FUNCTIONAL RELATIONSHIP BETWEEN YEAST VDAC PROTEINS AND THE SAK1 PROTEIN KINASE IN ACTIVATING THE SNF1 PROTEIN KINASE

by

Md Shaheduzzaman

A Thesis Submitted in

Partial Fulfillment of the

Requirements for the Degree of

Master of Science

in Biological Sciences

at

The University of Wisconsin-Milwaukee

May 2023

ABSTRACT

FUNCTIONAL RELATIONSHIP BETWEEN THE VDAC PROTEINS AND THE SAK1 PROTEIN KINASE IN ACTIVATING THE SNF1 PROTEIN KINASE IN YEAST

by

Md Shaheduzzaman

University of Wisconsin-Milwaukee, 2023
Under the Supervision of Dr. Sergei Kuchin

In mammalian cells, the AMPK protein kinase regulates energy metabolism to ensure better survival in energy-depleting conditions. AMPK monitors the cellular energy state through the AMP-to-ATP ratio. Defects in the function of AMPK have been implicated in diseases such as diabetes, cancer, and obesity. Snf1 is the yeast ortholog of AMPK and is activated under conditions of energy stress through phosphorylation by upstream kinases, among which Sak1 is the primary one. When energy is abundant, Snf1 is dephosphorylated by protein phosphatases, among which the Reg1-Glc7 complex plays a primary role. Previous studies have suggested that the activation by upstream kinases and nuclear localization of Snf1 are regulated by mitochondrial voltage dependent anion channel (VDAC) proteins Por1 and Por2. VDACs are present in the mitochondrial outer membrane and conserved among eukaryotes. Por1 and Por2 physically interact with Snf1. Their presence in the outer membrane of mitochondria, the "powerhouse" of the cell, and the presence of binding sites for adenine nucleotides make them

ideal candidates to sense cellular energy status and signal the activation of Snf1. In this study, we present genetic evidence that Por1 and Por2 contribute to the positive regulation of Snf1 by functioning in the same pathway as the primary Snf1-activating kinase, Sak1. Our findings may have implications for Snf1/AMPK regulation in other eukaryotes.

TABLE OF CONTENTS

Introduction 1

The AMP-activated protein kinase (AMPK) family 1

Structure and regulation of AMPK 1

Yeast as a model organism 3

Structure of Snf1 3

Activation by kinases 5

Snf1 is deactivated by phosphatases 6

Function of Snf1 6

Mitochondrial voltage-dependent anion channel (VDAC) proteins, a. k. a. mitochondrial porins 6

Por1 and Por2 physically interact with Snf1 7

Por1 and Por2 regulate Snf1 activation by Thr210 phosphorylation 8

Por1 and Por2 regulate Snf1 nuclear localization 8

Hypothesis 9

Significance of study 10

Aims of the study 11

Materials and methods 12

Media 12

Strains and genetic methods 13

Creating mutants by crossing and tetrad analysis 16

Preparation of protein extracts and immunoblotting assays of Snf1 phosphorylation 17

Results 18

Generating the *sak1*Δ single mutants 18

Generating *por1*Δ *por2*Δ double mutants 19

Generating *por1*Δ *por2*Δ *sak1*Δ triple mutants 20

PCR confirmation tests for *por1*Δ and *sak1*Δ mutations 22

Por1/2 function in the same pathway as Sak1 24

Discussion 26

Acknowledgements 29

References 30

Introduction

The AMP-activated protein kinase (AMPK) family

Evolution has selected for cells with the ability to survive environmental stresses through different mechanisms. In humans, disruption of stress management processes can lead to various health problems such as diabetes, obesity, and cancer (Ronnett et al., 2005; Winder & Hardie, 1999), which are major health issues not only in the US but also worldwide. AMPK-mediated stress management governs a complex combination of anabolic and catabolic pathways in order to balance them. Mammalian AMPK plays an important role in this process and is considered to be the main regulator of metabolism in the cell. AMPK acts as a sensor that regulates responses to energy stress, which includes switching off energy-consuming processes and up-regulating pathways involved in energy production (Hardie 1999; 2003). As a result, members of the AMPK family are called the 'fuel gauge' of the eukaryotic cell. They can sense the increase in the AMP (and/or ADP) pool in the cell, which is an indicator of energy depletion. AMP binding makes AMPK available for activation by upstream kinases, while ATP inhibits AMPK (Carling and Grahame Hardie 1989, Winder and Hardie 1999). In mammals, AMPK responds to increased AMP-to-ATP or ADP-to-ATP ratios and is implicated in diabetes, heart disease, and cancer.

Structure and regulation of AMPK

AMPK is a heterotrimer comprised of a catalytic α subunit, along with regulatory β and γ subunits, all of which may have multiple isoforms. Humans have two α, two β, and three γ

subunits (Hardie et al., 1998; Kahn et al., 2005). Thus, there can be twelve possible heterotrimers, and the composition of the heterotrimer can affect how AMPK is activated, the scale of its activation, and its localization inside the cell (Kim et al., 2016). Under stress conditions, activation of AMPK through AMP happens in two steps: first, the conformation of AMPK changes because of AMP binding, and second, the new conformation makes AMPK a more suitable substrate for activation through phosphorylation by upstream kinases. At the same time, AMPK is also protected from dephosphorylation by protein phosphatases. The phosphorylation necessary for activation happens on a highly conserved residue in the kinase domain (Thr172). Activation by AMP and inactivation by ATP make AMPK a highly efficient regulator of energy homeostasis as it controls energy-consuming and energy-generating processes (Figure 1).

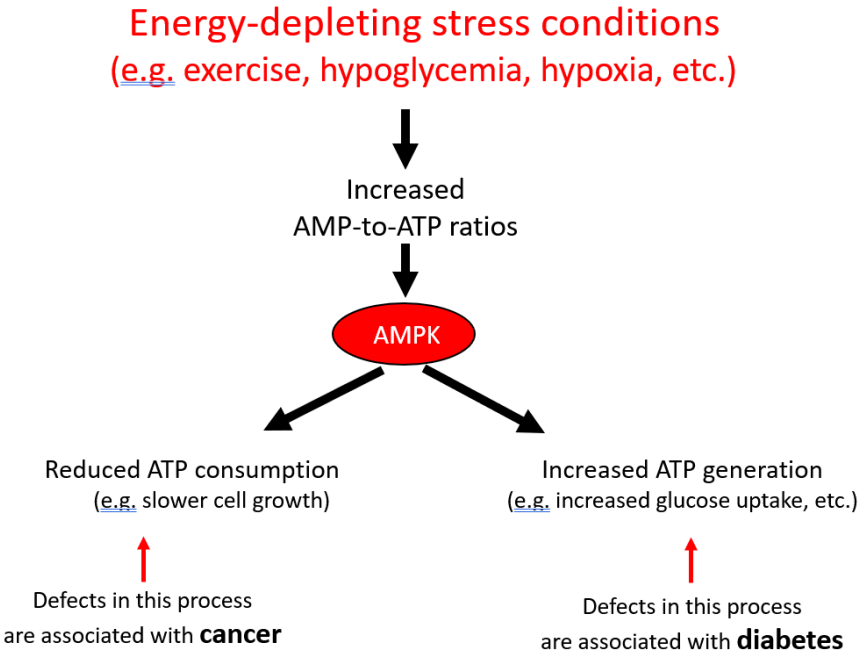

Figure 1: Role of AMPK in maintaining cellular energy homeostasis. Disruption of AMPK function can lead to the occurrence of diseases such as cancer and diabetes. [Adapted from (Barrett, 2011)].

Yeast as a model organism

Saccharomyces cerevisiae, more commonly known as baker's yeast, has been a vital contributor to the manufacturing process of many foods for a long time. Besides being an essential part of the food industries such as bread and alcohol production, it is also an excellent model eukaryote and has been used extensively for scientific research. Yeast cells reproduce quickly, can stably exist in haploid and diploid forms, have a sexual cycle, and share a lot of genetic and proteomic similarities with humans and other mammals. Yeast cells have served as a powerful model to study normal and "abnormal" metabolism. For example, the ability for aerobic fermentation (Crabtree effect) displayed by yeast cells is of significant interest because a similar process (Warburg effect) occurs in cancer cells and hence stands as an interesting target for novel drugs (Diaz-Ruiz et al., 2011). Thus, yeast represents a beneficial model for studying general mechanisms of controlling energy metabolism in eukaryotes.

