Mitochondrial Regulation of Yeast AMPK During Energy Stress

Aishwarya Shevade

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MITOCHONDRIAL REGULATION OF YEAST AMPK DURING ENERGY STRESS

by

Aishwarya Shevade

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ABSTRACT

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by

Aishwarya Shevade

The University of Wisconsin-Milwaukee, 2017
Under the Supervision of Dr. Sergei Kuchin

In eukaryotes, members of the conserved AMP-activated protein kinase (AMPK) family play a pivotal role in sensing and responding to energy stress. Mammalian AMPK becomes activated when the AMP:ATP ratio is too high, and functions to prevent unnecessary ATP spending and to increase ATP production. Due to their role in ATP production through aerobic respiration, mitochondria are known to play an indirect role in the negative control of AMPK. The conserved voltage-dependent anion channel (VDAC) proteins, also known as mitochondrial porins, mediate the passage of small metabolites between the mitochondria and cytoplasm, including the release of ATP. One would therefore expect VDACs to play a role in the negative regulation of AMPK. Contrary to this expectation, our results in budding yeast (Saccharomyces cerevisiae) provide evidence that mitochondria and VDACs play a role in the positive control of Snf1, the yeast homolog of AMPK.

In yeast, Snf1 protein kinase stimulates the utilization of alternate carbon/energy sources when the preferred source – glucose – becomes limiting. Under carbon/energy stress conditions, Snf1 is activated and enriches in the nucleus to elicit various transcriptional responses.
Our results indicate that Snf1 physically interacts with the yeast VDAC proteins Por1 and Por2. Interestingly, Por1 and Por2 contribute to the positive - rather than negative - control of Snf1. We present evidence indicating that Por1 and Por2 function redundantly to promote Snf1 catalytic activation, presumably as receptors of an intracellular glucose/energy signal. We also present evidence for novel mechanisms by which mitochondria positively regulate Snf1 nuclear localization.

In summary, our experiments in yeast reveal an array of mechanisms by which mitochondria positively regulate Snf1/AMPK, i.e. in a way that would be entirely counter-intuitive to researchers in the mammalian field. Due to the evolutionary conservation of the players involved, further studies of these novel mechanisms in the yeast model could provide invaluable insights into the etiology and therapy of AMPK-, VDAC-, and mitochondria-associated diseases including cancer, diabetes, obesity, and cardiac disorders.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>II</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>VII</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>IX</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>X</td>
</tr>
<tr>
<td>CHAPTER 1</td>
<td>1</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>AMP-activated protein kinase</td>
<td>1</td>
</tr>
<tr>
<td>Snf1 protein kinase</td>
<td>7</td>
</tr>
<tr>
<td>Voltage dependent anion channels</td>
<td>14</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>16</td>
</tr>
<tr>
<td>CHAPTER 2</td>
<td>22</td>
</tr>
<tr>
<td>Mitochondrial porin Por1 and its homolog Por2 contribute to the positive control of Snf1 protein kinase in <em>Saccharomyces cerevisiae</em></td>
<td></td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>22</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>22</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>25</td>
</tr>
<tr>
<td>RESULTS</td>
<td>28</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>34</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>36</td>
</tr>
<tr>
<td>CHAPTER 3</td>
<td>40</td>
</tr>
<tr>
<td>Mitochondrial porins as sensors of a signal that regulates the Snf1/AMPK pathway</td>
<td></td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>40</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>41</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>43</td>
</tr>
<tr>
<td>RESULTS</td>
<td>46</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>52</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>56</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Fig. 1. Role of AMP-activated protein kinase (AMPK) in maintaining energy equilibrium 2
Fig. 2. Structure of AMPK. AMPK is a heterotrimer of α, β, and γ subunits 3
Fig. 3. A simplified diagram of AMPK regulation 5
Fig. 4. AMPK of yeast cells: Snf1 7
Fig. 5. Snf1 structure and regulation 9
Fig. 6. Structure of yVDAC1 15

CHAPTER 2

MITOCHONDRIAL PORIN POR1 AND ITS HOMOLOG POR2 CONTRIBUTE TO THE POSITIVE CONTROL OF SNF1 PROTEIN KINASE IN SACCHAROMYCES CEREVISIAE

Fig. 7. Snf1 and Por1 interact in the two-hybrid system 28
Fig. 8. Snf1 coimmunoprecipitates with Por1 and Por2 30
Fig. 9. Lack of Por1 and Por2 affects Snf1 activation 33

CHAPTER 3

MITOCHONDRIAL PORINS AS SENSORS OF A SIGNAL THAT REGULATES THE SNF1/AMPK PATHWAY

Fig. 10. Roles of Por1 and Por2 in the activation of the Snf1 kinase domain 47
Fig. 11. Two-hybrid analysis of the interaction between Por1 and the Snf1 kinase domain 48
Fig. 12. Role of hexokinase-2 in the negative control of Snf1 49
Fig. 13. Functional relationship between mitochondrial porins and hexokinase-2 50
Fig. 14. Sequence analysis of Por1 and Por2 54
CHAPTER 4

MITOCHONDRIAL PORINS, POR1 AND POR2, POSITIVELY REGULATE SNF1-GAL83 NUCLEAR LOCALIZATION IN SACCHAROMYCES CEREVISIAE

Fig. 15. The por1Δ mutation strongly affects lexAop-lacZ reporter activation by LexA-Snf1-G53R 67

Fig. 16. Snf1 does not enrich in the nucleus of a por1Δ mutant 69

Fig. 17. Por1 interacts with Gal83 in the two-hybrid system 70

Fig. 18. Gal83 does not enrich in the nucleus of a por1Δ mutant 71

Fig. 19. Gal83 does not enrich in the nucleus of a snf1Δ por1Δ mutant 73

Fig. 20. Overexpression of Por2 suppresses the por1Δ mutation for the Snf1 nuclear localization defect 75

Fig. 21. Effects of Por2 overexpression on localization of Gal83-GFP 76

Fig. 22. Overexpression of Por2 suppresses the por1Δ mutant defect in transcriptional activation by LexA-Snf1-G53R 77

Fig. 23. Overexpression of Por2 does not bypass the requirement of Gal83 for lexAop-lacZ reporter activation by LexA-Snf1-G53R 78

Fig. 24. Expression of hyperactive LexA-Snf1-G53R restores the ability of the por1Δ mutant to grow on non-fermentable carbon sources in a Gal83-dependent manner 80

CHAPTER 5

MITOCHONDRIAL RESPIRATION REGULATES SNF1 LOCALIZATION

Fig. 25. Effect of the rho0 mutation on "shortcut" reporter activation 93

Fig. 26. Effects of respiratory deficiency on the localization of Snf1 and Gal83 95

Fig. 27. Roles of the β subunits in Snf1 localization in respiratory-deficient cells 96

Fig. 28. Effect of respiratory deficiency on β subunit expression 97


## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TABLE 1.</td>
<td><em>S. CEREVISIAE</em> STRAINS 1</td>
<td>35</td>
</tr>
<tr>
<td>TABLE 2.</td>
<td><em>S. CEREVISIAE</em> STRAINS 2</td>
<td>55</td>
</tr>
<tr>
<td>TABLE 3.</td>
<td><em>S. CEREVISIAE</em> STRAINS 3</td>
<td>83</td>
</tr>
<tr>
<td>TABLE 4.</td>
<td><em>S. CEREVISIAE</em> STRAINS 4</td>
<td>100</td>
</tr>
</tbody>
</table>
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Chapter 1

Introduction

Overview

Eukaryotic cells prefer glucose as their carbon and energy source. Glucose starvation creates energy stress. Cells have developed mechanisms to sense and cope with such a critical stress, and carefully turn on their energy-conservation mode under glucose-limiting conditions. Cells can pick up cues from outside and from within, such as from the mitochondria, the “powerhouse of the cell”, to determine the energy status and mount an appropriate response. The highly conserved Snf1/AMPK stress response pathway regulates the energy levels of eukaryotic cells from yeast to humans.

