability (Jones et al., 2005) and has been implicated in health conditions ranging from type II diabetes to cancer (Fogarty & Hardie, 2009; Hardie, 2007). In yeast, lack of Snf1 leads to inability to utilize carbon sources that are less preferred than glucose, including sucrose, galactose, maltose, and starch (Carlson, Osmond, & Botstein, 1981; Celenza & Carlson, 1984; S. V. Kuchin, Kartasheva, & Benevolensky, 1993). Genetic manipulation of Snf1 and/or its targets has been a useful strategy for achieving various practical goals in yeast biotechnology, such as improved maltose utilization (C. Y. Zhang, Bai, Lin, Liu, & Xiao, 2015), galactose utilization and ethanol production (Ostergaard, Olsson, Johnston, & Nielsen, 2000), fatty acid production (Choi & Da Silva, 2014; Shi, Chen, Siewers, & Nielsen, 2014), and biomass yields (Raab, Hlavacek,

Bolotina, & Lang, 2011). Thus, further understanding Snf1 regulation could help identify additional approaches for rational design of yeast strains.

When glucose is limiting, Snf1 is activated through the action of three partially redundant protein kinases (Sak1, Tos3, and Elm1), which phosphorylate its conserved T-loop threonine residue (Thr210) (Hong, Leiper, Woods, Carling, & Carlson, 2003; McCartney, Rubenstein, & Schmidt, 2005; McCartney & Schmidt, 2001; Nath, McCartney, & Schmidt, 2003; Sutherland et al., 2003). When glucose becomes abundant, Snf1 is inhibited by dephosphorylation, which involves the function of PP2A-like protein phosphatase Sit4 and type 1 protein phosphatase Glc7 (Hedbacker & Carlson, 2008; Ruiz, Liu, Xu, & Carlson, 2012; Ruiz, Xu, & Carlson, 2011b).

Type 1 protein phosphatase Glc7 associates with various regulatory subunits that target it to appropriate substrates, thereby allowing it to have multiple cellular functions. One of these subunits, Reg1 (Tu & Carlson, 1995), is known to play a major role in enabling Glc7 to recognize and dephosphorylate Thr210 of Snf1 (Ludin, Jiang, & Carlson, 1998; McCartney & Schmidt, 2001; Y. Zhang, McCartney, Chandrashekarappa, Mangat, & Schmidt, 2011).

Reg2 is another regulatory subunit of Glc7 that shares homology with Reg1 (Frederick & Tatchell, 1996; Jiang, Tatchell, Liu, & Michels, 2000). In fact, *REG1* and *REG2* are a pair of paralogous genes that arose during an ancient whole-genome duplication event in an ancestral yeast species (Byrne & Wolfe, 2005). Our previous work has shown that Reg2 has a fine-tuning role in the regulation of Snf1, but it also remained possible that Reg2 could have a more prominent role in Snf1 regulation.

In this study, we examined the possible role of Reg2 in the negative control of Snf1 following prolonged glucose deprivation. We present evidence that Reg2 accumulates during prolonged glucose deprivation in a Snf1-dependent manner, and glucose-starved cells lacking

Reg2 exhibit a delay in Snf1 Thr210 dephosphorylation and growth recovery upon glucose replenishment. We also show that cells lacking Reg2 are outcompeted by wild-type cells during glucose starvation/replenishment cycles, supporting the evolutionary significance of Reg2 for *S. cerevisiae*.