Structure of Snf1

Snf1, the yeast ortholog of AMPK and a founding member of the Snf1/AMPK protein kinase family, was identified genetically in *S. cerevisiae* in 1981 [reviewed in (Hedbacker & Carlson, 2008)]. Like mammalian AMPK, the yeast Snf1 kinase complex has three subunits – a catalytic subunit (α) with a kinase domain (KD) and a regulatory domain (RD), one of three (Sip1, Sip2 and Gal83) scaffolding/targeting subunits (β) and a stimulatory subunit (γ) (Hedbacker & Carlson, 2008). In the inactive conformation, the kinase domain is inhibited from activation by

the autoinhibitory sequence (AIS) in the regulatory domain. Under glucose limiting conditions, the γ subunit binds to the AIS and allows Snf1 activation (Celenza & Carlson, 1986; Estruch et al., 1992; Jiang & Carlson, 1996) (Figure 2). Snf1 is primarily required for yeast to adapt to environmental stresses and glucose limitation. As is the case with AMPK, Snf1 activity correlates with the AMP-to-ATP ratio in vivo (Wilson et al., 1996). However, mutations in the adenine nucleotide binding sites of the yeast γ subunit have no effect on Snf1 regulation by glucose (Chandrashekarappa et al., 2011). Thus, it remains unknown how the yeast Snf1 complex senses the energy status of the cell.

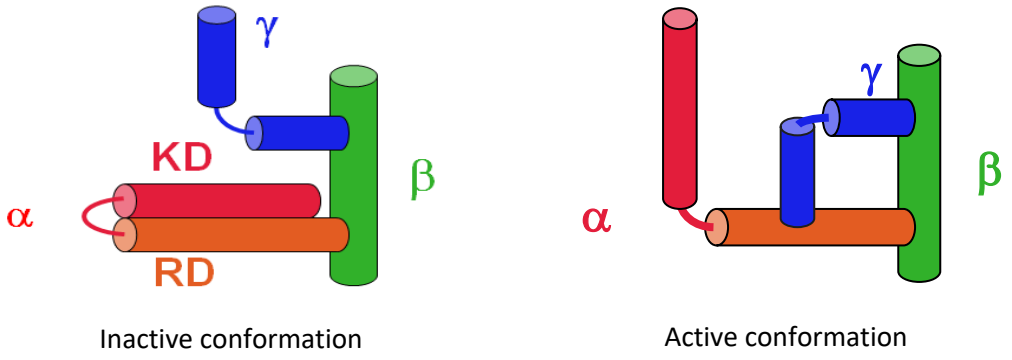

Figure 2. A simplified schematic of the structural differences in the active and inactive states of the Snf1 complex. In the active state, the γ subunit counteracts the auto-inhibitory effect of the regulatory domain (RD) on the kinase domain (KD) [Adapted from (Barrett, 2011)].

Activation by kinases

Upon stress, Snf1 is activated by the upstream kinases Sak1, Tos3 and Elm1, all of which can phosphorylate the conserved threonine residue (Thr210) in the activation loop of the α subunit, Snf1 (Figure 3). The Sak1 protein kinase, previously called Pak1, appears to play the most important role (Nath et al., 2003). The ability of Sak1 to phosphorylate Snf1 was demonstrated by affinity purifying Sak1 from yeast and assaying its ability to phosphorylate Snf1 *in vitro* (Nath et al., 2003). It was shown that when incubated with limiting quantities of Snf1, purified Sak1 promotes phosphorylation of the Snf1 protein.

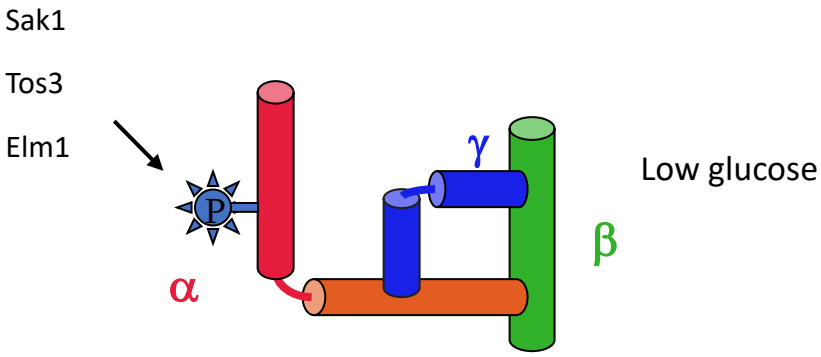

Figure 3: Snf1 is activated by upstream kinases in low glucose conditions [Adapted from (Barrett, 2011)].

Sak1 also associates with the Snf1 kinase complex *in vivo*, which was demonstrated by co-expressing a Sak1-myc protein and a Snf1-HA protein in a *snf1*Δ *sak1*Δ double mutant strain (Nath et al., 2003). When the Snf1 kinase complex is immunoprecipitated with anti-HA and probed by western blot assay with anti-myc, it is seen that in glucose-starved cells, Sak1 co-precipitates with Snf1. In contrast, little Sak1 is detected in Snf1 complexes from cells grown in high glucose.

Snf1 is deactivated by phosphatases

When glucose is added to cells growing in a glucose-depleted environment, Snf1 is dephosphorylated and inactivated. The Reg1-Glc7 phosphatase complex plays a major role in this process (Tu & Carlson, 1995). This was demonstrated by growing cells in high glucose, and then shifting them to low glucose, and then to high glucose again. In such a situation, the *reg1*Δ mutant fails to show dephosphorylation of Snf1, whereas WT cells show rapid and complete Snf1 dephosphorylation (Ruiz et al., 2011).

Function of Snf1

Regulation of the Snf1 kinase affects the metabolic pathways of yeast cells in many ways. Snf1 activates genes required for catabolic pathways (Garcia & Shaw, 2017). Snf1 regulates the transcription of a large set of genes, modifies metabolic enzyme activity, and controls various nutrient-responsive cellular development processes.

Mitochondrial voltage-dependent anion channel (VDAC) proteins, a. k. a. mitochondrial porins

This study intends to approach one of the main questions regarding Snf1 activation. Our previous results have shown that normal activation of Snf1 in response to low glucose requires two mitochondrial voltage-dependent anion channel (VDAC) proteins, Por1 and Por2 (Strogolova et al., 2012). VDACs are evolutionarily conserved proteins located in the

mitochondrial outer membrane (MOM) that mediate the transport of metabolites, including adenine nucleotides, between mitochondria and cytosol. In eukaryotic cells, mitochondria produce ATP during respiration, which is then exported to the cytosol. The yeast VDAC protein Por1 facilitates the transport of nucleotides and other metabolites in and out of mitochondria (Shoshan-Barmatz et al., 2018). Besides ATP, VDACs also mediate the transport of other metabolites up to ~5000 Da through the MOM (Shoshan-Barmatz et al., 2010). In addition, VDACs are strategically located on the boundary between mitochondria and cytosol, which allows them to interact with proteins that control cellular activities beyond the mitochondria (Shoshan-Barmatz et al., 2006, 2010; Shoshan-Barmatz & Mizrachi, 2012). In yeast cells, VDACs also show intriguing properties related to Snf1 regulation, which initiated this study and are discussed here.

Por1 and Por2 physically interact with Snf1

Por1 was identified in a previous two-hybrid screen for Snf1-interacting proteins (Vyas et al., 2001). This interaction was further demonstrated by using fusions to the LexA DNA-binding domain in combination with the viral VP16 or Gal4 transcription activating domain (GAD) to activate a LexA reporter (*lexAop-lacZ*) showing that LexA-Por1 interacted with VP16-Snf1 (Strogolova et al., 2012). This was confirmed and extended using coimmunoprecipitation assays that provided additional evidence that Snf1 physically interacts with both Por1 and Por2 (Strogolova et al., 2012).

Por1 and Por2 regulate Snf1 activation by Thr210 phosphorylation

Activation of Snf1 in low glucose is dramatically reduced when both Por1 and Por2 are absent. The absence of only one of these VDAC proteins does not affect Snf1 activation (Strogolova et al., 2012). This was demonstrated by checking the presence of activated Snf1 in *por1*Δ, *por2*Δ and *por1*Δ *por2*Δ mutants in high and low glucose conditions. Double mutants lacking Por1 and Por2 show an approximately 5-fold reduction in the level of phospho-Thr210-Snf1 in low glucose conditions, but Snf1 activation remains normal in the mutants lacking only Por1 or Por2. Thus, Por1 and Por2 function redundantly in promoting Snf1 activation.

Our lab has proposed that Por1 and Por2 function as energy sensors upstream of Snf1 (Strogolova et al., 2012). The magnitude of the effect produced by the *por1*Δ *por2*Δ double mutation is comparable to the effect produced by the *sak1*Δ mutation, raising the possibility that Por1 and Por2 function via Sak1.

Por1 and Por2 regulate Snf1 nuclear localization

Under glucose-limiting conditions, Snf1 undergoes not only catalytic activation, but also nuclear translocation (Vincent et al., 2001). Evidence indicates that Sak1, the primary Snf1 upstream kinase, is required for such translocation (Hedbacker et al., 2004). Interestingly, the nuclear localization of Snf1 is also facilitated by Por1. Its homolog Por2, when over-expressed *in vivo*, can compensate for the *por1*Δ mutant defect (Shevade et al., 2018). Effects of the *por1*Δ mutation on nuclear localization of Snf1 were shown using a Snf1-Green Fluorescent Protein (Snf1-GFP) fusion expressed from the native *SNF1* promoter on a low-copy centromeric

plasmid. During growth in high glucose, Snf1-GFP was excluded from the nuclei of WT, *por1*Δ and *por2*Δ cells. However, upon shifting to glucose-limiting conditions (ethanol-glycerol), Snf1-GFP enriched to the nucleus in WT and *por2*Δ cells, but failed to enrich to the nucleus in *por1*Δ cells. As mentioned above, overexpression of Por2 suppresses the *por1*Δ mutation for this defect. Thus, Por1 and Por2 (when the latter is overexpressed) promote the nuclear localization of Snf1. These results provide an additional line of evidence that Por1 and Por2 are functionally related to Sak1.

