Università degli Studi di Milano-Bicocca
Dipartimento di Biotecnologie e Bioscienze
Dottorato di ricerca in Biotecnologie Industriali – XXVII Ciclo

Role of Snf1/AMPK as regulator of cell cycle, signal transduction and metabolism in *Saccharomyces cerevisiae*

Raffaele Nicastro
Matr. 071877

Anno Accademico 2013-2014

The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny...'

Isaac Asimov

Contents

Riassunto

Riassunto

Snf1 è una serina/treonina chinasi necessaria per il lievito gemmante *Saccharomyces cerevisiae* per la crescita in condizioni di limitazione di nutrienti e per l'utilizzo di fonti di carbonio alternative al glucosio, quali saccarosio ed etanolo (Hedbacker and Carlson, 2008). L'attivazione di Snf1 in seguito alla limitazione di nutrienti ne causa la traslocazione nel nucleo dove è in grado di fosforilare, inattivandolo, il repressore trascrizionale Mig1, causando l'espressione di oltre 400 geni (Young et al., 2003).

Nel nostro laboratorio è stato precedentemente dimostrato che la mancanza di Snf1 causa un difetto nella transizione G_1/S del ciclo cellulare e un difetto nell'espressione del gene *CLB5*, codificante per la principale ciclina di fase S, anche in condizioni di sufficienza nutrizionale (2% glucosio) (Pessina et al., 2010). E' stato inoltre dimostrato che in un ceppo *snf1Δ* vi è un difetto globale di espressione dei geni di fase G_1, nonché del legame ai promotori di questi geni dei fattori trascrizionali SBF ed MBF, che ne determinano la trascrizione ciclo regolata, e della stessa RNA polimerasi II. Per dimostrare la necessità dell'attività catalitica di Snf1 per una corretta transizione G_1/S è stato utilizzato un ceppo Snf1-I132G, in cui l'attività della chinasi può essere inibita utilizzando l'inibitore specifico 2NM-PP1. Il grave difetto nell'effettuare la transizione G_1/S e nella trascrizione dei geni di fase G_1 in presenza dell'inibitore è stata dimostrata sia con esperimenti di blocco con *α-factor* sia mediante elutriazione (Busnelli et al., 2013).

Riassunto

Nell'ottica dello studio del coinvolgimento di Snf1 nella regolazione di altri *pathway* di trasduzione del segnale è stata identificata, mediante esperimenti di co-immunoprecipitazione accoppiata a spettrometria di massa, l'interazione tra Snf1 e l'adenilato ciclasi (Cyr1), l'enzima responsabile della produzione di AMP ciclico (cAMP), attivatore di PKA. Abbiamo inoltre dimostrato che in un ceppo Snf1-G53R, in cui la chinasi è costitutivamente attivata, risulta deregolata l'espressione di alcuni geni PKA-dipendenti. E' stata quindi ipotizzata l'esistenza di un nuovo meccanismo di *crosstalk* fra i *pathway* di Snf1 e PKA, considerando che fino ad ora sono note soltanto regolazioni di PKA sul *pathway* di Snf1 o regolazioni di entrambe le chinasi su *target* comuni (Barrett et al., 2012; Cherry et al., 1989; Görner et al., 1998, 2002; Hedbacker et al., 2004; Ratnakumar et al., 2009; De Wever et al., 2005). La sequenza di Cyr1 presenta cinque possibili siti consenso di fosforilazione per Snf1, dei quali due risultano essere fosforilati *in vivo*. Il dominio della proteina adenilato ciclasi contenente il *RAS Associating Domain* (RAD) e 2 dei 5 siti consenso è stato purificato in *E. coli* e ne è stata dimostrata la fosforilazione *in vitro* da parte di Snf1. È stato quindi dimostrato che in un ceppo Snf1-G53R si osserva una notevole riduzione nel contenuto di cAMP intracellulare (circa il 50%) paragonato al *wt*. È stato quindi ipotizzato che questa riduzione causi una minore attivazione di PKA e, in conseguenza, la deregolazione dei geni da essa controllati. Sono stati inoltre effettuati esperimenti di

mappatura dei siti dell'adenilato ciclasi fosforilati *in vivo* mediante analisi di spettrometria di massa, identificandone alcuni, prevalentemente concentrati nel dominio regolatorio della proteina, la cui fosforilazione è dipendente dalla presenza di Snf1.

Per chiarire il ruolo globale di Snf1 in condizioni di sufficienza nutrizionale è stata quindi effettuata un'analisi trascrittomica (*gene-chip*) di cellule *wt* e *snf1Δ* cresciute in 2% e 5% glucosio. Mediante questa analisi è stato evidenziato che Snf1 in 2% glucosio, ma non in 5%, sia importante per garantire l'espressione di geni coinvolti in traduzione mitocondriale, replicazione del DNA, biosintesi della parete cellulare e omeostasi del ferro. É stato viceversa dimostrato che la mancanza di Snf1 causi la de-repressione dei geni coinvolti nel trasporto degli aminoacidi, nella glicolisi e in alcuni *step* della biosintesi degli aminoacidi. Sono quindi state approfondite le deregolazioni metaboliche presenti in cellule prive di Snf1. L'analisi dei metaboliti secreti da cellule *snf1Δ* in crescita in presenza di 2% glucosio ha permesso di dimostrare che queste, in relazione alla propria velocità di crescita, producono più etanolo ed acetato in confronto a cellule *wt*. Questa maggiore attività glicolitica è abolita in presenza di 5% glucosio, nonostante cellule sia *wt* che mutate, in queste condizioni, producano più metaboliti glicolitici in confronto a cellule *wt* in 2% glucosio. Mediante analisi di spettrometria di massa è stato dimostrato che anche nelle condizioni di crescita dei nostri esperimenti (2% e 5%

glucosio, fase esponenziale), cellule *snf1Δ* presentano un notevole accumulo di acidi grassi, fenotipo già osservato in cellule in crescita con bassi livelli di glucosio e dovuto all'assenza di fosforilazione Snf1-dipendente dell'enzima acetyl-CoA carbossilasi (Shirra et al., 2001). Una più estesa analisi metabolica, sia mediante spettrometria di massa che mediante NMR, ha inoltre permesso di descrivere in dettaglio i riarrangiamenti metabolici che cellule *snf1Δ* subiscono perché la crescita sia garantita nonostante i processi anabolici sopra descritti. Cellule *snf1Δ* in crescita in 2% glucosio accumulano glutammato, l'amminoacido più abbondante in cellula, in funzione di un maggiore consumo degli amminoacidi forniti nel mezzo di crescita (leucina e metionina), evento che risulta essere necessario per il mantenimento della velocità di crescita del mutante. Il mutante inoltre, accumula intermedi del ciclo degli acidi tricarbossilici (in particolare acido malico), permettendo di ipotizzare che l'attività mitocondriale sia importante per l'omeostasi energetica di cellule *snf1Δ*. Questa ipotesi è stata testata trattando le cellule con antimicina A, un inibitore del complesso III della catena di trasporto degli elettroni. Coerentemente a quanto ipotizzato, cellule *snf1Δ* in 2% glucosio, ma non in 5% glucosio, subiscono un effetto deleterio dall'aggiunta dell'inibitore, che influenza negativamente crescita e contenuto di ATP, e causa nel mutante aumento di NADH, dimostrandone la mancata riossidazione mitocondriale. Riassumendo, il mutante risulta

massimizzare l'utilizzo di amminoacidi come fonte di carbonio, attraverso la respirazione dei relativi prodotti di degradazione come substrati anaplerotici del ciclo degli acidi tricarbossilici, per bilanciare la "leva metabolica" rappresentata dalla deregolata sintesi di acidi grassi.

In conclusione, gli studi compiuti hanno permesso di esplorare il coinvolgimento di Snf1 in condizioni di sufficienza nutrizionale nella regolazione di importanti processi cellulari, approfondendone l'importanza nella transizione G_1/S del ciclo cellulare, determinandone il *crosstalk* con il *pathway* della PKA e ricostruendo i riarrangiamenti subiti dal *network* metabolico in assenza della chinasi.

Abstract

Abstract

Snf1 is a serine/threonine kinase required by the yeast *S. cerevisiae* to grow in nutrient-limited conditions and to utilize carbon sources alternative to glucose, as sucrose and ethanol (Hedbacker and Carlson, 2008). Following nutrient deprivation Snf1 is activated and translocated to the nucleus where it can phosphorylate and inactivate the transcriptional repressor Mig1, causing the expression of over 400 genes (Young et al., 2003).

In our laboratory we previously demonstrated that lack of Snf1 causes an impairment of the G_1/S transition of the cell cycle and a defect in the expression of the *CLB5* gene, coding for the major S-phase cyclin, even in condition of glucose sufficiency (2% glucose) (Pessina et al., 2010). It was moreover demonstrated that in a *snf1Δ* strain there is a global defect in the expression of G_1 genes and of the binding to the promoters of these genes of the SBF and MBF transcription factors and of RNA polymerase II. To demonstrate the necessity of the catalytic activity of Snf1 for a proper G_1/S transition was utilized a Snf1-I132G strain, whose catalytic activity of the kinase can be inhibited by the specific inhibitor 2NM-PP1. The impairment of the G_1/S transition and of the transcription of G_1 genes in this strain in the presence of the inhibitor was demonstrated performing α-factor release and elutriation experiments (Busnelli et al., 2013).

Studying the involvement of Snf1 in the regulation of other signaling pathways we identified, through CoIP/MS experiments, the interaction

between Snf1 and adenylate cyclase (Cyr1), the enzyme responsible for the synthesis of cyclic AMP (cAMP), activator of PKA. Furthermore, we demonstrated that in a Snf1-G53R strain, in which the kinase is constitutively activated, the expression of PKA-dependent genes is deregulated. We therefore hypothesized the existence of a new crosstalk mechanism between the Snf1 and PKA pathways, regarding which only the PKA regulation on the Snf1 pathway and the synergistic action of both kinases on common targets were known (Barrett et al., 2012; Cherry et al., 1989; Görner et al., 1998, 2002; Hedbacker et al., 2004; Ratnakumar et al., 2009; De Wever et al., 2005). The sequence of Cyr1 presents five possible phosphorylation consensus sites, of which two are phosphoserines *in vivo*. The RAS Associating Domain of Cyr1, containing 2 putative Snf1 phosphorylation sites, was purified in *E. coli* and its *in vitro* phosphorylation by Snf1 was demonstrated. Moreover, in a Snf1-G53R strain we found a reduction of 50% of intracellular cAMP, compared to the wt. We therefore hypothesized that this reduction could be responsible for a reduced activation of PKA and, as a consequence, for the deregulation of the expression of PKA-dependent genes. Moreover, we performed *in vivo* mapping of phosphorylated sites of adenylate cyclase by mass spectrometry, identifying several sites, mainly in the regulatory domain of the protein, for which the phosphorylation is Snf1-dependent.

Abstract

To investigate the global role of Snf1 in conditions of nutritional sufficiency we performed a transcriptomic analysis (gene chip) of wt and *snf1Δ* cells grown in 2% and 5% glucose, evidencing that Snf1 in 2%, but not in 5% glucose, is important to guarantee the expression of genes involved in mitochondrial translation, DNA replication, cell wall biosynthesis and iron homeostasis. On the other hand, lack of Snf1 causes the derepression of genes involved in amino acid transport and biosynthesis and in glycolysis. Therefore, possible metabolic deregulations in the absence of Snf1 were investigated. *snf1Δ* cells grown in 2% glucose secrete more ethanol and acetate, in proportion to their growth rate, compared to the wt. This enhanced glycolytic activity is abolished, as observed for transcripts, in 5% glucose. We further demonstrated that in our growth condition (2% and 5% glucose) *snf1Δ* cells accumulate fatty acids, as previously observed in low glucose, due to the lack of Snf1-dependent phosphorylation of acetyl-CoA carboxylase (Shirra et al., 2001). An extended metabolic analysis, both through mass spectrometry and NMR, revealed in detail the metabolic rewiring occurring in *snf1Δ* cells to guarantee the growth in spite of the enhanced anabolic processes. *snf1Δ* cells in 2% glucose accumulate glutamate, coming from the degradation of supplemented amino acids (leucine and methionine), in process essential to maintain the growth rate of the mutant. Moreover, the mutant accumulates TCA cycle intermediates

(notably malate), giving us the possibility to hypothesize that mitochondrial activity could be important for the energetic homeostasis of the mutant. This hypothesis was positively tested since in 2%, but not 5% glucose, *snf1Δ* cells are negatively affected by treatment with antimycin A, inhibitor of the complex III of the electron transport chain. The treatment impairs growth and ATP content and increases NADH in the mutant, demonstrating the necessity of its mitochondrial reoxidation. In sum, the mutant maximizes the utilization of amino acids as carbon source, through the respiration of their degradation products as anaplerotic substrates of the TCA cycle, to balance the "metabolic pull" represented by the deregulated synthesis of fatty acids.

In conclusion, the studies here reported were focused on the exploration of the involvement of Snf1, in condition of nutrient sufficiency, in the regulation of important cellular processes. The results deepened the knowledge of the role of the kinase in the G_1/S transition of the cell cycle, evidenced the crosstalk between the PKA and Snf1 pathways and permitted the dissection of the rearrangements occurring to the metabolic network following Snf1 loss.

Introduction

1. Glucose sensing and metabolism in yeast

Glucose, the most abundant monosaccharide in nature, in addition to being the preferred carbon source by microorganisms, among which the budding yeast *Saccharomyces cerevisiae*, acts also as a signal molecule (Santangelo, 2006). Yeasts possess various glucose sensing and transport mechanisms, and finely regulate the metabolism of the hexose to achieve proper growth. Hereafter, the basics of glucose sensing, transport and metabolism, as well as the main signaling pathway involved, are presented to describe the framework in which acts the protein kinase Snf1/AMPK, major subject of this thesis.

1.1. Glucose sensing and transport

In yeast, two main sensing mechanisms of extracellular glucose exist: the GPCR (G Protein-Coupled Receptor) system and the Snf3/Rgt2 pathway.

The GPCR is constituted by the G-protein Gpa2, associated to the receptor Gpr1, which structure presents seven transmembrane domains and senses both glucose and sucrose, while it does not detect fructose (Kraakman et al., 1999; Rolland et al., 2000). Gpa2 is the α subunit of a trimeric G-protein, whose GTPase activity is positively regulated by Asc1 and negatively regulated by Rgs2 (Versele et al., 1999; Zeller et al., 2007). The binding of glucose to the receptor Gpr1 triggers the activity of the G-protein which binds and activates adenylate cyclase (Cyr1) (Peeters et al.,

2006), which in turn synthesizes cyclic AMP (cAMP) that, as described below, activates protein kinase A (PKA). The GPCR system is important for the production of cAMP spikes, while the stimulation of the synthesis of cAMP basal levels is almost fully dependent from the RAS proteins (Kraakman et al., 1999). However, the overexpression of *GPA2* alleviates the growth defect of a *ras2Δ* strain and a *ras2Δgpa2Δ* presents an increased impairment of growth, indicating that these pathways act on parallel pathways sharing common downstream targets (Xue et al., 1998). Snf3 and Rgt2 are proteins with twelve transmembrane domain, evolutionary related to hexose transporters but unable to transport glucose (Neigeborn et al., 1986; Ozcan et al., 1996, 1998). They form a transmembrane receptor which acts as an extracellular glucose sensor (Bisson et al., 1993). Snf3 senses glucose at low concentrations while Rgt2 detects the sugar at high concentrations (Coons et al., 1997; Ozcan et al., 1996). Both sensors transudce the signal to casein kinase I (CK1), which phosphorylates, thus causing its ubiquitination and degradation, the corepressors Std1 and Mth1 (Moriya and Johnston, 2004). The corepressors stabilize the binding of transcription factor Rgt1 to the promoters of a number of glucose-induced genes (Lakshmanan et al., 2003), among which those coding for glucose transporters (*HXT* genes) (Johnston and Kim, 2005).

The *HXT* genes code for a set of 18 hexose transporters (Hxt1-17 and Gal2), which allow the transport of glucose, fructose and mannose through passive transport or facilitated diffusion (Bisson et al., 1993). The transporters could be divided in high affinity/low capacity transporters (Hxt2,4,6,7), expressed when glucose is scarce, and low affinity/high capacity transporters (Hxt1,3), expressed when glucose concentration is high (Reifenberger et al., 1997). The remaining Hxt transporters perform less relevant functions (Ozcan and Johnston, 1999), while Gal2 transports with an equal efficiency both galactose and glucose (Nehlin et al., 1989).

1.2. The RAS/PKA pathway

The main signaling pathway activated by glucose in *Saccharomyces cerevisiae* is the PKA pathway, which shows extended involvement in growth, proliferation and metabolism (Broach, 2012). The second messenger responsible for the activation of PKA, as mentioned above, is cAMP, which is synthesized by adenylate cyclase (Cyr1) upon stimulus from Gpa2, already discussed, and the RAS proteins (Figure 1) (Santangelo, 2006; Thevelein and de Winde, 1999).

Ras1,2 are small monomeric G-proteins with redundant functions, anchored to the inner layer of the plasma membrane (Dong et al., 2003). The Guanine nucleotide Exchange Factor (GEF) of RAS proteins, responsible for the exchange of GDP with GTP and thus for their

activation, is Cdc25 (Martegani et al., 1986). On the contrary the IRA proteins (Ira1,2) act as GTPase Activating Proteins (GAPs), favoring GTP hydrolysis by RAS (Thevelein and de Winde, 1999). It is not until now perfectly clear how glucose activates the RAS proteins, but mechanisms involving intracellular acidification and glucose-6-phosphate metabolism have been proposed (Colombo et al., 1998; Rolland et al., 2001).

Active RAS proteins stimulate cAMP synthesis by adenylate cyclase (Cyr1) interacting with its Ras Associating Domain (RAD) (Kataoka et al., 1985). *CYR1* is an essential gene, and its loss causes the arrest in the G_1 phase of the cell cycle (Van Dijck et al., 2000), nonetheless it is possible to rescue the lethality of a cyr1Δ mutant with the deletion of *PDE2*, which codes for the phosphodiesterase responsible for the degradation of basal cAMP, *YAK1*, which codes for a kinase with inverse transcriptional involvement as compared to PKA, or *MSN2,4*, which code for the main PKA-repressed transcription factors (Van de Velde and Thevelein, 2008).

cAMP binds to an heterodimer of PKA catalytic subunits (composed either by the partially redundant proteins Tpk1, Tpk2 and Tpk3), causing their detachment from the homodimer of regulatory subunits (two Bcy1 molecules) and thus relieving from their inhibitory function (Toda et al., 1987). The *tpk1Δtpk2Δtpk3Δ* mutant is lethal even in cells growing in glucose, demonstrating that PKA activity is required also at basal levels and not only to respond to glucose refeeding (Toda et al., 1987).

Notably, the PKA pathway is finely regulated by feedback mechanisms and several targets of feedback inhibition by PKA have been proposed: Cdc25 (Munder and Küntzel, 1989), RAS (Resnick and Racker, 1988), IRA (Tanaka et al., 1989) and Cyr1 (De Vendittis et al., 1986).

Active PKA has a broad influence on transcription. During exponential growth and in the absence of stress PKA phosphorylates the transcription factors Msn2,4, thus causing their confinement to the cytoplasm (Görner et al., 1998). Msn2,4 activate the transcription of genes which have promoters containing STRE sequences (Stress Response Element), comprising genes coding for chaperones, antioxidant proteins, proteases and in general for elements necessary to respond to various stress types (Gasch et al., 2000). Besides Msn2,4, PKA controls several other transcription factors, here not exhaustively described for conciseness, including Rim15 (Wei et al., 2008) and Rap1 (Klein and Struhl, 1994). Moreover, as will be discussed in following sections, PKA targets transcription factors which are also regulated by Snf1 and phosphorylates enzymes to directly tune metabolic pathways.

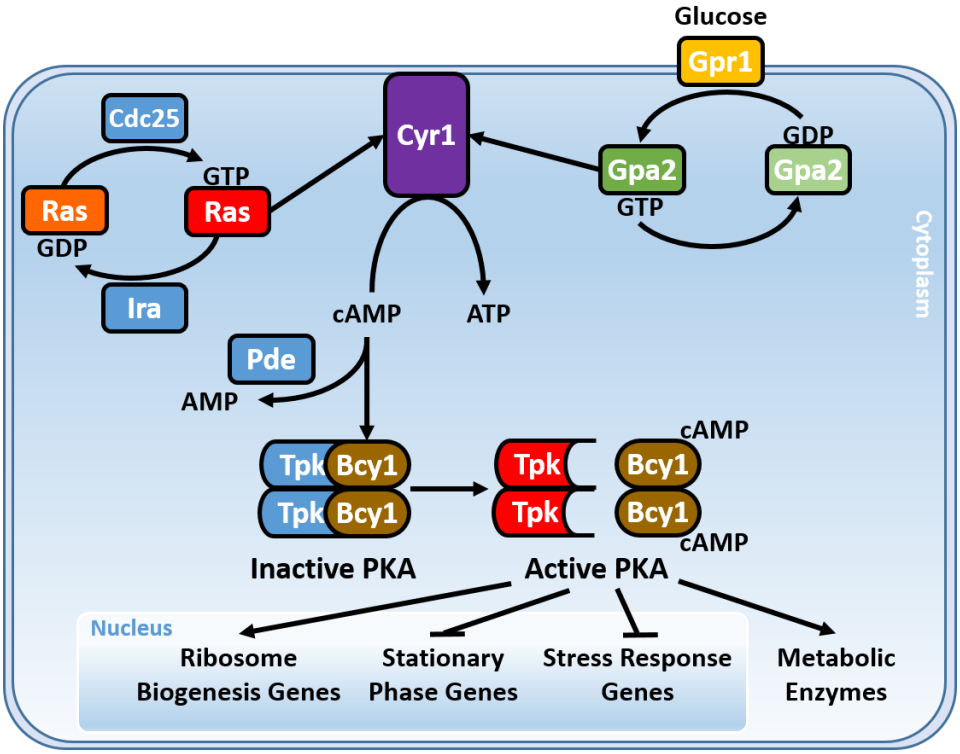


Figure 1. Protein Kinase A pathway

Schematization of the activation of PKA and of its main functions.

1.3. Glucose metabolism

The budding yeast *Saccharomyces cerevisiae* preferentially uses glucose and fructose over other carbon sources as they can directly enter the glycolytic pathway (Turcotte et al., 2010). An overview of the central carbon metabolism is provided in Figure 2. Glucose is converted to pyruvate through the glycolysis and then fermented to give ethanol even

in the presence of oxygen (Crabtree effect), leading to the production of ATP, metabolic intermediates and NADH to be used for other biosynthetic pathways. However, when glucose is unavailable, a wide variety of alternative and non-fermentable carbon sources (such as galactose, sucrose, maltose, ethanol, glycerol, acetate and others) can be used for the production of metabolic energy and cellular biomass. Indeed, yeast cells can rapidly switch between respiratory and fermentative metabolism in response to variations in the availability of oxygen and fermentable sugars. This response is achieved by changes in the pattern of gene expression and protein regulation, allowing optimal adaptation to the most convenient substrate available in a certain situation and ensuring that enzymes needed for a specific pathway are produced only when required (Schüller, 2003; Turcotte et al., 2010). Yet, although glycolysis and gluconeogenesis are two opposite pathways for glucose metabolism, a number of enzymes are common to both pathways while only a few enzymes are specific for gluconeogenesis.

The tricarboxylic acid (TCA) cycle occurs in the mitochondrial matrix and plays a pivotal role in utilizing non-fermentable carbon sources via generation of NADH, driving aerobic respiration to yield ATP. However, the TCA cycle is important also under fermentative conditions, since it is a source of biosynthetic building blocks, such as α-ketoglutarate, succinyl-CoA and oxaloacetate required for the synthesis of amino acids, glucose

and of the prosthetic heme group. When cells are grown on two-carbon compounds, such as acetate, the TCA cycle by itself cannot supply adequate amounts of biosynthetic precursors unless alternative reactions are available. In this case, yeast cells use the glyoxylate cycle, which converts two-carbon units into four-carbon dicarboxylic acids bypassing oxidative decarboxylation. The glyoxylate cycle shares three of the five reactions with the TCA cycle, which are catalyzed by malate dehydrogenase, aconitase and citrate synthase. Instead, the first two enzymes, isocitrate lyase and malate synthase, are unique to the glyoxylate cycle and are encoded by *ICL1* (Fernández et al., 1992; Taylor et al., 1996) and *MLS1* (Kunau and Hartig, 1992; Kunze et al., 2002), respectively.

There are other biosynthetic pathways that branch from glycolysis. The pentose phosphate pathway starts from glucose-6-phosphate through the cytoplasmic glucose-6-phosphate dehydrogenase Zwf1, which catalyzes the first irreversible and rate-limiting step of this pathway. It is required for generating NADPH, which is a source of reducing energy and of sugar molecules that are needed for the biosynthesis of nucleic acids and amino acids. It is also important for protecting yeast cells from oxidative stress, since NADPH is an essential cofactor for glutathione- and thioredoxin-dependent enzymes, that defend cells against oxidative damage (Halliwell, 1994; Wells et al., 1993).

Glucose-6-phosphate is also the debranching point for the synthesis of the storage carbohydrate glycogen, a high molecular mass branched polysaccharide and of the stress protectant trehalose, a non-reducing disaccharide. Their concentration is high during nutrient limitations and in resting cells. The large variations in the cell content of these two compounds in response to different environmental changes indicate that their metabolism is controlled by complex regulatory systems (François and Parrou, 2001). Moreover, a short branch of glycolysis consisting of NAD-dependent glycerol-3-phosphate dehydrogenase (Gpd1, Gpd2) and glycerol-1-phosphatase (Rhr2, Hor2), produces glycerol from dihydroxyacetone phosphate. Glycerol synthesis is not only required under osmotic stress, but plays an important role in lipid synthesis and it is necessary in anaerobiosis, since conversion of NADH excess to yield NAD^+ is essential for balancing the redox potential (Brisson et al., 2001; Hohmann et al., 2007).

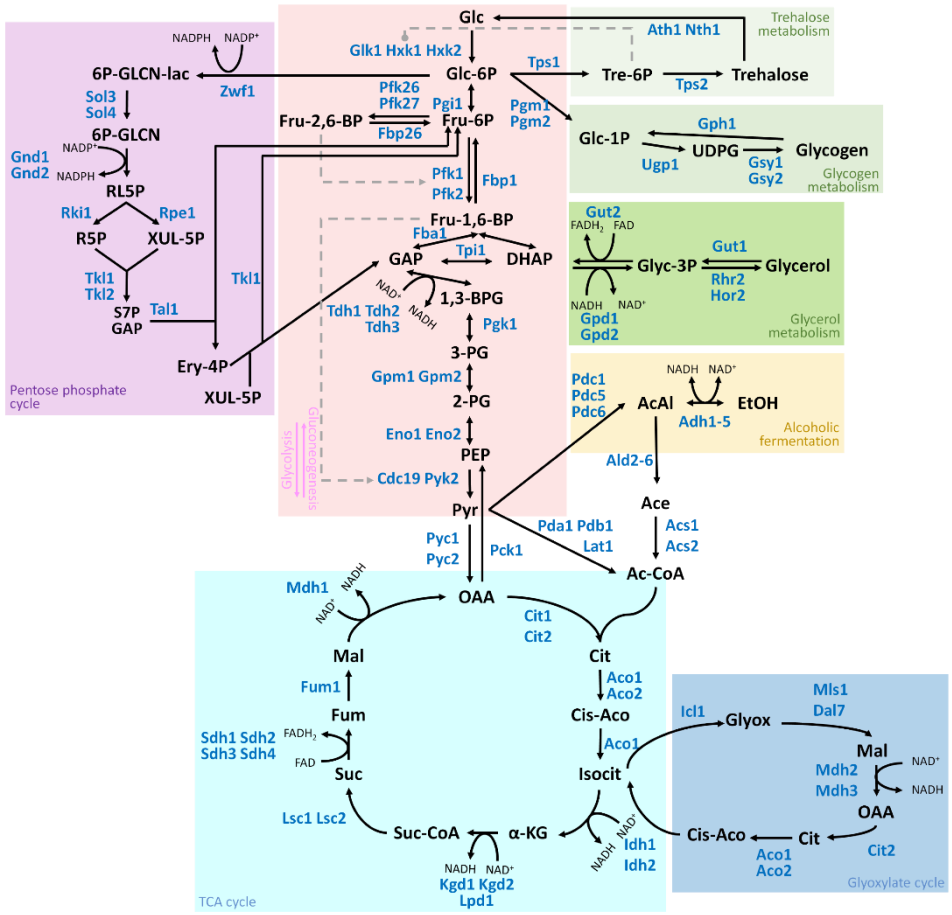


Figure 2. Metabolic pathways involved in central carbon metabolism

Metabolic pathways for carbons utilization are schematically shown as well as key enzymes (in blue) involved in these processes. Compartmentalization information was omitted for graphical reasons. Black arrows indicate enzymatic reactions while dashed grey arrows correspond to regulatory steps. Colored boxes group metabolic reactions and enzymes of the same pathway.

1.4. Regulation of metabolism by signaling pathways

Yeast cells have to adjust their metabolism in response to genetic and environmental perturbations and this can be achieved in many different ways. Widely studied mechanisms include transcriptional regulation of metabolic genes (Turcotte et al., 2010), control of mRNA stability (Lombardo et al., 1992), allosteric regulations (Jurica et al., 1998) and several post-translational modifications of metabolic enzymes (Tripodi et al., 2015). More interestingly, in the context of this thesis, is the regulation of metabolism by various signaling pathways. Regulations by the Snf1/AMPK pathway are discussed in a specific section below.

Most of the metabolic enzymes of the central carbon metabolism which are targeted by signaling pathways are subject to phosphorylation events (Figure 3). One of the more studied positive regulators of glycolysis is protein kinase A (PKA) (Busti et al., 2010; Thevelein et al., 2008; Zaman et al., 2008). It phosphorylates proteins catalyzing key enzymatic steps, such as both isoforms of pyruvate kinase, Cdc19 and Pyk2 (Cytryńska et al., 2001; Portela et al., 2002, 2006). PKA phosphorylation on Ser22 was shown to stimulate Cdc19 activity, although cells expressing Cdc19-S22A are still able to grow in glucose (Portela et al., 2006). PKA also phosphorylates the 6-phosphofructo-2-kinase Pfk26 on Ser644, increasing its enzymatic activity (Dihazi et al., 2003; François et al., 1988; Müller et

al., 1997), as well as on some other residues, whose physiological relevance have not been fully understood (Dihazi et al., 2003).

Pfk26 is phosphorylated by several other kinases. For instance, it was reported as a substrate of protein kinase C (Pkc1), a Ser/Thr protein kinase involved in the regulation of cell wall remodeling, through the activation of a mitogen-activated protein (MAP) kinase cascade (Levin, 2005). Under hypotonic stress conditions, Pkc1 is activated to respond to the environmental change (Davenport et al., 1995) and phosphorylates the 6-phosphofructo-2-kinase Pfk26 on Ser8 and Ser652, causing its inactivation (Dihazi et al., 2001). This inactivation could be part of the cellular response to the osmotic stress, since the reduction of the glycolysis could lead to an accumulation of glucose-6-phosphate for the synthesis of glucane (the main constituents of the yeast cell wall (Shahinian and Bussey, 2000)), as well as to a reduction of the synthesis of the strong osmolyte glycerol (Dihazi et al., 2001). Moreover, hypotonic stress stimulates Pfk26 acetylation on Lys3, which affects its N-terminal phosphorylation under hypo-osmotic stress and slightly inhibits its activity (Dihazi et al., 2005). On the contrary, hyper-osmotic stress stimulates the HOG-MAPK pathway (Saito and Posas, 2012) and leads to multiple phosphorylations on Pfk26 within the peptide 67–101, causing its activation and possibly rerouting glycolytic flux towards lower glycolysis (Dihazi et al., 2004; Petelenz-Kurdziel et al., 2013).