Materials and Methods

Strains and growth conditions. The *S. cerevisiae* strains used in this study are listed in Table 3. All strains were in the Σ 1278b genetic background and were descendants of strains MY1384 (*MATa*, prototroph), MY1401 (*MATa ura3 Δ leu2 Δ his3 Δ*), and MY1402 (*MATa ura3 Δ leu2 Δ trp1 Δ*) of the isogenic Sigma2000 series (Microbia, Cambridge, MA). To generate Σ 1278b derivatives with *reg2 Δ ::KanMX6*, the *KanMX6* marker sequence was amplified by PCR with primers flanking the *REG2* open reading frame (ORF). The mutant allele was first introduced into a wild-type diploid (MKY324 X MKY341) by transformation; all yeast transformations were performed using the standard lithium acetate method (Rose, Winston, & Hieter, 1990). The genotype of the heterozygous *REG2/reg2 Δ ::KanMX6* diploid was confirmed by PCR analysis of genomic DNA, and haploid *reg2 Δ ::KanMX6* segregants were recovered from the heterozygous diploid by tetrad analysis. Construction of the *reg1 Δ ::KanMX6* and *snf1 Δ ::KanMX6* derivatives was described previously (S. Kuchin, Vyas, & Carlson, 2002; Orlova, Kanter, Krakovich, & Kuchin, 2006).

Rich medium was yeast extract-peptone (YEP) supplemented with extra tryptophan (40 mg/liter) and adenine (20 mg/liter); synthetic complete (SC) medium lacking appropriate supplements was used to select for plasmids (Rose et al., 1990). Unless indicated otherwise, the

media contained 2% glucose, and yeast cells were grown at 30°C.

Analysis of regulation of Reg2 expression. To analyze Reg2 expression from the native *REG2* promoter, a low copy number plasmid, pReg2-L-HA, was used. This plasmid encodes a Reg2 protein C-terminally tagged with LexA followed by triple HA (hereafter referred to as Reg2-L-HA), and was constructed as follows. First, the *REG2* gene, including its 0.52-kb upstream regulatory sequence (the entire intergenic region between *REG2* and its upstream neighbor *RFS1*), was amplified by PCR using wild-type genomic DNA as template; the reverse primer used for this PCR also included a BspEI site and a sequence encoding a single HA tag. The resulting PCR fragment was then inserted into the BamHI site of low copy number *CEN-URA3* vector pRS316 (Sikorski & Hieter, 1989), yielding plasmid pReg2-Bsp-HA. Next, the *lexA* ORF was amplified by PCR using plasmid pEG202 as template; the reverse primer used for this PCR also included sequences for two consecutive HA tags. The resulting fragment was inserted into the BspEI site of plasmid pReg2-Bsp-HA, yielding pReg2-L-HA. The plasmid was then transformed into wild-type and mutant yeast strains, and transformants were subjected to various culture conditions as specified in the text. Protein extracts were prepared using the boiling/alkaline lysis method (Orlova, Barrett, & Kuchin, 2008), and Reg2-L-HA expression levels were analyzed by immunoblotting with anti-HA antibody HA-7.

Mixed culture competition assays. Strains MKY323 (*MATa ura3*) and MMY9 (*MATa ura3 reg2Δ::KanMX6*) were grown to mid-log phase in YEP with 2% glucose. Cultures were adjusted to an OD₆₀₀ = 0.15 with YEP-2% glucose medium, mixed together in equal proportions, and grown for 4 h to an OD₆₀₀ ~ 0.6. The mixed-culture cells were then subjected to ten cycles of

glucose starvation/glucose replenishment. For glucose starvation, cells were shifted to YEP medium lacking glucose for 24 h. For glucose replenishment, the starved cells were shifted to YEP containing 2% glucose (at an initial OD₆₀₀ of 0.15) and grown for 5-6 h; all incubations were at 30°C with shaking at 200 rpm. To determine the proportion of *reg2Δ::KanMX6* cells, culture aliquots were plated on YEP with 2% glucose, and following colony formation, the number of kanamycin-resistant colonies was determined by replica-plating onto YEP containing 2% glucose and 200 μg/mL kanamycin.