Hypothesis

These Snf1 activation and localization results strongly suggest the existence of a functional relationship between Por1/Por2 and the principal Snf1-activating kinase Sak1. We therefore hypothesized that Por1 and Por2 work in the same pathway as Sak1 in promoting Snf1 catalytic activation. This hypothesis predicts that the *por1*Δ *por2*Δ double mutation should not significantly enhance the effect of the *sak1*Δ single mutation on Snf1 activation, as modeled in Figure 4, lane 4. However, if Por1/Por2 function in a pathway that is different from the Sak1 pathway, we would expect a synergistic effect on Snf1 activation (Figure 4, lane 5).

As will be shown further below, the *por1*Δ *por2*Δ double mutation did not exacerbate the effect of the *sak1*Δ single mutation, supporting our hypothesis that Por1/2 work in the same pathway as Sak1.

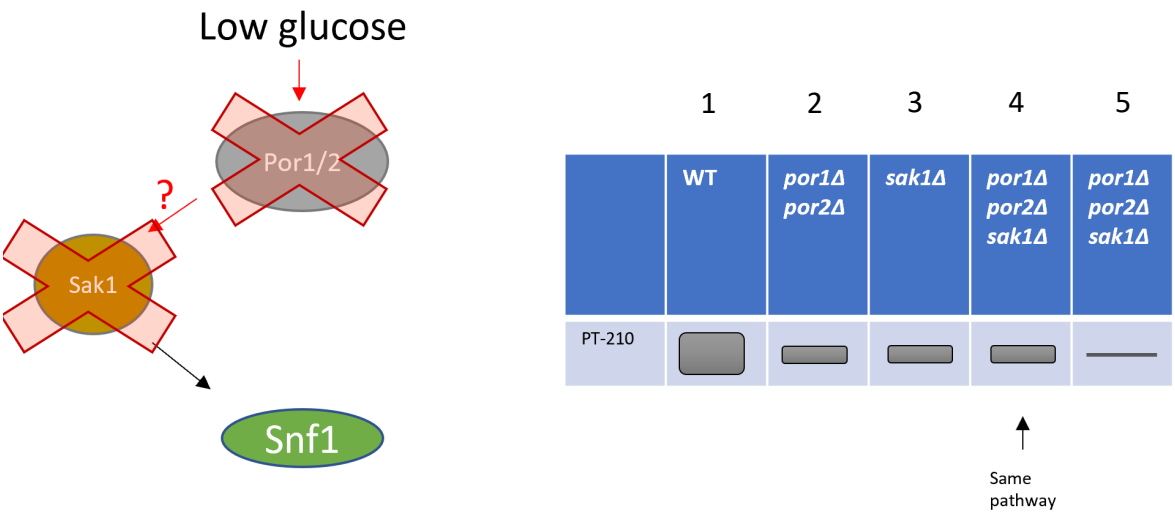

Figure 4: Possible results of the Snf1 activation analysis according to the hypothesis. If Por1 and Por2 work in the same pathway with Sak1 to activate Snf1 in low glucose conditions, then the lack of Por1 and Por2 should not exacerbate the effect caused by the lack of Sak1.

Significance of study

AMPK, the human equivalent of yeast Snf1, maintains metabolic homeostasis and fitness of the cell, and several human diseases are associated with AMPK dysregulation (Dasgupta et al., 2012). Thus, better understanding the signaling mechanisms that regulate AMPK is important and might open windows to designing novel therapeutics. Yeast Por1 and Por2 influence the activation and nuclear localization of Snf1. Gathering more insights into their exact method of promoting Snf1 activation will increase our understanding of Snf1's role in responding to nutrient stress and may have implications for Snf1/AMPK regulation in other eukaryotes, including humans.

Aims of the study

In previous studies it was demonstrated that Snf1 is phosphorylated and activated by Sak1, Tos3 and Elm1, among which Sak1 plays the primary role. This, together with the result that the absence of Por1 and Por2 affects Snf1 activation, leads us to the hypothesis that Por1/2 function in the same pathway with Sak1 to activate Snf1. To test this hypothesis, we propose to check the activation level of Snf1 in WT, *sak1*Δ, *por1*Δ *por2*Δ, and *por1*Δ *por2*Δ *sak1*Δ cells. This will be achieved by constructing the necessary mutants and assessing Thr210 phosphorylation (activation) of Snf1 in response to carbon/energy stress.

Materials and methods

The general methods used here were as described previously (Rose et al., 1990). Other more specific and customized methods are described below.

Media

The most common medium to grow yeast in laboratory conditions is yeast extract/peptone/ dextrose medium (YPD; 1% yeast extract, 2% peptone, 2% glucose), which is a nutrient-rich medium. For glucose-limiting conditions, the glucose level was reduced from 2% to 0.05% (YPLD; 2% peptone, 1% yeast extract, 0.05% glucose). For solid media, 1.7% agar was added before autoclaving. A 40% stock solution of glucose was separately filtered using sterile filters and added after autoclaving so that the sugar did not caramelize. All yeast strains used for this study were grown on solid media prior to their inoculation into liquid YPD and further growth with shaking. Glucose starvation of the cells was achieved by washing them in the low-glucose YPLD medium (0.05% glucose), resuspending them in YPLD, and incubating for 1 hour with shaking. Solid synthetic complete (SC) medium lacking histidine and containing 2% glucose (Rose et al., 1990) and solid YPD with 200 µg/mL gentamycin were used to select for gene knock-outs. Solid sporulation medium (1% potassium acetate, 0.1% yeast extract, 0.05% glucose) was used to induce meiosis/formation of tetrads in diploid strains. Solid synthetic defined (SD) medium (2% glucose, 6.7g/L yeast nitrogen base without amino acids) (Rose et al., 1990) plates spread with *MAT***a** and *MAT*$\boldsymbol{\alpha}$ tester strains were used to determine the mating types of the haploid segregants. All yeast cultures were grown at 30°C.

Strains and genetic methods

The yeast strains used in this study are listed in Table 1. All strains are in the W303 genetic background (Thomas & Rothstein, 1989). The strain carrying the *por1*Δ::*KanMX6* allele (YSK1279) was described previously (Shevade et al., 2018). This strain is *MAT***a** and was crossed under a dissection microscope with HSY76 (*MAT**α*** *sak1*Δ::*KanMX6 por2*Δ::*HIS3*), which was constructed by another lab member, Hemanth Singuluri. This cross resulted in a triply heterozygous diploid MSY61 with the genotype *POR1*/*por1*Δ *POR2*/*por2*Δ *SAK1*/*sak1*Δ. The resultant strain was then sporulated and the tetrads were dissected using a dissection microscope. The four haploid cells from each tetrad were grown on YPD medium and then screened through replica plating. The gene knockouts were confirmed by PCR using confirmation primers. Haploid triple mutants with *por1*Δ *por2*Δ *sak1*Δ mutations were recovered by this method.

The haploid *sak1*Δ single mutants were obtained by dissecting tetrads of strain HSY78 (*MAT***a**/***α*** *SAK1*/*sak1*Δ::*KanMX6 POR2*/*por2*Δ::*HIS3*). The resultant segregants were screened by replica plating on YPD+KAN plates.

The haploid *por1*Δ *por2*Δ double mutants were obtained by dissecting tetrads of strain AMS02 (*MAT***a**/***α*** *POR1*/*por1*Δ::*KanMX6 POR2*/*por2*Δ::*KanMX6)* and examining the growth patterns of the segregants. Colonies that showed slower growth were confirmed to be double mutants and selected for further processing.