AMP-activated protein kinase

AMP – activated protein kinase (AMPK) is the key regulator of many metabolic processes in humans and is widely known for its effects on metabolism and regulation of cell growth and proliferation (HARDIE 2011a; HARDIE et al. 2012b). AMPK monitors levels of energy nucleotides such as ATP, ADP, and AMP. During metabolic stresses such as hypoglycemia, exercise, and hypoxia AMPK is activated by sensing the AMP:ATP ratio (and also ADP:ATP ratio), and hence is aptly referred to as the “fuel gauge” of the cell (HARDIE et al. 1998). AMPK maintains energy equilibrium by balancing energy-consuming and energy-generating processes (Fig. 1). AMPK stimulates processes such as glucose uptake, glycolysis, fatty acid uptake and fatty acid oxidation, and mitochondrial biogenesis that generate more ATP (HARDIE 2008; HARDIE 2011a). At the same time, active AMPK inhibits anabolic pathways such as fatty acid synthesis, glycogen synthesis and gluconeogenesis, thus reducing energy
expenditure (HARDIE 2008; HARDIE 2011b). AMPK performs its function by phosphorylating key enzymes in these pathways, which are sensitive to energy levels, and by regulating the expression of various genes (HARDIE et al. 1998; HARDIE 2011a; HARDIE et al. 2012b).

**Fig. 1.** Role of AMP-activated protein kinase (AMPK) in maintaining energy equilibrium. AMPK senses depleting energy reserves (high AMP:ATP ratio) and gets activated. Active AMPK responds to energy stress by stimulating catabolic ATP-generating processes and inhibiting anabolic ATP-consuming processes. Adapted from (BARRETT 2011).

**Structure and regulation of AMPK**

AMPK is a heterotrimeric protein complex consisting of the catalytic α subunit, regulatory β subunit, and sensory γ subunit (HARDIE 2011a; HARDIE 2011b; HARDIE et al. 2012b) (Fig. 2). There are multiple isoforms of these subunits in mammals, making for twelve combinations of heterotrimers. Present at the N terminus of the α subunit is the protein kinase domain containing the conserved threonine (Thr172) in the activation loop, whose phosphorylation by upstream kinases results in AMPK activation (see below) (HAWLEY et al. 1996). The kinase domain is followed by the auto-inhibitory domain (AID) that is connected to the C-terminal domain of the catalytic subunit (α-CTD) by a linker peptide. AID keeps the
kinase domain inactive and lowers kinase activity in non-stressful conditions (Pang et al. 2007).

The C-terminal domain of the β subunit (β-CTD)

![Diagram of AMPK structure]

**Fig. 2.** Structure of AMPK. AMPK is a heterotrimer of α, β, and γ subunits. The catalytic α subunit contains Thr172 in the kinase domain that is activated by phosphorylation, which is otherwise protected by autoinhibitory domain (AID). The regulatory β subunit binds to α and γ subunits via its C terminus. γ subunit acts as the sensor that binds to AMP when the AMP:ATP ratio is high under low glucose conditions. Adapted from (Hardie et al. 2012a).

... binds to α-CTD and the γ subunit, thus holding the heterotrimeric complex together. There are carbohydrate-binding modules (CBM) in the β subunits that possibly allow AMPK to sense energy reserves in the form of glycogen levels (Polekhina et al. 2003). Such CBMs present in enzymes, such as glycogen synthase, help in the co-localization of AMPK and its downstream targets bound to glycogen. The γ subunit of AMPK contains four cystathione β synthase (CBS) repeats that bind to adenine nucleotides (Kemp 2004; Scott et al. 2004). AMP and ATP bind to
two sites in the γ subunit causing opposite effects (Oakhill et al. 2011; Xiao et al. 2011). AMPK senses increased AMP:ATP ratio during low energy conditions, and AMP binds to the γ subunit changing the conformation of AMPK and releasing autoinhibition, allowing activation of AMPK by phosphorylation of the Thr172 residue in the kinase domain of the α subunit (Hawley et al. 1996; Hardie and Hawley 2001). Moreover, AMP binding protects AMPK from dephosphorylation by phosphatases (Davies et al. 1995; Suter et al. 2006); it has also been shown that β subunit myristoylation is a prerequisite for Thr172 phosphorylation (Oakhill et al. 2010).

Together, the binding of AMP (at the exclusion of ATP) to the γ subunit and subsequent phosphorylation of Thr172 lead to a more than 1000-fold activation of AMPK (Fig. 3) (Hardie 2011b). During conditions that correspond to low energy (high AMP:ATP), such as hypoglycemia and hypoxia, and metabolic stresses that increase ATP consumption, AMPK is activated by upstream kinase liver kinase B1 (LKB1) (Hawley et al. 2003; Hong et al. 2003; Woods et al. 2003). Apart from LKB1, which is the major upstream kinase, AMPK can also be activated by Ca^{2+}/calmodulin-dependent kinase kinase β (CaMKKβ) in response to a rise in Ca^{2+} levels that is normally associated with ATP-consuming processes such as contraction of skeletal muscles (Hawley et al. 2005; Hong et al. 2005; Woods et al. 2005). Evidence suggests that toxic reactive oxygen species (ROS) such as hydrogen peroxide H_{2}O_{2}, and xenobiotic compounds such as metformin, a drug commonly used for treating type 2 diabetes, also activate AMPK (Hawley et al. 2010). The upstream kinases may continually phosphorylate AMPK, but in the absence of metabolic stresses, removal of the phosphate group by phosphatases such as protein phosphatase 2C (PP2C), and protein phosphatase 1 (PP1), will maintain the inactive state of AMPK (Davies et al. 1995; Garcia-Haro et al. 2010).
**Fig. 3.** A simplified diagram of AMPK regulation. Top panel shows AMPK in its inactive conformation. Under low energy conditions, AMP binds to the γ subunit creating a conformational change in AMPK, and activation-loop Thr172 is phosphorylated (bottom panel). KD, kinase domain. Adapted from (BARRETT 2011).

**Downstream targets of AMPK**

Active AMPK phosphorylates downstream targets at Ser/Thr residues to turn on energy-generating catabolic pathways and turn off energy-consuming anabolic pathways (HARDIE *et al.* 2012b; HARDIE *et al.* 2016). AMPK promotes glucose and fatty acid uptake in the cells by increased translocation of transporters GLUT1 and CD36, respectively, to the plasma membrane (BARNES *et al.* 2002; HABETS *et al.* 2009). Subsequently, by phosphorylating rate-limiting enzymes, AMPK stimulates glycolysis, mitochondrial respiration and fatty acid oxidation.
Mitochondrial biogenesis and maintenance of healthy mitochondria is also regulated by AMPK, ultimately leading to increased breakdown of glucose and fatty acids (Egan et al. 2011; O'Neill et al. 2011) (O’Neill 2011; Egan et al 2011). On the other hand, AMPK downregulates anabolic processes by phosphorylating proteins and enzymes in fatty acid, glycogen, and protein biosynthesis pathways (reviewed in (Hardie et al. 2012b)). AMPK achieves energy conservation by slowing down cell growth and proliferation, thus exerting its anti-tumor effects (Hawley et al. 2003).