Remarkably, PKA also modulates gluconeogenesis. It phosphorylates the fructose-1,6-bisphosphatase Fbp1 on Ser11 in a glucose-induced manner, leading to its inactivation through a proteolytic mechanism (Gancedo et al., 1983; Hung et al., 2004; Jiang et al., 1998; Mazón et al., 1982; Rittenhouse et al., 1987; Schork et al., 1994). PKA is also involved in glucose storage mobilization and inhibition of their synthesis. In fact, PKA inhibits glycogen synthase activity through phosphorylation of the C-terminal tail of Gsy2 (Hardy and Roach, 1993), the most important glycogen synthase involved in the accumulation of glycogen upon entry into stationary phase (Farkas et al., 1991). Gsy2 is also inhibited by two other protein kinases: PAS kinases, which phosphorylate Gsy2 on Ser654, (Rutter et al., 2002) and Pho85/Pcl8,10 complexes, which phosphorylate Gsy2 on Ser654 and Thr667, preventing hyper-accumulation of glycogen during exponential phase, when its function is not required (Huang et al., 1996, 1998; Wilson et al., 1999). In addition, PKA stimulates glucose storage mobilization through phosphorylation of the glycogen phosphorylase Gph1, on Thr31 and Ser333 (Albuquerque et al., 2008; Holt et al., 2009), as well as through phosphorylation of several residues of the *NTH1*-encoded neutral trehalase, which is responsible for trehalose breakdown (Wera et al., 1999).

Finally, the metabolism of storage carbohydrates is regulated by the nutrient sensing PAS kinases (Cardon and Rutter, 2012; DeMille and

Grose, 2013). They phosphorylate Ugp1, the UDP-glucose pyrophosphorylase, which produces UDP-glucose, a substrate for both glucan and glycogen synthesis (Daran et al., 1995).

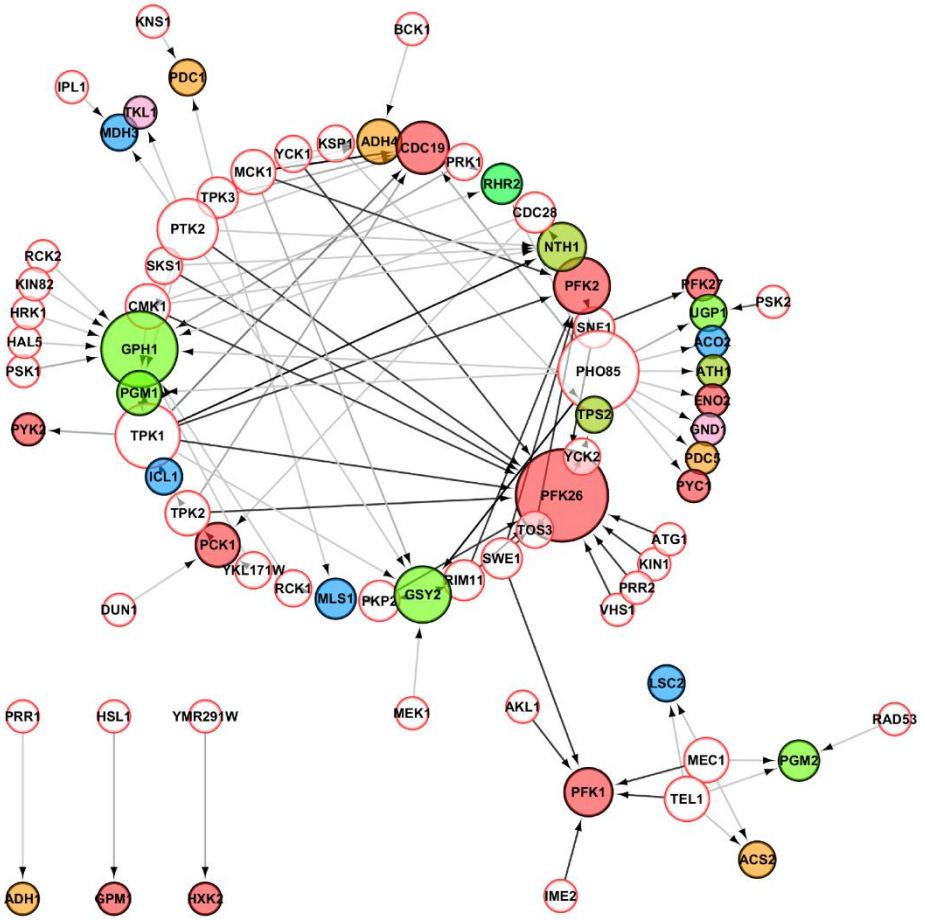


Figure 3. Phosphorylation network of the central carbon metabolism enzymes

The network reports the known target-kinase associations. Red bordered nodes indicate a kinase, filled nodes indicate a substrate. Red nodes indicate glycolytic enzymes, different shades of green indicate enzymes of glycolysis-branching pathways (trehalose, glycogen and glycerol biosynthesis), pink nodes indicate pentose phosphate enzymes, orange nodes indicate fermentation enzymes and blue nodes indicate TCA/glyoxylate cycle enzymes. Node size represents the degree of connection. The network was generated querying the Yeast Kinase Interaction Database (KID; (Sharifpoor et al., 2011); updated 2014-01-10).

2. Protein kinase Snf1/AMPK

All proliferating cells need to match metabolism, growth and cell cycle progression with nutrient availability to guarantee cell viability in spite of a changing environment. In yeast, a signaling pathway centered on effector kinase Snf1 is required to adapt to nutrient limitation and to utilize alternative carbon sources, such as sucrose and ethanol (Hedbacker and Carlson, 2008). Snf1 shares evolutionary conserved functions with the AMP-activated Kinase (AMPK) in higher eukaryotes, constituting what has been defined as a family of regulators of cellular energy homeostasis in eukaryotes (Hardie, 2007).

2.1. Snf1 structure

Protein kinase Snf1 in yeast is as a heterotrimeric complex made by the catalytic α subunit Snf1, a regulatory β subunit (alternatively Gal83, Sip1 and Sip2) and the γ subunit Snf4 (Hedbacker and Carlson, 2008)

The catalytic α subunit (encoded by the *SNF1*, Sucrose Not Fermenting gene) was identified in a screening of mutants unable to grow in presence of sucrose (Carlson et al., 1981). The Snf1 subunit is constitutively expressed and constituted by a catalytic N-terminal domain and a C-terminal regulatory region. The regulatory region presents a short autoinhibitory sequence (AIS) (380-415 aa) and the domain which mediates the interactions with the β subunits of the complex, such as Sip1,

Sip2 and Gal83. The autoinhibitory domain interacts with both the regulatory subunit Snf4 and the kinase domain of Snf1. The interaction with Snf4 relieves the inhibition of the AIS allowing the phosphorylation of Thr210 residue of Snf1 that determines its activation (Chen et al., 2009; Rudolph et al., 2005).

In S. cerevisiae, three β subunits (Gal83, Sip1 and Sip2) are present (Erickson and Johnston, 1993). The β subunits share partially redundant functions, since only the triple mutant *sip1Δsip2Δgal83Δ* strain shows growth defects growing with glycerol or ethanol as carbon sources and is unable to present phosphorylation of Snf1 targets such as Mig1 (Schmidt and McCartney, 2000). The β subunits contain a conserved C-terminal sequence in which two domains are present: the KIS domain (Kinase Interacting Sequence) that mediates the interaction with the α-subunit Snf1 (Yang et al., 1994) and the ASC domain (Association with SNF1 kinase complex) that allows the interaction with Snf4 (Jiang and Carlson, 1997). Differently, the N-terminal sequence is specific for each β subunit and confers a different subcellular localization patterns to each protein. All three proteins are cytoplasmic in presence of high glucose concentrations. Upon glucose depletion, Sip1 relocalizes to the vacuolar membrane, Gal83 relocalizes to the nucleus, and Sip2 remains cytoplasmic (Vincent et al., 2001). Thus, the role of the β subunits is to interact with Snf1 and to modulate its subcellular localization (Hedbacker et al., 2004; Vincent et

al., 2001). The particular localization of the kinase complexes including a different β subunit confers specialized functions. For example, Sip1 alone is not able to sustain growth on ethanol or glycerol and determines a very low kinase activity of the complex (Nath et al., 2002), Sip2 function seems to be involved in the mechanism of cellular aging (Ashrafi et al., 2000) while Gal83 seems to play the main role in the Snf1 dependent transcriptional regulation, since in low glucose Gal83 determines the nuclear localization of the Snf1 complex thank to its NLS (Nuclear Localization Sequences). On the contrary, NES (Nuclear Export Signals) present in the sequence of Gal83 allows the exit from the nucleus of the complex when high glucose concentrations are present (Hedbacker and Carlson, 2006). In addition, Gal83 mediates the interaction of Snf1 with some substrates, such as the transcription activator Sip4 (Vincent and Carlson, 1999) and the transcriptional apparatus (Kuchin et al., 2000). More recently, it has also been shown that deletion of the glycogen binding domain (GBD) of Gal83 leads to a constitutive activation of Snf1 which results able to modulate the expression of some Snf1-regulated genes also in high glucose concentrations. In addition, the GBD domain of Gal83 interacts with the Reg1/Glc7 phosphatase complex (Momcilovic et al., 2008). Taken together those data suggest that Gal83 plays a dual role regulating nuclear localization of Snf1 in low glucose and guaranteeing the inactivation of Snf1 in high glucose.

Similarly to *SNF1*, the gene encoding the γ subunit, *SNF4*, was identified by isolation of a sucrose non fermenting mutant (Carlson et al., 1981). Snf4 is a constitutively expressed protein that binds both the α and β subunits of the Snf1 complex (Celenza and Carlson, 1989; Jiang and Carlson, 1997). The role of Snf4 is to relieve the inhibition of Snf1 interacting with its AIS domain, stabilizing the Snf1 complex in the active conformation (Leech et al., 2003). In fact, *SNF4* deletion causes a decreased kinase activity of Snf1, whereas deletion of the AIS domain of Snf1 fully complement the phenotype of a *snf4Δ* strain (Celenza and Carlson, 1989; Leech et al., 2003). Remarkably, the activating phosphorylation of Thr210 residue of Snf1 is still detectable in a *snf4Δ* strain (McCartney and Schmidt, 2001) and in high glucose Snf4 seems to be required for the proper inactivation of Snf1 mediated by the phosphatase complex Reg1/Glc7 (Momcilovic et al., 2008). Thus, these findings indicate that Snf4 plays a complex role in the regulation of Snf1.

2.2. Snf1 activity regulation

Snf1 complex is activated, as schematized in Figure 4, through the phosphorylation of the Thr210 of the α subunit by one of the three constitutively active upstream kinases Sak1, Tos3 and Elm1 (Hong et al., 2003; Sutherland et al., 2003). This phosphorylation is essential for Snf1 activity, since the *sak1Δtos3Δelm1Δ* strain shows the same phenotype of

a *snf1Δ* strain, such as growth defects in presence of limiting glucose or carbon sources like glycerol or ethanol (Hong et al., 2003).

Although Snf1 phosphorylation is a key step of its activation, a non-phosphorylatable Snf1 mutant (Snf1-T210A) retains a low catalytic activity, originating intermediate phenotypes (Pessina et al., 2010). Also the mutation of the lysine which constitutes the ATP binding site in the kinase domain (Snf1-K84R), which for many aspects mimics the loss of Snf1 protein (Portillo et al., 2005; Shinoda and Kikuchi, 2007; Wade et al., 2009), still confers a slight catalytic activity *in vitro* (our not shown data).

On the other side, in response to high glucose concentrations Snf1 is inactivated through dephosphorylation of Thr210 by the Glc7 protein phosphatase (also known as PP1), which is targeted to Snf1 by the adaptor subunit Reg1 (Ludin et al., 1998; Sanz et al., 2000). Protein Reg1 interacts both with Glc7 and Snf1 when glucose is largely available in the culture medium and loss of Reg1 leads to the constitutive activation of Snf1 (Frederick and Tatchell, 1996; Huang et al., 1996; Rubenstein et al., 2008). It has been reported that in high glucose concentration, Hxk2 (Hexokinase 2) regulates the activity of PP1 and thus indirectly the activation of Snf1 kinase (Sanz et al., 2000).

Active Snf1 phosphorylates serines and threonines contained in the consensus pattern Φ-x-R-x-x-S/T-x-x-x-Φ, where Φ is a hydrophobic residue (Dale et al., 1995).

Differently from its mammalian homolog AMPK, protein kinase Snf1 is not allosterically activated by AMP (Wilson et al., 1996). However, recently it has been demonstrated that ADP molecules are able to bind the γ subunit Snf4 preventing Snf1 dephosphorylation mediated by Glc7 (Chandrashekarappa et al., 2011; Mayer et al., 2011).

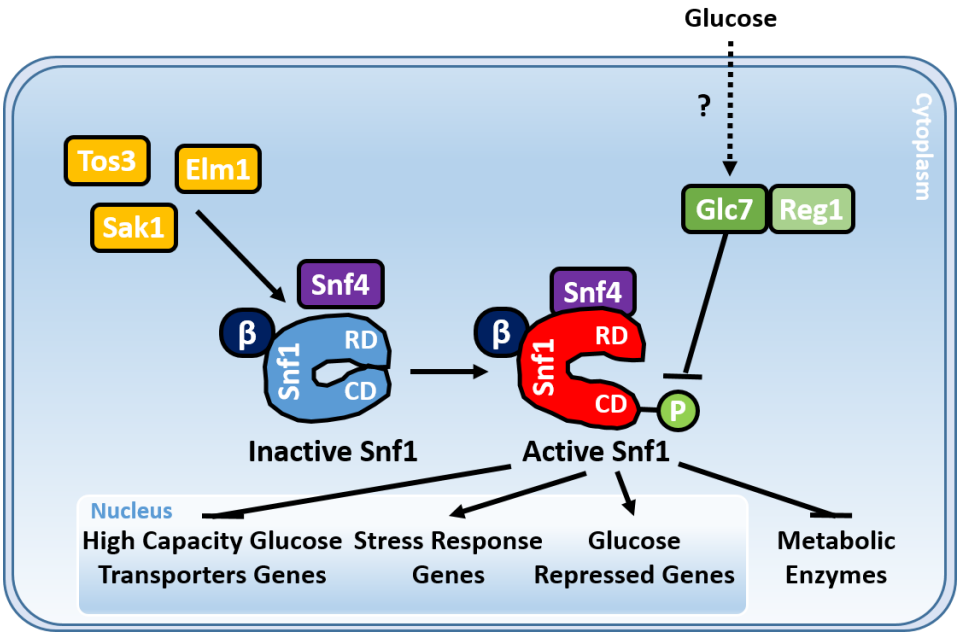


Figure 4. Snf1 pathway

Schematization of the activation of Snf1 and of its main functions.

2.3. Snf1-dependent transcriptional regulation

The most studied function of Snf1 is the regulation of transcription, involving more than 400 genes (Young et al., 2012). Snf1 acts both on

transcription factors and directly on the chromatin (Hedges et al., 1995; Lesage et al., 1996; Lo et al., 2001; Treitel et al., 1998).

Mig1 is the most important glucose-regulated transcriptional repressor (Nehlin and Ronne, 1990). Mig1 is phosphorylated by Snf1 on four sites when glucose is scarce, causing the activation of a NES sequence that causes the translocation of Mig1 from the nucleus to the cytoplasm through the exportin Msn5 (DeVit and Johnston, 1999; Papamichos-Chronakis et al., 2004; Treitel et al., 1998). Important in the regulation of Mig1 is the glycolytic enzyme Hxk2, which interacts with the transcriptional repressor directly in the nucleus to avoid its phosphorylation by Snf1, thus providing a link between glucose metabolism and transcription of glucose-repressed genes (Ahuatzi et al., 2007). Mig1 represses about 90 genes, notably including those coding for enzymes required for the metabolism of sucrose (*SUC2*), maltose (the *MAL* regulon) and galactose (*GAL4*) (Ostergaard et al., 2001). Furthermore, Mig1 controls the expression of high-affinity glucose transporters, required when glucose is scarce (*HXT2*, *HXT4*) (van Oevelen et al., 2006), represses TPS1, essential for the metabolism of trehalose (Hohmann et al., 1994) and genes coding for enzymes of the TCA cycle (Jin et al., 2007).

Besides Mig1, Snf1 regulates the activity of various transcription factors. Cat8 and Sip4, which bind Carbon Source Response Elements (CSRE),

mostly regulate the expression of gluconeogenic genes (Vincent and Carlson, 1998) and are targeted by activatory phosphorylations by Snf1 (Lesage et al., 1996; Randez-Gil et al., 1997). Cat8 activates the expression of glucose-repressed genes alongside transcription factor Adr1, which is itself a target of Snf1 (Kacherovsky et al., 2008; Young et al., 2002). Moreover, in a fine mechanism of positive feedback, the *CAT8* gene is activated by Snf1 through inhibition of Mig1 (Hedges et al., 1995). Gcn4, transcription factor responsible for the expression of amino acid biosynthesis, is regulated in a peculiar way by Snf1, which acts through a uORF upstream of *GCN4* to control its expression at a post-transcriptional level (Shirra et al., 2008).

As for the regulation of chromatin structure, it has been shown that protein kinase Snf1 phosphorylates Ser10 residue of histone H3, promoting its acetylation on Lys14 residue by Gcn5, a component of SAGA complex (Lo et al., 2001). Recently, it has been demonstrated that the Snf1-mediated regulation of histone H3 is involved in the expression of *ADY2* gene. In fact, Snf1 stimulates the binding of Gcn5 and the acetylation of histone H3 at *ADY2* promoter, promoting the transcription of this gene (Abate et al., 2012).

In our laboratory, we recently highlighted the role of Snf1 in the regulation of transcription of cell cycle genes of the G_1 phase. The G_1 regulon is controlled by transcription factors SBF and MBF (constituted by the Swi4-

Swi6 and Mbp1-Swi6 heterodimers, respectively), with SBF controlling mostly genes involved in the budding process and MBF controlling the expression of genes involved in DNA replication (Koch et al., 1993). In particular we demonstrated that a delay in the G_1 to S transition in *snf1Δ* cells is accompanied by impaired expression of *CLB5*, MBF-regulated gene coding for the main S-phase cyclin (Pessina et al., 2010). More recently, the defect was shown to be extended to a wide set of both SBF and MBF-regulated genes, together with an impaired recruitment to the promoters of the co-activator Swi6 and RNA polymerase II (Busnelli, 2013).

2.4. Snf1 and the regulation of metabolism

Snf1, besides its role in regulating the transcription of several genes involved in metabolism, directly regulates through phosphorylation important metabolic enzymes.

Pfk27, the second isoform of 6-phosphofructo-2-kinase, was shown to be phosphorylated by protein kinase Snf1 (Benanti et al., 2007). Upon glucose removal, Snf1 phosphorylates Pfk27 in its N-terminal domain, leading to the SCF^{Grr1}-dependent degradation of Pfk27 (Benanti et al., 2007). In particular, Snf1-dependent phosphorylation is required to promote Pfk27 association with the F-box protein Grr1 (Benanti et al., 2007), thus leading to Pfk27 turnover and consequently to a reduction of fructose-2,6-bisphosphate. The importance of Pfk27 turnover is

highlighted by the fact that expression of a non-phosphorylatable and non-degradable Pfk27 protein inhibits growth on glycerol (Benanti et al., 2007).

Glycerol synthesis is inhibited during the diauxic shift by Snf1 phosphorylation on Gpd2, the glycerol-3-phosphate dehydrogenase required for anaerobic growth. In fact, it was recently reported that Snf1 phosphorylates Gpd2 on Ser72 priming Gpd2 for subsequent phosphorylation on Ser75, probably by Yck1 (Lee et al., 2012).

Probably the most impactful function exerted by Snf1 as a direct regulator of metabolism is the regulation of the acetyl-CoA carboxylase Acc1 (Shirra et al., 2001). In yeast, loss of Snf1 causes a dramatic accumulation of fatty acids, and the carbon overflow into the fatty acid biosynthetic pathway has been shown to cause inositol auxotrophy mediated by the impairment of *INO1* expression (Hofbauer et al., 2014; Shirra et al., 2001). Moreover, the excessive allocation of carbon into fatty acids causes a depletion of the intracellular acetyl-CoA pool, and thus a global reduction of histone acetylation (Zhang et al., 2013).

2.5. Crosstalk of Snf1/AMPK and PKA

In *Saccharomyces cerevisiae*, several crosstalks between the Snf1 and PKA pathways have been reported. PKA phophorylates and inactivates the Snf1-activating kinase Sak1 (Barrett et al., 2012) and the γ subunit Sip1,

preventing the localization of the Snf1 complex to the vacuole (Hedbacker et al., 2004). Remarkably, a direct regulation of the Snf1 orthologue AMPK by PKA has been demonstrated in human adipocytes, where PKA phosphorylates Ser173 of AMPK preventing the phosphorylation of the activatory residue Thr172 (Djouder et al., 2010). Both residues are conserved in yeast (activatory Thr210 and adjacent Ser211), thus a similar mechanism has been hypothesized (Barrett et al., 2012).

Snf1 and PKA share common downstream targets. PKA phosphorylates and deactivates Adr1, transcriptional activator of several glucose repressed genes, while Snf1 indirectly causes its dephosphorylation and activation (Cherry et al., 1989; Ratnakumar et al., 2009). Surprisingly, both PKA and Snf1 act as repressors of the transcription factor Msn2, but, while PKA is the main regulator of this factor, Snf1 targets it only to shut off transcription of the STRE regulon after prolonged stationary phase (Görner et al., 1998, 2002; De Wever et al., 2005).

Results

1. Snf1/AMPK catalytic activity promotes a proper G_1/S transition

1.1. Results

Deletion of *SNF1* causes a strong defect in the G_1/S transition in elutriated cells

We previously demonstrated the involvement of Snf1 in the cell cycle of cells growing in glucose non limiting condition, showing that *snf1Δ* cells have increased duplication time, decreased expression of the S-phase cyclin Clb5 and impaired G_1/S transition in α-factor block/release experiments (Busnelli, 2013; Pessina et al., 2010). However, *snf1Δ* cells are known to be less resistant to various types of treatments, and cell cycle arrest with α-factor could be itself a stress for some mutants (Ozeki-Miyawaki et al., 2005). We therefore wanted to exclude any artefact caused by α-factor treatment and chose to perform G_1 synchronization experiments through centrifugal elutriation.

After release in fresh pre-warmed medium, G_1 elutriated *snf1Δ* cells remained mostly unbudded until 240 min and presented a strongly impaired DNA replication, indicating that loss of Snf1 causes a severe delay in the beginning of a new cell cycle (Figure 5), and that this defect is not caused by a specific synchronization method.

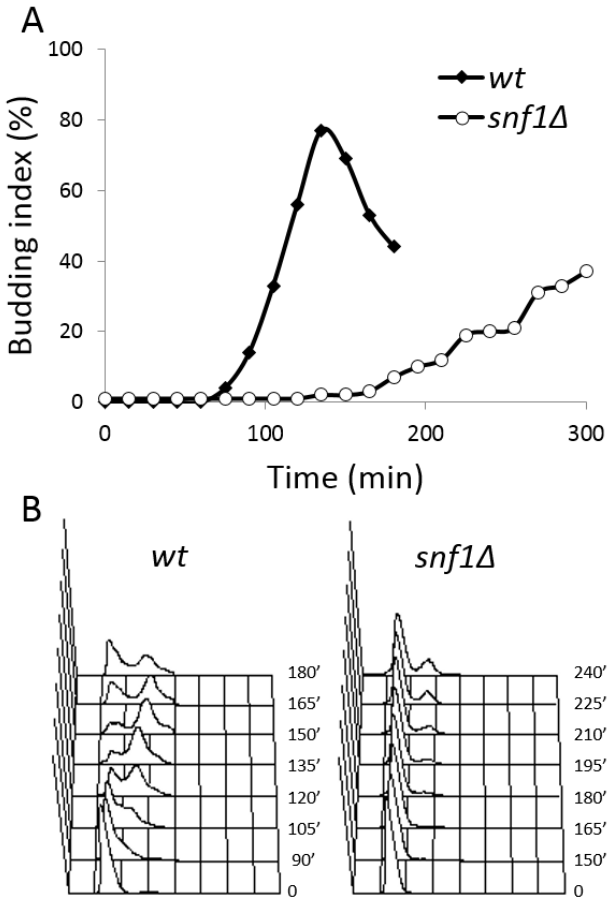


Figure 5. Snf1 is required for the G_1/S transition of elutriated cells

wt and *snf1Δ* cells were grown in synthetic medium containing 2% glucose until late exponential phase, elutriated to obtain cells in G_1 phase and then released in fresh pre-warmed medium. Samples were taken at the indicated time points to assay (A) budding index and (B) DNA content by FACS analysis.

Chemical inhibition of Snf1 catalytic activity causes a defect in the G_1/S transition and G_1 gene transcription

As shown, deletion of *SNF1* caused a severe defect in the G_1/S transition. To assess whether this effect was directly due to the lack of Snf1 catalytic activity we used a strain expressing an analog sensitive isoform of Snf1 (Snf1-I132G or Snf1^{as}), whose catalytic activity could be inhibited by treatment with the ATP analog 2NM-PP1 (Shirra et al., 2008). Treatment with 2NM-PP1 reduced the growth rate of exponentially growing Snf1^{as} cells, determining an increase of cells with 1C DNA content, already detectable 60 min after the treatment (Figure S1), rendering the Snf1^{as} strain very similar to a *snf1Δ* strain. To investigate this effect on a synchronous population, exponentially growing Snf1^{as} cells were synchronized with α-factor, after 1 h the culture was treated with 25 μM 2NM-PP1 for an additional hour and then released into fresh medium containing 25 μM 2NM-PP1. In the absence of the inhibitor, in accordance with the onset of bud emergence and DNA replication, the Snf1^{as} strain expressed G_1-specific genes as the wild type (Figure 6). Remarkably, the addition of 2NM-PP1 delayed S-phase entrance by affecting the expression G_1 phase genes (Figure 6). As expected, treatment with 2NM-PP1 on wt cells had no effect on budding index, DNA content and G_1 genes expression (Figure S2), confirming the specificity of 2NM-PP1.

As discussed above, deletion of *SNF1* affects G_1 elutriated cells. It has been reported that centrifugation could cause the activation of Snf1 (Wilson et al., 1996), thus causing a significantly different starting condition between wt and *snf1Δ* cells after elutriation. We therefore implemented the elutriation experiment with the use of the $Snf1^{as}$ strain. $Snf1^{as}$ cells were grown in synthetic medium with 2% glucose until late exponential phase and elutriated to obtain cells in G_1 phase in the absence of the inhibitor, to ensure that cells with an active Snf1 protein could respond to the g stress of centrifugation. Small unbudded cells were then re-inoculated in fresh prewarmed medium at time 0 with 0.1% DMSO (solvent control) or with 2NM-PP1 to inhibit Snf1 activity. Treatment with the inhibitor severely impaired budding, DNA replication, as well as the expression of *CLN2* and *RNR1* mRNA, with an increase of *CLN2* expression only 135 min after the release (Figure 7).

Taken together our results strongly support the notion that the catalytic activity of Snf1 is required to promote G_1-transcription.

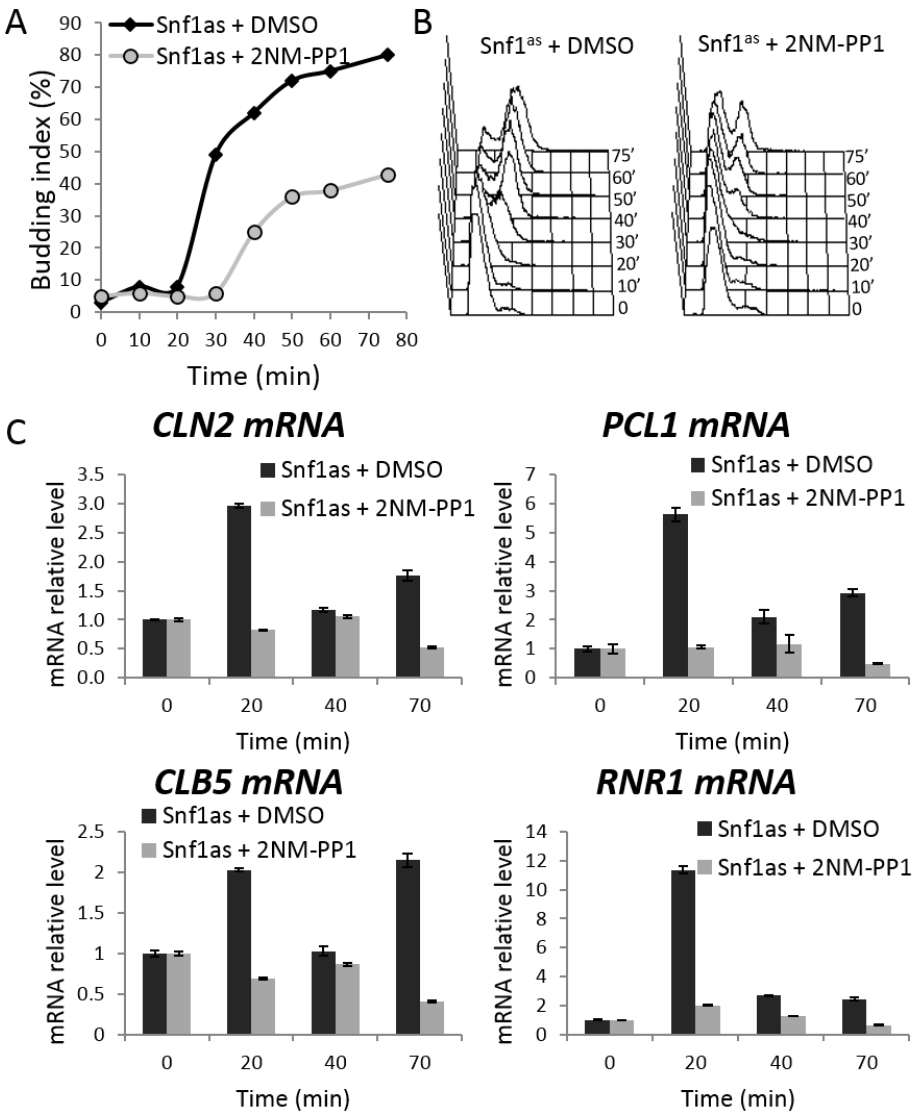

A
Snf1as + DMSO
Snf1as + 2NM-PP1
Budding index (%)
Time (min)
B
Snf1as + DMSO
Snf1as + 2NM-PP1
75′
60′
50′
40′
30′
20′
10′
0
C
CLN2 mRNA
PCL1 mRNA
CLB5 mRNA
RNR1 mRNA
mRNA relative level
Time (min)

Figure 6. Snf1 catalytic activity is required to promote G_1-gene transcription

Snf1^{as} strain was grown in synthetic medium containing 2% glucose until exponential phase and G_1-arrested by α-factor treatment. After 1 h, the culture was split in two: half of the culture was treated with 0.1% DMSO and the rest of the cells were treated with 25 µM 2NM-PP1 (final concentration of DMSO 0.1%) for an additional hour. Then the cultures were released in fresh medium containing 0.1% DMSO or 25 µM 2NM-PP1. Samples were taken at the indicated time points to assay (A) budding index, (B) DNA content by FACS analysis and (C) *CLN2*, *PCL1*, *CLB5* and *RNR1* mRNA level by quantitative relative Real-time PCR.