Table 1: *S. cerevisiae* W303 strains

Strain	Genotype	Reference/ source
W303-1A	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	Thomas & Rothstein, 1989
W303-1B	*MAT***α** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	Thomas & Rothstein, 1989
AMS02	*MAT***a/α** *ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3/leu2-3 trp1-1/trp1-1 ura3-1/ura3-1 POR1/por1*Δ::*KanMX6 POR2/por2*Δ::*KanMX6*	This laboratory
YSK1279	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3 trp1-1 ura3-1 por1*Δ::*KanMX6*	This laboratory
HSY76	*MAT***α** *ade2-1 can1-100 his3-11,15 leu2-3 trp1-1 ura3-1 sak1*Δ::*KanMX6 por2*Δ::*HIS3*	This laboratory
MSY61	*MAT***a/α** *ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3/leu2-3 trp1-1/trp1-1 ura3-1/ura3-1 SAK1/sak1*Δ::*KanMX6 POR1/por1*Δ::*KanMX6 POR2/por2Δ*::*HIS3*	This work

HSY78	*MAT***a/α** *ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3/leu2-3 trp1-1/trp1-1 ura3-1/ura3-1 SAK1/sak1*Δ::*KanMX6 POR2/por2*Δ::*HIS3*	This laboratory
MSY67	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3 trp1-1 ura3-1 sak1*Δ::*KanMX6 por1*Δ::*KanMX6 por2*Δ::*HIS3*	This work
MSY69	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3 trp1-1 ura3-1 sak1*Δ::*KanMX6 por1*Δ::*KanMX6 por2*Δ::*HIS3*	This work
MSY71	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3 trp1-1 ura3-1 sak1*Δ::*KanMX6 por1*Δ::*KanMX6 por2*Δ::*HIS3*	This work
MSY72	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3 trp1-1 ura3-1 por1*Δ::*KanMX6 por2*Δ::*KanMX6*	This work
MSY73	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3 trp1-1 ura3-1 por1*Δ::*KanMX6 por2*Δ::*KanMX6*	This work
MSY74	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3 trp1-1 ura3-1 por1*Δ::*KanMX6 por2*Δ::*KanMX6*	This work
MSY77	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3 trp1-1 ura3-1 sak1*Δ::*KanMX6*	This work
MSY78	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3 trp1-1 ura3-1 sak1*Δ::*KanMX6*	This work
MSY79	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3 trp1-1 ura3-1 sak1*Δ::*KanMX6*	This work

Creating mutants by crossing and tetrad analysis

For creating haploid triple mutants with the genotype *por1*Δ *por2*Δ *sak1*Δ, a crossing process was carried out by micro-manipulation to bring together individual cells of the opposite mating types with the following genotypes: *MAT***a** *por1*Δ::*KanMX6* (YSK1279) and *MAT***α** *sak1*Δ::*KanMX6 por2*Δ::*HIS3* (HSY76). After the cells mated, the resultant diploid cells were allowed to grow under optimal conditions (YPD, 30˚C) for 48 hours. These diploid cells were then subjected to meiosis/sporulation and tetrad analysis. The haploid segregants were patched on YPD plates first and then analyzed by replica plating for kanamycin resistance and histidine prototrophy.

The genotypes of the haploid segregants selected were subjected to PCR analysis of the genomic DNA. Genomic DNA was extracted, and gene knockouts were confirmed by PCR with confirmation primers specific to the flanking regions of the genes and internal regions of the knockout cassettes. PCR was performed using the Gene AmpPCR system 9700 thermocycler (Applied Biosystems) and Accuprime High Fidelity DNA polymerase (Invitrogen) according to the manufacturer's instructions.

The haploid *por1*Δ *por2*Δ double mutants were obtained by tetrad analysis of the doubly heterozygous mutant diploid with the genotype *MAT***a**/**α** *POR1*/*por1*Δ::*KanMX6* *POR2*/*por2*Δ::*KanMX6* (strain AMS02; Table 1). The slower-growing haploid segregants were selected because this is a known phenotype of *por1*Δ *por2*Δ double mutants.

The haploid *sak1*Δ single mutants were obtained by tetrad analysis of the heterozygous diploid with the genotype *sak1*Δ::*KanMX6* (strain HSY78; Table 1). The haploid segregants that showed kanamycin resistance were used for subsequent work.

Preparation of protein extracts and immunoblotting assays of Snf1 phosphorylation

Pre-cultures were grown overnight in optimal growth conditions using 3 mL of liquid YPD medium (2% glucose; 30°C) for each of the strains. The pre-cultures were then inoculated into fresh YPD medium at an OD_{600} of 0.2. The cells were grown at 30°C for 2-5 hours depending on the strain, until the OD_{600} reached 0.5. One-half of each culture was used as the high-glucose sample. The other half was shifted to the low-glucose YPLD medium (0.05% glucose) for 1 hour at 30°C. Protein extraction and immunoblotting were performed as described previously (Orlova et al., 2008).

Results

Generating the *sak1*Δ single mutants

We needed to generate the *sak1*Δ single*, por1*Δ *por2*Δ double, and *por1*Δ *por2*Δ *sak1*Δ triple mutants. For consistency, all strains used for Snf1 activation assay were of the mating type *MAT***a**. Generation of the single *sak1*Δ::*KanMX6* mutant was done by sporulating the strain HSY78 **(***MAT***a/α**, *SAK1/sak1*Δ*::KanMX6 POR2/por*2Δ*::HIS3*) and dissecting the tetrads.

1				2		3	4	5	6	7		8	9	10	Complete tetrads

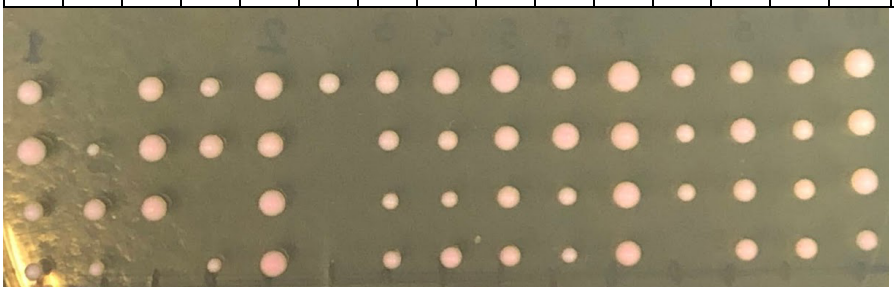

Figure 5: Complete tetrads dissected and grown on YPD agar from HSY78. Out of 15 tetrads, 10 showed four complete colonies and were selected for further analysis by replica plating.

The complete tetrads were then patched on a YPD agar plate, grown for 48 hours at 30˚C, and replica plated on YPD+KAN and SC-HIS plates. Segregants that showed growth only on YPD+KAN and not on SC-HIS plates were single *sak1*Δ*::KanMX6* mutants. The segregants marked with black boxes in Figure 6 were selected and taken to the next stage of the experiment.

Strain	Plate A: YPD	Plate B: SC-HIS	Plate C: YPD+KAN
MSY77			
MSY78			
MSY79			

Figure 6: Replica plating to identify *sak1*Δ::*KanMX6* single mutants. Plate A: Master plate with YPD medium where all the segregants from the 10 tetrads were patched. Plate B: SC-HIS plates to identify *por2*Δ haploid segregants. Strains that grew here were removed from consideration. Plate C: YPD+KAN plates to identify *sak1*Δ::*KanMX6* haploid segregants. Strains that grew here, but not on plate B, are *sak1*Δ single mutants.

Generating *por1*Δ *por2*Δ double mutants

Generation of the *por1*Δ *por2*Δ double mutants was done by sporulating and dissecting strain AMS02 (*MAT***a/α**, *POR1/por1Δ::KanMX6 POR2/por2Δ::KanMX6*). The haploid segregants that grew slower compared to the other colonies were selected as the double mutants (Figure 7). The slower colony growth happens because of the growth defect caused by the *por1*Δ *por2Δ* double mutation.

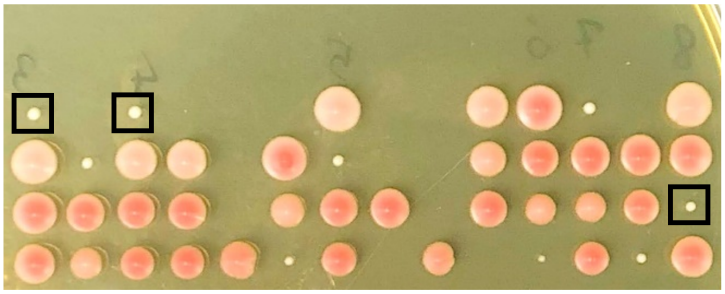

Figure 7: Tetrad analysis of a doubly heterozygous *POR1*/*por1Δ POR2*/*por2Δ* diploid strain. For consistency, the strains that were used in the subsequent steps were *MAT***a**. The segregants that were taken to the next stage of the experiment are highlighted with the black boxes.

Generating *por1*Δ *por2*Δ *sak1*Δ triple mutants

To generate the *por1*Δ *por2*Δ *sak1*Δ triple mutants, we crossed YSK1279 (*MAT***a**, *por1*Δ*::KanMX6*) with HSY76 (*MAT***α**, *sak1*Δ::*KanMX6 por2*Δ*::HIS3*). This way, the heterozygous diploid strain MSY61 with the genotype *MAT***a/α**, *SAK1/sak1*Δ::*KanMX6 POR1/por1*Δ::*KanMX6 POR2/por2Δ*::*HIS3* was generated that has all three mutations. This strain was then sporulated and dissected to obtain haploid segregants (Figure 8). The segregants were collected and analyzed by replica plating to identify the possible triple mutants (Figure 9).