Results

The Reg2 protein is expressed during prolonged glucose starvation

Our previous work showed that cells lacking Reg2 exhibited defects in Snf1 Thr210 dephosphorylation only when *REG1* was also knocked out. A simple explanation could be that the Reg2 protein levels are Snf1-dependent. If so, deletion of Reg1, which is known to cause Snf1 hyperactivity, could actually boost the levels of the Reg2 protein. We therefore compared the levels of a C-terminally tagged Reg2 protein (Reg2-L-HA) expressed from the native *REG2* promoter in both *REG1* and in *reg1Δ* cells. We observed that Reg2 is not detectably expressed in the wild type grown on high glucose, and is expressed only weakly after a 1 h shift to low glucose (Fig. 17). By contrast, Reg2 was expressed at much higher levels in *reg1Δ* cells, both under high and low glucose conditions (Fig. 17).

In a previous microarray study, *REG2* was listed among genes positively regulated by Snf1 at the mRNA level (Young, Dombek, Tachibana, & Ideker, 2003). To directly address the requirement of Snf1 for Reg2 protein expression, we examined the effects of the *snf1Δ* mutation in *REG1* and *reg1Δ* backgrounds. We observed a Reg2 expression defect in the *snf1Δ* mutant

after a 1 h shift to low glucose (Fig. 17). Moreover, we did not detect any Reg2 protein in the *reg1Δ snf1Δ* double mutant (Fig. 17), indicating that even in the absence of Reg1, Reg2 protein expression remains Snf1-dependent.



Fig. 17. Reg2 protein expression is Snf1-dependent. Cells of the indicated genotypes transformed with vector pRS316 (V) or pReg2-L-HA (Reg2-L-HA) were grown in SC medium lacking uracil and containing 2% glucose (H, high glucose) and then shifted for 1 h to an otherwise identical medium containing 0.05% glucose (L, low glucose). The levels of tagged Reg2 expressed from its native promoter (Reg2-L-HA) were analyzed by immunoblotting; Por1 was used as a loading control (Porin) and detected using an anti-Por1 antibody (Invitrogen). The strains were MKY191 (WT), MKY366 (*snf1Δ*), KBY266 (*reg1Δ*), and KBY238 (*snf1Δ reg1Δ*).

Reg2 plays a role in Snf1 inactivation after prolonged glucose starvation.

We considered the possibility that the *reg2Δ* mutation failed to produce detectable effects on its own (in *REG1* cells) because Reg2 protein expression is quite low under experimental conditions employed so far. In other words, the *reg2Δ* single mutation could produce an effect under conditions where Reg2 is naturally expressed at a sufficiently high level. Since Reg2 expression showed an increase in response to short-term glucose limitation (1 h; Fig. 17), we reasoned that its expression might increase even further in response to a longer starvation time. Indeed, we detected a higher Reg2 protein level after depriving cells of glucose for 24 h (Fig. 18A, N24), and expression still remained Snf1-dependent (Fig. 18B).

When we added glucose back to wild-type and *reg2Δ* mutant cells pre-starved for 24 h, we detected a difference between these cells. Specifically, Snf1 Thr210 dephosphorylation

occurred more slowly in the *reg2Δ* mutant, as there was a clear difference in phospho-Thr210-Snf1 levels at 30 sec, 1 min, and 5 min post-replenishment (Fig. 18C); in subsequent experiments, we also detected differences at later time points (see Fig. 19B and text below). These results strongly suggested that Reg2 has an important role in the rapid dephosphorylation of Snf1 when glucose is added back after prolonged starvation.