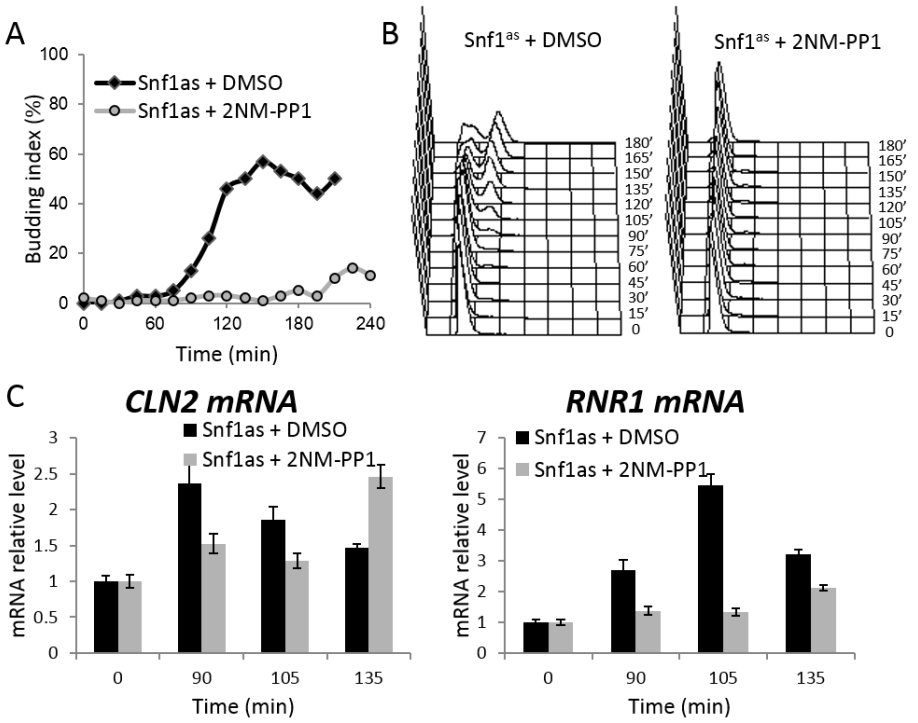


Figure 7. Snf1 is required for G_1-gene expression in elutriated cells

Snf1^{as} strain was grown in synthetic medium containing 2% glucose until late exponential phase, elutriated to obtain cells in G_1 phase that were then released in fresh pre-warmed medium containing 0.1% DMSO or 25 µM 2NM-PP1. Samples were taken at the indicated time points to assay (A) budding index, (B), DNA content by FACS analysis, and (C) *CLN2* and *RNR1* mRNA level by quantitative relative Real-time PCR.

1.2. Supplementary results

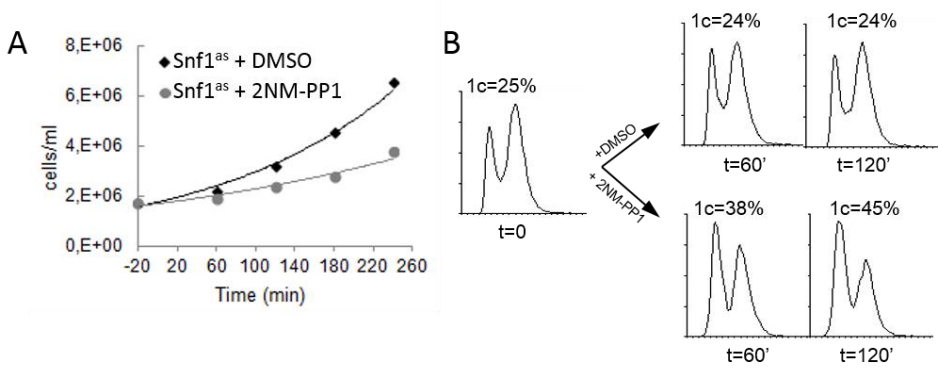


Figure S1. Inhibition of Snf1 catalytic activity affects cell growth

Snf1^{as} strain was grown in synthetic medium containing 2% glucose until early exponential phase. At time 0, the culture was split in two: half of the culture was treated with 0.1% DMSO and the rest of the cells were treated with 25 µM 2NM-PP1 (final concentration of DMSO 0.1%). Samples were taken at the indicated time points to assay (A) cell density, (B) DNA content by FACS analysis.

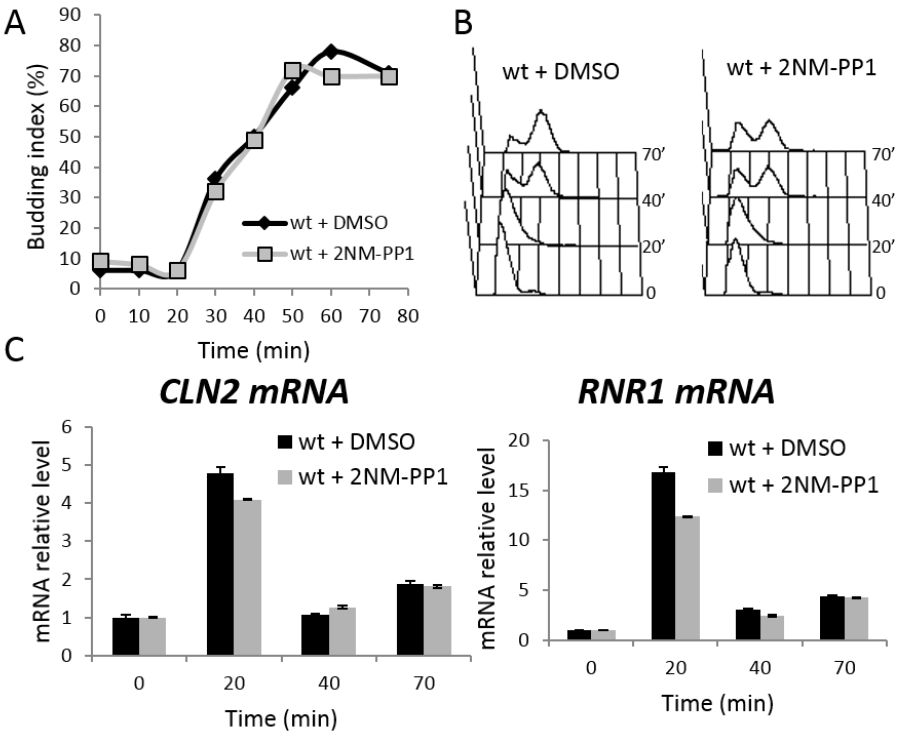


Figure S2. The inhibitor 2NM-PP1 has no effect on wild type cells

Cells expressing wild type *SNF1* were grown in synthetic medium containing 2% glucose until exponential phase and G_1-arrested by α-factor treatment. After 1 h, the culture was split in two: half of the culture was treated with 0.1% DMSO and the rest of the cells were treated with 25 µM 2NM-PP1 (final concentration of DMSO 0.1%) for an additional hour. Then the cultures were released in fresh medium containing 0.1% DMSO or 25 µM 2NM-PP1. Samples were taken at the indicated time points to assay (A) budding index, (B) DNA content by FACS analysis and (C) *CLN2* and *RNR1* mRNA level by quantitative relative Real-time PCR.

2. Snf1/AMPK physically and functionally interacts with the PKA pathway

2.1. Results

Snf1 interacts with adenylate cyclase

Snf1 is known to regulate glucose metabolism and to promote the utilization of alternative carbon sources mainly through the derepression of a large transcription regulon and the phosphorylation of metabolic enzymes (Hedbacker and Carlson, 2008). However, since the cellular metabolic behaviour could be the result of the action of different, and somehow opposite, signalling pathways, we wondered whether Snf1 could directly regulate such pathways. To detect possible new targets of Snf1, we choose to use a CoIP/MS approach, immunoprecipitating a tagged isoform of Snf1 and detecting co-immunoprecipitated proteins with mass spectrometry after resolution with SDS-PAGE. We performed this experiment with protein extracts of log phase cells growing in 2% glucose, since we already demonstrated that in this condition Snf1 is at least partially functional (Pessina et al., 2010).

Interestingly, we identified a total of 56 interacting proteins of which only 20 were already identified as Snf1 interactors (Table S1 and Figure S3), probably because in the studies in which these data were obtained the cells were grown in different conditions, thus originating a different

interaction pattern (Breitkreutz et al., 2010; Collins et al., 2007; Krogan et al., 2006). As a proof of the validity of our method in preserving existing interactions, we detected most of the components of the complex of Snf1 and of the Snf1 pathway (Table S1 and Figure S3).

Strikingly, one of the newly identified interactors was adenylate cyclase (Cyr1), the essential enzyme responsible for the synthesis of cAMP, activator of PKA (Table S1 and Figure S3). To validate the interaction, we performed co-immunoprecipitation experiments, clearly detecting myc-tagged Snf1 in Cyr1-TAP immunocomplexes (Figure 8A). The interaction, moreover, was not indirectly caused by Snf1 and Cyr1 being common substrates of the phosphatase complex Reg1-Glc7, since it was detectable also in extracts of *reg1Δ* cells (Figure 8B). Interestingly, the interaction was not dependent on the glucose concentration nor the carbon source (Figure S4), and was detectable in the presence of a catalytic-deficient isoform of Snf1, Snf1-K84R (Figure 8C). Furthermore, switching bait and prey proteins and detecting Cyr1-TAP in Snf1-HA immunocomplexes, we were able to demonstrate that the interaction is stronger in the presence of a Snf1-K84R isoform (Figure 8D). This finding was of particular interest, since it was previously shown that in glucose-repressed conditions the catalytic-deficient isoform of Snf1 interacts more strongly than the wt with Mig1, a well-known Snf1 substrate (Treitel et al., 1998).

Taken together, these data demonstrate the physical interaction of Snf1 with Cyr1 and support the possibility of adenylate cyclase being a candidate substrate of Snf1.

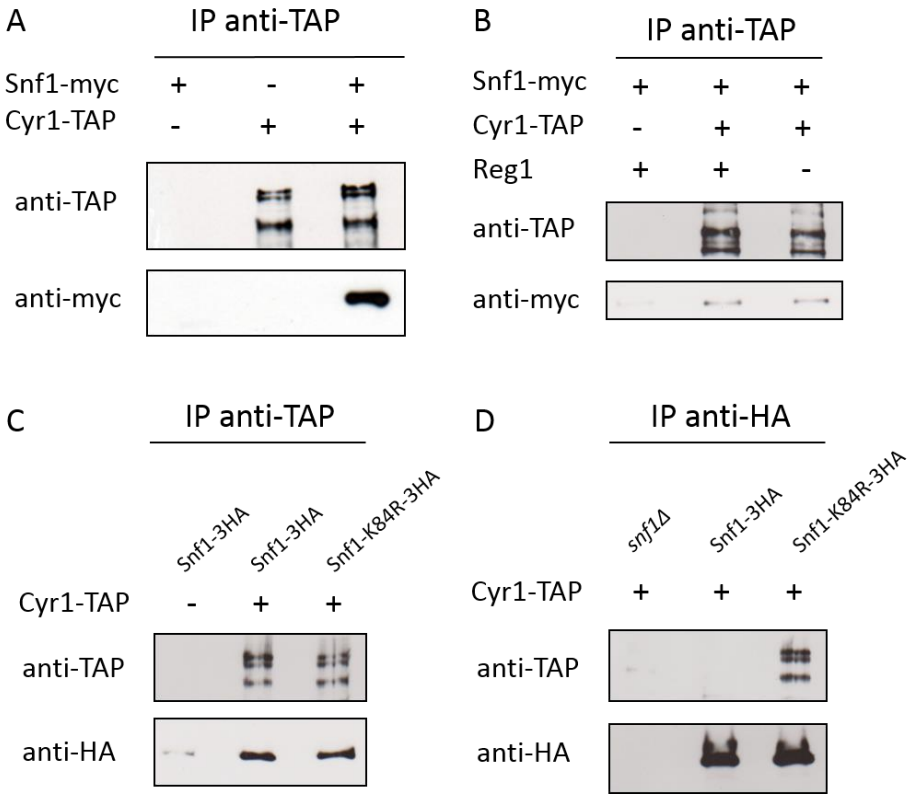


Figure 8. Snf1 interacts with adenylate cyclase

(A) Western blot showing the detection of Snf1-myc in Cyr1-TAP immunocomplexes. (B) Western blot showing the detection of Snf1-myc in Cyr1-

TAP immunocomplexes obtained from *reg1Δ* cells. (C) Western blot showing the detection of Snf1-HA and Snf1-K84R-HA in Cyr1-TAP immunocomplexes. (D) Western blot showing the detection of Cyr1-TAP in Snf1-HA and Snf1-K84R-HA immunocomplexes. Strains expressing untagged isoforms of either Snf1 or Cyr1 are shown as control.

Loss of Snf1 causes an alteration in the phosphorylation pattern of adenylate cyclase

Cyr1 is a phosphoprotein and several phosphorylated residues have been identified in proteomic studies (Gruhler et al., 2005; Holt et al., 2009; Li et al., 2007; Smolka et al., 2007). Moreover, the sequence of Cyr1 presents 5 phosphorylatable residues, 2 of which were identified as phosphosites *in vivo*, that are contained in perfect Snf1 phosphorylation consensus motifs (Figure S5). However, no specific function until now has been assigned to any phosphorylation site.

Since the physical interaction with Snf1 suggested Cyr1 as a possible target of the kinase, we aimed to verify whether the presence or the absence of Snf1 could influence the phosphorylation status of adenylate cyclase. We therefore immunoprecipitated TAP-tagged Cyr1 from wt and *snf1Δ* cell extracts and, after resolution on SDS-PAGE and silver staining, detected the phosphorylated residues by MS analysis (Figure 9). Remarkably, in a wt strain we identified 21 phosphosites, 7 of which were previously

reported, providing an extensive map of adenylate cyclase phosphorylation. Strikingly, a subset of these phosphosites, almost completely localized in the region flanking the RAS associating domain, resulted to be Snf1-dependent, not being detectable in the *snf1Δ* strain. Surprisingly, none of these sites is one of the 5 contained in Snf1 consensus sequences discussed above. Interestingly, we also identified 10 additional phosphosites which were exclusively present in a *snf1Δ* strain. However, we checked if the identified sites are contained in consensus sequences of a specific kinase, but failed to identify any obvious responsible of the phosphorylation events (data not shown).

These data provide a substantial advance in mapping possible regulatory post translational modifications of adenylate cyclase and demonstrate the necessity of Snf1 to preserve the proper phosphorylation pattern of Cyr1.

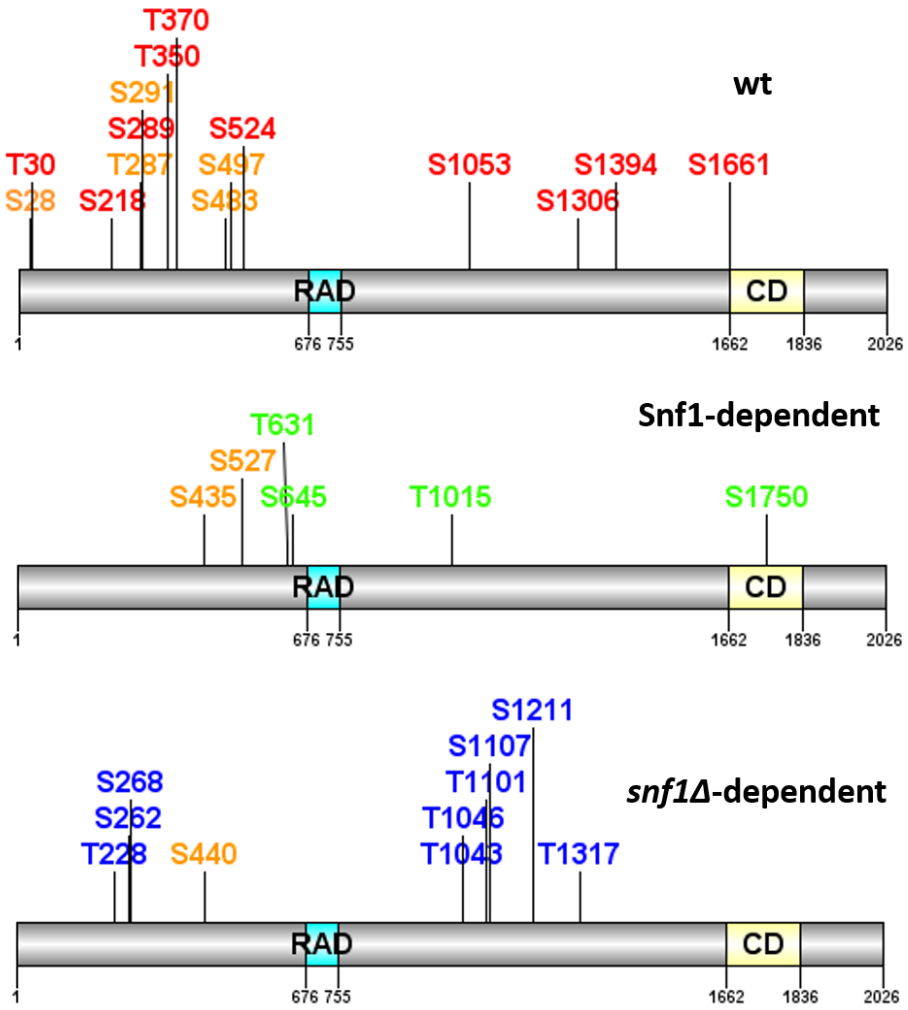


Figure 9. Schematization of the Cyr1 phosphorylated sites identified *in vivo*

Graphical schematization of the phosphorylated residues identified *in vivo* by MS analysis of immunoprecipitated adenylate cyclase. Residues are divided in the subclasses: wt, if present in a wt strain and not affected by *SNF1* deletion; Snf1-

dependent, if present in a wt strain and no longer retrievable in a *snf1Δ* mutant; *snf1Δ*-dependent, if not present in a wt strain and only retrievable in a *snf1Δ* mutant. Phosphosites already identified *in vivo*, as reported in PhosphoGRID, are highlighted in orange. RAD = RAS Associating Domain, CD = Catalytic Domain.

Snf1 phosphorylates *in vitro* the RAS associating domain of adenylate cyclase

Since we demonstrated that lack of Snf1 could influence the phosphorylation pattern of Cyr1 *in vivo*, we sought for direct kinase activity of Snf1 on adenylate kinase. We therefore purified from *E. coli* a His_6-tagged region of Cyr1 containing the whole RAS associating domain (RAD) and 5 out of 6 residues whose phosphorylation we have shown to be Snf1-dependent (Figure 9). We then performed an in vitro kinase assay with γ-^{32}P labelled ATP, using the purified protein as substrate and immunoprecipitated HA-tagged Snf1 as kinase (see Materials and methods for details). In the presence of the kinase, the His_6-Cyr1(335-1066) recombinant protein incorporates radiolabelled ATP, demonstrating that this domain of adenylate cyclase is a *bona fide* substrate of Snf1 *in vitro* (Figure 10).

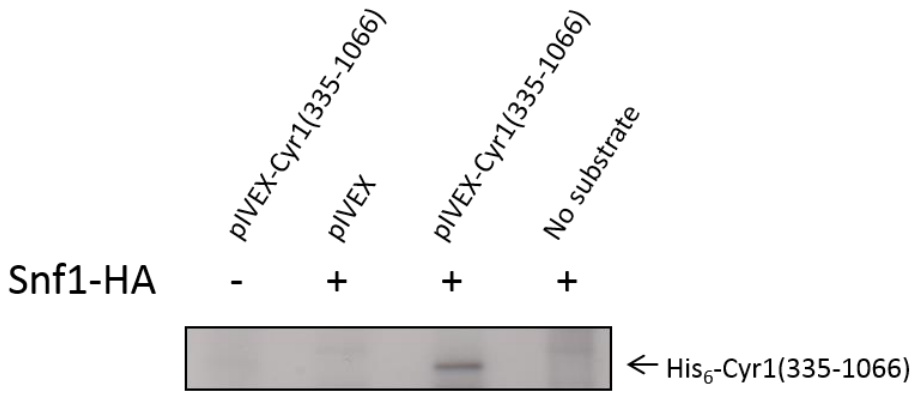


Figure 10. Snf1 phosphorylates *in vitro* the Cyr1 RAS associating domain

Autoradiography of the *in vitro* kinase assay with γ-^{32}P labelled ATP. The different lanes indicate the vector contained by host *E. coli* from which purification of the substrate protein was performed. A kinase reaction without substrate is shown as control.

Catalytic activation of Snf1 negatively regulates cAMP content

The function of adenylate kinase is to catalyze the synthesis of cAMP, the second messenger responsible for the activation of PKA, in response to stimuli from the RAS and GPCR systems (Santangelo, 2006). Having shown that lack of Snf1 influences the Cyr1 phosphorylation pattern and that Snf1 can phosphorylate Cyr1 in vitro, we wondered whether activation of Snf1 could influence the activity of adenylate cyclase. cAMP is rapidly synthesized at high levels after nutrient refeeding to starved cells, but is present at detectable basal levels also in log phase cells (Petkova et al.,

2010). Therefore we measured cAMP levels in log phase cells growing in 2% glucose, either lacking Snf1 or harboring wt Snf1 or a Snf1-G53R isoform, which is constitutively activated due to persistent phosphorylation of the activator Thr210 (Estruch et al., 1992).

Strikingly, while lack of Snf1 does not influence the cAMP content of cells growing in glucose repressed condition, the presence of the Snf1-G53R isoform nearly halves the cAMP levels, as compared to the wt (Figure 11).

As previously discussed, the sequence of Cyr1 presents 5 serine residues contained in motifs which perfectly match the Snf1 phosphorylation consensus sequence. Two of these residues, Ser554 and Ser736, are contained in the domain which we demonstrated to be phosphorylated *in vitro* by Snf1. We therefore performed site-specific mutagenesis of these residues to alanine to assess whether their phosphorylation could be relevant for the cell, in particular for the synthesis of cAMP. However, the strain transformed with the Cyr1-S554A,S736A isoform did not show altered cAMP levels (figure S6), compared to the wt, nor presented any phenotypic defect under standard growth conditions (data not shown).

These data demonstrate that the catalytic activity of Snf1 can reduce the intracellular content of cAMP in glucose repressed condition, but that the precise mechanism of regulation on adenylate cyclase is still to be elucidated.

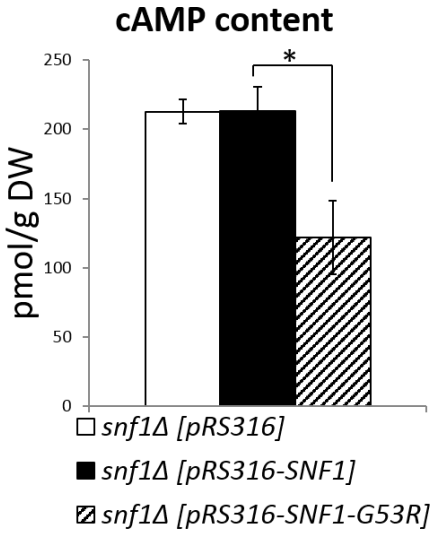


Figure 11. A constitutively activated isoform of Snf1 reduces intracellular cAMP levels

Histogram showing the intracellular cAMP content of the indicated strains. Data normalized on cellular dry weight. *p-value < 0.05

Catalytic activation of Snf1 negatively influences PKA-regulated transcriptional expression

PKA regulates several cellular processes including nutrient sensing, energy metabolism, cell cycle progression, thermotolerance, osmotic shock tolerance, sporulation, pseudohyphal growth, aging, and autophagy (Santangelo, 2006). Some of these processes, such as thermotolerance,

are usually used to indirectly measure the activity of the PKA pathway (Toda et al., 1987). However, Snf1 regulates these processes independently from PKA (Hong and Carlson, 2007), and the use of these methods to measure the influence of Snf1 catalytic activity on the PKA pathway was therefore rejected.

Therefore, we chose to indirectly measure the activity of the PKA pathway through the effect on the transcription of genes which are regulated by transcription factors that are PKA targets. Some transcription factors are shared as downstream targets by Snf1 and PKA (Cherry et al., 1989; Görner et al., 1998, 2002; Hedbacker et al., 2004; Ratnakumar et al., 2009; De Wever et al., 2005), thus the strategy required the identification of Snf1-independent target genes.

PKA-regulated genes have been mapped (Livas et al., 2011), and we checked the dependency of selected genes (*HXT1*, *HXT7* and *ATR1*) using a *cyr1Δpde2Δyak1Δ* strain, in which the disruption of adenylate cyclase impairs the activation of PKA (Görner et al., 2002) (Figure S7A-B). *HXT1*, whose expression is PKA-dependent, is also known to be regulated by Snf1 (Tomás-Cobos and Sanz, 2002), and therefore was included in subsequent analyses as a control.

To assess whether Snf1 catalytic activity influences the expression of PKA-regulated genes, we tested the expression of *HXT1*, *HXT7* and *ATR1* in the presence of the aforementioned Snf1-G53R isoform. *HXT1* resulted to be

down-regulated in a Snf1-G53R strain (Figure 12), thus proving the validity of the use of this isoform to mimic the activated kinase. The PKA-activated gene *ATR1* was slightly down-regulated in the presence of a Snf1-G53R isoform but, more strikingly, in the same strain the PKA-repressed gene *HXT7* was fivefold derepressed (Figure 12). *HXT7* is known to be Snf1-independent (Zaman et al., 2009), and furthermore we checked its dependency from the Snf1-inhibited repressor Mig1 using a *mig1Δ* strain (Figure S7C), demonstrating the usefulness of this gene as a PKA-dependent/Snf1-independent reporter gene.

Altogether, these data demonstrate that Snf1 catalytic activity functionally influences the PKA pathway, determining an alteration in the expression of PKA-dependent genes.

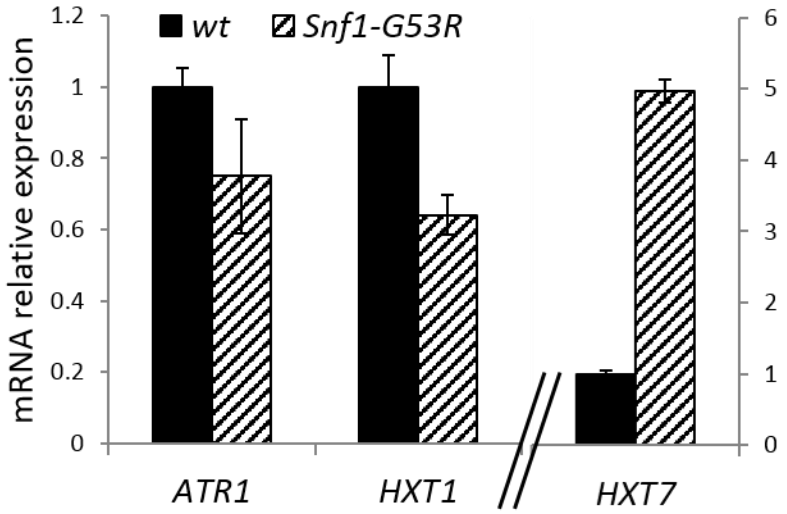


Figure 12. Expression of PKA-dependent genes in the presence of constitutively activated Snf1

Real-time PCR quantification of mRNAs of the indicated genes in cells in exponential phase of growth in 2% glucose.