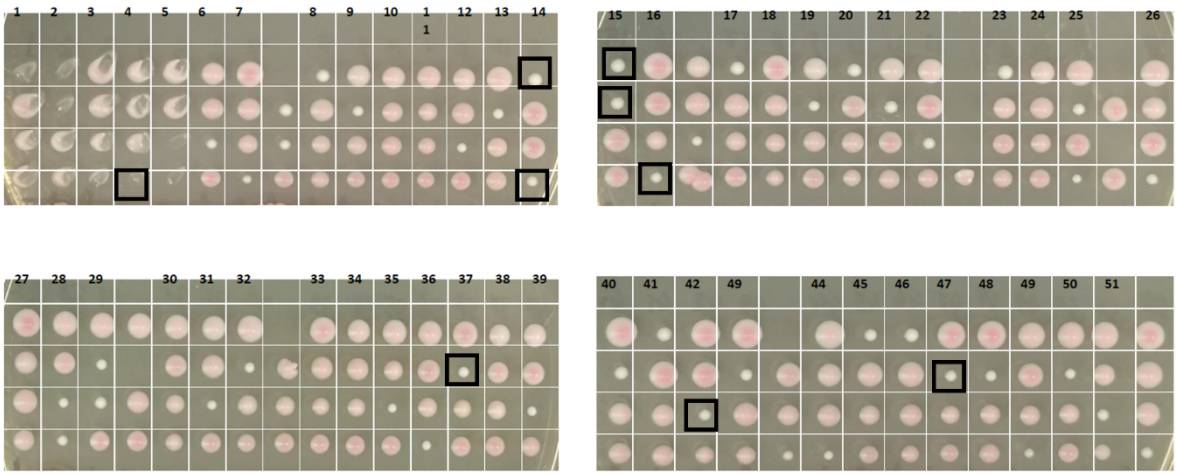

Figure 8: Tetrad dissection of the triply heterozygous *POR1*/*por1*Δ *POR2*/*por2*Δ *SAK1*/*sak1*Δ mutant. The segregants that were selected as possible triple mutants (after replica plating, see Figure 9 below) were chosen for PCR confirmation and are marked with black boxes.

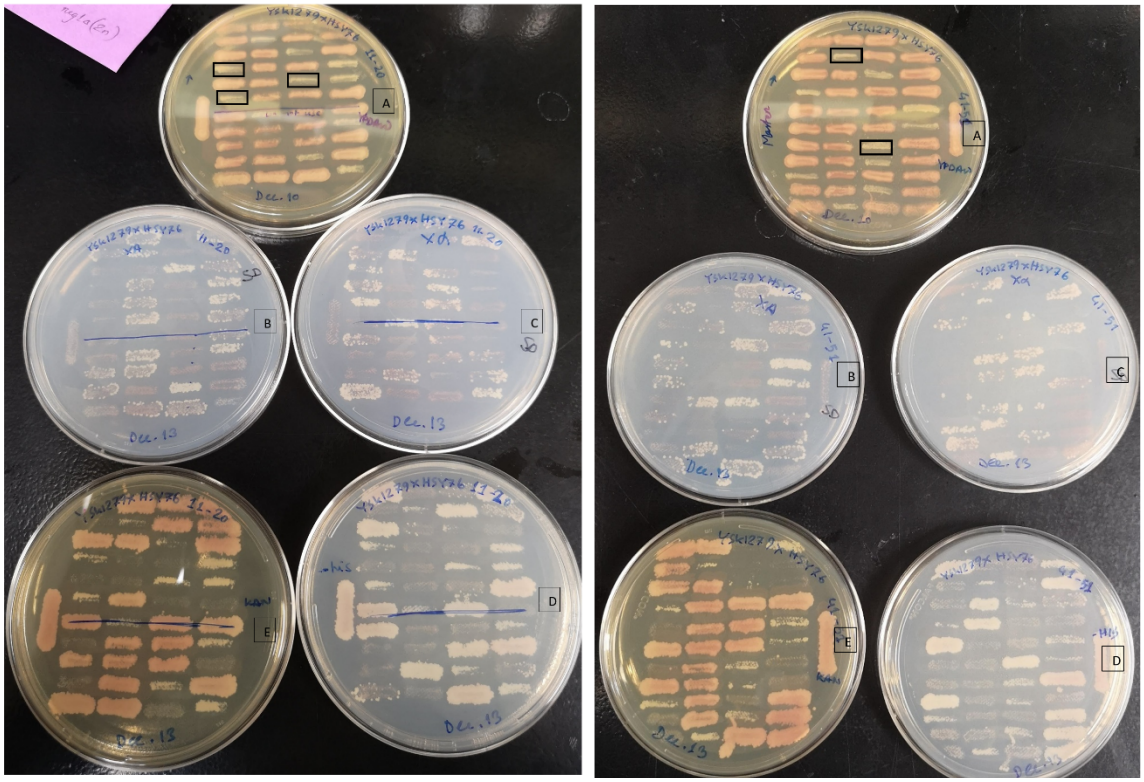

Figure 9: Tetrad analysis to identify potential *por1*Δ *por2*Δ *sak1*Δ triple mutants. 51 tetrads from Figure 8 above were patched onto several YPD master plates (8-10 tetrads/plate) and subsequently replica

plated to determine the mating types, kanamycin resistance, and histidine prototrophy. The results shown represent only those two master plates which were subsequently confirmed to contain triple mutants (segregants marked with black boxes). In both images, the plates are organized as follows. Plate A: Master plate containing YPD medium where the segregants were patched. Plates B and C: These plates were spread with haploid tester strains of the mating types *MAT***a** and *MATα*, respectively, and then the segregants were replica plated on top of those lawns to determine the mating types. Growth on these plates indicates that the segregant is of the opposite mating type to the strain of the lawn. Plate D: SC-HIS plate to identify *por2*Δ segregants. Plate E: YPD+KAN plate to identify *sak1*Δ and/or *por1*Δ single or double mutant segregants. Segregants that grew here as well as on SC-HIS plates were selected as potential *por1*Δ *por2*Δ *sak1*Δ triple mutants.

The potential triple mutants needed to be further tested for mutations, as both *por1*Δ and *sak1*Δ mutations had the same marker. To this end, PCR confirmation tests were conducted to confirm both mutations individually.

PCR confirmation tests for *por1*Δ and *sak1*Δ mutations

The preceding tetrad analysis identified nine potential triple mutant candidates. These candidates needed to be tested further as both *por1*Δ and *sak1*Δ mutations had the same marker (KAN resistance). To this end, PCR confirmation tests were conducted to confirm both mutations simultaneously. The parent strain MSY61 was used as a control. To conduct the PCR confirmation tests, two primers for *por1*Δ confirmation and two primers for *sak1*Δ confirmation were used. If the knockout is present, then when PCR is performed, the primers should be able

to produce a DNA fragment that will be of a different size than the fragment produced with the WT DNA template.