Due to its central role in maintaining energy homeostasis, AMPK is a therapeutic target for type 2 diabetes and many other conditions such as obesity, cardiovascular disorders, and cancer (Hardie 2007; Fogarty and Hardie 2009; Oakhill et al. 2011). Although much is known about the structure, function, and regulation of AMPK, a detailed understanding of these areas needs further investigation. AMPK structure and function are conserved across different species. *Saccharomyces cerevisiae* serves as an important model organism to study most aspects of AMPK.

**Yeast as a model organism**

*Saccharomyces cerevisiae*, commonly known as baker’s yeast (brewer’s yeast, or budding yeast), is a simple eukaryote that nonetheless shares many genes with humans. Yeast cells are easy to propagate, and – importantly – this organism has a sexual haploid-diploid life cycle, making it easy to make combinatorial mutants. Interestingly, yeast cells have the capacity to survive without mitochondrial respiration, facilitating the analysis of mutants with mitochondrial defects, which is highly relevant to this work.
Snf1 protein kinase

Snf1, the yeast homolog of AMPK, is a founding member of the Snf1/AMPK family of serine/threonine protein kinases (HARDIE et al. 1998). Yeast cells prefer glucose as their carbon/energy source. Snf1, like AMPK, plays important roles in the metabolic control during nutrient stress, especially glucose limitation (HEDBACKER AND CARLSON 2008). Snf1 activates catabolic processes and inhibits anabolic processes whenever yeast cells are starved for glucose (HEDBACKER AND CARLSON 2008). Active Snf1 switches on the transcription of genes required for the utilization of less preferred sugars such as sucrose, galactose, raffinose, etc. (CARLSON et al. 1981; CELENZA AND CARLSON 1986; TREITEL et al. 1998). In fact, Snf1 was identified in a screen for mutants that are unable to utilize sucrose, giving Snf1 its name, sucrose non-fermenting 1 (CARLSON et al. 1981). Moreover, Snf1 is essential to turn on mitochondrial respiration and utilize non-fermentable carbon sources such as glycerol and ethanol (CELENZA AND CARLSON 1984).

Glucose limitation

Snf1

Slower growth

Utilization of less preferred carbon/energy sources (e.g. sucrose, glycerol)

Fig. 4. AMPK of yeast cells: Snf1. Snf1 plays a central role in adaptation of yeast cells to nutrient stresses such as low glucose, by slowing down cell growth and utilizing alternative carbon sources. Adapted from (BARRETT 2011).
**Structure of Snf1**

Snf1 protein kinase is a heterotrimeric protein consisting of the catalytic α subunit Snf1, three alternative regulatory β subunits, Gal83, Sip1 and Sip2, and the γ subunit Snf4 ([Hedbacker and Carlson 2008](#)). The Snf1 catalytic subunit contains a kinase domain at the N terminus, and a regulatory domain at the C terminus ([Celenza and Carlson 1986; Estruch et al. 1992](#)). The kinase domain contains the activation loop Thr210 residue (equivalent to Thr172 of AMPK) that is phosphorylated by upstream kinases to activate Snf1 ([Celenza and Carlson 1986; Estruch et al. 1992; McCartney and Schmidt 2001; Hong et al. 2003; Nath et al. 2003; Sutherland et al. 2003](#)). Crystal structure analyses suggest that the kinase domain forms homodimers, and in the inactive state the activation loop is buried within the dimers; interestingly, the kinase domain itself can be phosphorylated and regulated, bypassing the need for regulation by β and γ subunits ([Rudolph et al. 2005; Nayak et al. 2006](#)). The C terminus contains the autoinhibitory sequence (AIS), which binds to the kinase domain as well as γ subunit Snf4. Deletion of the AIS region alleviates the requirement for Snf4 ([Celenza and Carlson 1989; Estruch et al. 1992; Jiang and Carlson 1996](#)). AIS binds to the kinase domain and inhibits its activation; however, under glucose limiting conditions, Snf4 binds to AIS and releases the inhibition allowing Snf1 activation ([Celenza and Carlson 1989; Estruch et al. 1992; Jiang and Carlson 1996; Leech et al. 2003](#)). The C terminus also contains a region that binds to the β subunits ([Jiang and Carlson 1997](#)).

There are three alternative β subunits: Gal83, Sip1, and Sip2, forming three possible Snf1 protein kinase complexes ([Yang et al. 1994; Schmidt and McCartney 2000](#)). The β subunits are responsible for unique subcellular localization of Snf1 ([Vincent et al. 2001](#)) (Fig. 5). This is achieved by the presence of divergent N termini in the β subunits. All three Snf1 complexes are
cytoplasmic when abundant glucose is available. However, under metabolic stress such as glucose limitation, redistribution of Snf1 complexes occurs depending on the β subunit: Gal83 enriches in the nucleus, Sip1 relocalizes to the vacuole, and Sip2 remains cytoplasmic (Vincent et al. 2001). The C terminus of the β subunits has a conserved sequence that mediates their interaction with Snf1 and Snf4 (Yang et al. 1994; Jiang and Carlson 1997). The C terminus also contains a glycogen-binding domain (GBD), similar to CBM of the β subunits in AMPK. The Snf1-Gal83 complex is the major isoform observed during growth on glucose and contributes the most in response to glucose limitation (Hedbacker et al. 2004). Levels of Sip2 appear to somewhat increase when cells are shifted to non-fermentable carbon sources, whereas Sip1 levels remain constant (Vincent et al. 2001).

Fig. 5. Snf1 structure and regulation. Snf1 protein kinase is a heterotrimer consisting of α (Snf1), β (Gal83, Sip1, Sip2), and γ (Snf4) subunits. The catalytic α subunit, Snf1, contains a conserved activation-loop Thr-210 in its kinase domain, which is phosphorylated by upstream kinases in response to low glucose. Snf1 complex is cytoplasmic when abundant glucose is available. However, under glucose limitation, Gal83 relocates active Snf1 to the nucleus, Sip1 takes Snf1 to the vacuolar membrane, and the complex containing Sip2 remains cytoplasmic. Adapted from (Hedbacker and Carlson 2008).
The γ subunit, Snf4, binds to Snf1 and the different β subunits. Binding of Snf4 to AIS prevents AIS binding to the kinase domain of Snf1, thus allowing activation of Snf1 by upstream kinases (Celenza and Carlson 1989; Estruch et al. 1992; Jiang and Carlson 1996; Leech et al. 2003). Snf4 contains four CBS repeats that are known to bind to adenine nucleotides (Bateman 1997). However, unlike γ subunits of AMPK, which bind to AMP and act as sensors of energy stress, Snf4 is not known to bind to AMP to allosterically activate Snf1 (Mitchelhill et al. 1994; Wilson et al. 1996; Adams et al. 2004). Instead, in vitro studies suggest that although AMP, ADP, and ATP bind to Snf4, Snf4 bound to ADP (not AMP) protects it from dephosphorylation by phosphatases, thus maintaining the Thr210 residue in the phosphorylated state (Chandrashekarappa et al. 2011; Mayer et al. 2011).