2.2. Supplementary results

Interactor	Description	Known
	Snf1 complex	
SIP1	Alternate beta-subunit of the Snf1p kinase complex, may confer substrate specificity; vacuolar protein containing KIS (Kinase-Interacting Sequence) and ASC (Association with Snf1 kinase Complex) domains involved in protein interactions	yes
SIP2	One of three beta subunits of the Snf1 kinase complex; involved in the response to glucose starvation; null mutants exhibit accelerated aging; N-myristoylprotein localized to the cytoplasm and the plasma membrane	yes
GAL83	One of three possible beta-subunits of the Snf1 kinase complex; allows nuclear localization of the Snf1 kinase complex in the presence of a nonfermentable carbon source; contains glycogen-binding domain	yes
SNF4	Activating gamma subunit of the AMP-activated Snf1p kinase complex; additional subunits of the complex are Snf1p and a Sip1p/Sip2p/Gal83p family member; activates glucose-repressed genes, represses glucose-induced genes; role in sporulation, and peroxisome biogenesis; protein abundance increases in response to DNA replication stress	yes
SAK1	Upstream serine/threonine kinase for the SNF1 complex; partially redundant with Elm1p and Tos3p; members of this family have functional orthology with LKB1, a mammalian kinase associated with Peutz-Jeghers cancer-susceptibility syndrome; SAK1 has a paralog, TOS3, that arose from the whole genome duplication	yes
REG1	Regulatory subunit of type 1 protein phosphatase Glc7p; involved in negative regulation of glucose-repressible genes; involved in regulation of the nucleocytoplasmic shuttling of Hxk2p	yes

Interactor	Description	Known
	Glucose metabolism	
HXK2	Hexokinase isoenzyme 2; catalyzes phosphorylation of glucose in the cytosol; predominant hexokinase during growth on glucose; functions in the nucleus to repress expression of HXK1 and GLK1 and to induce expression of its own gene; phosphorylation/dephosphorylation at serine-14 by protein kinase Snf1p and protein phosphatase Glc7p-Reg1p regulates nucleocytoplasmic shuttling of Hxk2p	yes
PFK1	Alpha subunit of heterooctameric phosphofructokinase involved in glycolysis, indispensable for anaerobic growth, activated by fructose-2,6-bisphosphate and AMP, mutation inhibits glucose induction of cell cycle-related genes	no
PFK2	Beta subunit of heterooctameric phosphofructokinase involved in glycolysis, indispensable for anaerobic growth, activated by fructose-2,6-bisphosphate and AMP, mutation inhibits glucose induction of cell cycle-related genes	yes
TDH2	Glyceraldehyde-3-phosphate dehydrogenase, isozyme 2; involved in glycolysis and gluconeogenesis; tetramer that catalyzes the reaction of glyceraldehyde-3-phosphate to 1,3 bis-phosphoglycerate; detected in the cytoplasm and cell wall; protein abundance increases in response to DNA replication stress	no
TDH3	Glyceraldehyde-3-phosphate dehydrogenase, isozyme 3; involved in glycolysis and gluconeogenesis; tetramer that catalyzes the reaction of glyceraldehyde-3-phosphate to 1,3 bis-phosphoglycerate; detected in the cytoplasm and cell wall	no
PDC1	Major of three pyruvate decarboxylase isozymes, key enzyme in alcoholic fermentation, decarboxylates pyruvate to acetaldehyde; subject to glucose-, ethanol-, and autoregulation; involved in amino acid catabolism	no
CDC19	Pyruvate kinase; functions as a homotetramer in glycolysis to convert phosphoenolpyruvate to pyruvate, the input for aerobic (TCA cycle) or anaerobic (glucose fermentation) respiration; regulated via allosteric activation by fructose bisphosphate	no

Interactor	Description	Known
ENO2	Enolase II, a phosphopyruvate hydratase; catalyzes conversion of 2-phosphoglycerate to phosphoenolpyruvate during glycolysis and the reverse reaction during gluconeogenesis; expression induced in response to glucose	no
ALD6	Cytosolic aldehyde dehydrogenase, activated by Mg2+ and utilizes NADP+ as the preferred coenzyme; required for conversion of acetaldehyde to acetate; constitutively expressed; locates to the mitochondrial outer surface upon oxidative stress	no
ATP2	Beta subunit of the F1 sector of mitochondrial F1F0 ATP synthase, which is a large, evolutionarily conserved enzyme complex required for ATP synthesis; F1 translationally regulates ATP6 and ATP8 expression to achieve a balanced output of ATP synthase genes encoded in nucleus and mitochondria; phosphorylated	no
TAL1	Transaldolase, enzyme in the non-oxidative pentose phosphate pathway; converts sedoheptulose 7-phosphate and glyceraldehyde 3-phosphate to erythrose 4-phosphate and fructose 6-phosphate	no
	Ribosomes and Translation	
RPS8A	Protein component of the small (40S) ribosomal subunit; homologous to mammalian ribosomal protein S8, no bacterial homolog	no
RPS6A	Protein component of the small (40S) ribosomal subunit; homologous to mammalian ribosomal protein S6, no bacterial homolog	no
RPL1A	Ribosomal 60S subunit protein L1A; N-terminally acetylated; homologous to mammalian ribosomal protein L10A and bacterial L1	no
RPL3	Ribosomal 60S subunit protein L3; homologous to mammalian ribosomal protein L3 and bacterial L3; involved in the replication and maintenance of killer double stranded RNA virus	no

Interactor	Description	Known
RPL4A	Ribosomal 60S subunit protein L4A; N-terminally acetylated; homologous to mammalian ribosomal protein L4 and bacterial L4	no
RPL6A	Ribosomal 60S subunit protein L6A; N-terminally acetylated; binds 5.8S rRNA; homologous to mammalian ribosomal protein L6, no bacterial homolog	no
RPL6B	Ribosomal 60S subunit protein L6B; binds 5.8S rRNA; homologous to mammalian ribosomal protein L6, no bacterial homolog	no
RPL8A	Ribosomal 60S subunit protein L8A; mutation results in decreased amounts of free 60S subunits; homologous to mammalian ribosomal protein L7A, no bacterial homolog	yes
RPL17B	Ribosomal 60S subunit protein L17B; homologous to mammalian ribosomal protein L17 and bacterial L22	no
RPL20B	Ribosomal 60S subunit protein L20B; homologous to mammalian ribosomal protein L18A, no bacterial homolog	no
RPP0	Conserved ribosomal protein P0 of the ribosomal stalk; involved in interaction between translational elongation factors and the ribosome; phosphorylated on serine 302; homologous to mammalian ribosomal protein LP0 and bacterial L10	no
EFT1	Elongation factor 2 (EF-2), also encoded by EFT2; catalyzes ribosomal translocation during protein synthesis; contains diphthamide, the unique posttranslationally modified histidine residue specifically ADP-ribosylated by diphtheria toxin	no
EFT2	Elongation factor 2 (EF-2), also encoded by EFT1; catalyzes ribosomal translocation during protein synthesis; contains diphthamide, the unique posttranslationally modified histidine residue specifically ADP-ribosylated by diphtheria toxin	no

Interactor	Description	Known
TEF1	Translational elongation factor EF-1 alpha; also encoded by TEF2; functions in the binding reaction of aminoacyl-tRNA (AA-tRNA) to ribosomes; may also have a role in tRNA re-export from the nucleus	no
TEF2	Translational elongation factor EF-1 alpha; also encoded by TEF1; functions in the binding reaction of aminoacyl-tRNA (AA-tRNA) to ribosomes; may also have a role in tRNA re-export from the nucleus	yes
SRO9	Cytoplasmic RNA-binding protein; associates with translating ribosomes; involved in heme regulation of Hap1p as a component of the HMC complex, also involved in the organization of actin filaments; contains a La motif	yes
CFT1	RNA-binding subunit of the mRNA cleavage and polyadenylation factor; involved in poly(A) site recognition and required for both pre-mRNA cleavage and polyadenylation, 51% sequence similarity with mammalian AAUAA-binding subunit of CPSF	no
NIP1	eIF3c subunit of the eukaryotic translation initiation factor 3 (eIF3), involved in the assembly of preinitiation complex and start codon selection	no
STM1	Protein required for optimal translation under nutrient stress; perturbs association of Yef3p with ribosomes; involved in TOR signaling; binds G4 quadruplex and purine motif triplex nucleic acid; helps maintain telomere structure; protein abundance increases in response to DNA replication stress; serves as a ribosome preservation factor both during quiescence and recovery	yes

Interactor	Description	Known
	A.a. biosynthesis and transport	
LYS20	Homocitrate synthase isozyme; catalyzes the condensation of acetyl-CoA and alpha-ketoglutarate to form homocitrate, which is the first step in the lysine biosynthesis pathway	no
LYS21	Homocitrate synthase isozyme; catalyzes the condensation of acetyl-CoA and alpha-ketoglutarate to form homocitrate, which is the first step in the lysine biosynthesis pathway	no
CPA2	Large subunit of carbamoyl phosphate synthetase, which catalyzes a step in the synthesis of citrulline, an arginine precursor	no
NPR1	Protein kinase; stabilizes several plasma membrane amino acid transporters by antagonizing their ubiquitin-mediated degradation; phosphorylates Aly2p; negatively regulates Ldb19p-mediated endocytosis through phosphorylation of Ldb19p, which prevents its association with the plasma membrane; Npr1p activity is negatively regulated via phosphorylation by the TOR complex	yes
	Fatty acid synthesis	
FAS1	Beta subunit of fatty acid synthetase, which catalyzes the synthesis of long-chain saturated fatty acids; contains acetyltransacylase, dehydratase, enoyl reductase, malonyl transacylase, and palmitoyl transacylase activities	yes
FAS2	Alpha subunit of fatty acid synthetase, which catalyzes the synthesis of long-chain saturated fatty acids; contains the acyl-carrier protein domain and beta-ketoacyl reductase, beta-ketoacyl synthase and self-pantetheinylation activities	no

Interactor	Description	Known
	IMP dehydrogenases	
IMD1	Nonfunctional protein with homology to IMP dehydrogenase; blocked reading frame, located close to the telomere; not expressed at detectable levels; YAR073W and YAR075W comprise a continuous reading frame in most strains of S. cerevisiae	no
IMD2	Inosine monophosphate dehydrogenase; catalyzes the rate-limiting step in GTP biosynthesis, expression is induced by mycophenolic acid resulting in resistance to the drug, expression is repressed by nutrient limitation	no
IMD3	Inosine monophosphate dehydrogenase; catalyzes the first step of GMP biosynthesis, member of a four-gene family in S. cerevisiae, constitutively expressed	no
IMD4	Inosine monophosphate dehydrogenase; catalyzes the first step of GMP biosynthesis, member of a four-gene family in S. cerevisiae, constitutively expressed	no
YAR075W	Non-functional protein with homology IMP dehydrogenase; YAR073W/IMD1 and YAR075W comprise a continuous reading frame in most strains of S. cerevisiae	no
	Chaperones	
SSA2	ATP binding protein involved in protein folding and vacuolar import of proteins; member of heat shock protein 70 (HSP70) family; associated with the chaperonin-containing T-complex; present in the cytoplasm, vacuolar membrane and cell wall; 98% identical with paralog Ssa1p, but subtle differences between the two proteins provide functional specificity with respect to propagation of yeast [URE3] prions and vacuolar-mediated degradations of gluconeogenesis enzymes	yes

Interactor	Description	Known
SSB1	Cytoplasmic ATPase that is a ribosome-associated molecular chaperone; functions with J-protein partner Zuo1p; may be involved in folding of newly-made polypeptide chains; member of the HSP70 family; interacts with phosphatase subunit Reg1p	yes
SSB2	Cytoplasmic ATPase that is a ribosome-associated molecular chaperone; functions with J-protein partner Zuo1p; may be involved in the folding of newly-synthesized polypeptide chains; member of the HSP70 family	yes
BMH1	14-3-3 protein, major isoform; controls proteome at post-transcriptional level, binds proteins and DNA, involved in regulation of many processes including exocytosis, vesicle transport, Ras/MAPK signaling, and rapamycin-sensitive signaling; protein increases in abundance and relative distribution to the nucleus increases upon DNA replication stress	yes
	Others	
CYR1	Adenylate cyclase, required for cAMP production and cAMP-dependent protein kinase signaling; the cAMP pathway controls a variety of cellular processes, including metabolism, cell cycle, stress response, stationary phase, and sporulation	no
CKA1	Alpha catalytic subunit of casein kinase 2 (CK2), a Ser/Thr protein kinase with roles in cell growth and proliferation; CK2, comprised of CKA1, CKA2, CKB1 and CKB2, has many substrates including transcription factors and all RNA polymerases; regulates Fkh1p-mediated donor preference during mating-type switching	yes
COP1	Alpha subunit of COPI vesicle coatomer complex, which surrounds transport vesicles in the early secretory pathway	no

Interactor	Description	Known
CHC1	Clathrin heavy chain, subunit of the major coat protein involved in intracellular protein transport and endocytosis; two heavy chains form the clathrin triskelion structural component; the light chain (CLC1) is thought to regulate function	no

Table S1. List of Snf1 interactors

List of identified interactors, grouped by pathway/complex. The last column indicates whether the interactions were already present in the database (BioGRID, as to 01/07/2015).

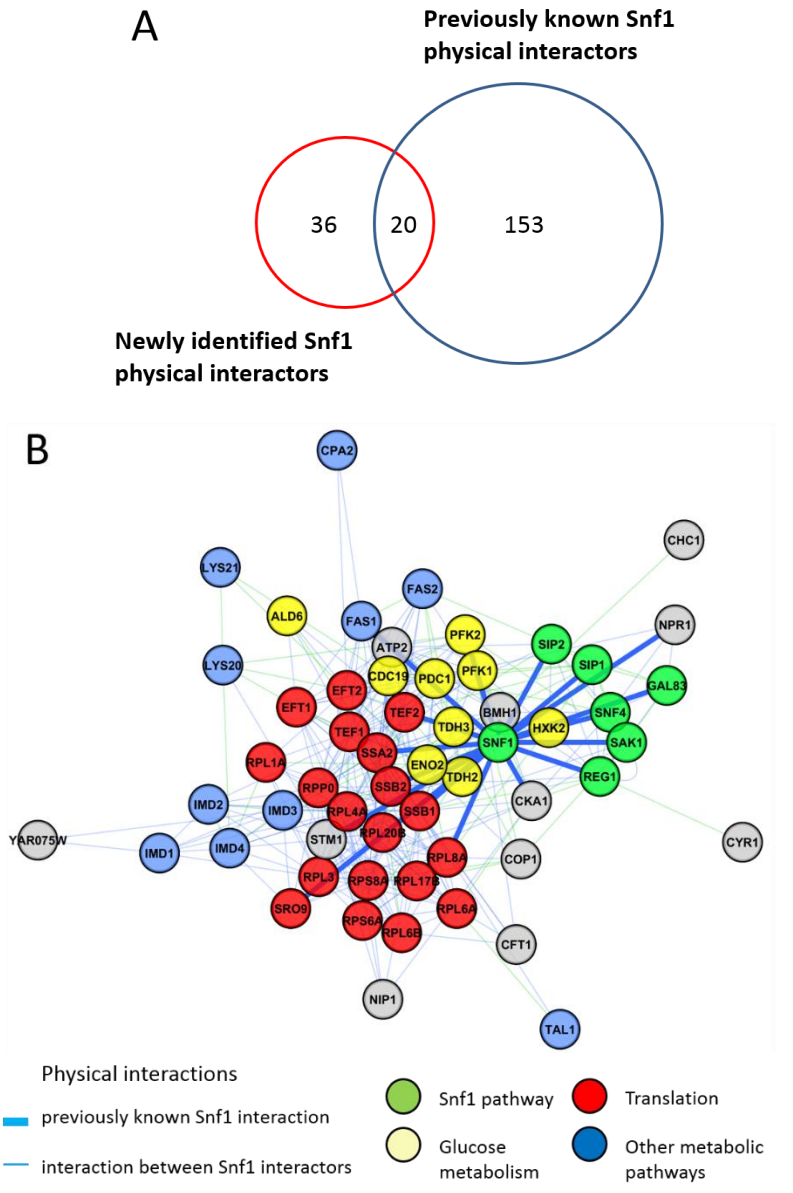

A
Previously known Snf1 physical interactors
36
20
153
Newly identified Snf1 physical interactors
B
CPA2
CHC1
LYS21
FAS2
ALD6
FAS1
NPR1
PFK2
ATP2
SIP2
LYS20
SIP1
CDC19
PDC1
PFK1
GAL83
EFT2
EFT1
TEF2
BMH1
SNF4
TDH3
HXK2
TEF1
SNF1
SAK1
SSA2
RPL1A
ENO2
TDH2
REG1
RPP0
SSB2
CKA1
IMD2
IMD3
RPL4A
SSB1
YAR075W
STM1
RPL20B
CYR1
IMD1
IMD4
RPL8A
COP1
RPL3
RPS8A
RPL17B
SRO9
RPL6A
RPS6A
RPL6B
CFT1
NIP1
TAL1
Physical interactions
previously known Snf1 interaction
interaction between Snf1 interactors
Snf1 pathway
Translation
Glucose metabolism
Other metabolic pathways

Figure S3. Data analysis of the identified Snf1 interactors

The known interactions were retrieved in the BioGRID database of physical interactions. Data visualization was performed with Cytoscape. (A) Intersection between the already known Snf1 physical interactors and the interactors identified in this study. (B) GO Biological Process enrichment analysis of the Snf1 physical interactors in 2% glucose.

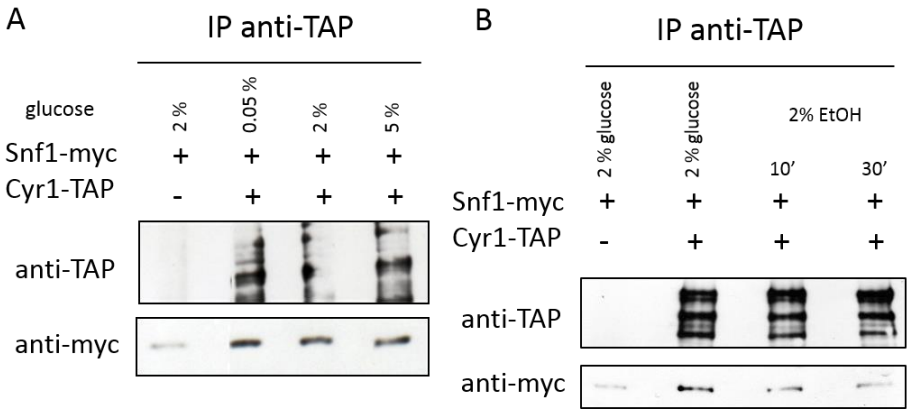


Figure S4. Snf1 interaction with adenylate cyclase is not dependent from glucose concentration or type of carbon source

(A) Western blot showing the detection of Snf1-myc in Cyr1-TAP immunocomplexes. Cells were grown with the indicated glucose concentration. (B) Western blot showing the detection of Snf1-myc in Cyr1-TAP immunocomplexes. Samples were collected at the indicated times after the cells

were shifted from 2% glucose to 2% ethanol containing medium. The strain expressing the untagged isoform of Cyr1 is shown as control.

Figure S5. Schematization of the putative Snf1 phosphorylation sites on Cyr1

Graphical schematization of the phosphorylatable residues contained in perfect Snf1 phosphorylation consensus sequences ([MVLIF]-X-R-X-X-[ST]-X-X-X-[MVLIF]). Phosphosites identified *in vivo*, as reported in PhosphoGRID, are highlighted in orange. Putative phosphorylation sites retrieved with PattInProt. RAD = RAS Associating Domain, CD = Catalytic Domain.

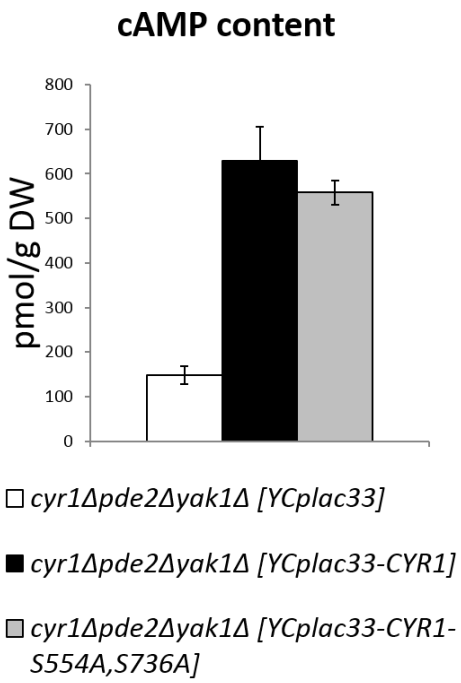


Figure S6. A Cyr1-S554A.S736A double mutation does not influence intracellular cAMP levels

Histogram showing the intracellular cAMP content of the indicated strains. Data normalized on cellular dry weight.

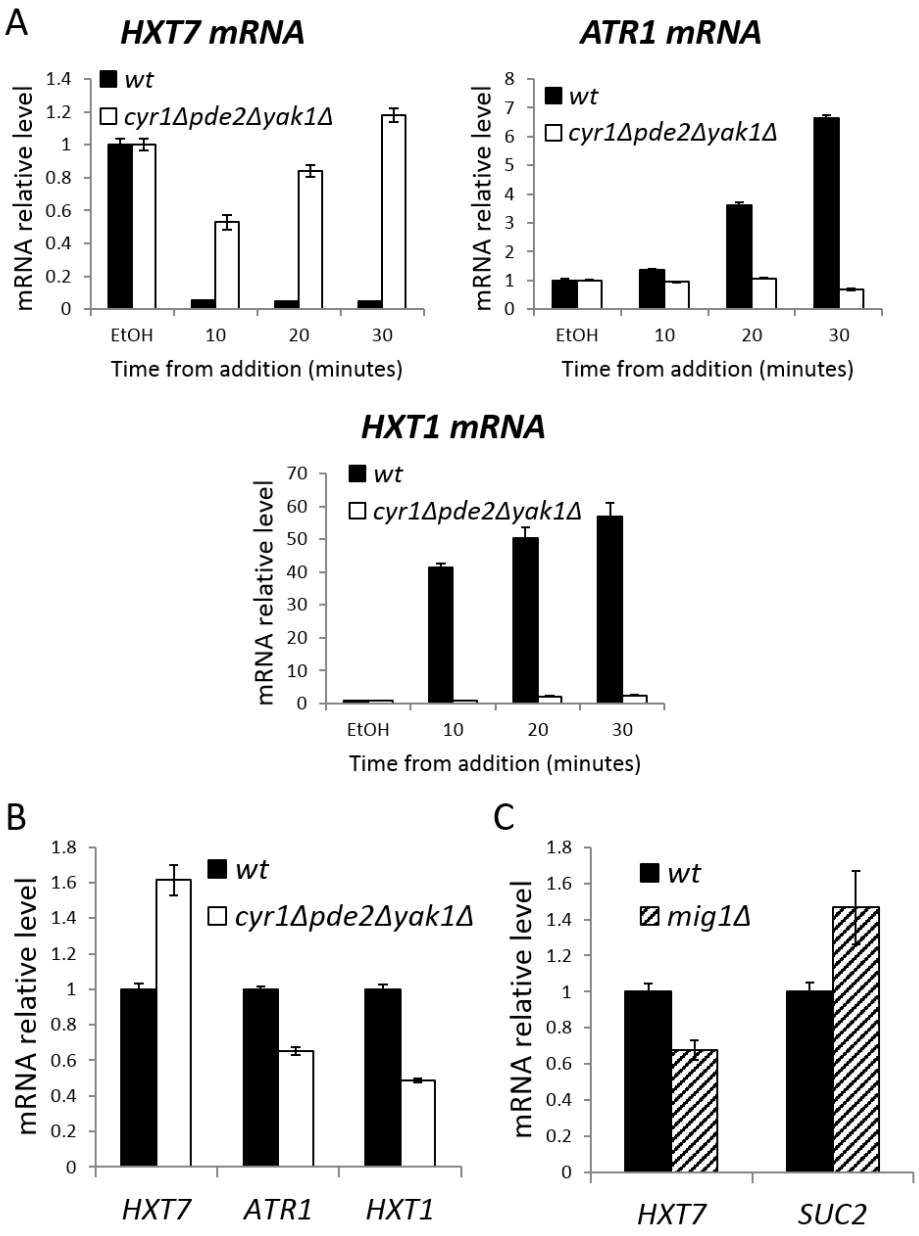

A
HXT7 mRNA
ATR1 mRNA
HXT1 mRNA
wt
cyr1Δpde2Δyak1Δ
mRNA relative level
EtOH
10
20
30
Time from addition (minutes)
B
HXT7
ATR1
HXT1
C
mig1Δ
HXT7
SUC2

Figure S7. Identification of PKA-dependent reporter genes

(A) Real-time PCR quantification of mRNAs of the indicated genes in nutritional shift-up experiments, in which the cells, growing with 2% ethanol as carbon source, were feed with 5% glucose (final concentration). Samples were taken at indicated time points. (B) Real-time PCR quantification of mRNAs of the indicated genes in cells in exponential phase of growth in 2% glucose. (C) Real-time PCR quantification of mRNAs of the indicated genes in cells in exponential phase of growth in 2% glucose. *SUC2* expression was evaluated as a control of Mig1 inactivation.

3. Enhanced amino acid utilization sustains growth of cells lacking Snf1/AMPK

3.1. Results

Deletion of *SNF1* causes a large transcriptional reprogramming in cells growing in 2% glucose

Protein kinase Snf1 is known for its role in response to stress conditions and nutrient limitations (Hedbacker and Carlson, 2008). We recently highlighted its requirement also for growth and cell cycle progression at standard glucose growth condition (2% glucose), requirement that disappears with a larger (5%) glucose supply (Busnelli et al., 2013; Pessina et al., 2010). To characterize the poor growth phenotype of *snf1Δ* cells in a 2% glucose synthetic medium, we performed transcriptome analysis (Affymetrix) of wild type and *snf1Δ* cells growing in this condition, as well as in the phenotype-reverting glucose supplemented medium (5% glucose).

*snf1*Δ cells growing in 2% glucose showed extensive transcriptional reprogramming as compared to the wild type, involving about 1000 genes, with a slight predominance of up-regulated transcripts (Figure S8A). Increasing the glucose concentration to 5% greatly reduced the number of differentially expressed genes to roughly 300 (Figure 13A). Strikingly, the DEGs (differential expressed genes) in 5% glucose resulted mostly in a

subset of the DEGs in 2% glucose (Figure 13A) and highly correlated with these, but with reduced fold changes (Figure 13B). This finding indicates that the reversion of the slow-growth phenotype of *snf1Δ* cells with higher glucose availability correlates with the reversion of the differential gene expression.

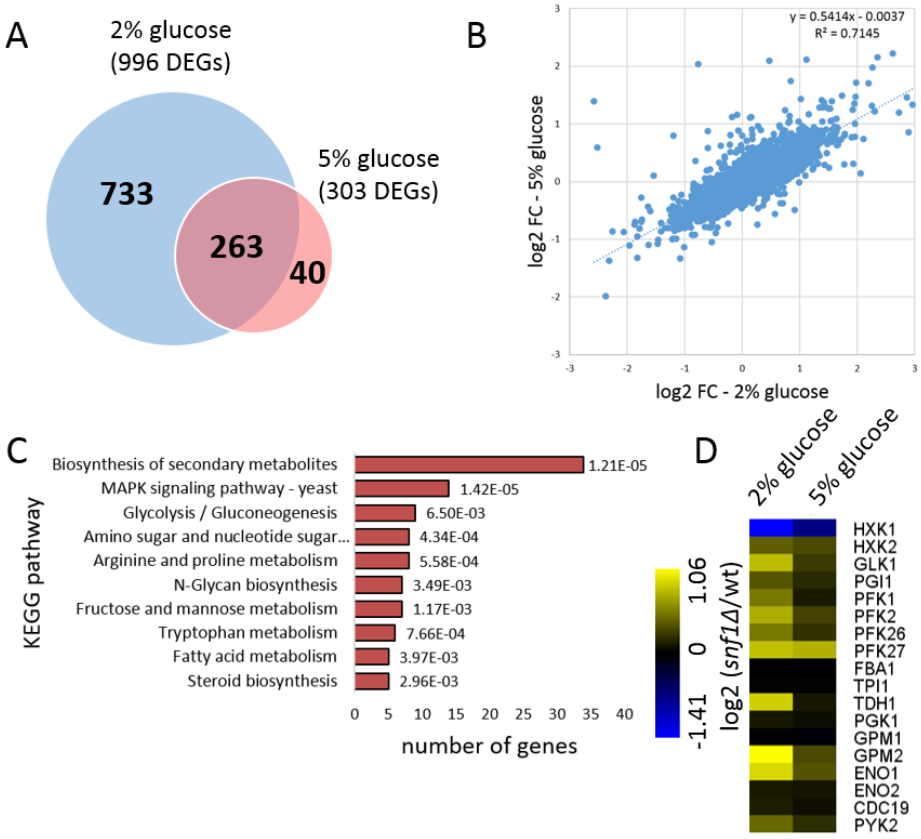
Gene Ontology enrichment analysis of the up-regulated transcripts in *snf1Δ* cells growing in 2% glucose showed de-repression of genes involved mainly in transmembrane transport and in a large number of metabolic processes (Figure S8B and S8C). In particular, more than 100 up-regulated genes encode for transmembrane transporters of amino acids and other metabolites, mostly targets of the Gcn4 transcription factor (Fig S8B). Interestingly, the inhibition of Snf1 catalytic activity has been shown to cause the de-repression of Gcn4-regulated genes through the regulation of translation of the transcription factor in cells growing in low glucose (Shirra et al., 2008). Our data indicate that this phenomenon could be extended also to glucose-repressed cells, further supporting the emerging role of Snf1 also in non-limiting nutrient conditions. The major part of the remaining up-regulated genes were involved in several metabolic processes such as redox metabolism, iron homeostasis and amino acids biosynthesis (Figure S8C). Further analysis of the subset of up-regulated genes in *snf1Δ* cells growing in 2% glucose for enrichment in KEGG pathways remarkably identified metabolic processes not previously linked

to Snf1, overall glycolysis/gluconeogenesis and associated sugar metabolisms, as well as known Snf1-controlled pathways such as fatty acid metabolism (Figure 13C). Gene Ontology enrichment of down-regulated genes revealed that the presence of Snf1 is required for the expression of genes important for mitochondrial functionality and cell cycle progression (Figure S8D). In particular, several genes coding for structural proteins of both the small and large subunits of the mitochondrial ribosomes were down-regulated in *snf1Δ* cells grown in 2% glucose, as well as cell cycle-related genes, mainly involved in microtubule assembly and mitosis (Figure S8D).

Notably, genes involved in phosphate metabolism present an interesting response in expression between 2% and 5% glucose in *snf1Δ* cells. Genes coding for high affinity phosphate transporters (*PHO84* and *PHO89*), the membrane glycoprotein *PHO11* and the transcription factor *SPL2* were inhibited by the deletion of *SNF1* in 2% glucose, while the *PHO87* gene coding for the low affinity transporter was up-regulated in the same condition (Figure S8B). The same genes switched behaviour in 5% glucose, possibly indicating that the restored growth due to the higher glucose availability was sensed as not matched by sufficient phosphate uptake.

Taken together, these data provide a wide picture of the transcriptional re-arrangement occurring in cells lacking Snf1 in a condition in which the activity of the kinase is not essential for the de-repression of genes subject

to catabolite repression but guarantees a proper growth, as well as in the presence of a larger glucose supply (5% glucose) in which the presence of Snf1 becomes dispensable.



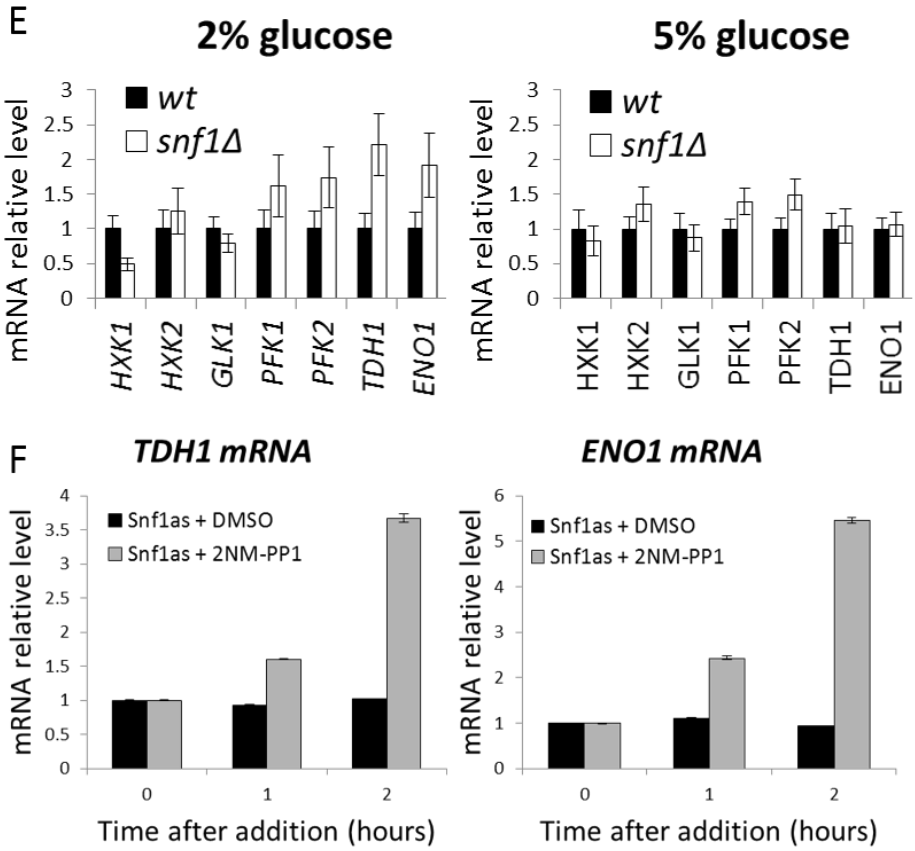


Figure 13. Transcriptional reprogramming of *snf1Δ* yeast cells growing in 2% glucose

(A) Euler diagram showing the overlap between the differential expressed genes (DEGs) in *snf1Δ* batch cultures supplemented either with 2% or 5% glucose, compared to the wt. (B) Dot-plot of the correlation of the fold change of DEGs in the *snf1Δ*/wt 2% glucose and *snf1Δ*/wt 5% glucose comparisons. (C) KEGG Pathways enrichment of the up-regulated genes in *snf1Δ* cells growing in 2%

glucose compared to the wt. Enrichment p-value is shown beside each bar. (D) Heat map showing the log2 fold change of expression of glycolytic genes in *snf1Δ* cells growing in 2% and 5% glucose, compared to the wt. (E) Real-time PCR quantification of mRNAs of the indicated genes in wt and *snf1Δ* cells growing respectively in 2% glucose and 5% glucose. (F) Real-time PCR quantification of *TDH1* and *ENO1* mRNAs in the Snf1^{as} strain following treatment with 2NM-PP1, DMSO was used as control.