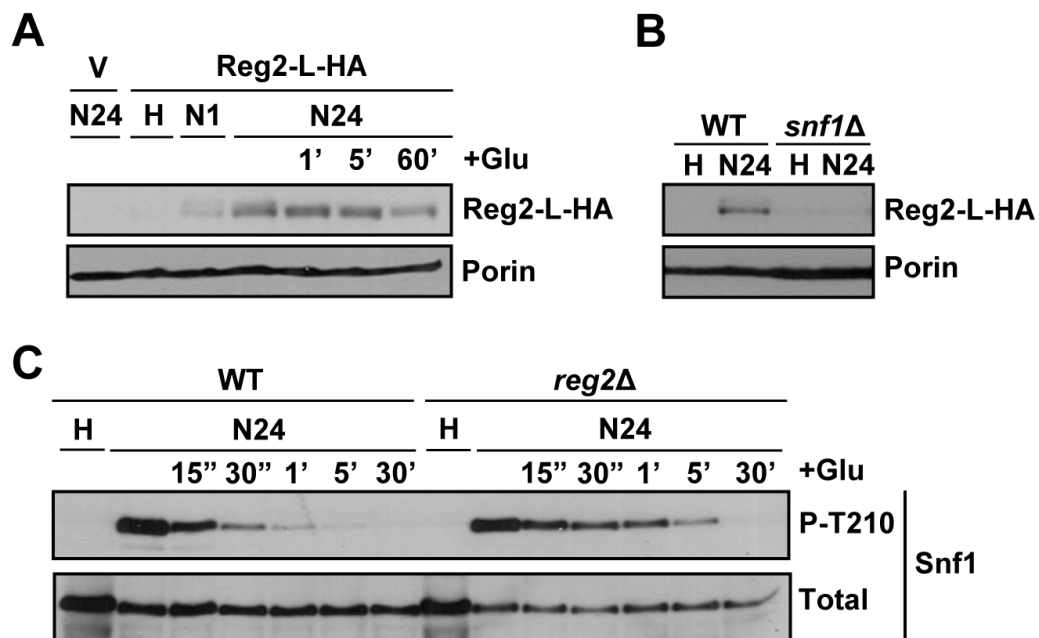


Fig. 18. Reg2 accumulation increases after prolonged glucose deprivation in a Snf1-dependent manner. (A) *reg2Δ* cells transformed with vector pRS316 (V) or pReg2-L-HA (Reg2-L-HA) were grown to mid-log phase in selective SC medium lacking uracil and containing 2% glucose (H, high glucose), and then shifted to an otherwise identical medium containing no glucose for either 1 h (N1) or 24 h (N24). After 24 h of glucose starvation, cultures were replenished with 2% glucose (+Glu) for either 1 min (1'), 5 min (5'), or 60 min (60'). (B) Cells of the indicated genotypes carrying pReg2-L-HA (Reg2-L-HA) were grown to mid-log phase in selective SC medium lacking uracil and containing 2% glucose (H, high glucose) and then shifted to an otherwise identical medium containing no glucose for 24 h (N24). In panels A and B, the levels of tagged Reg2 expressed from the native *REG2* promoter (Reg2-L-HA) and Por1 protein (Porin) were detected by immunoblotting as for Fig. 7E. (C) Cells of the indicated genotypes were grown to mid-log phase in YEP medium containing 2% glucose (H, high glucose) and shifted to an otherwise identical medium containing no glucose for 24 h (N24). Following 24 h in no glucose, cells were replenished with 2% glucose (+Glu) for either 15 sec (15''), 30 sec (30''), 1 min (1'), 5 min (5'), or 30 min (30'). Levels of phospho-Thr210-Snf1 (P-T210) and total Snf1 protein (Total) were analyzed by immunoblotting. The strains were MMY9 (*reg2Δ*), MKY323 (WT), and MKY366 (*snf1Δ*).

Starved cells lacking Reg2 exhibit a growth recovery delay following glucose replenishment.

Since increased Snf1 activity can cause slower growth, we compared the ability of glucose-starved wild-type and *reg2Δ* mutant cells to resume growth upon glucose replenishment. When 2% glucose was added back to cells pre-starved for 24 h, the *reg2Δ* mutant required ~ 20 min longer than the wild type to undergo the first doubling (*ca.* 220 min versus 200 min, respectively; Fig. 19A). There was no statistically significant difference in their second doubling time (*ca.* 130 min; Fig. 19A). The initial 20-min growth delay of the *reg2Δ* mutant also coincided with higher levels of phospho-Thr210-Snf1, which could be detected at 15, 20, and even 25 min after glucose re-addition (Fig. 19B).