**Regulation of Snf1**

Snf1 activation is regulated by glucose availability. Sak1, Tos3, and Elm3 are three upstream kinases that activate Snf1 by phosphorylation of Thr210 in the activation loop during glucose limitation, as well as other environmental stresses including alkaline pH, salt stress, and oxidative stress (Hong et al. 2003; Nath et al. 2003; Sutherland et al. 2003; Hong and Carlson 2007). While all three kinases have overlapping functions, Sak1 is the major kinase that activates Snf1; however, to observe a Snf1 null phenotype, all three kinases have to be deleted (Hong et al. 2003; Nath et al. 2003; Sutherland et al. 2003). Sak1 is not only important for activating Snf1, but also necessary and sufficient for nuclear localization of the Snf1-Gal83 complex, while Elm1 and Tos3 do not seem to play a major role (Hedbacker et al. 2004).

Snf1 is inactivated by Thr210 dephosphorylation, which primarily involves type 1 protein
phosphatase Glc7 in association with a regulatory protein Reg1 (Tu and Carlson 1994; Tu and Carlson 1995; McCartney and Schmidt 2001). In cells lacking Reg1, Snf1 is constitutively switched on during growth on high glucose (McCartney and Schmidt 2001). Reg2, another targeting subunit of Glc7, contributes to dephosphorylation of Snf1 when cells are replenished with glucose after prolonged starvation (Frederick and Tatchell 1996; Maziarz et al. 2016). Apart from Glc7, other phosphatases including Sit4, a type 2A-like protein phosphatase, and Ptc1, a type 2C protein phosphatase, have been implicated in dephosphorylation and inactivation of Snf1 (Ruiz et al. 2011; Ruiz et al. 2013).

Recent evidence also suggests that ubiquitination (Wilson et al. 2011) and SUMOylation (Simpson-Lavy and Johnston 2013) affect Snf1 Thr210 phosphorylation and activity. While the identity of the kinases and phosphatases that regulate Snf1 has been established, the mechanisms that regulate the balance between Thr210 phosphorylation and dephosphorylation are not completely understood and need further investigation.

**Downstream targets of Snf1**

Snf1 is critical for yeast cells to respond to energy stress such as glucose limitation. To achieve this, active Snf1 regulates transcription of various genes by phosphorylating repressors and activators and modifies enzymatic activity by phosphorylating enzymes of rate-limiting steps (Hardy et al. 1994; Woods et al. 1994; Huang et al. 1996; Randez-Gil et al. 1997; Treitel et al. 1998). In the end, Snf1 maintains energy homeostasis by activating genes that help the cells utilize alternative carbon sources, promotes respiration, inhibits energy-consuming synthesis of large polymers such as fatty acids, and slows down cell growth (Hedbacker and Carlson 2008).
Snf1 regulates gene expression by phosphorylating transcription factors such as the Mig1 repressor (Ostling and Ronne 1998; Treitel et al. 1998). Inactivation of Mig1 leads to the release of Mig1-dependent repression of many genes including *SUC, GAL, MAL*, and other glucose-repressible genes whose expression is inhibited by Mig1 in high glucose (Treitel and Carlsson 1995; Tzamarias and Struhl 1995). Snf1 also regulates transcriptional activators such as Cat8, which is phosphorylated by Snf1 directly (Randez-Gil et al. 1997). Cat8 is responsible for activating transcription (de-repression) of gluconeogenic genes and for growth on non-fermentable carbon sources (Randez-Gil et al. 1997; Young et al. 2003). Snf1 turns on mitochondrial respiration, and the *snf1Δ* mutant is unable to grow on non-fermentable carbon sources such as ethanol and glycerol. It is known that transcription of many genes involved in the mitochondrial transport of metabolites is Snf1 dependent (Young et al. 2003). Evidence also suggests that Snf1 modifies histone H3 by phosphorylation, regulates histone acetylation, and affects global gene expression (Lo et al. 2000; Zhang et al. 2013).

Snf1 also regulates activity of many enzymes involved in metabolic pathways by direct phosphorylation. For example, active Snf1 phosphorylates acetyl-CoA carboxylase (Acc1) and inhibits fatty acid biosynthesis to save energy expenditure (Woods et al. 1994). Additionally, Snf1 regulates the activity of glycogen synthase responsible for the rate-limiting step in accumulation of glycogen in cells (Hardy et al. 1994; Huang et al. 1996).

Apart from responding to energy stress, Snf1 is activated in response to environmental stresses such as nitrogen limitation, sodium and lithium ion stress, pH stress, and oxidative stress (Orlova et al. 2006; Hong and Carlsson 2007). Activated Snf1 enriches in the nucleus in response to some stresses, such as glucose limitation and alkaline pH, but not others such as salt stress (Hong and Carlsson 2007).
Comparison of Snf1 and AMPK

Snf1 and AMPK are highly conserved in their structure, regulation, and function (HARDIE et al. 1998; HEBBACKER AND CARLSON 2008; GHILLEBERT et al. 2011). Snf1 and AMPK turn on ATP-generating catabolic processes and turn off ATP-consuming anabolic process during nutrient stress. Snf1 and AMPK regulate transcription of genes and activity of metabolic enzymes by phosphorylation. Activation of these signaling kinases stimulates respiration that leads to increased energy production.

Snf1 and AMPK are heterotrimers each consisting of a catalytic α subunit and regulatory β and γ subunits (GHILLEBERT et al. 2011). The kinase domain of the α subunit contains a conserved activation loop threonine that is phosphorylated by upstream kinases for activation of Snf1/AMPK signaling. The β subunits act as scaffolding proteins that hold together the heterotrimer, and the γ subunits have the CBS domains that bind to different adenine nucleotides.

The regulation of Snf1/AMPK by phosphorylation and dephosphorylation is also very conserved. As a matter of fact, establishing the identity of the kinases that activate Snf1 in yeast facilitated the discovery of mammalian kinases that phosphorylate and activate AMPK (HONG et al. 2003; MOMCILOVIC et al. 2006). Yeast Snf1 upstream kinases such as Tos3 can activate mammalian AMPK in vitro (HONG et al. 2003). Similarly, the AMPK-activating kinases, LKB1 and CaMKKβ, can activate Snf1 in yeast (HONG et al. 2005; MOMCILOVIC et al. 2006). AMP, a signal of low energy, binds to the γ subunit of AMPK (at the exclusion of ATP) and creates a conformational change that leads to allosteric activation of AMPK; AMP binding makes AMPK a better substrate for activation by upstream kinases and protects AMPK from dephosphorylation (HARDIE 2011b). Unlike with AMPK, however, AMP does not allosterically activate Snf1 and the signal that leads to activation of Snf1 remains elusive (HARDIE et al. 1998). It has been
shown that ADP binds to Snf4 and protects Snf1 from dephosphorylation \textit{in vitro} (MAYER et al. 2011). However, based on analysis of ADP and ATP binding affinities, it was suggested that the yeast Snf1 complex responds to absolute ADP levels rather than to ADP:ATP ratios (MAYER et al. 2011). According to an earlier report, however, the absolute ADP levels in yeast cells shifted from high to low glucose do not appear to change as much as the AMP:ATP or ADP:ATP ratios (WILSON et al. 1996). In other words, whether absolute ADP levels represent a true \textit{in-vivo} signal for Snf1 activation remains an open question, and the existence of additional mechanisms that could regulate Snf1 in response to AMP:ATP ratios remains a distinct possibility.

In an attempt to identify new regulators of Snf1, a yeast two-hybrid screen was performed, recovering a novel Snf1-interacting partner: the mitochondrial voltage-dependent anion channel yVDAC1/Por1 (VYAS et al. 2001).