Deletion of *SNF1* or inhibition of Snf1 catalytic activity causes the up-regulation of several glycolytic genes

Snf1 is known to regulate the transcription of several metabolic genes, enhancing the expression of enzymes involved in the utilization of low glucose levels or of alternative carbon sources (Hedbacker and Carlson, 2008). Here we show that Snf1 also functions as a regulator of glycolytic genes as there is de-repression of several of these genes in *snf1Δ* cells growing in 2% glucose (Figure 13D). To validate this finding we performed real-time PCR analyses of glycolytic genes mRNAs, confirming the up-regulation of *PFK2*, *TDH1* and *ENO1*, and showing that there is also up-regulation of *PFK1* and *HXK2*, which did not emerge from the gene chip analysis (Figure 13E). In the previous section, we highlighted that the reversion of the growth defect of *snf1Δ* cells once growth in 5% glucose was accompanied by a consistent normalization of the transcriptional

phenotype (Figure S8A). This phenomenon was confirmed also for glycolytic genes, since all of them showed nearly wild type expression levels in 5% glucose (Figure 13E).

To demonstrate that the de-repression of glycolytic genes was specifically due to the lack of Snf1 catalytic activity, we used a strain carrying an analog sensitive isoform of Snf1 (Snf1-I132G), which activity can be inhibited by the ATP analog 2NM-PP1 (Busnelli et al., 2013; Shirra et al., 2008) and checked the mRNA levels of representative genes following addition of 2NM-PP1 (Figure 13F). Higher levels of *TDH1* and *ENO1* mRNAs were detectable already after 1 hour following treatment with the inhibitor, with an even higher expression after 2 hours (Figure 13F).

Altogether, these data demonstrate that Snf1 catalytic activity is required for the transcriptional repression of glycolytic genes and, more intriguingly, that this response is performed in a glucose-dependent fashion.

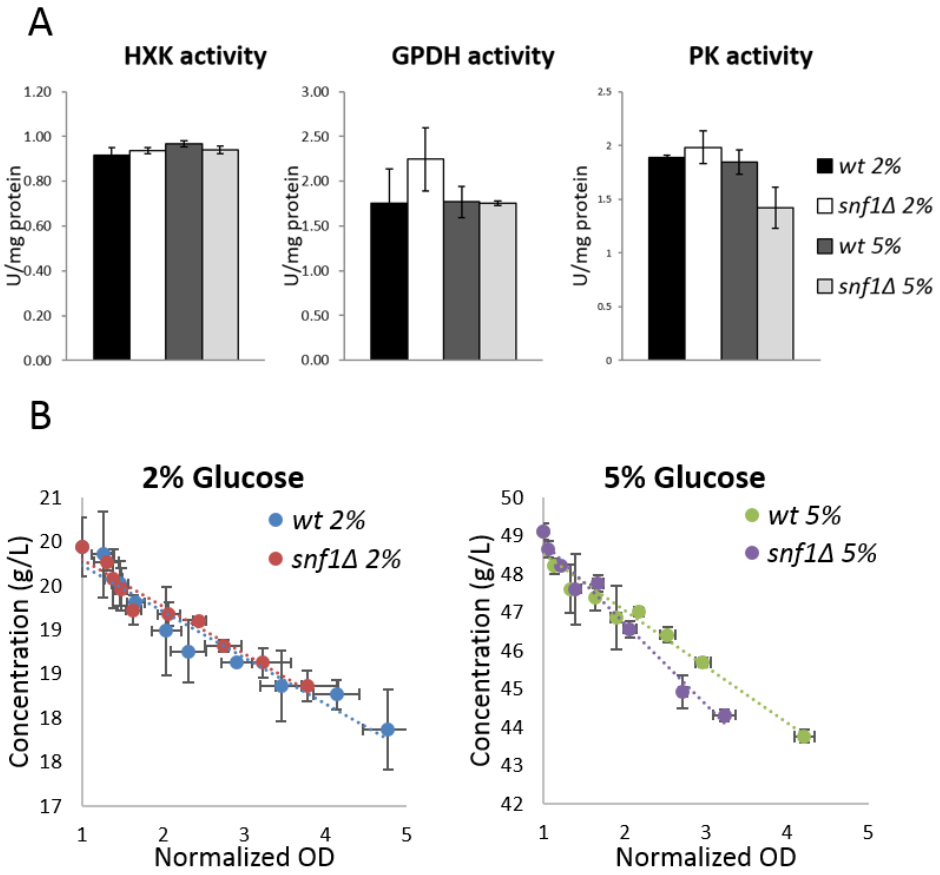
Lack of Snf1 causes enhanced glycolytic flux

Transcription of glycolytic genes has been reported to influence the enzymatic activity of the corresponding gene products, as shown using strains lacking the transcription factor Gcr1 or the co-activator Gcr2 (Sasaki et al., 2005). We therefore wondered whether the observed transcriptional up-regulation resulted in higher activity of glycolytic

enzymes. Surprisingly, we were unable to detect significant variations except for the GPDH (glyceraldehyde-3-phosphate dehydrogenase) activity, which showed a 20% increase in *snf1Δ* cells growing in 2% glucose and a subsequent reversion to wt activity in 5% glucose (Figure 14A). This increase is interesting, since *TDH1* was the only up-regulated isoform of glyceraldehyde-3-phosphate dehydrogenase, but it is the isoform expressed at lowest level in exponentially growing cells, compared to *TDH2* and *TDH3* (McAlister and Holland, 1985).

To determine whether the glycolytic flux was altered in *snf1Δ* cells, we measured the glucose consumption in log phase cultures by NMR spectroscopy. Interestingly, glucose uptake of the mutant strain was at wt levels in 2% glucose (consumption normalized with the specific growth rate), but was appreciably higher, as compared to the wt, in 5% glucose (Figure 14B). The unaltered glucose uptake in 2% glucose is surprising, given the up-regulation of the gene coding for the high capacity glucose transporter Hxt1 which depends on the impairment of a known transcriptional regulation exerted by Snf1 (Tomás-Cobos and Sanz, 2002). Nevertheless, *snf1Δ* cells produced more glycolytic output products, *i.e.* ethanol and acetate, when growing in 2% glucose, but secreted the same amount of these metabolites as the wt when growing in 5% glucose (Figure 14C).

These findings indicate that glycolysis is functionally enhanced in the absence of Snf1, in spite of unaltered glucose uptake, suggesting that the increased expression of glycolytic genes may be a transcriptional adaptation to the metabolic re-arrangement.



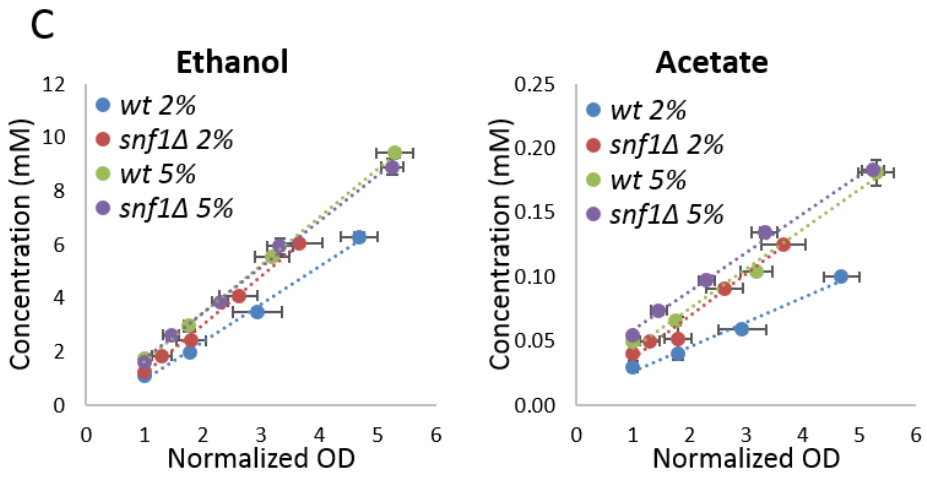


Figure 14. Lack of Snf1 enhances glucose uptake and glycolytic flux

(A) Enzymatic activity of the indicated enzymes in total protein extracts. (B) Glucose consumption of *snf1Δ* and wt strains in exponential phase of growth in 2% and 5% glucose, assayed by NMR analysis. (C) Ethanol and Acetate production of *snf1Δ* and wt cells in exponential phase of growth in 2% and 5% glucose, assayed by NMR analysis.

Growth in 5% glucose does not influence the *snf1Δ* fatty acid biosynthesis deregulation

One of the most extensively characterized phenotypes of *snf1Δ* cells is the accumulation of fatty acids, caused by the loss of Snf1-dependent phosphorylation of the acetyl-CoA carboxylase Acc1 (Hofbauer et al., 2014; Shi et al., 2014). This phenotype is of particular interest, since until

now has been basically a stand-alone in the comparison between the metabolic roles of Snf1 and its mammalian counterpart AMPK, which in other aspects diverged during evolution (Faubert et al., 2013). In yeast, loss of Snf1 and the consequent carbon overflow into the fatty acid biosynthetic pathway has been shown to cause inositol auxotrophy mediated by the impairment of *INO1* expression (Hofbauer et al., 2014; Shirra et al., 2001) and a depletion of the intracellular acetyl-CoA pool, causing a global reduction of histone acetylation (Zhang et al., 2013). However, no information about fatty acid biosynthesis in yeast at higher glucose concentration (>2%) is available.

We therefore quantitatively measured the fatty acid composition of wt and *snf1Δ* cells growing in 2% and 5% glucose by GC-MS analysis, to investigate whether the phenotype-reverting glucose supply (5% glucose) influences fatty acid accumulation in *snf1Δ*. As previously shown, lack of Snf1 caused accumulation of long chain fatty acids and interestingly this happened also in cells growing in 5% glucose (Figure 15).

It is well known that fatty acid elongation requires NADPH (Tehlivets et al., 2007), so we speculated that in *snf1Δ* cells the reducing equivalents for fatty acid elongation could be obtained from the pentose-phosphate pathway. Nevertheless, when we measured the activity of glucose-6-phosphate dehydrogenase (coded by *ZWF1*), we failed to observe any significant difference (Figure S9A). However, our gene chip analysis

evidenced that the genes coding for all the three NADH kinases (*POS5*, *UTR1*, *YEF1*) were up-regulated in *snf1Δ* cells growing in 2% glucose (Figure S9B), thus suggesting a compensatory mechanism involving *de novo* NADPH biosynthesis.

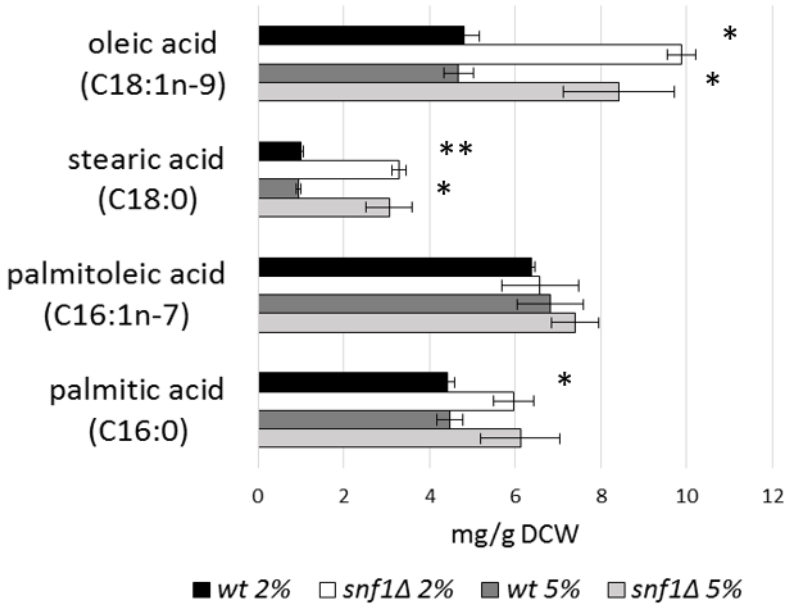


Figure 15. Fatty acids are equally accumulated in 2% and 5% glucose *snf1Δ* log phase cells

The most abundant yeast long chain saturated (C16:0 and C:18:0) and un-saturated (C16:1n-7 and C18:1n-9) fatty acids were quantified by GC-MS analysis and normalized on cellular dry weight. *p-value < 0.05; **p-value < 0.005.

Lack of Snf1 causes glutamate accumulation and fueling of carbon into the TCA cycle

Alteration of the Snf1 pathway in respiration-defective cells in stationary phase has been shown to cause a reshaping of the yeast metabolome, involving storage carbohydrates and amino acids (Friis et al., 2014). To assess whether the observed alterations in the glycolytic flux and the fatty acid biosynthetic pathway could be linked to broad alterations of the metabolism, we performed a metabolome profiling through ^{1}H-NMR spectroscopy, measuring a panel of intracellular metabolites. The most evident effect of the lack of Snf1 on metabolite levels in 2% glucose was the significant accumulation of glutamate, notably the most abundant metabolite (Figure 16A). Interestingly, the accumulation of glutamate was reduced in cells growing in 5% glucose, correlating with the reversion of the growth phenotype of *snf1Δ* cells (Figure 16A).

The profiling of intracellular amino acids also showed a slight but significant accumulation of intracellular methionine and a substantial reduction in the histidine content (Figure 16A). The wt and *snf1Δ* strains did not show a differential histidine uptake from the media (data not shown) and the amino acid itself was supplemented in large excess, thus the observed reduction in histidine content is attributable only to the alteration of intracellular processes. The only specific utilization of histidine, except for protein synthesis, is its use as a precursor for thiamine

biosynthesis. Thiamine, as thiamine diphosphate, is a cofactor in a number of biochemical reactions, but it is primarily required for the activity of pyruvate decarboxylase and pyruvate dehydrogenase (Kowalska and Kozik, 2008). Hence, the hyper-glycolytic phenotype of *snf1Δ* cells may cause an enhanced thiamine utilization by pyruvate decarboxylase isoforms, thus accounting for the reduction in the intracellular histidine pool.

Another major alteration in *snf1Δ* cells was the drastic reduction in intracellular glycerol (Figure 16B). This reduction was evident also for cells growing in 5% glucose, even if both wt and *snf1Δ* cells presented higher levels of glycerol at 5% glucose as compared to 2% glucose, consistent with the role of glycerol as redox sink and as recipient of glucose excess during exponential growth (Ansell et al., 1997). Snf1 is known to phosphorylate and inactivate the major glycerol-3-phosphate dehydrogenase isoform Gpd2 in nutrient limited conditions (Lee et al., 2012), but here we show a reduction rather than an increase in glycerol content, indicating that this repressive role of the kinase is negligible in a non-limiting glucose concentration.

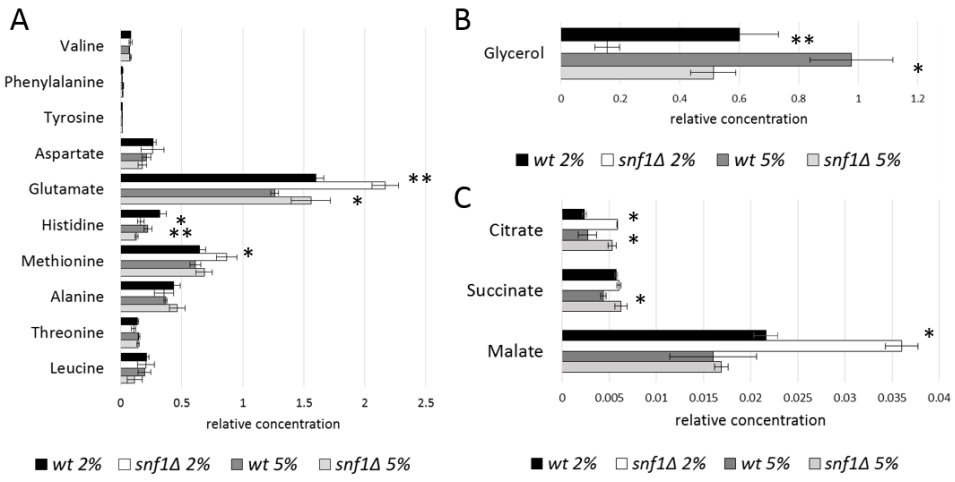
As mentioned above and shown in Figure 4A, *snf1Δ* cells accumulate glutamate. In fermenting yeast cells the biosynthesis of glutamate occurs via the reaction of ammonia and α-ketoglutarate, thus it seems likely that cells accumulating this amino acid could increase the activity of the TCA

cycle to supply biosynthetic building blocks. We therefore performed a targeted GC-MS analysis to measure intermediates of the TCA cycle and identified different levels of some of them in *snf1Δ* cells compared to the wt. In particular, *snf1Δ* cells accumulated more citrate both in 2% and 5% glucose, and even more clearly they accumulated malate when growing in 2% glucose (Figure 16C), thus suggesting the hypothesis of an increased fueling of the TCA cycle in the absence of Snf1.

During growth on glucose, glutamate biosynthesis from α-ketoglutarate is performed mainly by the NADPH-dependent glutamate dehydrogenase Gdh1 (DeLuna et al., 2001). We therefore measured the NADPH-dependent Gdh1 activity in total cell extracts, which interestingly was found lower in *snf1Δ* cells as compared to the wt (Figure 16D). This finding was not surprising in light of the drain of NADPH caused by the enhanced fatty acid biosynthesis in a *snf1Δ* mutant (Figure 16A), which adjusts the NADPH consuming Gdh1 activity accordingly. Notably, this lower activity was consistent with the reduction of glycerol accumulation (Figure 16B) and ethanol overproduction (Figure 14C), matching the results previously obtained with *gdh1Δ* cells (Bro et al., 2004).

We also measured the activity of NAD- and NADP-dependent isocitrate dehydrogenases (IDH and IDP, respectively), which perform the synthesis of α-ketoglutarate from isocitrate and thus can be considered shared reactions between the TCA cycle and the glutamate biosynthetic pathway.

Both enzymatic activities were lower in *snf1Δ* cells growing in 2% glucose, but the activity was not affected by deletion of Snf1 in 5% glucose. Besides, the activity of Glt1, the NADH-dependent glutamate synthase catalyzing the synthesis of two glutamate molecules from glutamine and α-ketoglutarate, was unaltered in *snf1Δ* cells (Figure 16D). However this alternative glutamate biosynthetic pathway, which biochemically could account for glutamate accumulation, is evidently a hotspot, since all the genes coding for the enzymes of the pathway were up-regulated in *snf1Δ* cells (Figure S10A-B).



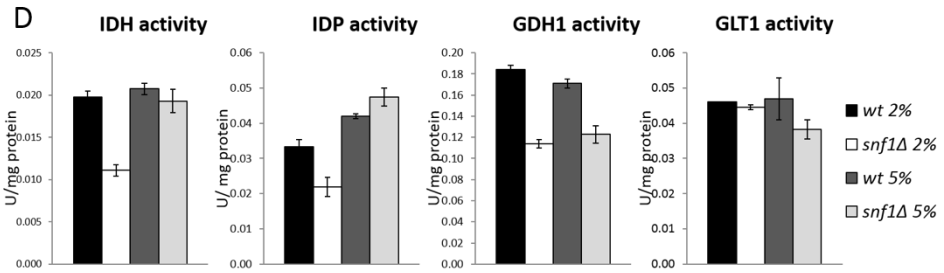


Figure 16. Absence of Snf1 promotes carbon flow into the TCA cycle and glutamate accumulation

(A) Intracellular concentration of amino acids evaluated by NMR. *p-value < 0.05; **p-value < 0.005. (B) Intracellular concentration of glycerol evaluated by NMR. *p-value < 0.05; **p-value < 0.005. (C) Intracellular concentration of TCA cycle intermediates evaluated by GC-MS. *p-value < 0.05. (D) Enzymatic activity of the indicated enzymes in total protein extracts.

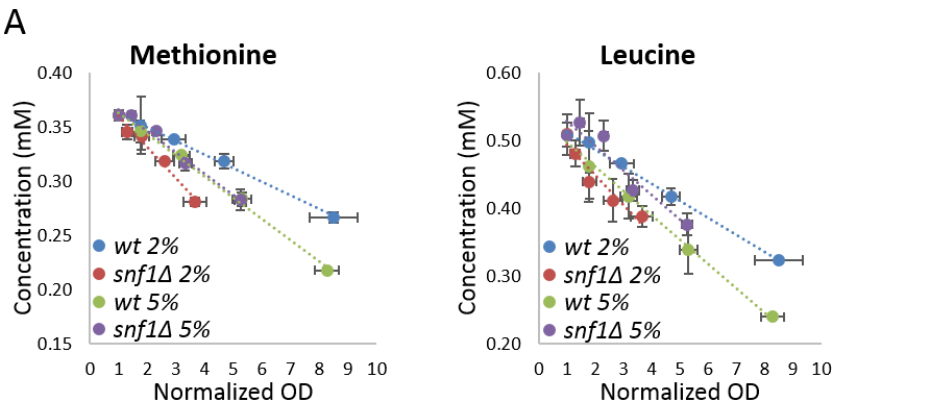
Increased uptake of amino acids in *snf1Δ* cells supports growth

The down-regulation of the main glutamate biosynthetic pathway through Gdh1 left unexplained the issue of the origin of exceeding glutamate in *snf1Δ* cells. We therefore tested the possibility of glutamate being a product of utilization of other amino acids assaying the consumption of leucine, methionine and histidine in the medium. While the consumption of histidine was negligible in every tested condition (data not shown), leucine and methionine uptake were significantly higher in *snf1Δ* cells growing in 2% glucose compared to the wt, consistently with

the enhanced expression of genes encoding for amino acid transporters (Figure S1B), while in 5% glucose the uptake of amino acids was comparable between the mutant strain and its control (Figure 17A). Interestingly, leucine is known to be degraded through the Ehrlich pathway to glutamate and fusels alcohols (Dickinson, 2000) and *snf1Δ* cells transcriptionally up-regulate genes (*BAT2*, *THI3*, *ADH4*, *ADH5*) belonging to this pathway (Figure S10C). Noteworthy, decreasing the concentration of leucine and methionine in the medium to 1/5th of the amount normally supplemented impaired the growth of *snf1Δ* cells only in 2% glucose, while having no effect on wt cells in this condition nor on wt and *snf1Δ* growing in 5% glucose (Figure 17B).

Strikingly, lowering the concentration of leucine and methionine in the medium abolished the accumulation of intracellular glutamate in *snf1Δ* cells growing in 2% glucose (Figure 17C).

Altogether, these data support the hypothesis that *snf1Δ* cells in 2% glucose fuel carbon into the TCA cycle from amino acids supplemented in the medium.



B

MDT (min)	LM 50 mg/L	LM 10 mg/L
wt 2%	119±5	121±7
snf1Δ 2%	180±16	228±10
wt 5%	117±4	119±1
snf1Δ 5%	154±5	146±7

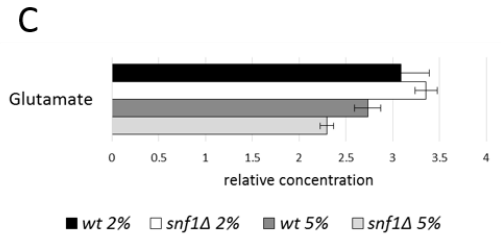


Figure 17. Enhanced amino acids uptake is necessary to sustain growth of *snf1Δ* cells

(A) Methionine and Leucine consumption of *snf1Δ* and wt cells in exponential phase of growth in 2% and 5% glucose, assayed by NMR analysis. OD normalized to time point 0. (B) Table showing the mean duplication time (MDT) of the indicated strains in standard medium (leucine and methionine 50 mg/L) or in amino acids limiting cultures (leucine and methionine 10 mg/L). (C) Glutamate intracellular content of the indicated strains in amino acids limiting cultures (leucine and methionine 10 mg/L).

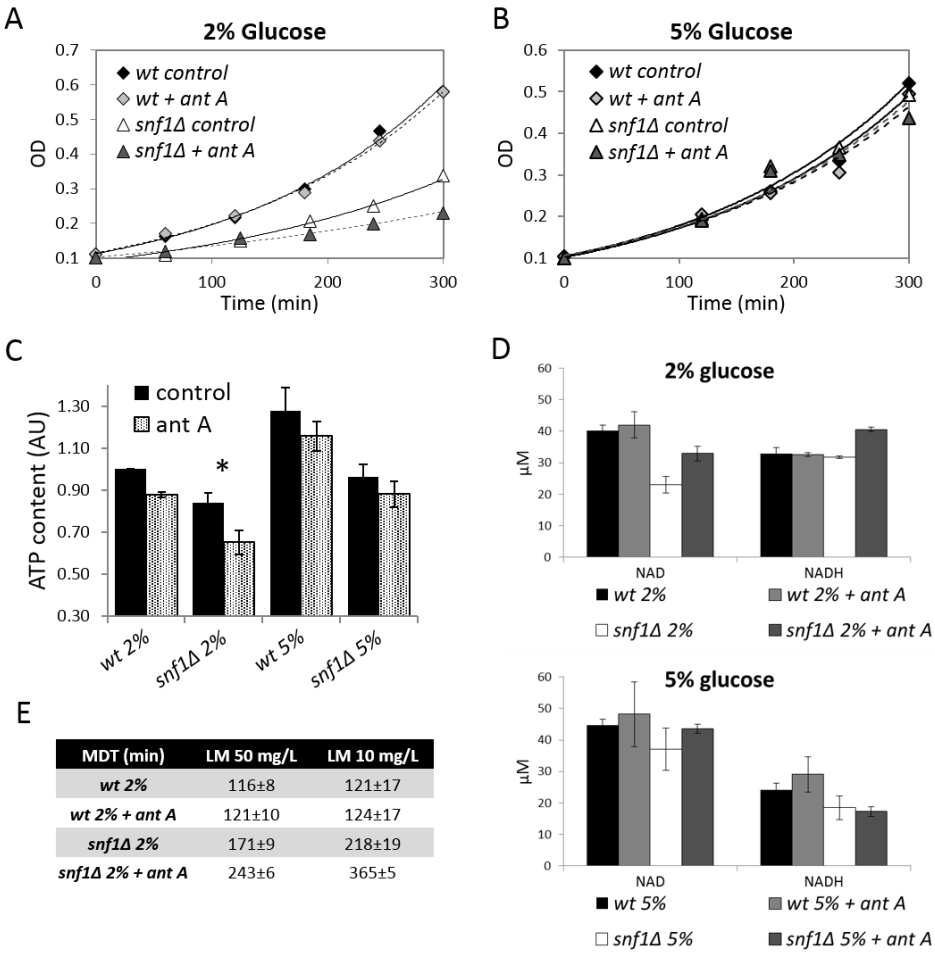
Oxidative phosphorylation sustains growth and energy production in the absence of Snf1

The previous findings that indicate an amino acid utilization through the TCA cycle in *snf1Δ* cells in 2% glucose, suggest a respiro-fermentative metabolism, thus requiring the re-oxidation of mitochondrial NADH, whose pool is well known to be separated from the cytoplasmic one (Kato and Lin, 2014). To assess the role of mitochondrial activity, *snf1Δ* and wt cells in exponential phase of growth were treated with antimycin A, an inhibitor of the complex III of the electron transport chain. In 2% glucose the drug further impaired the slow growth phenotype of *snf1Δ* cells, without affecting wt cells (Figure 18A). Remarkably, *snf1Δ* cells completely ceased to be affected by antimycin A when grown in 5% glucose (Figure 18B).

Electron flow through the respiratory chain ultimately leads to the synthesis of ATP, therefore we wondered whether the inhibition in a *snf1Δ* strain could influence energy supply. To assess this issue, we measured intracellular content of ATP in wt and *snf1Δ* cells treated with antimycin A for 5 hours, as well as in mock controls. Although antimycin A treatment slightly reduced ATP content of wt cells, indicating a limited role of oxidative phosphorylation in wt fermenting cells, *snf1Δ* cells growing in 2% glucose synthetized a significantly larger fraction of their ATP through this mechanism (Figure 18C). Consistently with the observed protective

role of glucose on the growth of *snf1Δ* cells treated with antimycin A, ATP synthesis was only slightly affected in 5% glucose, as for the wt strain (Figure 18C). In addition, *snf1Δ* cells contained lower basal ATP levels, indicating that unrequired energy consuming processes, possibly fatty acid biosynthesis, are draining energy.

Since a direct consequence of the inhibition of electron transport chain should be a reduction of the capability of mitochondrial NADH pool to be re-oxidized, we measured total NAD^+ and NADH intracellular content (mitochondrial and cytoplasmic) in the same conditions tested above for ATP content. *snf1Δ* cells contained significantly lower amounts of NAD when grown in 2% glucose, with a relative increase of the reduced form (Figure 18D). After treatment with antimycin A, NADH in *snf1Δ* cells significantly increased, consistent with the predicted effect of electron transport chain inhibition (Figure 18D). However, in the same condition NAD^+ content increased accordingly, indicating a compensatory mechanism probably involving the *de novo* NAD(H) biosynthesis. In agreement with the aforementioned results for growth and ATP, NAD^+/NADH levels of *snf1Δ* cells growing in 5% glucose were not influenced by antimycin A treatment (Figure 18D). Remarkably, antimycin A treatment of *snf1Δ* cells grown with lower concentrations of leucine and methionine greatly impairs their growth, in respect to that of cells grown in standard medium (Figure 18E).



E

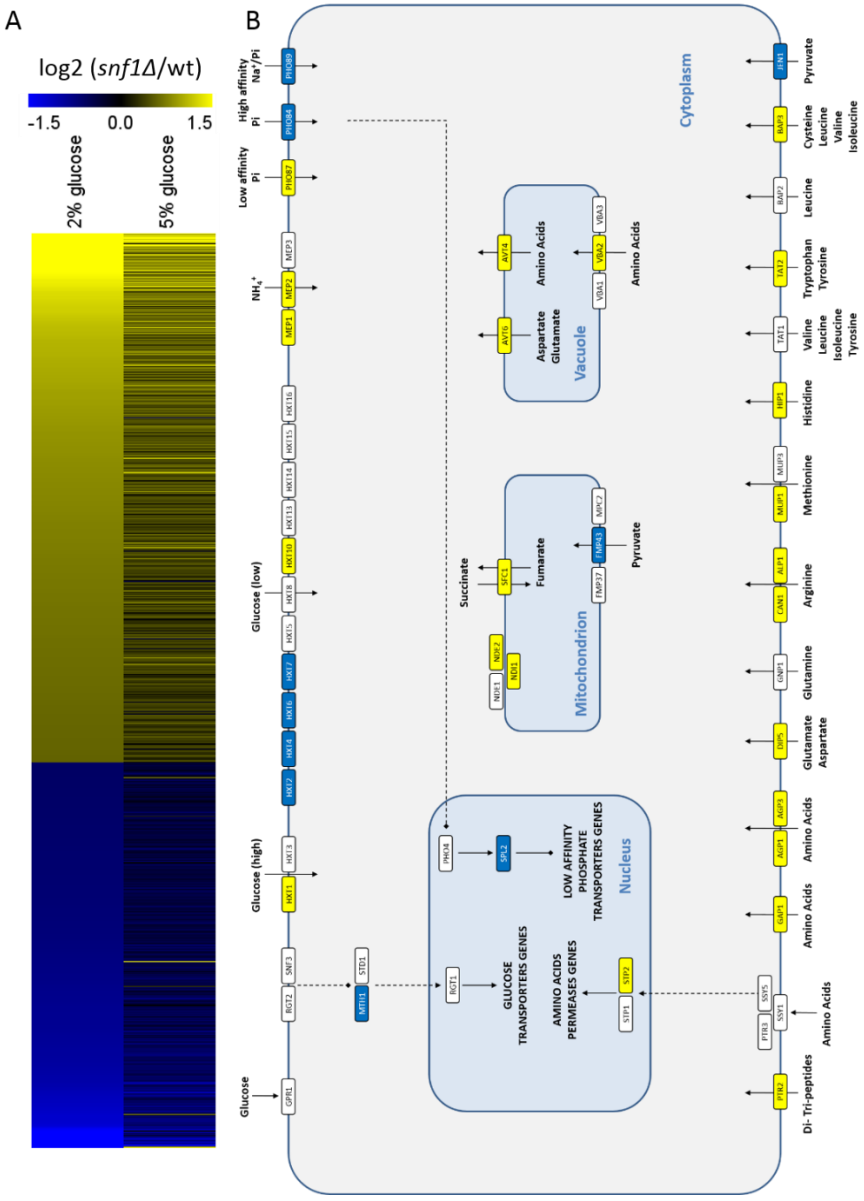
MDT (min)	LM 50 mg/L	LM 10 mg/L
wt 2%	116±8	121±17
wt 2% + ant A	121±10	124±17
snf1Δ 2%	171±9	218±19
snf1Δ 2% + ant A	243±6	365±5

Figure 18. *snf1Δ* cells require oxidative phosphorylation to sustain growth and generate ATP

(A) Growth curve of wt and *snf1Δ* cells in exponential phase in 2% glucose treated and un-treated with antimycin A. (B) Growth curve of wt and *snf1Δ* cells in exponential phase in 5% glucose treated and un-treated with antimycin A. (C)

Intracellular ATP concentration of the indicated strains after 5 hours treatment with antimycin A, un-treated sample shown as mock control. *p-value < 0.05. (D) Intracellular content of NAD and NADH of the indicated strains after 5 hours treatment with antimycin A, un-treated sample shown as mock control. (E) Table showing the mean duplication time (MDT) of wt and *snf1Δ* cells either treated or not with antimycin A and growing in standard medium (leucine and methionine 50 mg/L) or in amino acids limiting conditions (leucine and methionine 10 mg/L).