Although the 20-min growth recovery delay of the *reg2Δ* mutant may seem modest, if repeated over several starvation/replenishment cycles, it could translate into a substantial competitive disadvantage. To address this point, we performed mixed-culture competition assays. Wild-type and *reg2Δ* cells were grown to mid-log phase in glucose-rich medium, mixed in equal proportions, and subjected to repetitive cycles of 24-h glucose starvation followed by glucose replenishment. The proportion of the mutant cells in the culture progressively declined, dropping below 10% after 10 such cycles (Fig. 19C). Thus, these results underscore the evolutionary significance of the *REG2* gene.

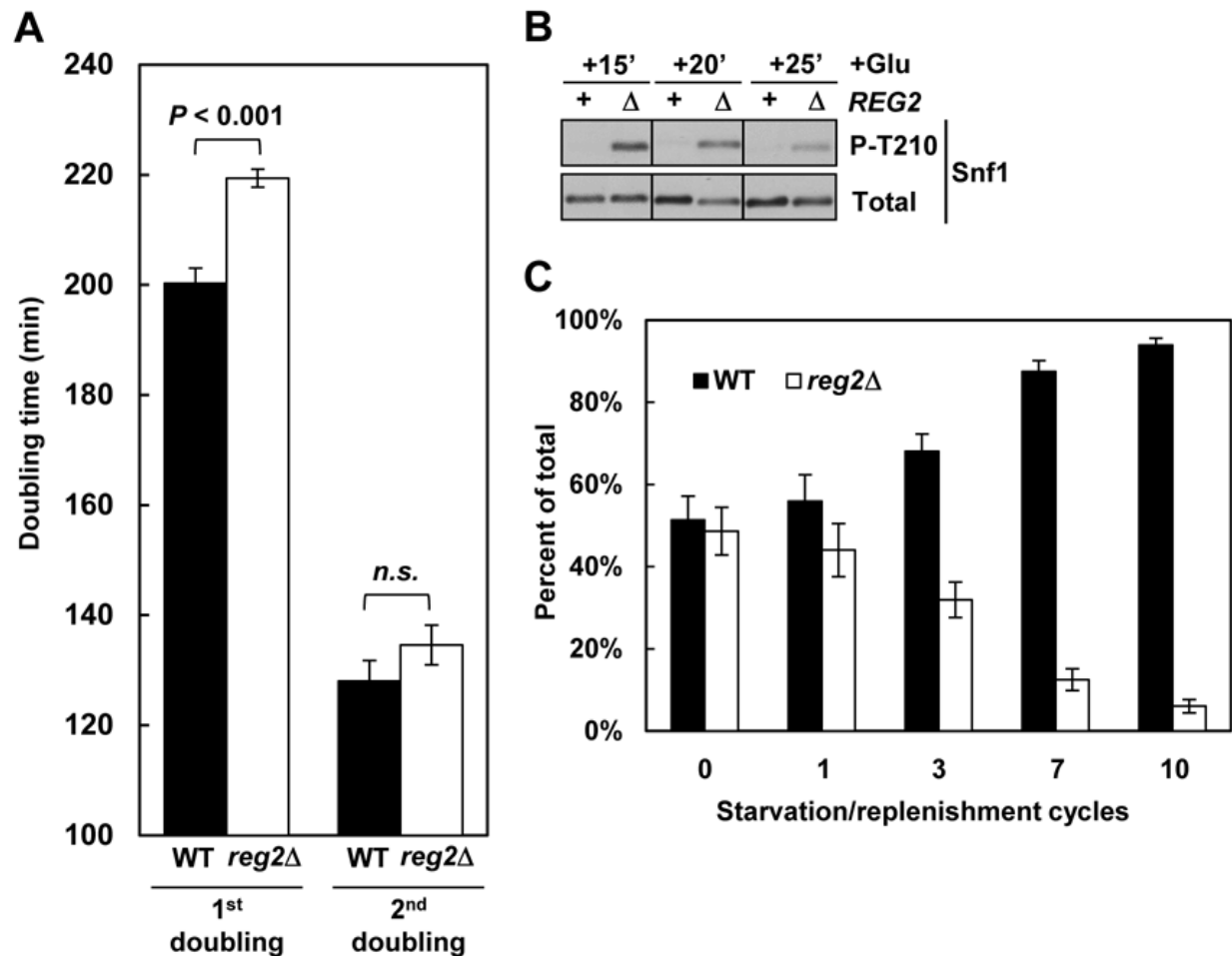


Fig. 19. Cells lacking Reg2 exhibit a delayed recovery from prolonged glucose deprivation. (A) Wild-type and *reg2Δ* cells were grown to mid-log phase in YEP containing 2% glucose and then shifted to YEP without glucose for 24 h. After this starvation period, the cultures were shifted (at an initial OD₆₀₀ of 0.15) to YEP containing 2% glucose, and the OD₆₀₀ of the cultures was monitored. Values are averages for three cultures of each genotype. The error bars indicate standard errors. (B) Wild-type (*REG2*, +) and *reg2Δ* cells (*REG2*, Δ) were grown to mid-log phase in YEP medium containing 2% glucose, shifted to YEP containing no glucose for 24 h, and glucose was then added at 2% (+Glu) for either 15 min (15'), 20 min (20'), or 25 min (+25'). The levels of phosphorylated Thr210 (P-T210) and total Snf1 protein (Total) were analyzed by immunoblotting. (C) Wild-type and *reg2Δ* cells were grown to mid-log phase in YEP medium containing 2% glucose, mixed in equal proportions, subjected to ten cycles consisting of a 24-h glucose starvation phase and a 5-6-h glucose replenishment phase, and the proportion of *reg2Δ* cells was determined as described in Materials and Methods. The strains were MKY323 (WT) and MMY9 (*reg2Δ*).

Discussion