\textbf{Voltage-dependent anion channels}

Voltage-dependent anion channel (VDAC) proteins, also called mitochondrial porins, are conserved proteins present in the outer membrane of mitochondria and allow passage of small metabolites including AMP, ADP, and ATP (COLOMBINI 2004; COLOMBINI AND MANNELLA 2012). As the name suggests, VDACs are voltage-gated and, depending on the potential applied, VDACs can exist in two states: open and closed (PENG et al. 1992; COLOMBINI 2004; COLOMBINI AND MANNELLA 2012). In their open state, VDACs prefer anions including adenine nucleotides and metabolites of the TCA cycle. In their closed state, cations such as Ca$^{2+}$ can be transported in and out of mitochondria. VDACs are not only important for metabolic flux between cytoplasm and mitochondria, but they are also involved in respiration, apoptosis, and
cancer (Colombini 2004; Lemasters and Holmuhamedov 2006; Shoshan-Barmatz and Ben-Hail 2011).

The structure of VDACs is conserved across eukaryotes (Fig. 6); VDACs are β barrel proteins containing ~12-19 β strands embedded in the membrane with hydrophobic residues exposed to the lipid bilayer and hydrophilic residues forming the core with a diameter of ~3nm. VDACs also contain an α helix at the N terminus, which is important for metabolite selectivity (Peng et al. 1992; Zeth 2010).

**Fig. 6.** Structure of yVDAC1. (A) Side view. (B) Top view. Source: http://www.uniprot.org/uniprot/P04840

In humans, there are three VDACs that show tissue specific expression and function (Shoshan-Barmatz and Ben-Hail 2011). Yeast cells express two VDAC isoforms, yVDAC1 and yVDAC2 (also called Por1 and Por2, respectively). Por1 is an abundant 283 amino-acid protein in the mitochondrial outer membrane (MOM) and its permeability has been studied over many years (Colombini 2004). Por2 is 49% identical to Por1 and localizes to the MOM, but is not believed to have channel function (Blachly-Dyson et al. 1997; Lee et al. 1998).

Based on the preliminary interaction between Snf1 and Por1, and the functional involvement of the porins with adenine nucleotides, we hypothesized that Por1 and Por2 may be
involved in the regulation of Snf1. This work primarily aims at elucidating the mechanisms by which mitochondria communicate with the Snf1 signaling pathway via porins.

References


Ghillebert, R., E. Swinnen, J. Wen, L. Vandesteene, M. Ramon et al., 2011 The AMPK/SNF1/SnRK1 fuel gauge and energy regulator: structure, function and regulation. FEBS J 278: 3978-3990.


as the major site at which it phosphorylates AMP-activated protein kinase. J Biol Chem 271: 27879-27887.


Nayak, V., K. Zhao, A. Wyce, M. F. Schwartz, W. S. Lo *et al.*, 2006 Structure and dimerization of the kinase domain from yeast Snf1, a member of the Snf1/AMPK protein family. Structure 14: 477-485.


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


Sutherland, C. M., S. A. Hawley, R. R. McCartney, A. Leech, M. J. Stark et al., 2003 Elm1p is one of three upstream kinases for the Saccharomyces cerevisiae SNF1 complex. Curr Biol 13: 1299-1305.

Treitel, M. A., and M. Carlson, 1995 Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein. Proc Natl Acad Sci USA 92: 3132-3136.


Tu, J., and M. Carlson, 1995 REG1 binds to protein phosphatase type 1 and regulates glucose repression in Saccharomyces cerevisiae. EMBO J 14: 5939-5946.


Yang, X., R. Jiang and M. Carlson, 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., K. M. Dombek, C. Tachibana and T. Ideker, 2003 Multiple pathways are co-regulated by the protein kinase Snf1 and the transcription factors Adr1 and Cat8. J Biol Chem 278: 26146-26158.


Chapter 2

Mitochondrial porin Por1 and its homolog Por2 contribute to the positive control of Snf1 protein kinase in *Saccharomyces cerevisiae*

[The results presented in this chapter have been published (STROGOLOVA et al. 2012)]

Abstract

*Saccharomyces cerevisiae* Snf1 is a member of the conserved Snf1/AMP-activated protein kinase (Snf1/AMPK) family involved in regulating responses to energy limitation, which is detected by mechanisms that include sensing adenine nucleotides. Mitochondrial voltage-dependent anion channel (VDAC) proteins, also known as mitochondrial porins, are conserved in eukaryotes from yeast to humans and play key roles in mediating mitochondrial outer membrane permeability to small metabolites including ATP, ADP, and AMP. We previously recovered the yeast mitochondrial porin Por1 (yVDAC1) from a two-hybrid screen for Snf1-interacting proteins. Here, we present evidence that Snf1 interacts with Por1 and its homolog Por2 (yVDAC2). Cells lacking Por1 and Por2, but not respiratory-deficient *rho0* cells lacking the mitochondrial genome, exhibit reduced Snf1 activation-loop phosphorylation in response to glucose limitation. Thus, Por1 and Por2 contribute to the positive control of Snf1 protein kinase. Physical proximity to the VDAC proteins and mitochondrial surface could facilitate Snf1’s ability to sense energy limitation.

Introduction

Mammalian AMP-activated protein kinase (AMPK) is often referred to as the cell’s “fuel gauge” (HARDIE AND CARLING 1997). According to the standard model, AMPK responds to decreases in cellular energy by sensing increased levels of AMP relative to ATP (HARDIE 2007c;
The AMPK activation mechanism involves AMP binding to the γ subunit of the AMPK complex at the exclusion of ATP, making the catalytic α subunit of AMPK a better substrate for activation-loop threonine (Thr172) phosphorylation by upstream kinases while interfering with its dephosphorylation and inactivation. More recent evidence indicates that besides AMP binding, ADP binding also stimulates Thr172 phosphorylation of AMPK and protects it from dephosphorylation (Oakhill et al. 2011; Xiao et al. 2011). Activated AMPK functions to balance the energy “budget” by cutting general energy spending (e.g. by inhibiting cell growth and proliferation) while activating specific energy-generating mechanisms (e.g. glucose uptake). In accord with these important roles, defects in AMPK signaling have been linked to diabetes, obesity, and cancer, making AMPK a good drug target (Hardie 2007a; Fogarty and Hardie 2009).

Decreases in cellular energy levels can be caused by factors ranging from reduced glucose availability to reduced mitochondrial respiration (Kahn et al. 2005). Metformin, a drug that is often prescribed to patients with type 2 diabetes, is thought to activate AMPK indirectly, by affecting the respiratory complex chain I (El-Mir et al. 2000; Brunmair et al. 2004). Metformin also exhibits anti-cancer action, an effect that is largely mediated by AMPK (Vakana and Platani 2011; Dowling et al. 2012). These findings indicate that a better understanding of the functional connection between AMPK and mitochondria could provide valuable clues to the molecular etiology, treatment, and prevention of metabolic disorders and cancer.

Snf1 protein kinase of the yeast Saccharomyces cerevisiae is the homolog of mammalian AMPK and is required for growth on carbon sources that are less preferred than glucose, including non-fermentable carbon sources (Hedbacker and Carlson 2008). Like AMPK,
Snf1 is activated in response to glucose/energy limitation, which is detected by mechanisms that include ADP binding to the γ subunit of the Snf1 complex (Snf4), which similarly protects Snf1 from dephosphorylation of the activation-loop threonine (Thr210) (MAYER et al. 2011). Snf1 serves as a good model for studying the general aspects of Snf1/AMPK regulation in eukaryotes, and S. cerevisiae is particularly suitable for studies of the relationship between this kinase and mitochondria because of the well-known ability of this organism to survive with its respiratory function completely disrupted.