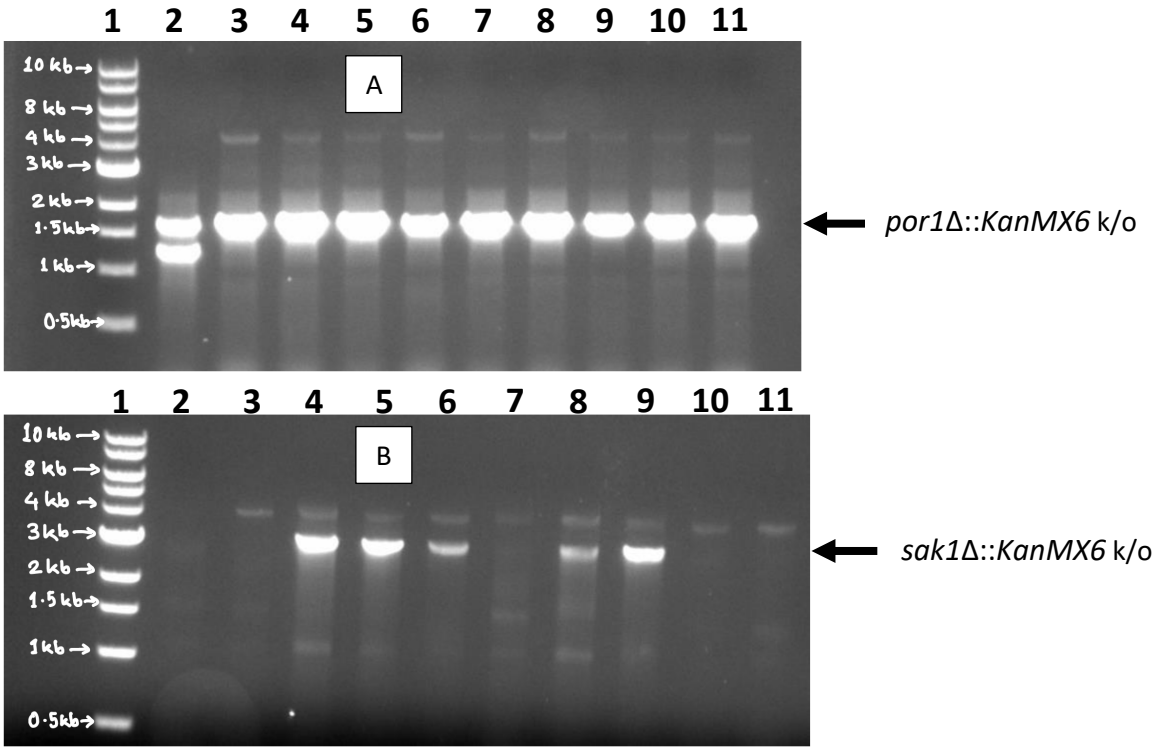

Figure 10: Confirmation of *por1*Δ and *sak1*Δ knock-outs. A. *por1*Δ::*KanMX6* knockout confirmation with primers DK23 por1 k/o conf. F and DK24 por1 k/o conf. R. The lanes are as follows. Lane 1: size markers; lane 2: triply heterozygous diploid parent containing the *por1*Δ, *por2*Δ, and *sak1*Δ mutations; lanes 3-11: potential haploid *por1*Δ *por2*Δ *sak1*Δ triple mutant candidates. The WT *POR1* allele shows a band at 1.25 kb, and the *por1*Δ::*KanMX6* allele shows a band at 1.6 kb (marked with the arrow). All candidates showed the *por1*Δ mutation. B. *sak1*Δ::*KanMX6* knockout confirmation with primers MK1 sak1 k/o conf. F and MK2 sak1 k/o conf. R. The lanes are as follows. Lane 1: size markers; lane 2: triply heterozygous diploid parent containing the *por1*Δ, *por2*Δ, and *sak1*Δ mutations; lanes 3-11: potential haploid *por1*Δ *por2*Δ *sak1*Δ triple mutant candidates. The WT *SAK1* allele would show a band at 4.9 kb, but this band is

too large to be amplified under the PCR conditions used. The *sak1*Δ::*KanMX6* allele shows a band at 3 kb (marked with an arrow). Segregants from lanes 4,5,6, 8, and 9 showed bands corresponding to the *sak1*Δ::*KanMX6* allele. Together with the tetrad analysis results in Figures 8 and 9 above, these results indicate that the segregants from lanes 4, 5, 6, 8, and 9 represent triple *por1*Δ *por2*Δ *sak1*Δ mutants.

Por1/2 function in the same pathway as Sak1

The results from the western blot (Figure 11) and its quantification (Figure 12) indicated that Por1 and Por2 work in the same pathway with Sak1 to regulate Snf1 protein kinase. This conclusion is drawn from our observation that *por1*Δ *por2*Δ double mutation did not exacerbate the effect of the *sak1*Δ single mutation on Snf1 activation.

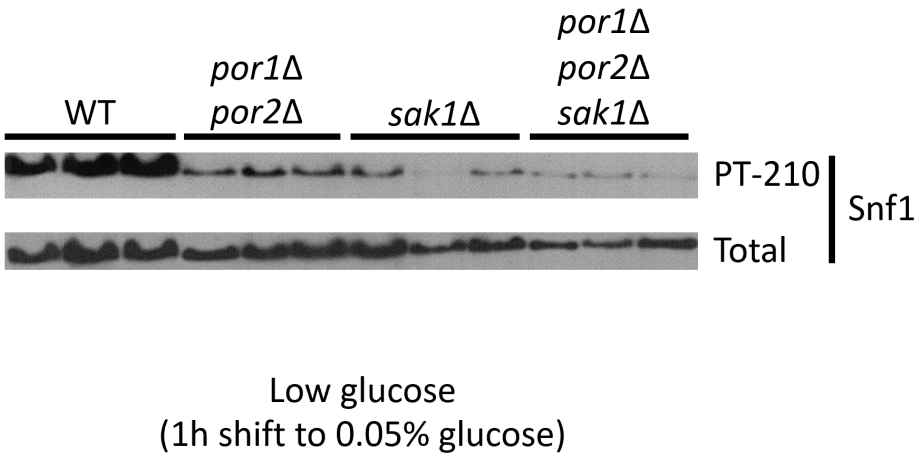

Figure 11: The *por1*Δ *por2*Δ double mutation did not exacerbate the effect of the *sak1*Δ mutation. Cells of the indicated genotypes were cultured under glucose-limiting conditions according to the methods described before. The levels of activated phospho-Thr210-Snf1 (PT-210) and total Snf1 (Total) were determined by immunoblotting as described previously (Orlova et al., 2008). The data represent results for three biological replicates per genotype.

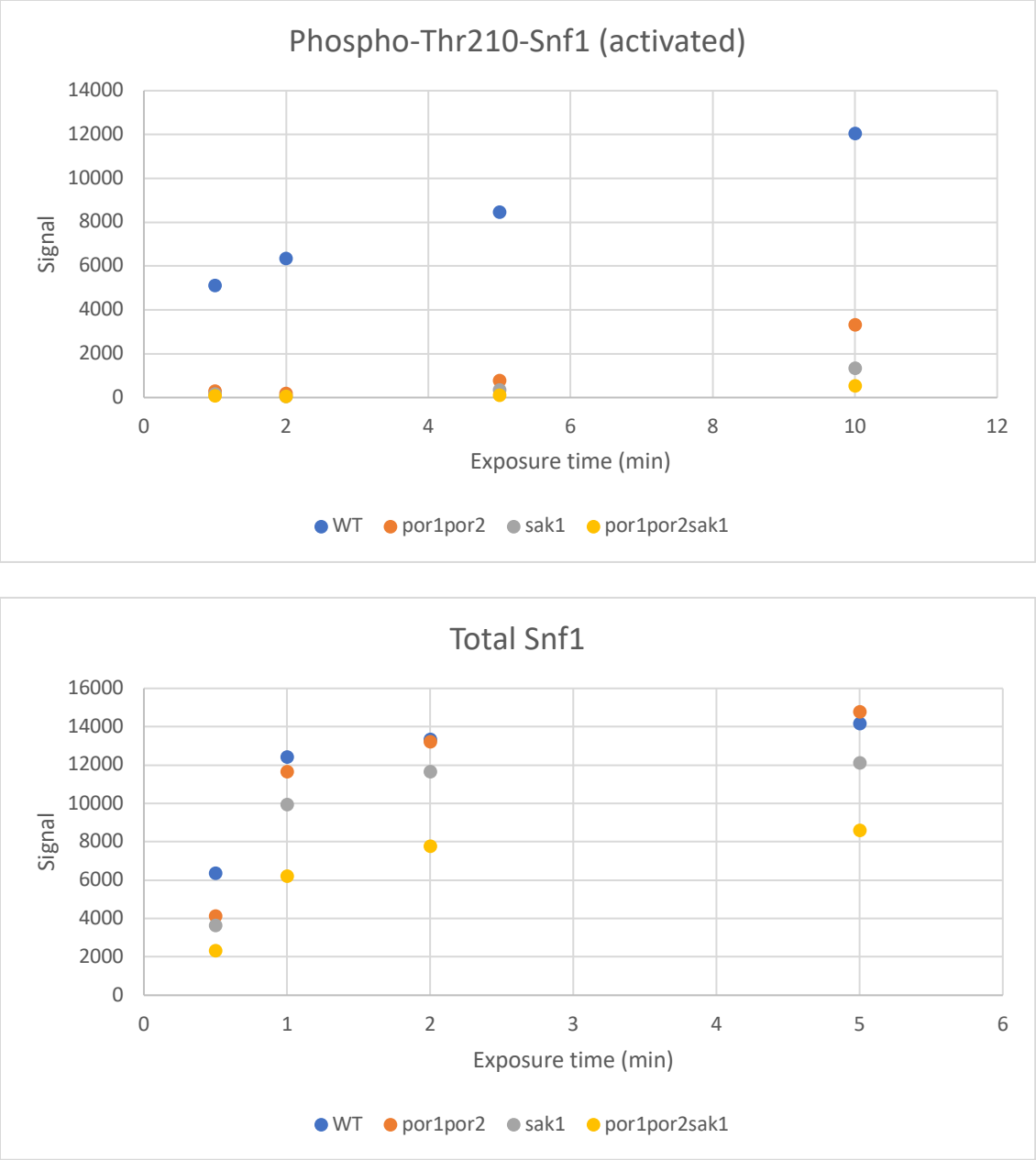

Figure 12: Quantification of Snf1 activation. Different exposures of the western blot shown in Figure 11 above were quantified using ImageJ software. The dots indicate the averages of three strains for each genotype. In summary, these quantification results show that the *por1*Δ *por2*Δ double mutation did not exacerbate the effect of the *sak1*Δ single mutation on Snf1 activation.

Discussion

The focus of this study was on the regulation of yeast Snf1 protein kinase, the major regulator of mechanisms of energy stress response. In yeast cells, the Snf1 protein kinase complex regulates metabolic processes, stress responses, and aging, and is regulated through the phosphorylation of the Thr210 residue in its α subunit by upstream kinases. It is dephosphorylated when Snf1 activation is not necessary anymore. We have presented evidence that the mitochondrial outer membrane proteins Por1 and Por2 work in tandem with the major Snf1-activating kinase, Sak1.