REG1 and *REG2* of *S. cerevisiae* are a pair of paralogous genes dating back to an ancient whole-genome duplication event in an ancestral yeast species (Wolfe & Shields, 1997). In the course of evolution, most duplicate gene copies were lost, and more than 80% of protein-coding genes in modern *S. cerevisiae* do not have a paralog [for a recent review, see reference (Wolfe, 2015)]. Of the preserved gene pairs, many function in energy metabolism and/or its regulation (Kuepfer, Sauer, & Blank, 2005), and one of the copies in such gene pairs has often developed a specialized function. For instance, *SNF3* and *RGT2* are paralogous genes whose protein products have functionally diverged into sensing differing concentrations of glucose (Ozcan, Dover, & Johnston, 1998). This bias toward the preservation of gene pairs involved in energy metabolism – together with the freedom of the paralogs to acquire a degree of specialization - may have been beneficial for the ability of *S. cerevisiae* to outcompete other species by rapid glucose fermentation (Conant & Wolfe, 2007). Rapid growth in glucose is associated with reduced Snf1 activity, emphasizing the importance of Snf1-inhibiting mechanisms.

As a regulatory subunit of Glc7 protein phosphatase, the role of Reg1 in Snf1 inhibition has been firmly established (Hedbacker & Carlson, 2008). Our previous work has shown that the homologous Reg2 also has a role in the regulation of Snf1, but whether Reg2 functions as just a redundant backup or has a unique role in Snf1 regulation had not yet been established.

Multiple lines of evidence suggest that Reg1 and Reg2 are not simply redundant proteins. A previous study showed that Reg1 and Reg2 have differing functions in maltose utilization, with Reg1 exhibiting a more prominent role in maltose transport (Jiang et al., 2000). Our prior work found that, although both Reg1 and Reg2 are *bona fide* regulatory subunits that depend on Glc7 interaction to act on Snf1, Reg2 does not seem to follow the same “competitive binding”

model as Reg1 (Tabba, Mangat, McCartney, & Schmidt, 2010) since the Reg2 RVxF motif is not necessary for interaction with Snf1 (see Fig. 16). We also found that, unlike deletion of *REG1*, deletion of *REG2* alone was not sufficient to cause a detectable change in Snf1 Thr210 phosphorylation levels (Fig. 9). We therefore considered the possibility that Reg2 protein expression could explain this lack of a phenotype and could provide evidence that Reg1 and Reg2 are functionally different.