Using two-hybrid screening for Snf1-interacting proteins, we previously recovered the yeast mitochondrial porin Por1 (MIHARA AND SATO 1985; FORTE et al. 1987; VYAS et al. 2001). Mitochondrial porins, also widely known as voltage-dependent anion channel (VDAC) proteins, are conserved in eukaryotes from yeast to humans. These proteins play key roles in mediating mitochondrial outer membrane permeability to small metabolites, notably adenine nucleotides, and are involved in mitochondrial respiration; VDACs also play important roles in apoptosis and represent attractive targets for anti-cancer therapy (COLOMBINI 2004; LEMASTERS AND HOLMUHAMEDOV 2006; SIMAMURA et al. 2008).

Here, we present evidence that the yeast mitochondrial porin Por1/yVDAC1 and its homolog Por2/yVDAC2 (hereafter referred to as Por1 and Por2, respectively) share a role in the positive control of Snf1, revealing a new and unexpected layer of regulation. Physical proximity to the VDAC proteins and mitochondrial surface could facilitate Snf1’s ability to sense energy limitation.
Materials and methods

Yeast strains and media. The *S. cerevisiae* strains used in this study are listed in Table 1. Yeast two-hybrid assays were performed in strain CTY10-5d (*MATα gal4 gal80 URA3::lexAop-lacZ his3 leu2 ade2 trp1-901*) (R. Sternglanz, SUNY, Stonybrook, NY). Except for CTY10-5d and its *por1Δ* derivative (see below), all strains were in the W303 background. The *snf1Δ::KanMX6* allele has been described (ORLOVA et al. 2006). To generate the *por1Δ::KanMX6* and *por2Δ::KanMX6* alleles, the marker sequences were amplified by PCR with primers flanking the corresponding open reading frames. The mutant alleles were introduced into W303-1A (*MATα ade2-1 can1–100 his3–11,15 leu2–3,112 trp1–1 ura3–1*) by transformation; all yeast transformations were performed using standard methods (ROSE et al. 1990). In addition, *por1Δ::KanMX6* was introduced into CTY10-5d, and *por2Δ::KanMX6* was introduced into W303-1B (*MATα ade2-1 can1–100 his3–11,15 leu2–3,112 trp1–1 ura3–1*). The genotypes of all knock-out strains were confirmed by PCR analysis of genomic DNA. Double mutants lacking Por1 and Por2 were constructed by tetrad analysis of diploid cells obtained by crossing *por1Δ* and *por2Δ* single mutants of the opposite mating types. The *rho0* cells were generated in W303-1A by ethidium bromide treatment (GOLDRING et al. 1970). The *rho0* status of the glycerol-non-utilizing cells thus obtained was verified by the loss of the mitochondrial *COX1* and *COX3* genes by PCR; the presence of the nuclear *COX4* gene was used as a positive control.

Rich medium was yeast extract/peptone (YEP) supplemented with extra tryptophan (40 mg/L) and adenine (20 mg/L); synthetic complete (SC) medium lacking appropriate supplements was used to select for plasmids (ROSE et al. 1990). Unless indicated otherwise, the media contained 2% glucose, and cells were grown at 30°C.
**Yeast two-hybrid interaction assays.** Plasmids pIT469 (Kuchin et al. 2000) and pRJ79 (Jiang and Carlson 1996) express LexA-Snf1 and VP16-Snf1 from vectors pEG202 (Golemis et al. 1997) and pVP16 (Vojtek et al. 1993), respectively. To construct pLexA-Por1 and pGAD-Por1, a PCR fragment encompassing the POR1 open reading frame was inserted at the BamHI site of pEG202 and pACTII (LeGrain et al. 1994), respectively. The pLexA-Por1 and pGAD-Por1 plasmids were confirmed for ability to complement the por1Δ mutant derivative of CTY10-5D for the growth defect on glycerol at 37°C (Blachly-Dyson et al. 1997). Strain CTY10-5d was co-transformed with pairs of plasmids expressing the protein pairs being tested. Transformants were grown to mid-log phase with plasmid selection in SC lacking histidine and leucine and containing 2% glucose, and then shifted for 3h to an otherwise identical medium containing 0.05% glucose. β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units, as described previously (Vyas et al. 2001).

**Coimmunoprecipitation assays.** Plasmids pHA-Por1 and pHA-Por2 express N-terminal triple hemagglutinin (HA) epitope-tagged Por1 (HA-Por1) and HA-Por2, respectively, and were constructed by inserting the POR1 and POR2 coding sequences into the BamHI site of vector pWS93 (Song and Carlson 1998). Cells of W303-1A carrying the snf1Δ mutation were transformed with plasmid pSK117 expressing Snf1 (Treitel et al. 1998) or with vector pSK134 (Treitel et al. 1998; Vincent et al. 2001a), and co-transformed with plasmids pHA-Por1, pHA-Por2, or with vector pWS93. Cells were grown to mid-log phase with plasmid selection in SC lacking leucine and uracil and containing 2% glucose; where indicated, cells were grown as above and then shifted for 1h to an otherwise identical medium containing 0.05% glucose. Protein extracts were prepared, and immunoprecipitations (from 200 mg of protein per reaction)
were performed with anti-HA 12CA5 essentially as described previously (TREITEL et al. 1998) in a buffer containing 0.1% Triton X-100 and 50 mM NaCl (VYAS et al. 2001). The immunoprecipitates were examined for the presence of Snf1 by immunoblot analysis using anti-polyhistidine antibody H1029 (Sigma-Aldrich), which strongly recognizes Snf1 due to the presence of a natural stretch of 13 consecutive histidines near its N terminus (amino acids 18-30) (ORLOVA et al. 2008). The presence of HA-Por1 and HA-Por2 was analyzed using anti-HA. Signals were detected by enhanced chemiluminescence using ECL Plus (Amersham Biosciences) or HyGlo (Denville Scientific). The presence of Snf1, HA-Por1, and HA-Por2 in the extracts was analyzed by immunoblotting similarly (10 mg protein per lane).

**Analysis of SUC2-LEU2-lacZ reporter expression.** To target its genomic integration, reporter plasmid pLS11 (SAROKIN and CARLSON 1985) was linearized at the NcoI site within the URA3 gene prior to transformation. pLS11 was first introduced into a double heterozygous diploid (AMS2) obtained by crossing por1Δ and por2Δ single mutants. Wild-type and mutant haploids carrying pLS11 (Ura') were then recovered by tetrad analysis. The rho0 derivatives were generated from pLS11-carrying wild-type cells as described above. Cells were grown to mid-log phase in YEP with 2% glucose and then shifted to YEP with 0.05% glucose for 3h. β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units.
Results

Snf1 and Por1 interact in the two-hybrid system

Por1 was recovered previously by S.K. from a two-hybrid screen for Snf1-interacting proteins (Vyas et al. 2001). To further demonstrate this interaction, fusions to the LexA DNA-binding protein were tested in combination with fusions to the viral VP16 or Gal4 transcription activation domain (GAD) for ability to activate a reporter with LexA binding sites (lexAop-lacZ) in strain CTY10-5d. LexA-Por1 interacted with VP16-Snf1 (Fig. 7A). In the reciprocal setting, LexA-Snf1 interacted with GAD-Por1 (Fig. 7B). In both cases, the interaction signals exhibited an apparent increase in response to glucose limitation. As discussed further below, we do not necessarily take this to suggest that the affinity of the Snf1-Por1 interaction is stimulated by glucose limitation.