The phosphorylated (activated) form of Snf1 can start or stop different metabolic pathways and thus controls the energy economy of the cell. Snf1 is phosphorylated at its Thr210 residue by upstream kinases Sak1, Tos3 and Elm1 (Hong et al., 2003; Sutherland et al., 2003), of which Sak1 is the primary one. On the other hand, Glc7 is the major protein phosphatase (PP1) that deactivates Snf1 by means of dephosphorylation in response to high concentrations of glucose (Alms et al., 1999; Ludin et al., 1998). Glc7 is targeted to Snf1 by the adaptor subunit Reg1, which can interact both with Glc7 and Snf1 in the presence of abundant glucose; loss of Reg1 causes permanent Snf1 activation (Frederick & Tatchell, 1996; Huang et al., 1996; Rubenstein et al., 2008; Tu & Carlson, 1995).

Snf1 has a significant and widely studied role in the regulation of transcription in yeast cells, and more than 400 genes are involved in this regulation (Young et al., 2012). Mig1 is the most important repressor protein of glucose-regulated genes and gets phosphorylated by Snf1 on four distinct sites when energy is scarce (Nehlin & Ronne, 1990). Snf1 is also involved in the direct regulation of important metabolic enzymes such as acetyl-CoA carboxylase Acc1, Pfk27,

and Gpd2 (Shirra et al., 2001; Benanti et al., 2007). Stress response mechanisms are another domain of Snf1's activities, and it actively protects the cell against the toxicity caused by different chemicals and heavy metals (Thorsen et al., 2009). It ensures cellular resistance to high temperature, oxidative stress and antagonizes the activity of transcriptional repressors such as Nrg1 (Vyas et al., 2001). Snf1 has also been shown to play intriguing roles in the regulation of DNA damage and aging (Coccetti et al., 2018). The data that have been gathered so far and continue to be amassed are sufficient to make a statement that understanding the regulation of Snf1 at a deeper level is of paramount importance.

Previous studies from our lab have shown that the mitochondrial outer membrane proteins Por1 and Por2, also known as voltage-dependent anion channel proteins (VDACs), play a role in the regulation and localization of Snf1 (Shevade et al., 2018; Strogolova et al., 2012). We decided to gain a deeper understanding of this role in this study. Mitochondrial VDAC proteins, or porins, are a conserved group of eukaryotic proteins and exhibit a slight preference for anions over cations in the high-conducting state, which is why they were given the name VDACs (Schein et al., 1976). These proteins make up the most abundant protein family present on the outer membrane of mitochondria (Gonçalves et al., 2007; Mannella, 2008), and play a major role in mediating the permeability of the mitochondrial outer membrane and the passage of small molecules such as ATP, ADP, and AMP. These molecules are important indicators of the energy levels inside the cellular environment, and they can be utilized by the appropriate sensing mechanism to down-regulate the metabolic processes that consume energy and activate processes that allow the use of alternative energy sources.

Yeast cells produce two VDAC isoforms, which are called yVDAC1 (or Por1), and yVDAC2 (or Por2). Por2 has a 49% sequence identity to Por1, and both are found in the mitochondrial outer membrane. Unlike Por1, however, Por2 is not thought to have a channel function (Blachly-Dyson et al., 1997; Lee et al., 1998). The previous knowledge of Snf1 being activated by upstream kinases, combined with the fact that porins have affinity for adenine nucleotides and are important in the activation and localization of Snf1, compelled us to investigate whether the porins function in the regulation of the Snf1 protein kinase in conjunction with the principal Snf1 activating kinase, Sak1. Our present work provides evidence that Por1/Por2 work in the same pathway as Sak1. Further studies will be necessary to determine how Por1/2 facilitate Snf1 activation by Sak1 and the relevance of the observed pathway for higher eukaryotes, including humans.

Acknowledgements

I thank my advisor Dr. Sergei Kuchin and my colleagues Kerry Brown, Vidhya Basak, and Hemanth Singuluri for their advice, help, and technical assistance throughout this project. I would like to thank my committee members Dr. Sonia Bardy, Dr. Mark McBride, Dr. Gyaneshwar Prasad, and Dr. Ching-Hong Yang for their helpful criticisms and suggestions.

I am grateful to my wife, Afreen, for being there with me during the rough times and providing me with encouragement to pursue the degree. I am thankful to my parents and my siblings for their support throughout my time at UWM.

This work was supported by a UWM Research Growth Initiative (UWM-RGI) grant to S. K.

References

Alms, G. R., Sanz, P., Carlson, M., & Haystead, T. A. J. (1999). Reg1p targets protein phosphatase 1 to dephosphorylate hexokinase II in *Saccharomyces cerevisiae*: Characterizing the effects of a phosphatase subunit on the yeast proteome. *EMBO Journal*, *18*(15). https://doi.org/10.1093/emboj/18.15.4157

Barrett, L. (2011). Regulation of the stress-response protein kinase Snf1 of *Saccharomyces cerevisiae*. *The University of Wisconsin-Milwaukee, Proquest Dissertations Publishing 3462831*.

Blachly-Dyson, E., Song, J., Wolfgang, W. J., Colombini, M., & Forte, M. (1997). Multicopy suppressors of phenotypes resulting from the absence of yeast VDAC encode a VDAC-like protein. *Molecular and Cellular Biology*, *17*(10). https://doi.org/10.1128/mcb.17.10.5727

Carling, D., & Grahame Hardie, D. (1989). The substrate and sequence specificity of the AMP-activated protein kinase. Phosphorylation of glycogen synthase and phosphorylase kinase. *BBA - Molecular Cell Research*, *1012*(1). https://doi.org/10.1016/0167-4889(89)90014-1

Celenza, J. L., & Carlson, M. (1986). A yeast gene that is essential for release from glucose repression encodes a protein kinase. *Science*, *233*(4769). https://doi.org/10.1126/science.3526554

Chandrashekarappa, D. G., McCartney, R. R., & Schmidt, M. C. (2011). Subunit and domain requirements for adenylate-mediated protection of Snf1 kinase activation loop from dephosphorylation. *Journal of Biological Chemistry*, *286*(52). https://doi.org/10.1074/jbc.M111.315895

Coccetti, P., Nicastro, R., & Tripodi, F. (2018). Conventional and emerging roles of the energy sensor Snf1/AMPK in *Saccharomyces cerevisiae*. In *Microbial Cell* (Vol. 5, Issue 11). https://doi.org/10.15698/mic2018.11.655

Dasgupta, B., Ju, J. S., Sasaki, Y., Liu, X., Jung, S.-R., Higashida, K., Lindquist, D., & Milbrandt, J. (2012). The AMPK β2 subunit Is required for energy homeostasis during metabolic stress. *Molecular and Cellular Biology*, *32*(14). https://doi.org/10.1128/mcb.05853-11

Diaz-Ruiz, R., Rigoulet, M., & Devin, A. (2011). The Warburg and Crabtree effects: On the origin of cancer cell energy metabolism and of yeast glucose repression. In *Biochimica et Biophysica Acta - Bioenergetics* (Vol. 1807, Issue 6). https://doi.org/10.1016/j.bbabio.2010.08.010

Estruch, F., Treitel, M. A., Yang, X., & Carlson, M. (1992). N-terminal mutations modulate yeast SNF1 protein kinase function. *Genetics*, *132*(3). https://doi.org/10.1093/genetics/132.3.639

Frederick, D. L., & 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. *Molecular and Cellular Biology*, *16*(6). https://doi.org/10.1128/mcb.16.6.2922

Garcia, D., & Shaw, R. J. (2017). AMPK: Mechanisms of cellular energy sensing and restoration of metabolic balance. In *Molecular Cell* (Vol. 66, Issue 6). https://doi.org/10.1016/j.molcel.2017.05.032

Gonçalves, R. P., Buzhynskyy, N., Prima, V., Sturgis, J. N., & Scheuring, S. (2007). Supramolecular assembly of VDAC in native mitochondrial outer membranes. *Journal of Molecular Biology*, *369*(2). https://doi.org/10.1016/j.jmb.2007.03.063

Hardie, D. G. (1999). Roles of the AMP-activated/SNF1 protein kinase family in the response to cellular stress. *Biochemical Society Symposium*, *64*. https://doi.org/10.1515/9781400865048.13

Hardie, D. G. (2003). Minireview: The AMP-activated protein kinase cascade: The key sensor of cellular energy status. In *Endocrinology* (Vol. 144, Issue 12). https://doi.org/10.1210/en.2003-0982

Hardie, D. G., Carling, D., & Carlson, M. (1998). The AMP-activated/SNF1 protein kinase subfamily: Metabolic sensors of the eukaryotic cell? In *Annual Review of Biochemistry* (Vol. 67). https://doi.org/10.1146/annurev.biochem.67.1.821

Hedbacker, K., & Carlson, M. (2008). SNF1/AMPK pathways in yeast. In *Frontiers in Bioscience* (Vol. 13, Issue 7). https://doi.org/10.2741/2854