Evidence indicates that the Reg1 protein is expressed at similar levels in glucose-rich and glucose-limiting conditions (Tabba et al., 2010). In contrast, *REG2* gene transcription (Lutfiyya et al., 1998; Young et al., 2003) was previously shown to increase in response to glucose limitation.

Here, we show that Reg2 protein abundance similarly increases in response to glucose limitation in a Snf1-dependent manner. Furthermore, Reg2 protein expression increases even further following long-term glucose deprivation. This elevated expression of Reg2 may have a physiological purpose: we show that Reg2 contributes to faster growth recovery when glucose is added back to cells after prolonged glucose deprivation. Collectively, these results support a model in which Snf1-dependent Reg2 accumulation during prolonged glucose deprivation prepares the cells to more quickly dephosphorylate and inactivate Snf1 upon glucose replenishment (Fig. 20).

It seems logical to assume that at least part of the mechanism controlling rapid growth recovery involves Snf1 Thr210 dephosphorylation, but it seems possible – and even likely – that Reg2-Glc7 also dephosphorylates other growth-related targets. As proposed previously (Frederick & Tatchell, 1996), some additional growth-related targets of Reg2-Glc7 could actually be downstream phosphorylation substrates of Snf1 itself. In this regard, it has been

recently reported that Snf1 phosphorylates and negatively regulates adenylate cyclase (Nicastro et al., 2015), a component of the pro-growth PKA pathway, and that it also inhibits the pro-growth TORC1 pathway in response to glucose starvation (Hughes Hallett, Luo, & Capaldi, 2014, 2015). These reports raise the possibility that Reg2-Glc7 might target components of the Ras-cAMP-PKA and/or TORC1 pathways for dephosphorylation. Multiple subunits of the PKA complex, including catalytic and inhibitory subunits, are an intriguing possibility since they are regulated by phosphorylation (Budhwar, Lu, & Hirsch, 2010; Steinberg, Cauthron, Symcox, & Shuntoh, 1993). It also remains possible that some relevant targets of Reg2-Glc7 could lie outside of the Snf1 pathway. Further studies will be required to address these possibilities.

Regardless of whether the role of Reg2-Glc7 is limited to dephosphorylation and inactivation of Snf1 itself or extends to other targets, it is clear that the function provided by Reg2 bears evolutionary significance. Results presented in this study show that cells lacking Reg2 are readily outcompeted by wild-type cells in the course of a few glucose starvation/replenishment cycles. The ability of starved yeast cells to rapidly activate metabolism and growth following nutrient replenishment is crucial for competing with microbial neighbors, and the *REG2* gene was likely preserved to help fulfill this function.

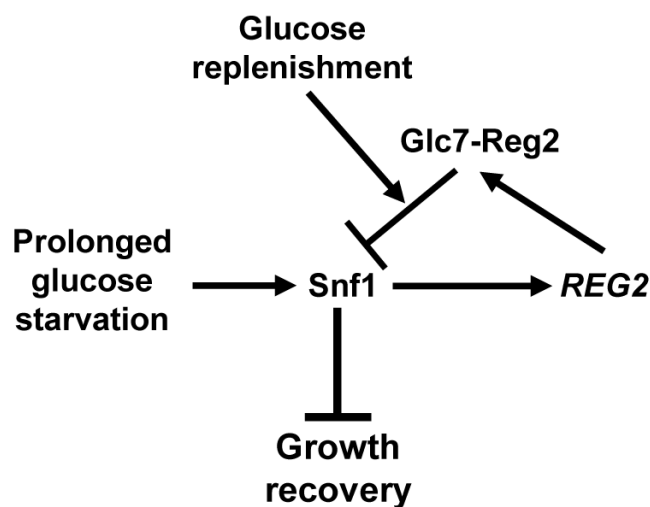


Fig. 20. Proposed model. During steady-state growth in high glucose, Snf1 is inactive and *REG2* is not expressed. Prolonged glucose starvation leads to Snf1-dependent accumulation of Reg2. Once glucose becomes abundant, Reg2 contributes to rapid dephosphorylation and inactivation of Snf1. By inactivating Snf1 (and/or a Snf1 target), Reg2 promotes quicker growth recovery.