Fig. 7. Snf1 and Por1 interact in the two-hybrid system. Strain CTY10-5d with an integrated lexAop-lacZ reporter was co-transformed with pairs of plasmids expressing the indicated proteins. β-Galactosidase activity was assayed in mid-log cultures grown in selective medium with high (2%) glucose (open bars) and shifted for 3h to an otherwise...
identical medium with low (0.05%) glucose (closed bars). Values are averages for at least three transformants and 
are expressed in Miller units (U). Error bars indicate standard errors. Asterisks indicate statistically significant 
difference from the corresponding controls ($p < 0.03$ both in high and low glucose). (A) LexA-Por1 interacts with 
VP16-Snf1. (B) LexA-Snf1 interacts with GAD-Por1.

In terms of β-galactosidase activity, the observed interaction was weak by comparison to 
other known interactions of Snf1 (e.g. within the classical Snf1 complex (JIANG AND CARLSON 
1996)), which was not surprising; in fact, it was more surprising that this interaction could be 
detected at all. In order for a two-hybrid interaction to be detected, both interacting partners 
must be present in the nucleus. While this is not a problem for Snf1, which is a 
cytoplasmic/nuclear protein (VINCENT et al. 2001b), Por1 is a major mitochondrial outer 
membrane protein (BLACHLY-DYSON et al. 1997). However, porin/VDAC proteins are 
apparently not absolutely restricted from the nucleus, allowing the yeast two-hybrid system to 
work and provide valuable preliminary information. For example, some interactions of 
eukaryotic VDAC proteins that were initially detected in the yeast two-hybrid system were 
subsequently shown to exist and to be physiologically relevant (FENG et al. 2007; CHEN et al. 
2009). We therefore decided to explore the Snf1-Por1 interaction further.

**Snf1 coimmunoprecipitates with Por1**

We next conducted coimmunoprecipitation experiments. A snf1Δ strain was co-
transformed with plasmids expressing Snf1 and HA-Por1. Cells were grown to mid-log phase in 
abundant glucose, protein extracts were prepared and subjected to immunoprecipitation with 
anti-HA. The immunoprecipitates were then tested for the presence of Snf1 by immunoblot 
analysis. Snf1 was detected in the immunoprecipitates from cells expressing both HA-Por1 and
Snf1, but not from cells expressing HA-Por1 but no Snf1, or expressing Snf1 but no HA-Por1 (Fig. 8A). Thus, these results provided further evidence that Snf1 interacts with Por1.

![Fig. 8. Snf1 coimmunoprecipitates with Por1 and Por2. (A, B) Snf1 coimmunoprecipitates with HA-Por1. (A) Cells carrying the snf1Δ mutation (isogenic to W303-1A) were transformed with a plasmid expressing Snf1 (Snf1: +) or the appropriate vector (Snf1: -), as well as with a plasmid expressing HA-Por1 (HA-Por1, +) or the appropriate vector (HA-Por1, -). Cells were grown to mid-log phase with plasmid selection in media containing 2% glucose, protein extracts were prepared, and HA-Por1 was immunoprecipitated with anti-HA. The presence of Snf1 and HA-Por1 in the extracts (Extract) and immunoprecipitates (IP: α-HA) was examined by immunoblotting. (B) Cells co-expressing Snf1 and HA-Por1 were grown in 2% glucose as for panel A (H, high glucose), and then shifted for 1h to an otherwise identical medium with 0.05% glucose (L, low glucose). Coimmunoprecipitation experiments were performed as for panel A. (C, D) Snf1 coimmunoprecipitates with HA-Por2. Experiments in panels C and D were performed as for panels A and B, respectively, except that HA-Por2 was expressed instead of HA-Por1.]

We also compared the ability of Snf1 to coimmunoprecipitate with Por1 in high and low glucose conditions. In contrast to the two-hybrid experiments, there was no apparent increase in interaction upon shift from high to low glucose (Fig. 8B). It should be noted that the apparent strength of a two-hybrid interaction reflects not only interaction affinity, but also the nuclear
content of the interaction partners. The latter factor could at least partly account for the increased two-hybrid signal in low glucose, since there is more Snf1 in the nucleus under carbon stress conditions (Vincent et al. 2001b). In addition, the lexAop-lacZ two-hybrid reporter could be more responsive to activation in low glucose, since this reporter is constructed on the basis of the GAL1 promoter, which is glucose-regulated (West et al. 1984; Johnston 1987).

Lack of Por1 and its homolog Por2 affects Snf1 activation

*S. cerevisiae* has two VDAC isoforms, Por1 and Por2. Por2 is 49% identical and 70% similar to Por1 and similarly localizes to the mitochondrial outer membrane (Blachly-Dyson et al. 1997; Zahedi et al. 2006). Unlike Por1, however, Por2 does not appear to possess channel function (Blachly-Dyson et al. 1997; Lee et al. 1998). Unlike the por1Δ mutation, the por2Δ mutation alone does not affect growth on non-fermentable carbon sources, but nonetheless enhances por1Δ for this defect (Blachly-Dyson et al. 1997). This and other evidence strongly suggested the existence of a mechanism where Por1 and Por2 share a role in non-fermentable carbon source utilization that is not directly related to channel function (Blachly-Dyson et al.; Lee et al. 1998). Since Snf1 is required for growth on non-fermentable carbon sources (Hedbacker and Carlson 2008), and since Snf1 also coimmunoprecipitates with Por2 (Fig. 8C, D), we considered the possibility that Por1 and Por2 share a role in the positive control of Snf1.

Catalytic activation of Snf1 requires phosphorylation of its conserved activation-loop threonine (Thr210, which corresponds to Thr172 of AMPK) that is performed by the upstream kinases Sak1, Tos3, and Elm1 (Hong et al. 2003; Nath et al. 2003; Sutherland et al. 2003). In the presence of abundant glucose, Snf1 is turned off by Thr210 dephosphorylation, which
involves type 1 protein phosphatase Glc7 together with its regulatory protein Reg1 (Tu and Carlson 1994; Tu and Carlson 1995; McCartney and Schmidt 2001). When wild-type cells are shifted from high to low glucose, the level of activated phospho-Thr210-Snf1 increases dramatically (McCartney and Schmidt 2001). In comparison to the wild type, double mutant cells lacking Por1 and Por2 exhibited a lower level of phospho-Thr210-Snf1, as hypothesized (Fig. 9A), with a reduction of approximately 5-fold upon normalization to total Snf1 levels. No such Snf1 activation defects were observed in cells lacking Por1 alone, Por2 alone, or in respiratory-deficient rho\(^0\) cells lacking the mitochondrial genome (Fig. 9B), with the normalized phospho-Thr210-Snf1 levels in these mutants and the wild type differing from each other by no more than 30%.

Thus, these results indicated that the combined lack of Por1 and Por2 confers a defect in Snf1 activation loop phosphorylation in low glucose, and that this defect is not directly related to compromised respiratory competence.