Hedbacker, K., Hong, S.-P., & Carlson, M. (2004). Pak1 protein kinase regulates activation and nuclear localization of Snf1-Gal83 protein kinase. *Molecular and Cellular Biology*, *24*(18). https://doi.org/10.1128/mcb.24.18.8255-8263.2004

Hong, S. P., Leiper, F. C., Woods, A., Carling, D., & Carlson, M. (2003). Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(15). https://doi.org/10.1073/pnas.1533136100

Huang, D., Farkas, I., & Roach, P. J. (1996). Pho85p, a cyclin-dependent protein kinase, and the Snf1p protein kinase act antagonistically to control glycogen accumulation in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*, *16*(8). https://doi.org/10.1128/mcb.16.8.4357

Jiang, R., & Carlson, M. (1996). Glucose regulates protein interactions within the yeast SNF1 protein kinase complex. *Genes and Development*, *10*(24). https://doi.org/10.1101/gad.10.24.3105

Kahn, B. B., Alquier, T., Carling, D., & Hardie, D. G. (2005). AMP-activated protein kinase: Ancient energy gauge provides clues to modern understanding of metabolism. In *Cell Metabolism* (Vol. 1, Issue 1). https://doi.org/10.1016/j.cmet.2004.12.003

Kim, J., Yang, G., Kim, Y., Kim, J., & Ha, J. (2016). AMPK activators: Mechanisms of action and physiological activities. In *Experimental and Molecular Medicine* (Vol. 48, Issue 4). https://doi.org/10.1038/emm.2016.16

Lee, A. C., Xu, X., Blachly-Dyson, E., Forte, M., & Colombini, M. (1998). The role of yeast VDAC genes on the permeability of the mitochondrial outer membrane. *Journal of Membrane Biology*, *161*(2). https://doi.org/10.1007/s002329900324

Ludin, K., Jiang, R., & Carlson, M. (1998). Glucose-regulated interaction of a regulatory subunit of protein phosphatase 1 with the Snf1 protein kinase in Saccharomyces cerevisiae. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(11). https://doi.org/10.1073/pnas.95.11.6245

Mannella, C. A. (2008). Structural diversity of mitochondria: Functional implications. *Annals of the New York Academy of Sciences*, *1147*. https://doi.org/10.1196/annals.1427.020

Nath, N., McCartney, R. R., & Schmidt, M. C. (2003). Yeast Pak1 kinase associates with and activates Snf1. *Molecular and Cellular Biology*, *23*(11). https://doi.org/10.1128/mcb.23.11.3909-3917.2003

Nehlin, J. O., & Ronne, H. (1990). Yeast MIG1 repressor is related to the mammalian early growth response and Wilms' tumour finger proteins. *EMBO Journal*, *9*(9). https://doi.org/10.1002/j.1460-2075.1990.tb07479.x

Orlova, M., Barrett, L. K., & Kuchin, S. (2008). Detection of endogenous Snfl and its activation state: Application to *Saccharomyces* and *Candida* species. *Yeast*, *25*(10). https://doi.org/10.1002/yea.1628

Ronnett, G. v., Kim, E. K., Landree, L. E., & Tu, Y. (2005). Fatty acid metabolism as a target for obesity treatment. *Physiology and Behavior*, *85*(1). https://doi.org/10.1016/j.physbeh.2005.04.014

Rose, M. D. ;, Winston, F. ;, & Hieter, P. (1990). Methods in yeast Genetics, a laboratory course manual. Cold Spring Harbor Laboratory. In *Biochemistry and Molecular Biology Education*.

Rubenstein, E. M., McCartney, R. R., Zhang, C., Shokat, K. M., Shirra, M. K., Arndt, K. M., & Schmidt, M. C. (2008). Access denied: Snf1 activation loop phosphorylation is controlled by availability of the phosphorylated threonine 210 to the PP1 phosphatase. *Journal of Biological Chemistry*, *283*(1). https://doi.org/10.1074/jbc.M707957200

Ruiz, A., Xu, X., & Carlson, M. (2011). Roles of two protein phosphatases, Reg1-Glc7 and Sit4, and glycogen synthesis in regulation of SNF1 protein kinase. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(16). https://doi.org/10.1073/pnas.1102758108

Schein, S. J., Colombini, M., & Finkelstein, A. (1976). Reconstitution in planar lipid bilayers of a voltage-dependent anion-selective channel obtained from paramecium mitochondria. *The Journal of Membrane Biology*, *30*(1). https://doi.org/10.1007/BF01869662

Shevade, A., Strogolova, V., Orlova, M., Yeo, C. T., & Kuchin, S. (2018). Mitochondrial voltage-dependent anion channel protein Por1 positively regulates the nuclear localization of *Saccharomyces cerevisiae* AMP-activated protein kinase. *MSphere*, *3*(1). https://doi.org/10.1128/msphere.00482-17

Shirra, M. K., Patton-Vogt, J., Ulrich, A., Liuta-Tehlivets, O., Kohlwein, S. D., Henry, S. A., & 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*. *Molecular and Cellular Biology*, *21*(17). https://doi.org/10.1128/mcb.21.17.5710-5722.2001

Shoshan-Barmatz, V., de Pinto, V., Zweckstetter, M., Raviv, Z., Keinan, N., & Arbel, N. (2010). VDAC, a multi-functional mitochondrial protein regulating cell life and death. In *Molecular Aspects of Medicine* (Vol. 31, Issue 3). https://doi.org/10.1016/j.mam.2010.03.002

Shoshan-Barmatz, V., Israelson, A., Brdiczka, D., & Sheu, S. (2006). The voltage-dependent anion channel (VDAC): function in intracellular signalling, cell life and cell death. *Current Pharmaceutical Design*, *12*(18). https://doi.org/10.2174/138161206777585111

Shoshan-Barmatz, V., Krelin, Y., & Shteinfer-Kuzmine, A. (2018). VDAC1 functions in Ca2+ homeostasis and cell life and death in health and disease. In *Cell Calcium* (Vol. 69). https://doi.org/10.1016/j.ceca.2017.06.007

Shoshan-Barmatz, V., & Mizrachi, D. (2012). VDAC1: from structure to cancer therapy. *Frontiers in Oncology*, *2*. https://doi.org/10.3389/fonc.2012.00164

Strogolova, V., Orlova, M., Shevade, A., & Kuchin, S. (2012). Mitochondrial porin Por1 and its homolog Por2 contribute to the positive control of snf1 protein kinase in *Saccharomyces cerevisiae*. *Eukaryotic Cell*, *11*(12). https://doi.org/10.1128/EC.00127-12

Sutherland, C. M., Hawley, S. A., McCartney, R. R., Leech, A., Stark, M. J. R., Schmidt, M. C., & Hardie, D. G. (2003). Elm1p is one of three upstream kinases for the *Saccharomyces cerevisiae* SNF1 complex. *Current Biology*, *13*(15). https://doi.org/10.1016/S0960-9822(03)00459-7

Thomas, B. J., & Rothstein, R. (1989). Elevated recombination rates in transcriptionally active DNA. *Cell*, *56*(4). https://doi.org/10.1016/0092-8674(89)90584-9

Thorsen, M., Perrone, G. G., Kristiansson, E., Traini, M., Ye, T., Dawes, I. W., Nerman, O., & Tamás, M. J. (2009). Genetic basis of arsenite and cadmium tolerance in *Saccharomyces cerevisiae*. *BMC Genomics*, *10*. https://doi.org/10.1186/1471-2164-10-105

Tu, J., & Carlson, M. (1995). REG1 binds to protein phosphatase type 1 and regulates glucose repression in *Saccharomyces cerevisiae*. *EMBO Journal*, *14*(23). https://doi.org/10.1002/j.1460-2075.1995.tb00282.x

Vincent, O., Townley, R., Kuchin, S., & Carlson, M. (2001). Subcellular localization of the Snf1 kinase is regulated by specific β subunits and a novel glucose signaling mechanism. *Genes and Development*, *15*(9). https://doi.org/10.1101/gad.879301

Vyas, V. K., Kuchin, S., & Carlson, M. (2001). Interaction of the repressors Nrg1 and Nrg2 with the Snf1 protein kinase in *Saccharomyces cerevisiae*. *Genetics*, *158*(2). https://doi.org/10.1093/genetics/158.2.563

Wilson, W. A., Hawley, S. A., & 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. *Current Biology*, *6*(11). https://doi.org/10.1016/S0960-9822(96)00747-6

Winder, W. W., & Hardie, D. G. (1999). AMP-activated protein kinase, a metabolic master switch: Possible roles in Type 2 diabetes. In *American Journal of Physiology - Endocrinology and Metabolism* (Vol. 277, Issues 1 40-1). https://doi.org/10.1152/ajpendo.1999.277.1.e1

Young, E. T., Zhang, C., Shokat, K. M., Parua, P. K., & 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*. *Journal of Biological Chemistry*, *287*(34). https://doi.org/10.1074/jbc.M112.380147