Table 3. *S. cerevisiae* strains

Strain	Genotype	Source
MKY191	<i>MATα ura3Δ leu2Δ his3Δ</i>	This laboratory
MKY323	<i>MATα ura3Δ</i>	This laboratory
MKY366	<i>MATα ura3Δ leu2Δ his3Δ snf1Δ::KanMX6</i>	This laboratory
KBY238	<i>MATα ura3Δ leu2Δ his3Δ reg1Δ::KanMX6</i> <i>snf1Δ::KanMX6</i>	This laboratory
KBY266	<i>MATα ura3Δ leu2Δ his3Δ reg1Δ::KanMX6</i>	This laboratory
MMY9	<i>MATα ura3Δ reg2Δ::KanMX6</i>	This study

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CURRICULUM VITAE

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EDUCATION:

University of Wisconsin-Milwaukee, Milwaukee, WI
Doctor of Philosophy in Biological Sciences – *expected* May 2016

Boston College, Chestnut Hill, MA
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RESEARCH

EXPERIENCE:

Graduate Student, Lab of Dr. Sergei Kuchin at University of Wisconsin-Milwaukee – Fall 2010 - Present

- Dissertation title: Role of protein phosphatase Reg2-Glc7 in the regulation of the yeast stress response kinase, Snf1
- Project: Identifying the role of a regulatory subunit of type 1 protein phosphatase (PP1) in the regulation of Snf1, a highly conserved protein kinase involved in the response to glucose limitation and other stresses
- Utilizing techniques including Western blotting, immunoprecipitation, yeast two-hybrid system, flow cytometry, and molecular cloning

Biochemistry Advanced Laboratory, Boston College – Fall 2009

- Worked in a research-oriented laboratory course with a team of students to study role of Msr proteins in the oxidative stress response
- Isolated and purified a methionine-rich protein
- Collaborated and presented data with a team of cell biology laboratory students

TEACHING

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Guest Lecturer: Genetics (BioSci 325) – Nov. 14, 2013 and Nov. 18, 2014

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Teaching Assistant: Genetics Discussion (BioSci 325) – Spring 2013 –Fall 2015

- Used hands-on problem-solving to emphasize critical Genetics topics
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Teaching Assistant: Experim. Microbiology (BioSci 580) Spring 2013 - Spring 2015

- Guided students through lab activities, assisted professor with demonstrations
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Teaching Assistant: Anatomy & Physiology I Lab (BioSci 202)– Fall 2010–Fall 2012

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Teaching Assistant: Molecular Cell Biology Laboratory & Genetics Laboratory (Boston College) – 2009-2010

- Ran writing workshops to aid students in writing laboratory reports
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Graduate Program Assistant, Biological Sciences - University of Wisconsin-Milwaukee – Jan-Mar 2016

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PUBLICATIONS:

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Barrett L, Orlova M, Maziarz M, Kuchin S. Protein kinase A contributes to the negative control of Snf1 protein kinase in *Saccharomyces cerevisiae*. *Eukaryot Cell* 2012; **11**(2):119-128.

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Maziarz M., & Kuchin S. (2012, April). *A novel glucose-regulated interaction fine-tunes Snf1 protein kinase in yeast*. Poster session presented at the Biological Sciences Research Symposium at the University of Wisconsin-Milwaukee, Milwaukee, WI.

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RELEVANT SKILLS:**Biochemical Techniques**

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Computer Skills

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