3.2. Supplementary results



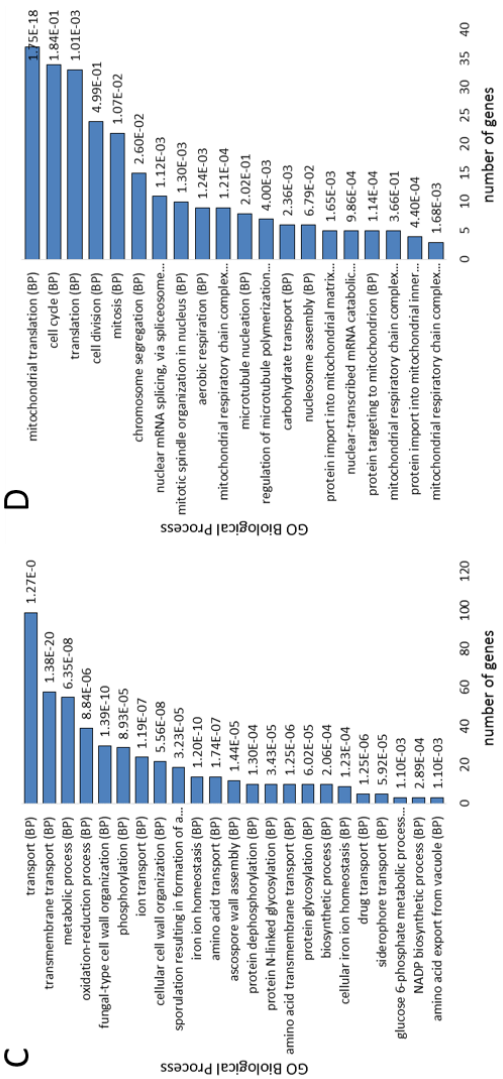


Figure S8.

(A) Heat map showing the log2 fold change of expression of all the differential expressed genes in the dataset. (B) Schematic representation of the differential expressed genes coding for extracellular and extracellular transporters. Yellow boxes indicate up-regulated genes, blue boxes indicate down-regulated genes. (C) GO Biological Process enrichment of the up-regulated genes in *snf1Δ* cells growing in 2% glucose compared to the wt. Enrichment p-value is shown beside each bar. (D) GO Biological Process enrichment of the down-regulated genes in *snf1Δ* cells growing in 2% glucose compared to the wt. Enrichment p-value is shown beside each bar.

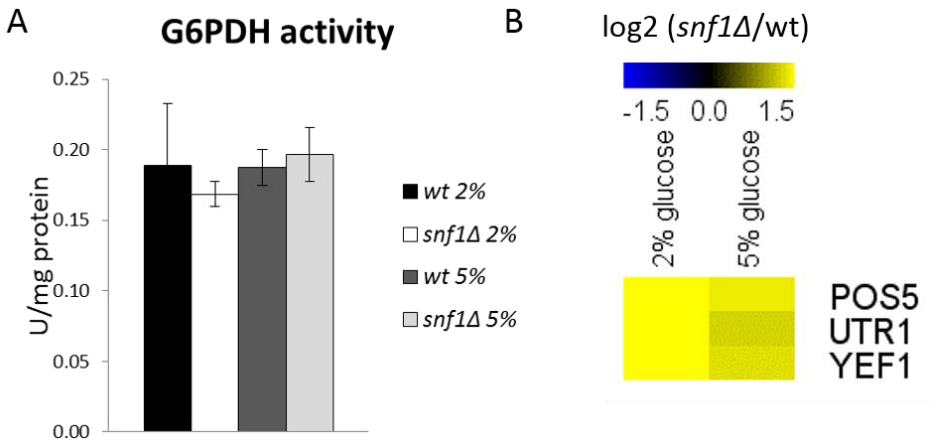


Figure S9.

(A) Enzymatic activity of glucose-6-phosphate dehydrogenase in total protein extracts of the indicated strains. (B) Heat map showing the log2 fold change of expression of the genes coding for the NADH protein kinases.

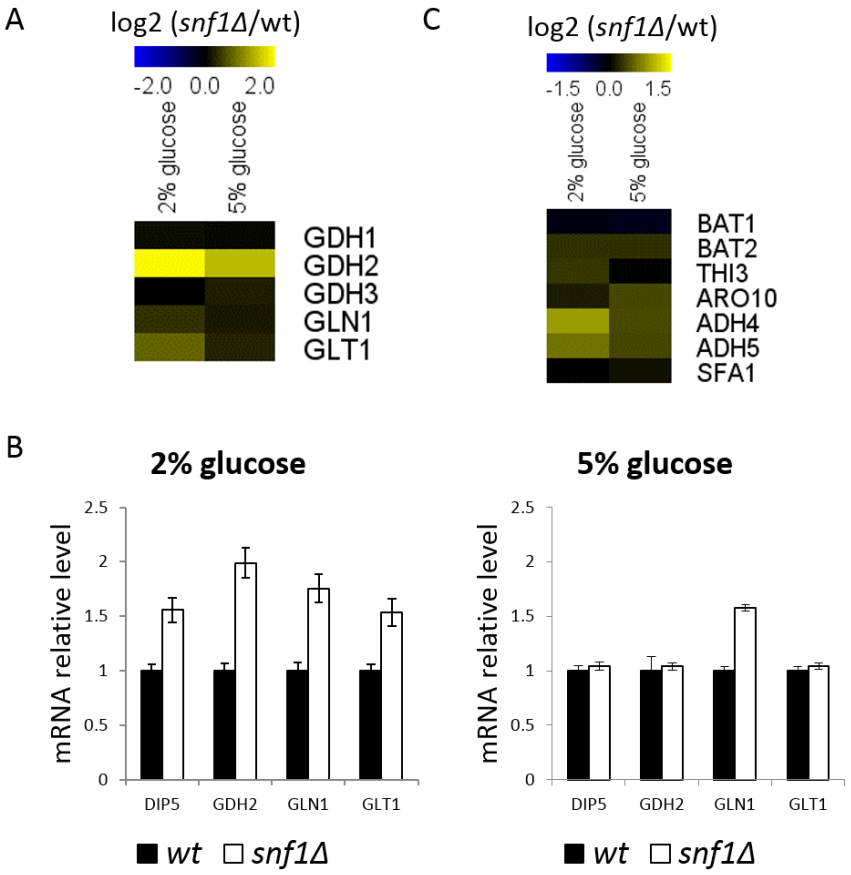


Figure S10.

(A) Heat map showing the log2 fold change of expression of the genes coding for enzymes involved in glutamate metabolism. (B) Real-time PCR validation of expression of genes involved in amino acids transport and glutamate metabolism. (C) Heat map showing the log2 fold change of expression of the genes coding for enzymes involved in the Ehrlich pathway of leucine degradation.

Materials and methods

Yeast strains and growth conditions

S. cerevisiae strains used in this study are listed in Table 1. Synthetic medium contained 2%, 5%, 0.05% glucose, or 2% ethanol (as indicated in Figures) 6.7 g/L of Yeast Nitrogen Base (Difco), 50 mg/L of required nutrients (or less, if indicated), at standard pH (5.5). Cell density of liquid cultures grown at 30°C was determined with a Coulter counter on mildly sonicated and diluted samples or spectrophotometrically at 600 nm. Antimycin A (Sigma) was added at a final concentration of 1 µg/ml (from a 2 mg/ml stock in 100% ethanol); as a control, a culture grown in the presence of the same concentration of ethanol was used. For experiments with the Snf1(I132G)^{as} strain, 25 µM 2NM-PP1 (from a 25 mM stock in 100% DMSO) was added to inhibit the activity of Snf1 (Shirra et al., 2008); as a control, a culture grown in the presence of the same concentration of solvent (0.1% DMSO) was used. Synthesis of compound 2NM-PP1 was carried out as previously described (Busnelli et al., 2013).

Table 1. Yeast strains used in this work

Strain	Genotype	Origin
BY wt[a]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
snf1Δ[a]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 snf1::HPH*	This study
Snf1-9myc[a]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SNF1-9myc:LEU2*	This study

Cyr1-TAP[a]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CYR1-TAP:HIS3*	Open Biosystems
Snf1-myc Cyr1-TAP[a]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SNF1-9myc:LEU2 CYR1-TAP:HIS3*	This study
Snf1-myc Cyr1-TAP reg1Δ[a]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SNF1-9myc:LEU2 CYR1-TAP:HIS3 reg1::URA3*	This study
Snf1-HA Cyr1-TAP[a]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Snf1::HPH [pRS316-SNF1-HA] CYR1-TAP:HIS3*	This study
Snf1-K84R-HA Cyr1-TAP[a]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Snf1::HPH [pRS316-SNF1-K84R-HA] CYR1-TAP:HIS3*	This study
snf1Δ [pRS316][b]	*MATa leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 snf1::HPH [pRS316]*	This study
Snf1-HA[b]	*MATa leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 snf1::HPH [pRS316-SNF1-HA]*	This study
Snf1-G53R-HA[b]	*MATa leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 snf1::HPH [pRS316-SNF1-G53R-HA]*	This study
cyr1Δ pde1Δ yak1Δ[b]	*MATa leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 cyr1::KanMX, pde2::TRP1, yak1::LEU2 [YCplac33]*	(Van de Velde and Thevelein, 2008)
cyr1Δ pde1Δ yak1Δ Cyr1wt[b]	*MATa leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 cyr1::KanMX, pde2::TRP1, yak1::LEU2 [YCplac33-CYR1]*	This study
cyr1Δ pde1Δ yak1Δ Cyr1-S554A,S736A[b]	*MATa leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 cyr1::KanMX, pde2::TRP1, yak1::LEU2 [YCplac33-CYR1-S554A,S736A]*	This study
mig1Δ[a]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mig1::KanMX4*	Euroscarf

[a] *Isogenic to BY4741*
[b] *Isogenic to W303-1A*

Recombinant and genetic techniques

DNA manipulation and yeast transformations were carried out according to standard techniques. To obtain the tagged strains, proteins were tagged with a C-terminal 4HA or 9myc epitope by an in locus 3' in-frame insertion. *E. coli* DH5α and BL21(DE3)[pLysE] were used in cloning

experiments and for expression of recombinant proteins, respectively. The HpaI-XhoI fragment of *CYR1*, originated from the Ycplac33-CYR1 plasmid (Vanhalewyn et al., 1999), was cloned in the HincII-XhoI site of pIVEX2.4a plasmid, originating plasmid pIVEX-Cyr1(335-1066) or pIVEX2.4a-Cyr1HX (Figure 19). Mutant CYR1-S554A,S736A gene was obtained by site-directed mutagenesis of plasmid pIVEX2.4a-Cyr1HX, using customly designed primers, and sub-cloning in the original Ycplac33-CYR1 plasmid.

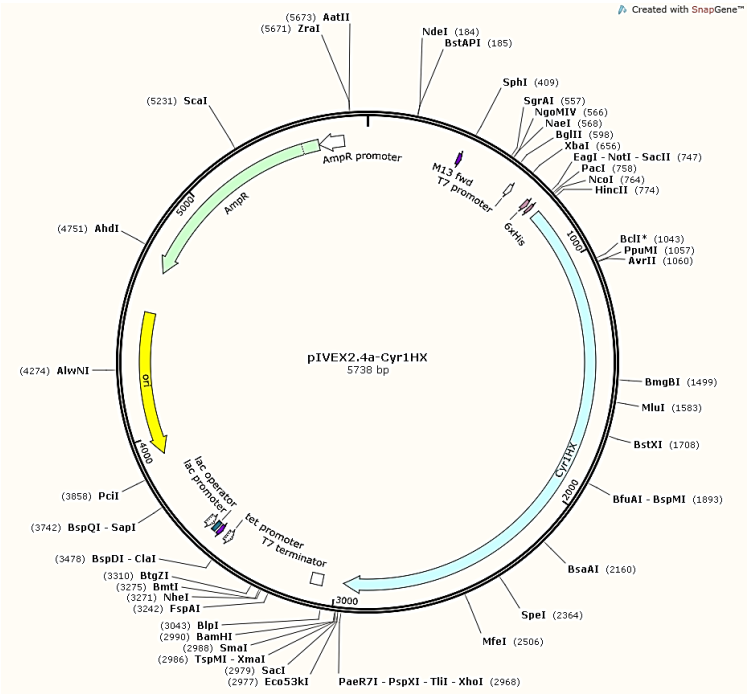


Figure 19. pIVEX2.4a-Cyr1HX plasmid

Recombinant proteins purification

Escherichia coli strain BL21(DE3)[pLysE] was transformed with pIVEX2.4a-Cyr1HX, cultured in Luria-Bertani broth with 100 mg/L of ampicillin and 34 mg/L of chloramphenicol at 37°C (OD_{600nm} = 0.3), and induced for 2 h with 1mM IPTG (isopropyl thio-β-d-galactoside) at 30°C. His_6-Cyr1HX was purified on Ni^{2+}/NTA beads (Qiagen) and eluted serially with 200, 400 and 800 mM imidazole. Protein concentration was measured by Bradford method using Bio-Rad protein assay kit (Bio-Rad). The purified proteins were dialyzed against a buffer containing 20 mM HEPES pH 7, 100 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 5mM MgAc, 5% glycerol and stored at -80°C.

Protein extraction, immunoblotting and immunoprecipitation.

Samples of cells were harvested and lysed using ice-cold lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% NP-40, 10% glycerol) plus 1 mM PMSF, proteases inhibitor mix (Complete EDTA free Protease Inhibitor Cocktail Tablets, Roche) and phosphatase inhibitor mix (Sigma). An equal volume of acid-washed glass beads (Sigma) was added and cells were broken by 20 vortex/ice cycles of 1 min each. Extracts were transferred to new tubes and clarified by centrifugation. Protein concentration was determined using the Bio-Rad protein assay. Crude protein extracts were incubated 2h at 4°C with anti-HA affinity matrix (Roche) to immunoprecipitate Snf1-HA

protein complexes or with IgG-agarose beads (Sigma) to immunopurify Cyr1-TAP. The immunocomplexes obtained after removal of supernatants were washed 3 times with lysis buffer and once with 25 mM MOPS pH 7.5. SDS-sample buffer was then added to the beads and the samples were heated for 5 min at 95°C. Western blot analysis was performed using anti-TAP antibody (1:2500, Thermo Scientific, Open BioSystems), anti-HA antibody (1:1000, 12CA5, Roche) or anti-myc antibody (1:1000, 9E10, Santa Cruz).

Gene Chip® Analysis

Total RNA concentration and purity of yeast samples was assessed by spectrophotometer (Nanodrop) evaluating 260/280 and 260/230 ratios. Total RNA integrity was assessed by Agilent Bioanalyzer. An aliquot (300 ng) of RNA was used for the preparation of targets for Affymetrix® Yeast Genome 2.0 arrays, according to the Ambion MessageAmp III RNA amplification kit manual. Affymetrix® Yeast Genome 2.0 arrays (which contain 10928 genes) were purchased from Affymetrix (Affymetrix, USA). The staining, washing and scanning of the arrays were conducted using the Fluidics 450 station, Command Console Software and GeneChip® Scanner 3000 7G, generating .CEL files for each array (Affymetrix, USA). The images were scanned by Affymetrix GeneChip Command Console (AGCC) and analyzed with the Affymetrix GeneChip Expression Console.

The quality control of the scanned data was first estimated by confirming the order of the signal intensities of the Poly-A and Hybridization controls using Expression Console Software (Affymetrix, USA). Raw expression values were imported as Affymetrix .CEL files into Partek Genomics Suite 6.6 (Partek Inc., St. Louis, MO, USA), were analyzed and normalized, including the Preprocessing, Differentially Expressed Genes (DEGs) Finding and Clustering modules. The .CEL files were uploaded and normalized in PM (perfect match)-only conditions as a PM intensity adjustment. A Robust Multichip Analysis (RMA) quantification method (Irizarry et al., 2003) was used as a probe set summarization algorithm for log transformation with base 2 (log2) and the Quantile normalization method was chosen to evaluate the preliminary data quality in the Preprocessing module, which functions as a data quality control through the Affymetrix Expression Console Software. The mean signal intensities of all genes were obtained using a group of arrays for each condition. After normalization, the differentially expressed genes (DEGs) satisfying the conditions of the Fold Change settings (FC = ± 1.5) from all of the genes probed in the array were selected in the DEGs Finding module. Hierarchical cluster analysis was also performed to see how data aggregated and to generate heat maps.

RNA purification and qReal-time PCR

Total RNA was isolated using a phenol-chloroform protocol, essentially as previously described (Pessina et al., 2010). Obtained data were normalized to both *ACT1* and *CDC34* reference genes and organized with CFX manager software (Bio-Rad). Data are presented as the mean value ± described (Pessina et al., 2010). Reverse transcription of 0.5 µg of mRNAs was carried out with iScript cDNA Synthesis Kit (BIO-RAD). Quantitative Real-time PCR for gene expression was performed using SsoFast EvaGreen Supermix standard deviation from at least three independent experiments performed in duplicates.

Metabolites extraction and analysis

Metabolic profiling by ^{1}H-NMR of intracellular and extracellular metabolites was performed as previously described (Airoldi et al., 2014). Fatty acids were extracted and analyzed by GC-MS as in (Khoomrung et al., 2012). Analysis of intracellular organic acids was carried out as in (Khoomrung et al., in preparation). Extracellular levels of glucose, ethanol, acetate and amino acids were evaluated by ^{1}H-NMR on growth media (Airoldi et al., 2014).

ATP and NAD^+/NADH assays

ATP was measured using the CellTiter-Glo® Luminescent Assay (Promega) following the manufacturer's instructions, using 100 µl of a 0.3 OD culture. NAD^+ and NADH were measured using the EnzyChrom™ NAD/NADH Assay Kit (Medibena), following the manufacturer's instructions.

Enzymatic assays

For enzymatic assays, cells were rapidly collected by filtration and frozen at -80°C. Total protein extracts were obtained disrupting the cells with glass beads in the buffer appropriate for each assay, protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad) and enzymatic assays were performed on equal amount of protein extracts. Hexokinase activity was assayed essentially as previously described (Bergmeyer et al., 1983). Enolase and isocitrate dehydrogenase activities were assayed essentially as described (Bergmeyer et al., 1983). Malate dehydrogenase was assayed essentially as previously described (Bergmeyer et al., 1983). Glyceraldehyde-3-phosphate dehydrogenase activity assay was performed essentially as described (Delgado et al., 2001). Glucose-6-phosphate dehydrogenase activity was measured essentially as in (Noltmann et al., 1961). Isocitrate lyase activity was assayed as in (Chell et al., 1978). Glutamate synthase (GOGAT) activity was

assayed as in (Cogoni et al., 1995). Glutamic dehydrogenase activity was assayed essentially as in (Shimizu et al., 1979).

cAMP assay

cAMP was measured using the cAMP Biotrak EIA Assay (GE Healthcare) following the manufacturer's instructions. Sample preparation was carried out as in (Petkova et al., 2010).

***In vitro* phosphorylation assay**

In vitro phosphorylation of recombinant His_6-Cyr1 (4 µg of purified protein) was performed in a buffer containing 20 mM HEPES pH 7, 100 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 5mM MgAc, 5% glycerol using protein kinase Snf1-HA immunopurified from exponentially growing yeast cells. The reaction was started by adding 0.24 µM [γ-^{32}P] ATP (specific radioactivity 2000 cpm/pmol) and incubated at 30°C for 30 min. The reaction was stopped by adding 4× SDS sample buffer, then heated for 5 min at 95°C and proteins were separated by SDS-PAGE. Phosphorylated bands were visualized by autoradiography.

Bioinformatic analyses and data visualization

GO and KEGG enrichment was performed with GeneCodis (Tabas-Madrid et al., 2012). Network data visualization was performed with Cytoscape

(Shannon et al., 2003). Protein sequence and phosphosites visualization was performed with DOG (Ren et al., 2009). Target-Kinase associations were retrieved in KID, Yeast Kinase Interaction Database (Sharifpoor et al., 2011).

IP/MS

Bands identified as Cyr1 by immunodetection, were excised after silver staining, reduced with dithiothreitol (DTT) and alkylated with 55 mM iodoacetamide at room temperature for 45 minutes. Bands were dried, soaked with ammonium bicarbonate 0.1 M and digested overnight with trypsin sequence grade, at 37 °C using a protease: protein ratio (1:10). Tryptic digests were extracted with 50% ACN in 0.1% TFA, desalted/concentrated on a µZipTipC18 (Millipore) and analyzed by mass spectrometry.

LC-ESI-MS/MS analysis was performed on a Dionex UltiMate 3000 HPLC System with a PicoFrit ProteoPrep C18 column (200 mm, internal diameter of 75 µm) (New Objective, USA). Gradient:1% ACN in 0.1 % formic acid for 10 min, 1-4 % ACN in 0.1% formic acid for 6 min, 4-30% ACN in 0.1% formic acid for 147 min and 30-50 % ACN in 0.1% formic for 3 min at a flow rate of 0.3 µl/min. The eluate was electrosprayed into an LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany) through a Proxeon nanoelectrospray ion source (Thermo Fisher Scientific). Data acquisition

was controlled by Xcalibur 2.0 and Tune 2.4 software (Thermo Fisher Scientific). The LTQ-Orbitrap was operated in positive mode in data-dependent acquisition mode to automatically alternate between a full scan (m/z 350-2000) in the Orbitrap (at resolution 60000, AGC target 1000000) and subsequent CID MS/MS in the linear ion trap of the 20 most intense peaks from full scan (normalized collision energy of 35%, 10 ms activation).

Data Base searching was performed using the Sequest search engine contained in the Proteome Discoverer 1.1 software (Thermo Fisher Scientific). The following parameters were used: 10 ppm for MS and 0.5 Da for MS/MS tolerance, carbamidomethylation of Cys as fixed modification; oxidation of Met and phosphorylation of Ser, Tyr and Thr, as variable modifications, trypsin (2 misses) as protease.

Discussion

Snf1 is a serine/threonine kinase widely recognized as required by the yeast *Saccharomyces cerevisiae* to adapt to nutrient limitation and to utilize alternative carbon sources, such as sucrose and ethanol (Hedbacker and Carlson, 2008). Being Snf1 a kinase whose orthologues in higher eukaryotes retain important functions as guardians of energy homeostasis, the study of the implications of alterations in the Snf1 pathway are important not only for yeast itself but also as a model, for example for human diseases (Hardie, 2007). Our recent finding of the cell cycle defect of *snf1Δ* cells in a condition of glucose sufficiency (Pessina et al., 2010) raised the challenge of a completely new investigation framework. The work performed and presented in this thesis, therefore, deals with different aspects of the role of Snf1 in the absence of nutritional distress.

To clarify the role of Snf1 in the regulation of cell cycle, we aimed to determine the extent of the defect of *snf1Δ* cells using a different method of synchronization from that used before (Pessina et al., 2010). Synchronizing the cells by centrifugal elutriation, we highlighted the strong delay in the G_1/transition of a *snf1Δ* strain (Figure 5). In consequence of the observation that a catalytic deficient isoform of Snf1 (Snf1-K84R) presents a somewhat intermediate cell cycle phenotype (Busnelli, 2013), we sought to determine the precise involvement of the catalytic activity of the enzyme in guaranteeing a proper G_1/S transition.

By using a combinatory approach of cell synchronization (both in α-factor and elutriation experiments) and chemical inhibition of an ATP-analog sensitive isoform of Snf1, we demonstrated that the cell cycle defect is an immediate consequence of the loss of Snf1 catalytic activity (Figure 6 and 7). These findings, together with a detailed analysis carried out in our lab of the alterations occurring at the molecular level at the promoters of G_1 genes in *snf1Δ* cells (Busnelli et al., 2013), provided a neat picture of the involvement of Snf1 in the regulation of the G_1/S transition of the cell cycle.

A major challenge in the understanding of a signaling pathway is the discovery of crosstalks with other pathways. Being Snf1 mainly known for its role in shaping metabolism to guarantee cell growth in nutritional scarceness, it seemed likely that it could regulate other signaling pathways that cause opposite outcomes. In search for new targets of Snf1, we identified a set of previously unknown Snf1 interacting proteins (Table S1 and Figure S3), among which adenylate cyclase (Cyr1), suggesting a possible crosstalk between the Snf1 and PKA pathways. PKA was a possible candidate as a Snf1-regulated pathway, because it is activated in conditions of nutrient abundance. The two pathways share common downstream targets, such as transcription factor Adr1 (Cherry et al., 1989; Görner et al., 1998, 2002; Ratnakumar et al., 2009; De Wever et al., 2005), and PKA has been reported to regulate the recruitment of Snf1 to the

vacuole and inactivate the Snf1-activating kinase Sak1 (Barrett et al., 2012; Hedbacker et al., 2004). This regulation seem to be evolutionary conserved, since in mammalian cells PKA phosphorylates and inactivates the Snf1 orthologue AMPK (Djouder et al., 2010). Thus, by hypothesizing a regulation of the PKA pathway by Snf1, we were suggesting a novel crosstalk mechanism. Indeed, we demonstrated that the absence of Snf1 could alter the phosphorylation status of Cyr1 *in vivo*, and that it can phosphorylate an important regulatory domain of adenylate cyclase *in vitro* (Figure 9 and 10). However, the lack of evidence for most of the identified phosphosites of being Snf1 targets, introduced a higher degree of complexity to the situation, suggesting the existence of other kinases acting as Snf1 downstream effectors. Strikingly, by using a constitutively activated isoform of Snf1, we highlighted an inverse correlation between the activation state of the kinase and the intracellular content of cAMP (Figure 11). On the contrary, we failed to determine any alteration in a mutant carrying a double serine to alanine substitution of two putative Snf1 phosphorylation sites on the RAD domain of Cyr1 (Figure S6). However, in this case the cAMP content was evaluated, for technical reasons, in a strain lacking the phosphodiesterase Pde2, which has been reported to have increased basal levels of the cyclic nucleotide (Ma et al., 1999), thus possibly flattening the differences between the strains. To determine whether the activity of Snf1 had actual influence on PKA

activity, we have chosen to establish a method based on the expression of an endogenous gene used as a reporter. The gene *HXT7*, identified as a target of the PKA-repressed transcription factors Msn2 and Msn4 (Livas et al., 2011), demonstrated to be suitable for the objective, since we checked the PKA-dependent repression of *HXT7* by using a strain lacking adenylate cyclase (Figure S7A-B), and its independence from direct Snf1 activity in a *mig1Δ* strain (Figure S7C). Moreover, *HXT7* has already been reported to be Snf1-independent (Zaman et al., 2009), neither the interaction of transcription factors such as Mig1 with the promoter of *HXT7* is reported in specialized databases. *HXT7* resulted to be dramatically derepressed in a Snf1-G53R strain (Figure 12), thus linking Snf1 activation and transcription of PKA-dependent genes. *HXT7* codes for a high affinity glucose transporter, specifically expressed in low glucose media (Reifenberger et al., 1995), and the dependence of its expression from an interplay between the PKA and Snf1 opposing pathways seems therefore biologically consistent. Altogether, these findings (summarized in Figure 20) added another link in the circuitry involving two major signaling pathways. The regulation of PKA pathway by Snf1 seems likely to occur in nutrient limiting conditions, but the study in glucose abundance medium allowed the use of the otherwise lethal mutant *snf1Δ* and the activated mutant Snf1-G53R in the absence of a high basal activity of Snf1.

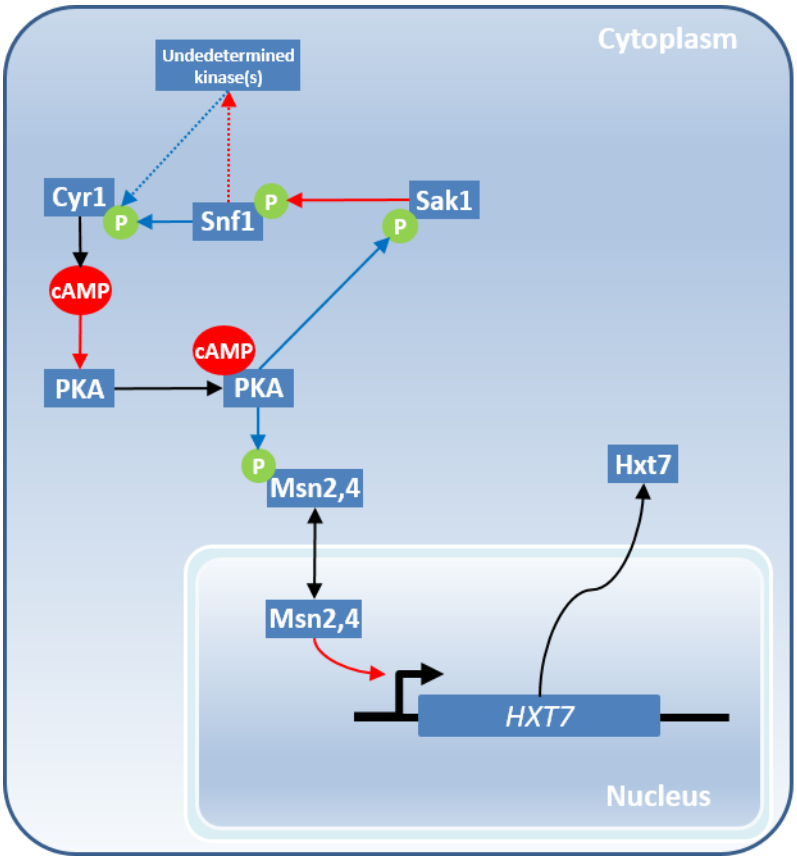


Figure 20. Map of the crosstalk between the Snf1 and PKA pathways

Schematization of the basic circuitry of the crosstalk between the Snf1 and PKA pathway, applied to the expression of the PKA-repressed gene *HXT7*. Subsidiary pathways were omitted for simplification. Red and blue arrows indicate activatory and inhibitory functions, respectively.

The last part of the presented work deepens the understanding of the involvement of signaling pathways in the regulation of metabolism of proliferating cells, by taking a snapshot of Snf1 deficient yeast cells without nutritional distress. Our analysis showed that even in condition of nutrient availability, the lack of Snf1 causes rearrangements at multiple levels (summarized in Figure 21), notably resembling the lack of proper AMPK activity in mammalian cells (Faubert et al., 2014a).

Recent studies evidenced that the activation of AMPK is involved in growth control and tumorigenesis and that ablation of the catalytic subunit AMPKα1 promotes glucose uptake and aerobic glycolysis to support proliferation of cancer cells (Faubert et al., 2013, 2014b). Here, we show that glycolysis of *snf1Δ* yeast cells is functionally enhanced (Figure 14), with increased secretion in the media of ethanol and acetate as compared to the wt, although no enhanced glucose consumption can be observed in 2% glucose. This enhanced glycolytic function is accompanied by transcriptional up-regulation of several genes of the pathway in *snf1Δ* cells growing in 2% glucose (Figure 13), but we suggest that this could be an adaptation phenomenon induced by the glycolytic flux, as observed also in mammalian cells (Roche et al., 1997). Notably, it was recently observed that mutants of the Snf1 pathway present a thinner cell wall and a higher susceptibility to agents targeting this structure (Backhaus et al., 2013). This effect is nicely reverted by deletion of *PFK1*,

which restores the glucose-6-phosphate pool, which is reduced in *snf1Δ* cells. In light of our findings, we propose that the reduced accumulation of cell wall glucose derivatives could be due to the draining of glycolytic intermediates and their funneling to the ending products.

In proliferating cells, a conspicuous aliquot of carbon is diverted into lipids to support membrane biosynthesis and as biomass and energy reservoir (Vander Heiden et al., 2009). Consistent with its role of keeper of cellular energy, AMPK has evolved as a negative regulator of fatty acids biosynthesis to avoid this energetically expensive process when nutrients are scarce (Hardie, 2007) and this feature is conserved also in yeast cells (Borklu Yucel and Ulgen, 2013; Usaite et al., 2009). In particular, both Snf1 and AMPK regulate the first step of fatty acid biosynthesis in a conserved way by direct phosphorylation of acetyl-CoA carboxylase (Acc1 and ACC1, respectively) (Davies et al., 1990; Shi et al., 2014; Shirra et al., 2001). Noteworthy, yeast exclusively use acetate activated in the cytoplasm to provide acetyl-CoA for fatty acids synthesis, since unlike plants and animals it lacks the citrate lyase activity to fuel this process through the TCA cycle (Hynes and Murray, 2010). Therefore, given that yeast fully relies on glycolysis for this process, we propose that *snf1Δ* cells need to increase the glycolytic flux (Figure 14) to sustain the hyper-accumulation of fatty acids (Figure 15).

The synthesis of fatty acids is not only expensive in terms of carbon and energy, but it also exploits the reducing equivalent (NADPH) pool of the cell (Tehlivets et al., 2007), therefore it is likely that *snf1Δ* cells face a problem in balancing its NADPH/$NADP^+$ ratio. Even if the pentose-phosphate shunt is the main pathway for $NADP^+$ reduction, it is disadvantageous for energy generation and we lack direct evidence of its enhancement in *snf1Δ* cells. Instead, the limited NADPH pool probably drives *snf1Δ* cells to down-regulate the processes which utilize the cofactor, as we show for glutamate biosynthesis via Gdh1 (Figure 16D). Interestingly, exceeding glutamate is known to activate its degradation to succinate via γ-aminobutyric acid (GABA), involving the reduction of $NADP^+$ to NADPH (Ramos et al., 1985). Therefore, it is possible that this phenomenon is occurring in *snf1Δ* cells, concomitantly obtaining NADPH and fueling the TCA cycle.

In the absence of glucose, Snf1 negatively regulates the translation of the transcription factor Gcn4, thus inhibiting the expression of a regulon of genes involved in amino acids biosynthesis (Shirra et al., 2008). In our gene chip analysis, that regulon is de-repressed even in *snf1Δ* cells growing in 2% glucose, suggesting that Snf1 effect on Gcn4 can be extended also to non-limiting nutrient conditions (Figure S8B). Furthermore, we show that alterations of amino acids metabolism are actually relevant for *snf1Δ* cells, the most evident being a significant intracellular accumulation of

glutamate (Figure 16A). Glutamate is pivotal in the biosynthesis of many other amino acids, and conversely it is one of the products of their degradation. Amino acids supplemented in the medium can be used by the cells not only as nitrogen source, but also as carbon source, via degradation to glutamate, and this has been described especially for leucine and methionine (Hothersall and Ahmed, 2013; Perpète et al., 2006). In this framework, the reliance of *snf1Δ* cells on leucine and methionine uptake (Figure 17A) demonstrated by the impairment of growth when these amino acids are reduced in the medium (Figure 17B-C), links leucine and methionine assimilation to compensation of carbon and energy waste (Figure 21).

Utilization of glutamate as carbon source requires its conversion to α-ketoglutarate and this process seems to be favored in *snf1Δ* cells at least at the transcriptional level (Figure S10A-B). Continuation of this process essentially requires the execution of a part of the TCA cycle, in keeping with the accumulation of malate in *snf1Δ* cells (Figure 16C). Moreover, inhibition of re-oxidation of mitochondrial NADH (treating with antimycin A) is detrimental for *snf1Δ* cells, since it coordinately affects growth, ATP and NAD^+/NADH contents (Figure 18). It was recently reported that Snf1 is required to face the impaired respiratory function caused by loss of mitochondrial DNA in stationary phase (Friis et al., 2014). Our findings

complement this notion, indicating that lack of Snf1 causes an increase of cellular dependence of mitochondrial function.

The common feature of the phenotypes described for *snf1Δ* cells in this paper is their reversion when cells grow in the presence of 5% glucose. Even if 2% glucose is non-limiting for fermentative growth, increase of glucose concentration to 5% results in enhanced glycolytic flux in wt cells (Figure 2C). In addition, glucose uptake is higher in *snf1Δ* cells grown in 5% glucose (Figure 2B). This finding strongly suggests that enhanced glucose metabolism satisfies the requirements of *snf1Δ* cells and abolishes their reliance on amino acids utilization. Noteworthy, *snf1Δ* cells have been shown to have impaired intracellular acetyl-CoA levels and subsequently reduced histone acetylation, a phenotype that could be at least partially reverted by the replenishment of acetyl-CoA pool (Zhang et al., 2013). Increased carbon flux provided in 5% glucose could therefore account for increased supply of acetyl-CoA, which, as described above, is directly subtracted for fatty acids biosynthesis (Hynes and Murray, 2010). This could relieve *snf1Δ* cells from effects of reduced histone acetylation on gene expression, which has been pointed out as particularly important at growth genes such as Cln3 (Shi and Tu, 2013). This hypothesis well fits our previous findings of *snf1Δ* cells having a cell cycle defect in the G_1 to S transition, accompanied by the impairment of the transcription of G_1-specific genes (Busnelli et al., 2013; Pessina et al., 2010).

In conclusion, here we describe the rewiring occurring in the metabolic network of cells lacking Snf1, showing remarkable resemblances with mammalian cells deprived of AMPK and identifying a strategy adopted by these cells to guarantee growth. It has been pointed out that although AMPK loss of function alone is not sufficient to cause transformation, mutations of the kinase are not infrequent in cancers (Faubert et al., 2014a). However, lack of functional AMPK causes ambivalent effects, because despite the loss of its catalytic activity enhances tumorigenesis, it also deprives the cells of a critical pathway for the response to environmental stresses. It is not surprising, therefore, that AMPK has been indicated as a target for cancer therapy (Hardie, 2013). In this work, we establish the usefulness of yeast in the study of a signaling pathway which until now showed marginal similarities with its relative in higher eukaryotes. The metabolic strategies adopted by Snf1/AMPK deficient cells to support proliferation, as the increase of the utilization of amino acids as carbon source reported here, are reminiscent of the increased glutamine utilization that, together with an enhanced glycolysis, is the hallmark of several cancer cells (Alberghina and Gaglio, 2014)

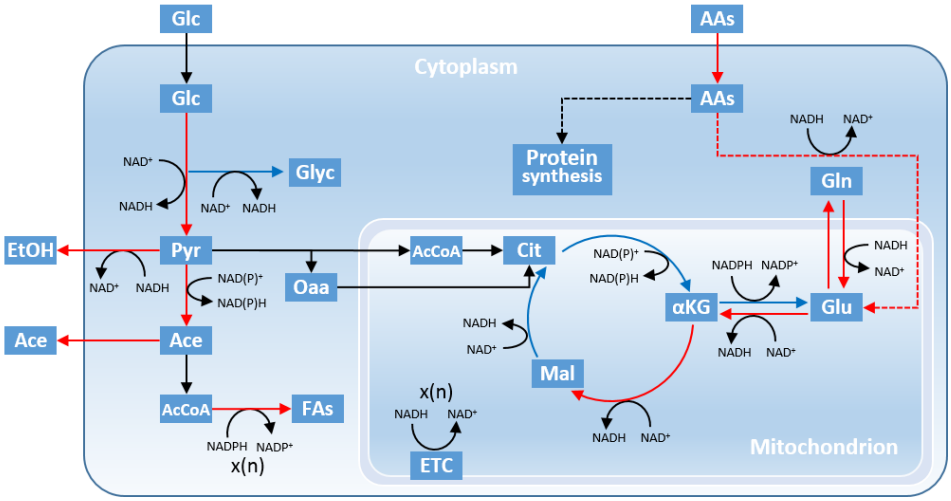


Figure 21. Map of the metabolic rewiring occurring upon Snf1 loss

Schematization of the metabolic rewiring occurring upon Snf1 loss. Red and blue arrows indicate processes for which there is evidence at different levels (transcriptional, enzymatic, product concentration) of enhancement or reduction, respectively.

Summarizing, this work provided new insights in both the causes and the consequences of several issues of cells with altered Snf1 function, establishing the kinase as a strong candidate as a coordinator of several cellular processes. Furthermore, we generated datasets whose further exploitation will be interesting in the future perspective of the project. For example, we provided a high resolution map of the phosphorylation pattern of adenylate cyclase, which indeed will be useful in further studies

of the regulation of the enzyme, also by feedback mechanisms. Moreover, the identified Snf1 interactors, among which several glycolytic genes, show the up to now not tackled opportunity of an integrated analysis together with the mapped transcriptional deregulation.

References

References

Abate, G., Bastonini, E., Braun, K.A., Verdone, L., Young, E.T., and Caserta, M. (2012). Snf1/AMPK regulates Gcn5 occupancy, H3 acetylation and chromatin remodelling at S. cerevisiae ADY2 promoter. Biochim. Biophys. Acta *1819*, 419–427.

Ahuatzi, D., Riera, A., Peláez, R., Herrero, P., and Moreno, F. (2007). Hxk2 regulates the phosphorylation state of Mig1 and therefore its nucleocytoplasmic distribution. J. Biol. Chem. *282*, 4485–4493.

Airoldi, C., Tripodi, F., Guzzi, C., Nicastro, R., and Coccetti, P. (2014). NMR analysis of budding yeast metabolomics: a rapid method for sample preparation. Mol. Biosyst.

Alberghina, L., and Gaglio, D. (2014). Redox control of glutamine utilization in cancer. Cell Death Dis. *5*, e1561.

Albuquerque, C.P., Smolka, M.B., Payne, S.H., Bafna, V., Eng, J., and Zhou, H. (2008). A multidimensional chromatography technology for in-depth phosphoproteome analysis. Mol. Cell. Proteomics *7*, 1389–1396.

Ansell, R., Granath, K., Hohmann, S., Thevelein, J.M., and Adler, L. (1997). The two isoenzymes for yeast NAD+-dependent glycerol 3-phosphate dehydrogenase encoded by GPD1 and GPD2 have distinct roles in osmoadaptation and redox regulation. EMBO J. *16*, 2179–2187.

Ashrafi, K., Lin, S.S., Manchester, J.K., and Gordon, J.I. (2000). Sip2p and its partner snf1p kinase affect aging in S. cerevisiae. Genes Dev. *14*, 1872–1885.

Backhaus, K., Rippert, D., Heilmann, C.J., Sorgo, A.G., de Koster, C.G., Klis, F.M., Rodicio, R., and Heinisch, J.J. (2013). Mutations in SNF1 complex genes affect yeast cell wall strength. Eur. J. Cell Biol. *92*, 383–395.

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

Benanti, J.A., Cheung, S.K., Brady, M.C., and Toczyski, D.P. (2007). A proteomic screen reveals SCFGrr1 targets that regulate the glycolytic-gluconeogenic switch. Nat. Cell Biol. *9*, 1184–1191.

Bergmeyer, H., Grassl, M., and Walter, H. (1983). Methods of Enzymatic Analysis (Verlag Chemie).

Bisson, L.F., Coons, D.M., Kruckeberg, A.L., and Lewis, D.A. (1993). Yeast sugar transporters. Crit. Rev. Biochem. Mol. Biol. *28*, 259–308.

Borklu Yucel, E., and Ulgen, K.O. (2013). Assessment of crosstalks between the Snf1 kinase complex and sphingolipid metabolism in S. cerevisiae via systems biology approaches. Mol. Biosyst. *9*, 2914–2931.

Breitkreutz, A., Choi, H., Sharom, J.R., Boucher, L., Neduva, V., Larsen, B., Lin, Z.-Y., Breitkreutz, B.-J., Stark, C., Liu, G., et al. (2010). A global protein kinase and phosphatase interaction network in yeast. Science *328*, 1043–1046.

Brisson, D., Vohl, M.C., St-Pierre, J., Hudson, T.J., and Gaudet, D. (2001). Glycerol: a neglected variable in metabolic processes? Bioessays *23*, 534–542.

Bro, C., Regenberg, B., and Nielsen, J. (2004). Genome-wide transcriptional response of a Saccharomyces cerevisiae strain with an altered redox metabolism. Biotechnol. Bioeng. *85*, 269–276.

Broach, J.R. (2012). Nutritional control of growth and development in yeast. Genetics *192*, 73–105.

Busnelli, S. (2013). Protein Kinase Snf1/AMPK: a new regulator of G1/S transition in Saccharomyces cerevisiae. Università degli Studi di Milano-Bicocca.

Busnelli, S., Tripodi, F., Nicastro, R., Cirulli, C., Tedeschi, G., Pagliarin, R., Alberghina, L., and Coccetti, P. (2013). Snf1/AMPK promotes SBF and MBF-dependent transcription in budding yeast. Biochim. Biophys. Acta *1833*, 3254–3264.

Busti, S., Coccetti, P., Alberghina, L., and Vanoni, M. (2010). Glucose Signaling-Mediated Coordination of Cell Growth and Cell Cycle in Saccharomyces Cerevisiae. Sensors *10*, 6195–6240.

Cardon, C.M., and Rutter, J. (2012). PAS kinase: integrating nutrient sensing with nutrient partitioning. Semin. Cell Dev. Biol. *23*, 626–630.

Carlson, M., Osmond, B.C., and Botstein, D. (1981). Mutants of yeast defective in sucrose utilization. Genetics *98*, 25–40.

Celenza, J.L., and Carlson, M. (1989). Mutational analysis of the Saccharomyces cerevisiae SNF1 protein kinase and evidence for functional interaction with the SNF4 protein. Mol. Cell. Biol. *9*, 5034–5044.

Chandrashekarappa, D.G., McCartney, R.R., and Schmidt, M.C. (2011). Subunit and domain requirements for adenylate-mediated protection of Snf1 kinase activation loop from dephosphorylation. J. Biol. Chem. *286*, 44532–44541.

Chell, R.M., Sundaram, T.K., and Wilkinson, A.E. (1978). Isolation and characterization of isocitrate lyase from a thermophilic Bacillus sp. Biochem. J. *173*, 165–177.

Chen, L., Jiao, Z.-H., Zheng, L.-S., Zhang, Y.-Y., Xie, S.-T., Wang, Z.-X., and Wu, J.-W. (2009). Structural insight into the autoinhibition mechanism of AMP-activated protein kinase. Nature *459*, 1146–1149.

Cherry, J.R., Johnson, T.R., Dollard, C., Shuster, J.R., and Denis, C.L. (1989). Cyclic AMP-dependent protein kinase phosphorylates and inactivates the yeast transcriptional activator ADR1. Cell *56*, 409–419.

Cogoni, C., Valenzuela, L., González-Halphen, D., Olivera, H., Macino, G., Ballario, P., and González, A. (1995). Saccharomyces cerevisiae has a single glutamate synthase gene coding for a plant-like high-molecular-weight polypeptide. J. Bacteriol. *177*, 792–798.

Collins, S.R., Kemmeren, P., Zhao, X.-C., Greenblatt, J.F., Spencer, F., Holstege, F.C.P., Weissman, J.S., and Krogan, N.J. (2007). Toward a comprehensive atlas of the physical interactome of Saccharomyces cerevisiae. Mol. Cell. Proteomics *6*, 439–450.

Colombo, S., Ma, P., Cauwenberg, L., Winderickx, J., Crauwels, M., Teunissen, A., Nauwelaers, D., de Winde, J.H., Gorwa, M.F., Colavizza, D., et al. (1998). Involvement of distinct G-proteins, Gpa2 and Ras, in glucose- and intracellular acidification-induced cAMP signalling in the yeast Saccharomyces cerevisiae. EMBO J. *17*, 3326–3341.

Coons, D.M., Vagnoli, P., and Bisson, L.F. (1997). The C-terminal domain of Snf3p is sufficient to complement the growth defect of snf3 null mutations in Saccharomyces cerevisiae: SNF3 functions in glucose recognition. Yeast *13*, 9–20.

Cytryńska, M., Frajnt, M., and Jakubowicz, T. (2001). Saccharomyces cerevisiae pyruvate kinase Pyk1 is PKA phosphorylation substrate in vitro. FEMS Microbiol. Lett. *203*, 223–227.

Dale, S., Wilson, W.A., Edelman, A.M., and Hardie, D.G. (1995). Similar substrate recognition motifs for mammalian AMP-activated protein kinase, higher plant HMG-CoA reductase kinase-A, yeast SNF1, and mammalian calmodulin-dependent protein kinase I. FEBS Lett. *361*, 191–195.

Daran, J.M., Dallies, N., Thines-Sempoux, D., Paquet, V., and François, J. (1995). Genetic and biochemical characterization of the UGP1 gene encoding the UDP-glucose pyrophosphorylase from Saccharomyces cerevisiae. Eur. J. Biochem. *233*, 520–530.

Davenport, K.R., Sohaskey, M., Kamada, Y., Levin, D.E., and Gustin, M.C. (1995). A second osmosensing signal transduction pathway in yeast. Hypotonic shock activates the PKC1 protein kinase-regulated cell integrity pathway. J. Biol. Chem. *270*, 30157–30161.

Davies, S.P., Sim, A.T., and Hardie, D.G. (1990). Location and function of three sites phosphorylated on rat acetyl-CoA carboxylase by the AMP-activated protein kinase. Eur. J. Biochem. *187*, 183–190.

Delgado, M.L., O'Connor, J.E., Azorín, I., Renau-Piqueras, J., Gil, M.L., and Gozalbo, D. (2001). The glyceraldehyde-3-phosphate dehydrogenase polypeptides encoded by the Saccharomyces cerevisiae TDH1, TDH2 and TDH3 genes are also cell wall proteins. Microbiology *147*, 411–417.

DeLuna, A., Avendano, A., Riego, L., and Gonzalez, A. (2001). NADP-glutamate dehydrogenase isoenzymes of Saccharomyces cerevisiae. Purification, kinetic properties, and physiological roles. J. Biol. Chem. *276*, 43775–43783.

DeMille, D., and Grose, J.H. (2013). PAS kinase: a nutrient sensing regulator of glucose homeostasis. IUBMB Life *65*, 921–929.

DeVit, M.J., and Johnston, M. (1999). The nuclear exportin Msn5 is required for nuclear export of the Mig1 glucose repressor of Saccharomyces cerevisiae. Curr. Biol. *9*, 1231–1241.

Dickinson, J.R. (2000). Pathways of leucine and valine catabolism in yeast. Methods Enzymol. *324*, 80–92.

Dihazi, H., Kessler, R., and Eschrich, K. (2001). Phosphorylation and inactivation of yeast 6-phosphofructo-2-kinase contribute to the regulation of glycolysis under hypotonic stress. Biochemistry *40*, 14669–14678.

Dihazi, H., Kessler, R., and Eschrich, K. (2003). Glucose-induced stimulation of the Ras-cAMP pathway in yeast leads to multiple phosphorylations and activation of 6-phosphofructo-2-kinase. Biochemistry *42*, 6275–6282.

Dihazi, H., Kessler, R., and Eschrich, K. (2004). High osmolarity glycerol (HOG) pathway-induced phosphorylation and activation of 6-phosphofructo-2-kinase are essential for glycerol accumulation and yeast cell proliferation under hyperosmotic stress. J. Biol. Chem. *279*, 23961–23968.

Dihazi, H., Kessler, R., Müller, G.A., and Eschrich, K. (2005). Lysine 3 acetylation regulates the phosphorylation of yeast 6-phosphofructo-2-kinase under hypo-osmotic stress. Biol. Chem. *386*, 895–900.

Van Dijck, P., Ma, P., Versele, M., Gorwa, M.F., Colombo, S., Lemaire, K., Bossi, D., Loïez, A., and Thevelein, J.M. (2000). A baker's yeast mutant (fil1) with a specific, partially inactivating mutation in adenylate cyclase maintains a high stress resistance during active fermentation and growth. J. Mol. Microbiol. Biotechnol. *2*, 521–530.

Djouder, N., Tuerk, R.D., Suter, M., Salvioni, P., Thali, R.F., Scholz, R., Vaahtomeri, K., Auchli, Y., Rechsteiner, H., Brunisholz, R.A., et al. (2010). PKA phosphorylates and inactivates AMPKalpha to promote efficient lipolysis. EMBO J. *29*, 469–481.

Dong, X., Mitchell, D.A., Lobo, S., Zhao, L., Bartels, D.J., and Deschenes, R.J. (2003). Palmitoylation and plasma membrane localization of Ras2p by a nonclassical trafficking pathway in Saccharomyces cerevisiae. Mol. Cell. Biol. *23*, 6574–6584.

Erickson, J.R., and Johnston, M. (1993). Genetic and molecular characterization of GAL83: its interaction and similarities with other genes involved in glucose repression in Saccharomyces cerevisiae. Genetics *135*, 655–664.

Estruch, F., Treitel, M.A., Yang, X., and Carlson, M. (1992). N-terminal mutations modulate yeast SNF1 protein kinase function. Genetics *132*, 639–650.

Farkas, I., Hardy, T.A., Goebl, M.G., and Roach, P.J. (1991). Two glycogen synthase isoforms in Saccharomyces cerevisiae are coded by distinct genes that are differentially controlled. J. Biol. Chem. *266*, 15602–15607.

Faubert, B., Boily, G., Izreig, S., Griss, T., Samborska, B., Dong, Z., Dupuy, F., Chambers, C., Fuerth, B.J., Viollet, B., et al. (2013). AMPK is a negative regulator of the Warburg effect and suppresses tumor growth in vivo. Cell Metab. *17*, 113–124.

Faubert, B., Vincent, E.E., Poffenberger, M.C., and Jones, R.G. (2014a). The AMP-activated protein kinase (AMPK) and cancer: Many faces of a metabolic regulator. Cancer Lett.

Faubert, B., Vincent, E.E., Griss, T., Samborska, B., Izreig, S., Svensson, R.U., Mamer, O.A., Avizonis, D., Shackelford, D.B., Shaw, R.J., et al. (2014b). Loss of the tumor suppressor LKB1 promotes metabolic reprogramming of cancer cells via HIF-1α. Proc. Natl. Acad. Sci. U. S. A. *111*, 2554–2559.

Fernández, E., Moreno, F., and Rodicio, R. (1992). The ICL1 gene from Saccharomyces cerevisiae. Eur. J. Biochem. *204*, 983–990.

François, J., and Parrou, J.L. (2001). Reserve carbohydrates metabolism in the yeast Saccharomyces cerevisiae. FEMS Microbiol. Rev. *25*, 125–145.

François, J., Van Schaftigen, E., and Hers, H.G. (1988). Characterization of phosphofructokinase 2 and of enzymes involved in the degradation of fructose 2,6-bisphosphate in yeast. Eur. J. Biochem. *171*, 599–608.

Frederick, D.L., and Tatchell, K. (1996). The REG2 gene of Saccharomyces cerevisiae encodes a type 1 protein phosphatase-binding protein that functions with Reg1p and the Snf1 protein kinase to regulate growth. Mol. Cell. Biol. *16*, 2922–2931.

Friis, R.M.N., Glaves, J.P., Huan, T., Li, L., Sykes, B.D., and Schultz, M.C. (2014). Rewiring AMPK and mitochondrial retrograde signaling for metabolic control of aging and histone acetylation in respiratory-defective cells. Cell Rep. *7*, 565–574.

Gancedo, J.M., Mazón, M.J., and Gancedo, C. (1983). Fructose 2,6-bisphosphate activates the cAMP-dependent phosphorylation of yeast fructose-1,6-bisphosphatase in vitro. J. Biol. Chem. *258*, 5998–5999.

Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., Botstein, D., and Brown, P.O. (2000). Genomic expression programs in the response of yeast cells to environmental changes. Mol. Biol. Cell *11*, 4241–4257.

Görner, W., Durchschlag, E., Martinez-Pastor, M.T., Estruch, F., Ammerer, G., Hamilton, B., Ruis, H., and Schüller, C. (1998). Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. Genes Dev. *12*, 586–597.

Görner, W., Durchschlag, E., Wolf, J., Brown, E.L., Ammerer, G., Ruis, H., and Schüller, C. (2002). Acute glucose starvation activates the nuclear localization signal of a stress-specific yeast transcription factor. EMBO J. *21*, 135–144.

Gruhler, A., Olsen, J. V, Mohammed, S., Mortensen, P., Faergeman, N.J., Mann, M., and Jensen, O.N. (2005). Quantitative phosphoproteomics applied to the yeast pheromone signaling pathway. Mol. Cell. Proteomics *4*, 310–327.

Halliwell, B. (1994). Free radicals and antioxidants: a personal view. Nutr. Rev. *52*, 253–265.

Hardie, D.G. (2007). AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. Nat. Rev. Mol. Cell Biol. *8*, 774–785.

Hardie, D.G. (2013). AMPK: a target for drugs and natural products with effects on both diabetes and cancer. Diabetes *62*, 2164–2172.

Hardy, T.A., and Roach, P.J. (1993). Control of yeast glycogen synthase-2 by COOH-terminal phosphorylation. J. Biol. Chem. *268*, 23799–23805.

Hedbacker, K., and Carlson, M. (2006). Regulation of the nucleocytoplasmic distribution of Snf1-Gal83 protein kinase. Eukaryot. Cell *5*, 1950–1956.

Hedbacker, K., and Carlson, M. (2008). SNF1/AMPK pathways in yeast. Front. Biosci. *13*, 2408–2420.

Hedbacker, K., Townley, R., and Carlson, M. (2004). Cyclic AMP-dependent protein kinase regulates the subcellular localization of Snf1-Sip1 protein kinase. Mol. Cell. Biol. *24*, 1836–1843.

Hedges, D., Proft, M., and Entian, K.D. (1995). CAT8, a new zinc cluster-encoding gene necessary for derepression of gluconeogenic enzymes in the yeast Saccharomyces cerevisiae. Mol. Cell. Biol. *15*, 1915–1922.

Vander Heiden, M.G., Cantley, L.C., and Thompson, C.B. (2009). Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science *324*, 1029–1033.

Hofbauer, H.F., Schopf, F.H., Schleifer, H., Knittelfelder, O.L., Pieber, B., Rechberger, G.N., Wolinski, H., Gaspar, M.L., Kappe, C.O., Stadlmann, J., et al. (2014). Regulation of gene expression through a transcriptional repressor that senses acyl-chain length in membrane phospholipids. Dev. Cell *29*, 729–739.

Hohmann, S., Van Dijck, P., Luyten, K., and Thevelein, J.M. (1994). The byp1-3 allele of the Saccharomyces cerevisiae GGS1/TPS1 gene and its multi-copy suppressor tRNA(GLN) (CAG): Ggs1/Tps1 protein levels restraining growth on fermentable sugars and trehalose accumulation. Curr. Genet. *26*, 295–301.

Hohmann, S., Krantz, M., and Nordlander, B. (2007). Yeast osmoregulation. Methods Enzymol. *428*, 29–45.

Holt, L.J., Tuch, B.B., Villén, J., Johnson, A.D., Gygi, S.P., and Morgan, D.O. (2009). Global analysis of Cdk1 substrate phosphorylation sites provides insights into evolution. Science *325*, 1682–1686.

Hong, S.-P., and Carlson, M. (2007). Regulation of snf1 protein kinase in response to environmental stress. J. Biol. Chem. *282*, 16838–16845.

Hong, S.-P., Leiper, F.C., Woods, A., Carling, D., and Carlson, M. (2003). Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. Proc. Natl. Acad. Sci. U. S. A. *100*, 8839–8843.

Hothersall, J.S., and Ahmed, A. (2013). Metabolic fate of the increased yeast amino Acid uptake subsequent to catabolite derepression. J. Amino Acids *2013*, 461901.

Huang, D., Farkas, I., and Roach, P.J. (1996). Pho85p, a cyclin-dependent protein kinase, and the Snf1p protein kinase act antagonistically to control glycogen accumulation in Saccharomyces cerevisiae. Mol. Cell. Biol. *16*, 4357–4365.

Huang, D., Moffat, J., Wilson, W.A., Moore, L., Cheng, C., Roach, P.J., and Andrews, B. (1998). Cyclin partners determine Pho85 protein kinase substrate specificity in vitro and in vivo: control of glycogen biosynthesis by Pcl8 and Pcl10. Mol. Cell. Biol. *18*, 3289–3299.

Hung, G.-C., Brown, C.R., Wolfe, A.B., Liu, J., and Chiang, H.-L. (2004). Degradation of the gluconeogenic enzymes fructose-1,6-bisphosphatase and malate dehydrogenase is mediated by distinct proteolytic pathways and signaling events. J. Biol. Chem. *279*, 49138–49150.

Hynes, M.J., and Murray, S.L. (2010). ATP-citrate lyase is required for production of cytosolic acetyl coenzyme A and development in Aspergillus nidulans. Eukaryot. Cell *9*, 1039–1048.

Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, Y.D., Antonellis, K.J., Scherf, U., and Speed, T.P. (2003). Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics *4*, 249–264.

Jiang, R., and Carlson, M. (1997). The Snf1 protein kinase and its activating subunit, Snf4, interact with distinct domains of the Sip1/Sip2/Gal83 component in the kinase complex. Mol. Cell. Biol. *17*, 2099–2106.

Jiang, Y., Davis, C., and Broach, J.R. (1998). Efficient transition to growth on fermentable carbon sources in Saccharomyces cerevisiae requires signaling through the Ras pathway. EMBO J. *17*, 6942–6951.

Jin, C., Barrientos, A., Epstein, C.B., Butow, R.A., and Tzagoloff, A. (2007). SIT4 regulation of Mig1p-mediated catabolite repression in Saccharomyces cerevisiae. FEBS Lett. *581*, 5658–5663.

Johnston, M., and Kim, J.-H. (2005). Glucose as a hormone: receptor-mediated glucose sensing in the yeast Saccharomyces cerevisiae. Biochem. Soc. Trans. *33*, 247–252.

Jurica, M.S., Mesecar, A., Heath, P.J., Shi, W., Nowak, T., and Stoddard, B.L. (1998). The allosteric regulation of pyruvate kinase by fructose-1,6-bisphosphate. Structure *6*, 195–210.

Kacherovsky, N., Tachibana, C., Amos, E., Fox, D., and Young, E.T. (2008). Promoter binding by the Adr1 transcriptional activator may be regulated by phosphorylation in the DNA-binding region. PLoS One *3*, e3213.

Kataoka, T., Broek, D., and Wigler, M. (1985). DNA sequence and characterization of the S. cerevisiae gene encoding adenylate cyclase. Cell *43*, 493–505.

Kato, M., and Lin, S.-J. (2014). Regulation of NAD+ metabolism, signaling and compartmentalization in the yeast Saccharomyces cerevisiae. DNA Repair (Amst).

Khoomrung, S., Chumnanpuen, P., Jansa-ard, S., Nookaew, I., and Nielsen, J. (2012). Fast and accurate preparation fatty acid methyl esters by microwave-assisted derivatization in the yeast Saccharomyces cerevisiae. Appl. Microbiol. Biotechnol. *94*, 1637–1646.

Klein, C., and Struhl, K. (1994). Protein kinase A mediates growth-regulated expression of yeast ribosomal protein genes by modulating RAP1 transcriptional activity. Mol. Cell. Biol. *14*, 1920–1928.

Koch, C., Moll, T., Neuberg, M., Ahorn, H., and Nasmyth, K. (1993). A role for the transcription factors Mbp1 and Swi4 in progression from G1 to S phase. Science *261*, 1551–1557.

Kowalska, E., and Kozik, A. (2008). The genes and enzymes involved in the biosynthesis of thiamin and thiamin diphosphate in yeasts. Cell. Mol. Biol. Lett. *13*, 271–282.

Kraakman, L., Lemaire, K., Ma, P., Teunissen, A.W., Donaton, M.C., Van Dijck, P., Winderickx, J., de Winde, J.H., and Thevelein, J.M. (1999). A Saccharomyces cerevisiae G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose. Mol. Microbiol. *32*, 1002–1012.

Krogan, N.J., Cagney, G., Yu, H., Zhong, G., Guo, X., Ignatchenko, A., Li, J., Pu, S., Datta, N., Tikuisis, A.P., et al. (2006). Global landscape of protein complexes in the yeast Saccharomyces cerevisiae. Nature *440*, 637–643.

Kuchin, S., Treich, I., and Carlson, M. (2000). A regulatory shortcut between the Snf1 protein kinase and RNA polymerase II holoenzyme. Proc. Natl. Acad. Sci. U. S. A. *97*, 7916–7920.

Kunau, W.H., and Hartig, A. (1992). Peroxisome biogenesis in Saccharomyces cerevisiae. Antonie Van Leeuwenhoek *62*, 63–78.

Kunze, M., Kragler, F., Binder, M., Hartig, A., and Gurvitz, A. (2002). Targeting of malate synthase 1 to the peroxisomes of Saccharomyces cerevisiae cells depends on growth on oleic acid medium. Eur. J. Biochem. *269*, 915–922.

Lakshmanan, J., Mosley, A.L., and Ozcan, S. (2003). Repression of transcription by Rgt1 in the absence of glucose requires Std1 and Mth1. Curr. Genet. *44*, 19–25.

Lee, Y.J., Jeschke, G.R., Roelants, F.M., Thorner, J., and Turk, B.E. (2012). Reciprocal phosphorylation of yeast glycerol-3-phosphate dehydrogenases in adaptation to distinct types of stress. Mol. Cell. Biol. *32*, 4705–4717.

Leech, A., Nath, N., McCartney, R.R., and Schmidt, M.C. (2003). Isolation of mutations in the catalytic domain of the snf1 kinase that render its activity independent of the snf4 subunit. Eukaryot. Cell *2*, 265–273.

Lesage, P., Yang, X., and Carlson, M. (1996). Yeast SNF1 protein kinase interacts with SIP4, a C6 zinc cluster transcriptional activator: a new role for SNF1 in the glucose response. Mol. Cell. Biol. *16*, 1921–1928.

Levin, D.E. (2005). Cell wall integrity signaling in Saccharomyces cerevisiae. Microbiol. Mol. Biol. Rev. *69*, 262–291.

Li, X., Gerber, S.A., Rudner, A.D., Beausoleil, S.A., Haas, W., Villén, J., Elias, J.E., and Gygi, S.P. (2007). Large-scale phosphorylation analysis of alpha-factor-arrested Saccharomyces cerevisiae. J. Proteome Res. *6*, 1190–1197.

Livas, D., Almering, M.J., Daran, J.-M., Pronk, J.T., and Gancedo, J.M. (2011). Transcriptional responses to glucose in Saccharomyces cerevisiae strains lacking a functional protein kinase A. BMC Genomics *12*, 405.

Lo, W.S., Duggan, L., Emre, N.C., Belotserkovskya, R., Lane, W.S., Shiekhattar, R., and Berger, S.L. (2001). Snf1--a histone kinase that works in concert with the histone acetyltransferase Gcn5 to regulate transcription. Science *293*, 1142–1146.

Lombardo, A., Cereghino, G.P., and Scheffler, I.E. (1992). Control of mRNA turnover as a mechanism of glucose repression in Saccharomyces cerevisiae. Mol. Cell. Biol. *12*, 2941–2948.

Ludin, K., Jiang, R., and Carlson, M. (1998). Glucose-regulated interaction of a regulatory subunit of protein phosphatase 1 with the Snf1 protein kinase in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U. S. A. *95*, 6245–6250.

Ma, P., Wera, S., Van Dijck, P., and Thevelein, J.M. (1999). The PDE1-encoded low-affinity phosphodiesterase in the yeast Saccharomyces cerevisiae has a specific function in controlling agonist-induced cAMP signaling. Mol. Biol. Cell *10*, 91–104.

Martegani, E., Baroni, M.D., Frascotti, G., and Alberghina, L. (1986). Molecular cloning and transcriptional analysis of the start gene CDC25 of Saccharomyces cerevisiae. EMBO J. *5*, 2363–2369.

Mayer, F. V, Heath, R., Underwood, E., Sanders, M.J., Carmena, D., McCartney, R.R., Leiper, F.C., Xiao, B., Jing, C., Walker, P.A., et al. (2011). ADP regulates SNF1, the Saccharomyces cerevisiae homolog of AMP-activated protein kinase. Cell Metab. *14*, 707–714.

Mazón, M.J., Gancedo, J.M., and Gancedo, C. (1982). Phosphorylation and inactivation of yeast fructose-bisphosphatase in vivo by glucose and by proton ionophores. A possible role for cAMP. Eur. J. Biochem. *127*, 605–608.

McAlister, L., and Holland, M.J. (1985). Differential expression of the three yeast glyceraldehyde-3-phosphate dehydrogenase genes. J. Biol. Chem. *260*, 15019–15027.

McCartney, R.R., and Schmidt, M.C. (2001). Regulation of Snf1 kinase. Activation requires phosphorylation of threonine 210 by an upstream kinase as well as a distinct step mediated by the Snf4 subunit. J. Biol. Chem. *276*, 36460–36466.

Momcilovic, M., Iram, S.H., Liu, Y., and Carlson, M. (2008). Roles of the glycogen-binding domain and Snf4 in glucose inhibition of SNF1 protein kinase. J. Biol. Chem. *283*, 19521–19529.

Moriya, H., and Johnston, M. (2004). Glucose sensing and signaling in Saccharomyces cerevisiae through the Rgt2 glucose sensor and casein kinase I. Proc. Natl. Acad. Sci. U. S. A. *101*, 1572–1577.

Müller, S., Zimmermann, F.K., and Boles, E. (1997). Mutant studies of phosphofructo-2-kinases do not reveal an essential role of fructose-2,6-bisphosphate in the regulation of carbon fluxes in yeast cells. Microbiology *143 (Pt 9*, 3055–3061.

Munder, T., and Küntzel, H. (1989). Glucose-induced cAMP signaling in Saccharomyces cerevisiae is mediated by the CDC25 protein. FEBS Lett. *242*, 341–345.

Nath, N., McCartney, R.R., and Schmidt, M.C. (2002). Purification and characterization of Snf1 kinase complexes containing a defined Beta subunit composition. J. Biol. Chem. *277*, 50403–50408.

Nehlin, J.O., and Ronne, H. (1990). Yeast MIG1 repressor is related to the mammalian early growth response and Wilms' tumour finger proteins. EMBO J. *9*, 2891–2898.

Nehlin, J.O., Carlberg, M., and Ronne, H. (1989). Yeast galactose permease is related to yeast and mammalian glucose transporters. Gene *85*, 313–319.

Neigeborn, L., Schwartzberg, P., Reid, R., and Carlson, M. (1986). Null mutations in the SNF3 gene of Saccharomyces cerevisiae cause a different phenotype than do previously isolated missense mutations. Mol. Cell. Biol. *6*, 3569–3574.

Noltmann, E.A., Gubler, C.J., and Kuby, S.A. (1961). Glucose 6-phosphate dehydrogenase (Zwischenferment). I. Isolation of the crystalline enzyme from yeast. J. Biol. Chem. *236*, 1225–1230.

Van Oevelen, C.J.C., van Teeffelen, H.A.A.M., van Werven, F.J., and Timmers, H.T.M. (2006). Snf1p-dependent Spt-Ada-Gcn5-acetyltransferase (SAGA) recruitment and chromatin remodeling activities on the HXT2 and HXT4 promoters. J. Biol. Chem. *281*, 4523–4531.

Ostergaard, S., Walløe, K.O., Gomes, S.G., Olsson, L., and Nielsen, J. (2001). The impact of GAL6, GAL80, and MIG1 on glucose control of the GAL system in Saccharomyces cerevisiae. FEMS Yeast Res. *1*, 47–55.

Ozcan, S., and Johnston, M. (1999). Function and regulation of yeast hexose transporters. Microbiol. Mol. Biol. Rev. *63*, 554–569.

Ozcan, S., Dover, J., Rosenwald, A.G., Wölfl, S., and Johnston, M. (1996). Two glucose transporters in Saccharomyces cerevisiae are glucose sensors that generate a signal for induction of gene expression. Proc. Natl. Acad. Sci. U. S. A. *93*, 12428–12432.

Ozcan, S., Dover, J., and Johnston, M. (1998). Glucose sensing and signaling by two glucose receptors in the yeast Saccharomyces cerevisiae. EMBO J. *17*, 2566–2573.

Ozeki-Miyawaki, C., Moriya, Y., Tatsumi, H., Iida, H., and Sokabe, M. (2005). Identification of functional domains of Mid1, a stretch-activated channel component, necessary for localization to the plasma membrane and Ca2+ permeation. Exp. Cell Res. *311*, 84–95.

Papamichos-Chronakis, M., Gligoris, T., and Tzamarias, D. (2004). The Snf1 kinase controls glucose repression in yeast by modulating interactions between the Mig1 repressor and the Cyc8-Tup1 co-repressor. EMBO Rep. *5*, 368–372.

Peeters, T., Louwet, W., Geladé, R., Nauwelaers, D., Thevelein, J.M., and Versele, M. (2006). Kelch-repeat proteins interacting with the Galpha protein Gpa2 bypass adenylate cyclase for direct regulation of protein kinase A in yeast. Proc. Natl. Acad. Sci. U. S. A. *103*, 13034–13039.

Perpète, P., Duthoit, O., De Maeyer, S., Imray, L., Lawton, A.I., Stavropoulos, K.E., Gitonga, V.W., Hewlins, M.J.E., and Dickinson, J.R. (2006). Methionine catabolism in Saccharomyces cerevisiae. FEMS Yeast Res. *6*, 48–56.

Pessina, S., Tsiarentsyeva, V., Busnelli, S., Vanoni, M., Alberghina, L., and Coccetti, P. (2010). Snf1/AMPK promotes S-phase entrance by controlling CLB5 transcription in budding yeast. Cell Cycle *9*, 2189–2200.

Petelenz-Kurdziel, E., Kuehn, C., Nordlander, B., Klein, D., Hong, K.-K., Jacobson, T., Dahl, P., Schaber, J., Nielsen, J., Hohmann, S., et al. (2013). Quantitative analysis of glycerol accumulation, glycolysis and growth under hyper osmotic stress. PLoS Comput. Biol. *9*, e1003084.

Petkova, M.I., Pujol-Carrion, N., Arroyo, J., García-Cantalejo, J., and Angeles de la Torre-Ruiz, M. (2010). Mtl1 is required to activate general stress response through Tor1 and Ras2 inhibition under conditions of glucose starvation and oxidative stress. J. Biol. Chem. *285*, 19521–19531.

Portela, P., Howell, S., Moreno, S., and Rossi, S. (2002). In vivo and in vitro phosphorylation of two isoforms of yeast pyruvate kinase by protein kinase A. J. Biol. Chem. *277*, 30477–30487.

Portela, P., Moreno, S., and Rossi, S. (2006). Characterization of yeast pyruvate kinase 1 as a protein kinase A substrate, and specificity of the phosphorylation site sequence in the whole protein. Biochem. J. *396*, 117–126.

Portillo, F., Mulet, J.M., and Serrano, R. (2005). A role for the non-phosphorylated form of yeast Snf1: tolerance to toxic cations and activation of potassium transport. FEBS Lett. *579*, 512–516.

Ramos, F., el Guezzar, M., Grenson, M., and Wiame, J.M. (1985). Mutations affecting the enzymes involved in the utilization of 4-aminobutyric acid as nitrogen source by the yeast Saccharomyces cerevisiae. Eur. J. Biochem. *149*, 401–404.

Randez-Gil, F., Bojunga, N., Proft, M., and Entian, K.D. (1997). Glucose derepression of gluconeogenic enzymes in Saccharomyces cerevisiae correlates with phosphorylation of the gene activator Cat8p. Mol. Cell. Biol. *17*, 2502–2510.

Ratnakumar, S., Kacherovsky, N., Arms, E., and Young, E.T. (2009). Snf1 Controls the Activity of Adr1 Through Dephosphorylation of Ser230. Genetics *182*, 735–745.

Reifenberger, E., Freidel, K., and Ciriacy, M. (1995). Identification of novel HXT genes in Saccharomyces cerevisiae reveals the impact of individual hexose transporters on glycolytic flux. Mol. Microbiol. *16*, 157–167.

Reifenberger, E., Boles, E., and Ciriacy, M. (1997). Kinetic characterization of individual hexose transporters of Saccharomyces cerevisiae and their relation to the triggering mechanisms of glucose repression. Eur. J. Biochem. *245*, 324–333.

Ren, J., Wen, L., Gao, X., Jin, C., Xue, Y., and Yao, X. (2009). DOG 1.0: illustrator of protein domain structures. Cell Res. *19*, 271–273.

Resnick, R.J., and Racker, E. (1988). Phosphorylation of the RAS2 gene product by protein kinase A inhibits the activation of yeast adenylyl cyclase. Proc. Natl. Acad. Sci. U. S. A. *85*, 2474–2478.

Rittenhouse, J., Moberly, L., and Marcus, F. (1987). Phosphorylation in vivo of yeast (Saccharomyces cerevisiae) fructose-1,6-bisphosphatase at the cyclic AMP-dependent site. J. Biol. Chem. *262*, 10114–10119.

Roche, E., Assimacopoulos-Jeannet, F., Witters, L.A., Perruchoud, B., Yaney, G., Corkey, B., Asfari, M., and Prentki, M. (1997). Induction by glucose of genes coding for glycolytic enzymes in a pancreatic beta-cell line (INS-1). J. Biol. Chem. *272*, 3091–3098.

Rolland, F., De Winde, J.H., Lemaire, K., Boles, E., Thevelein, J.M., and Winderickx, J. (2000). Glucose-induced cAMP signalling in yeast requires both a G-protein coupled receptor system for extracellular glucose detection and a separable hexose kinase-dependent sensing process. Mol. Microbiol. *38*, 348–358.

Rolland, F., Wanke, V., Cauwenberg, L., Ma, P., Boles, E., Vanoni, M., de Winde, J.H., Thevelein, J.M., and Winderickx, J. (2001). The role of hexose transport and

phosphorylation in cAMP signalling in the yeast Saccharomyces cerevisiae. FEMS Yeast Res. *1*, 33–45.

Rubenstein, E.M., McCartney, R.R., Zhang, C., Shokat, K.M., Shirra, M.K., Arndt, K.M., and Schmidt, M.C. (2008). Access denied: Snf1 activation loop phosphorylation is controlled by availability of the phosphorylated threonine 210 to the PP1 phosphatase. J. Biol. Chem. *283*, 222–230.

Rudolph, M.J., Amodeo, G.A., Bai, Y., and Tong, L. (2005). Crystal structure of the protein kinase domain of yeast AMP-activated protein kinase Snf1. Biochem. Biophys. Res. Commun. *337*, 1224–1228.

Rutter, J., Probst, B.L., and McKnight, S.L. (2002). Coordinate regulation of sugar flux and translation by PAS kinase. Cell *111*, 17–28.

Saito, H., and Posas, F. (2012). Response to hyperosmotic stress. Genetics *192*, 289–318.

Santangelo, G.M. (2006). Glucose signaling in Saccharomyces cerevisiae. Microbiol. Mol. Biol. Rev. *70*, 253–282.

Sanz, P., Alms, G.R., Haystead, T.A., and Carlson, M. (2000). Regulatory interactions between the Reg1-Glc7 protein phosphatase and the Snf1 protein kinase. Mol. Cell. Biol. *20*, 1321–1328.

Sasaki, H., Kishimoto, T., Mizuno, T., Shinzato, T., and Uemura, H. (2005). Expression of GCR1, the transcriptional activator of glycolytic enzyme genes in the yeast Saccharomyces cerevisiae, is positively autoregulated by Gcr1p. Yeast *22*, 305–319.

Schmidt, M.C., and McCartney, R.R. (2000). beta-subunits of Snf1 kinase are required for kinase function and substrate definition. EMBO J. *19*, 4936–4943.

Schork, S.M., Bee, G., Thumm, M., and Wolf, D.H. (1994). Catabolite inactivation of fructose-1,6-bisphosphatase in yeast is mediated by the proteasome. FEBS Lett. *349*, 270–274.

Schüller, H.-J. (2003). Transcriptional control of nonfermentative metabolism in the yeast Saccharomyces cerevisiae. Curr. Genet. *43*, 139–160.

Shahinian, S., and Bussey, H. (2000). beta-1,6-Glucan synthesis in Saccharomyces cerevisiae. Mol. Microbiol. *35*, 477–489.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., and Ideker, T. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. *13*, 2498–2504.

Sharifpoor, S., Nguyen Ba, A.N., Youn, J.-Y., Young, J.-Y., van Dyk, D., Friesen, H., Douglas, A.C., Kurat, C.F., Chong, Y.T., Founk, K., et al. (2011). A quantitative literature-curated gold standard for kinase-substrate pairs. Genome Biol. *12*, R39.

Shi, L., and Tu, B.P. (2013). Acetyl-CoA induces transcription of the key G1 cyclin CLN3 to promote entry into the cell division cycle in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U. S. A. *110*, 7318–7323.

Shi, S., Chen, Y., Siewers, V., and Nielsen, J. (2014). Improving production of malonyl coenzyme A-derived metabolites by abolishing Snf1-dependent regulation of Acc1. MBio *5*, e01130–14.

Shimizu, H., Kuratsu, T., and Hirata, F. (1979). Purification and some properties of glutamate dehydrogenase from Proteus inconstans. J. Ferment Technol. *57*, 428–433.

Shinoda, J., and Kikuchi, Y. (2007). Rod1, an arrestin-related protein, is phosphorylated by Snf1-kinase in Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. *364*, 258–263.

Shirra, M.K., Patton-Vogt, J., Ulrich, A., Liuta-Tehlivets, O., Kohlwein, S.D., Henry, S.A., and Arndt, K.M. (2001). Inhibition of acetyl coenzyme A carboxylase activity restores expression of the INO1 gene in a snf1 mutant strain of Saccharomyces cerevisiae. Mol. Cell. Biol. *21*, 5710–5722.

Shirra, M.K., McCartney, R.R., Zhang, C., Shokat, K.M., Schmidt, M.C., and Arndt, K.M. (2008). A chemical genomics study identifies Snf1 as a repressor of GCN4 translation. J. Biol. Chem. *283*, 35889–35898.

Smolka, M.B., Albuquerque, C.P., Chen, S., and Zhou, H. (2007). Proteome-wide identification of in vivo targets of DNA damage checkpoint kinases. Proc. Natl. Acad. Sci. U. S. A. *104*, 10364–10369.

Sutherland, C.M., Hawley, S.A., McCartney, R.R., Leech, A., Stark, M.J.R., Schmidt, M.C., and Hardie, D.G. (2003). Elm1p is one of three upstream kinases for the Saccharomyces cerevisiae SNF1 complex. Curr. Biol. *13*, 1299–1305.

Tabas-Madrid, D., Nogales-Cadenas, R., and Pascual-Montano, A. (2012). GeneCodis3: a non-redundant and modular enrichment analysis tool for functional genomics. Nucleic Acids Res. *40*, W478–W483.

Tanaka, K., Matsumoto, K., and Toh-E, A. (1989). IRA1, an inhibitory regulator of the RAS-cyclic AMP pathway in Saccharomyces cerevisiae. Mol. Cell. Biol. *9*, 757–768.

Taylor, K.M., Kaplan, C.P., Gao, X., and Baker, A. (1996). Localization and targeting of isocitrate lyases in Saccharomyces cerevisiae. Biochem. J. *319 (Pt 1*, 255–262.

Tehlivets, O., Scheuringer, K., and Kohlwein, S.D. (2007). Fatty acid synthesis and elongation in yeast. Biochim. Biophys. Acta *1771*, 255–270.

Thevelein, J.M., and de Winde, J.H. (1999). Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast Saccharomyces cerevisiae. Mol. Microbiol. *33*, 904–918.

Thevelein, J.M., Bonini, B.M., Castermans, D., Haesendonckx, S., Kriel, J., Louwet, W., Thayumanavan, P., Popova, Y., Rubio-Texeira, M., Schepers, W., et al. (2008). Novel mechanisms in nutrient activation of the yeast protein kinase A pathway. Acta Microbiol. Immunol. Hung. *55*, 75–89.

Toda, T., Cameron, S., Sass, P., Zoller, M., Scott, J.D., McMullen, B., Hurwitz, M., Krebs, E.G., and Wigler, M. (1987). Cloning and characterization of BCY1, a locus encoding a regulatory subunit of the cyclic AMP-dependent protein kinase in Saccharomyces cerevisiae. Mol. Cell. Biol. *7*, 1371–1377.

Tomás-Cobos, L., and Sanz, P. (2002). Active Snf1 protein kinase inhibits expression of the Saccharomyces cerevisiae HXT1 glucose transporter gene. Biochem. J. *368*, 657–663.

Treitel, M.A., Kuchin, S., and Carlson, M. (1998). Snf1 protein kinase regulates phosphorylation of the Mig1 repressor in Saccharomyces cerevisiae. Mol. Cell. Biol. *18*, 6273–6280.

Tripodi, F., Nicastro, R., Reghellin, V., and Coccetti, P. (2015). Post-translational modifications on yeast carbon metabolism: Regulatory mechanisms beyond transcriptional control. Biochim. Biophys. Acta *1850*, 620–627.

Turcotte, B., Liang, X.B., Robert, F., and Soontorngun, N. (2010). Transcriptional regulation of nonfermentable carbon utilization in budding yeast. FEMS Yeast Res. *10*, 2–13.

Usaite, R., Jewett, M.C., Oliveira, A.P., Yates, J.R., Olsson, L., and Nielsen, J. (2009). Reconstruction of the yeast Snf1 kinase regulatory network reveals its role as a global energy regulator. Mol. Syst. Biol. *5*, 319.

Vanhalewyn, M., Dumortier, F., Debast, G., Colombo, S., Ma, P., Winderickx, J., Van Dijck, P., and Thevelein, J.M. (1999). A mutation in Saccharomyces cerevisiae adenylate cyclase, Cyr1K1876M, specifically affects glucose- and acidification-induced cAMP signalling and not the basal cAMP level. Mol. Microbiol. *33*, 363–376.

Van de Velde, S., and Thevelein, J.M. (2008). Cyclic AMP-protein kinase A and Snf1 signaling mechanisms underlie the superior potency of sucrose for induction of filamentation in Saccharomyces cerevisiae. Eukaryot. Cell *7*, 286–293.

De Vendittis, E., Vitelli, A., Zahn, R., and Fasano, O. (1986). Suppression of defective RAS1 and RAS2 functions in yeast by an adenylate cyclase activated by a single amino acid change. EMBO J. *5*, 3657–3663.

Versele, M., de Winde, J.H., and Thevelein, J.M. (1999). A novel regulator of G protein signalling in yeast, Rgs2, downregulates glucose-activation of the cAMP pathway through direct inhibition of Gpa2. EMBO J. *18*, 5577–5591.

Vincent, O., and Carlson, M. (1998). Sip4, a Snf1 kinase-dependent transcriptional activator, binds to the carbon source-responsive element of gluconeogenic genes. EMBO J. *17*, 7002–7008.

Vincent, O., and Carlson, M. (1999). Gal83 mediates the interaction of the Snf1 kinase complex with the transcription activator Sip4. EMBO J. *18*, 6672–6681.

Vincent, O., Townley, R., Kuchin, S., and Carlson, M. (2001). Subcellular localization of the Snf1 kinase is regulated by specific beta subunits and a novel glucose signaling mechanism. Genes Dev. *15*, 1104–1114.

Wade, S.L., Poorey, K., Bekiranov, S., and Auble, D.T. (2009). The Snf1 kinase and proteasome-associated Rad23 regulate UV-responsive gene expression. EMBO J. *28*, 2919–2931.

Wei, M., Fabrizio, P., Hu, J., Ge, H., Cheng, C., Li, L., and Longo, V.D. (2008). Life span extension by calorie restriction depends on Rim15 and transcription factors downstream of Ras/PKA, Tor, and Sch9. PLoS Genet. *4*, e13.

Wells, W.W., Yang, Y., Deits, T.L., and Gan, Z.R. (1993). Thioltransferases. Adv. Enzymol. Relat. Areas Mol. Biol. *66*, 149–201.

Wera, S., De Schrijver, E., Geyskens, I., Nwaka, S., and Thevelein, J.M. (1999). Opposite roles of trehalase activity in heat-shock recovery and heat-shock survival in Saccharomyces cerevisiae. Biochem. J. *343 Pt 3*, 621–626.

De Wever, V., Reiter, W., Ballarini, A., Ammerer, G., and Brocard, C. (2005). A dual role for PP1 in shaping the Msn2-dependent transcriptional response to glucose starvation. EMBO J. *24*, 4115–4123.

Wilson, W.A., Hawley, S.A., and Hardie, D.G. (1996). Glucose repression/derepression in budding yeast: SNF1 protein kinase is activated by phosphorylation under derepressing conditions, and this correlates with a high AMP:ATP ratio. Curr. Biol. *6*, 1426–1434.

Wilson, W.A., Mahrenholz, A.M., and Roach, P.J. (1999). Substrate targeting of the yeast cyclin-dependent kinase Pho85p by the cyclin Pcl10p. Mol. Cell. Biol. *19*, 7020–7030.

Xue, Y., Batlle, M., and Hirsch, J.P. (1998). GPR1 encodes a putative G protein-coupled receptor that associates with the Gpa2p Galpha subunit and functions in a Ras-independent pathway. EMBO J. *17*, 1996–2007.

Yang, X., Jiang, R., and Carlson, M. (1994). A family of proteins containing a conserved domain that mediates interaction with the yeast SNF1 protein kinase complex. EMBO J. *13*, 5878–5886.

Young, E.T., Kacherovsky, N., and Van Riper, K. (2002). Snf1 protein kinase regulates Adr1 binding to chromatin but not transcription activation. J. Biol. Chem. *277*, 38095–38103.

Young, E.T., Dombek, K.M., Tachibana, C., and Ideker, T. (2003). Multiple pathways are co-regulated by the protein kinase Snf1 and the transcription factors Adr1 and Cat8. J. Biol. Chem. *278*, 26146–26158.

Young, E.T., Zhang, C., Shokat, K.M., Parua, P.K., and Braun, K.A. (2012). The AMP-activated protein kinase Snf1 regulates transcription factor binding, RNA

Polymerase II activity and mRNA stability of glucose-repressed genes in Saccharomyces cerevisiae. J. Biol. Chem. *287*, 29021–29034.

Zaman, S., Lippman, S.I., Zhao, X., and Broach, J.R. (2008). How Saccharomyces responds to nutrients. Annu. Rev. Genet. *42*, 27–81.

Zaman, S., Lippman, S.I., Schneper, L., Slonim, N., and Broach, J.R. (2009). Glucose regulates transcription in yeast through a network of signaling pathways. Mol. Syst. Biol. *5*, 245.

Zeller, C.E., Parnell, S.C., and Dohlman, H.G. (2007). The RACK1 ortholog Asc1 functions as a G-protein beta subunit coupled to glucose responsiveness in yeast. J. Biol. Chem. *282*, 25168–25176.

Zhang, M., Galdieri, L., and Vancura, A. (2013). The yeast AMPK homolog SNF1 regulates acetyl coenzyme A homeostasis and histone acetylation. Mol. Cell. Biol. *33*, 4701–4717.

Publications

List of publications (2013-2015)

The thesis is based on the following publications:

I. Busnelli, S., Tripodi, F., Nicastro, R., Cirulli, C., Tedeschi, G., Pagliarin, R., Alberghina, L., and Coccetti, P. (2013). Snf1/AMPK promotes SBF and MBF-dependent transcription in budding yeast. Biochim. Biophys. Acta *1833*, 3254–3264.

II. Nicastro, R., Tripodi, F., Gaggini, M., Reghellin, V., Tedeschi, G., Alberghina, L., and Coccetti, P. Snf1/AMPK interacts with adenylate cyclase and regulates cAMP synthesis and PKA activity in Saccharomyces cerevisiae. (In preparation).

III. Nicastro, R., Tripodi, F., Guzzi, C., Reghellin, V., Khoomrung, S., Airoldi, C., Nielsen, J., Alberghina, L., and Coccetti, P. Enhanced amino acid utilization sustains growth of cells lacking Snf1/AMPK. (Submitted).

IV. Tripodi, F., Nicastro, R., Reghellin, V., and Coccetti, P. (2015). Post-translational modifications on yeast carbon metabolism: Regulatory mechanisms beyond transcriptional control. Biochim. Biophys. Acta *1850*, 620-627.

Additional publications not discussed in this thesis:

V. Tripodi, F., Nicastro, R., Busnelli, S., Cirulli, C., Maffioli, E., Tedeschi, G., Alberghina, L., and Coccetti, P. (2013). Protein kinase CK2 holoenzyme promotes Start-specific transcription in Saccharomyces cerevisiae. Eukaryot. Cell *12*, 1271–1280.

VI. Airoldi, C., Tripodi, F., Guzzi, C., Nicastro, R., and Coccetti, P. (2014). NMR analysis of budding yeast metabolomics: a rapid method for sample preparation. Mol. Biosyst. (In press).

VII. Khoomrung, S., Martinez, J.L., Tippmann, S., Jansa-Ard, S., Buffing, M., Nicastro, R., and Nielsen, J. Improved chemical derivatization of tert-butyldimethylsilyation for the determination of amino/non-amino acids by GC-MS in Saccharomyces cerevisiae. (In preparation).

Publications I, IV, V and VI are attached below.