We also examined the effects of the mutations on the regulation of an integrated SUC2-LEU2-lacZ reporter; expression of this reporter is activated by glucose limitation in a Snf1-dependent manner (Sarokin and Carlson 1985). Under glucose-limiting conditions, the por1\(\Delta\) por2\(\Delta\) double mutation caused a 13-fold reporter activation defect relative to the wild type (Fig. 9C). Interestingly, the por1\(\Delta\) and rho\(^0\) mutations also affected activation, albeit to a lesser extent (2.5-fold and 4.1-fold, respectively), raising the possibility that these mutations affect yet another aspect of Snf1 pathway activity that is distinct from Thr210 phosphorylation, as discussed in Chapters 4 and 5.
Fig. 9. Lack of Por1 and Por2 affects Snf1 activation. (A, B) Cells of the indicated genotypes (isogenic to W303-1A) were grown to mid-log phase in YEP containing 2% glucose (H, high glucose) and shifted for 60 min (A) or 90 min (B) to YEP containing 0.05% glucose (L, low glucose). The activation-loop Thr210 phosphorylation status of Snf1 was analyzed by immunoblotting as described previously (ORLOVA et al. 2008): phospho-Thr210-Snf1 (PT210) was detected with anti-phospho-Thr172-AMPK (Cell Signaling Technology), and the total Snf1 protein (Snf1) was detected by re-probing with anti-polyhistidine antibody H1029 (Sigma-Aldrich), which recognizes the natural stretch of 13 histidines present in Snf1. (C) Cells of the indicated genotypes with an integrated SUC2-LEU2-lacZ reporter were grown to mid-log phase in YEP with 2% glucose (H, high glucose) and then shifted to YEP with 0.05% glucose for 3h (L, low glucose). β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units (U). Values are averages for four to six assays. Error bars indicate standard errors. For low glucose conditions, the value for the por1Δ por2Δ double mutant was significantly different from any other value ($p < 0.001$), and the values for por1Δ and rho0 were significantly different from any other value and from each other ($p < 0.003$). Under high glucose conditions, the values were 4.7 units (for the wild type) or lower.
Discussion

In this study, we present evidence that the yeast mitochondrial VDAC proteins Por1 and Por2 contribute to the positive control of Snf1 activation-loop phosphorylation, and this role is not directly mediated by the respiratory proficiency of the cell.

The VDAC proteins represent a major component of the interface between the mitochondria and cytoplasm of the eukaryotic cell (COLOMBINI 2004). Proteomic studies in yeast indicate that Por1 and Por2 physically interact with numerous proteins, including components of major nutrient-sensing pathways such as TOR, PKA, casein kinase II, and others (Ho et al. 2002); the ability of Snf1 to interact with Por1 and Por2 is also consistent with the previous finding that Snf1 associates with the mitochondria (SICKMANN et al. 2003). If the mitochondrial surface serves as a major substratum for interactions between global regulatory pathways, the Snf1 activation defect observed in the por1Δ por2Δ mutant could represent an indirect consequence of major pathway miscommunication caused by mitochondrial surface perturbation.

However, Por1 and Por2 could also at least partly contribute to Snf1 regulation by a more direct mechanism. Similar to mammalian AMPK, Snf1 activation correlates with increased AMP-to-ATP ratios in vivo; unlike AMPK, however, the yeast Snf1 complex does not respond to regulation by AMP in vitro (WILSON et al. 1996; ADAMS et al. 2004). The identity of the relevant in-vivo AMP and/or ATP sensor, if it exists, remains unknown. Following the recent findings that mammalian AMPK is positively regulated by ADP (OAKHILL et al. 2011; XIAO et al. 2011), it was shown that ADP binding to Snf4 protects yeast Snf1 from Thr210 dephosphorylation in vitro (MAYER et al. 2011). Based on analysis of ADP and ATP binding affinities, it was suggested that the yeast Snf1 complex responds to absolute ADP levels rather
than to ADP-to-ATP ratios (Mayer et al. 2011). At the same time, according to an earlier report (Wilson et al. 1996), the absolute ADP levels in yeast cells grown in high and low glucose do not appear to differ as much as the AMP-to-ATP or ADP-to-ATP ratios. These considerations suggest a possible functional niche for Por1 and Por2. We envision that an ability to interact with the VDAC proteins and proximity to the mitochondrial surface could put Snf1 complex in a better position to sense glucose/energy limitation. For example, as glucose levels decrease, the ADP levels could rise more sharply at the mitochondrial surface, making it a good location to monitor. In other words, the VDAC proteins could assist the γ subunit, Snf4, in sensing the local change in ADP levels. Alternatively, the VDAC proteins could be more directly involved in the regulation of Snf1 in a Snf4-independent manner, i.e. they may themselves serve as “sensors” of an energy stress signal/ligand. For example, the VDACs could serve as sensors of nucleotides, e.g. AMP, ADP, ATP, NADH, NAD+, etc., or other metabolites, e.g. glucose-6-phosphate. Further experiments will be necessary to address these and other possibilities.

**Table 1.** *S. cerevisiae* strains

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<th>Strain</th>
<th>Genotype</th>
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<td><em>MATa his3–11,15 leu2–3,112 trp1–1 ura3–1 por1Δ::KanMX6</em></td>
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<td>This study</td>
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<td>This study</td>
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<tr>
<td>AMS2</td>
<td>YSK1280 X YSK1283</td>
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AMS10  \( \text{MAT}a \text{his}3\text{--}11,15 \text{leu}2\text{--}3,112 \text{trp}1\text{--}1 \text{ura}3\text{--}1 \text{por}1\Delta\text{::KanMX6} \)  
\( \text{por}2\Delta\text{::KanMX6} \)

AMS-R0  \( \text{MAT}a \text{his}3\text{--}11,15 \text{leu}2\text{--}3,112 \text{trp}1\text{--}1 \text{ura}3\text{--}1 \text{rho}^0 \)  
This study

CTY10-5d  \( \text{MAT}a \text{gal}4 \text{gal}80 \text{URA3}\text{::lexAop-lacZ} \text{his}3 \text{leu}2 \text{ade}2 \text{trp}1 \)  
R. Sternglanz

YSK1271  \( \text{MAT}a \text{gal}4 \text{gal}80 \text{URA3}\text{::lexAop-lacZ} \text{his}3 \text{leu}2 \text{ade}2 \text{trp}1 \)  
This study

\( \text{por}1\Delta\text{::KanMX6} \)

References


Strogolova, V., M. Orlova, A. Shevade and S. Kuchin, 2012 Mitochondrial porin Por1 and its homolog Por2 contribute to the positive control of Snf1 protein kinase in Saccharomyces cerevisiae. Eukaryot Cell 11: 1568-1572.

Sutherland, C. M., S. A. Hawley, R. R. McCartney, A. Leech, M. J. Stark et al., 2003 Elm1p is one of three upstream kinases for the Saccharomyces cerevisiae SNF1 complex. Curr Biol 13: 1299-1305.


Tu, J., and M. Carlson, 1995 REG1 binds to protein phosphatase type 1 and regulates glucose repression in Saccharomyces cerevisiae. EMBO J 14: 5939-5946.


Chapter 3

Mitochondrial porins as sensors of a signal that regulates the Snf1/AMPK pathway

Abstract

The heterotrimeric Snf1 protein kinase in the yeast *Saccharomyces cerevisiae* plays an important role in sensing and responding to nutrient/energy stress such as during glucose limitation. The N-terminal region of the catalytic a subunit, Snf1, contains the kinase domain (Snf1-KD). Snf1-KD contains the activation-loop Thr210 residue that is phosphorylated during glucose limitation. Previous *in-vitro* experiments showed that ADP binding to the so-called “sensory” γ subunit, Snf4, protects Snf1 Thr210 from dephosphorylation by phosphatases. However, the physiological relevance of this mechanism of sensing energy/glucose stress remains controversial. The voltage dependent anion channel (VDAC) proteins allow the flux of small metabolites, including ATP, ADP, and AMP, between the cytoplasm and the outer membrane of mitochondria. As such, VDACs are in an excellent position to sense the energy status of the cell.

In the previous chapter we have shown that yeast cells lacking mitochondrial VDAC proteins, Por1 and Por2, exhibit a defect in the catalytic activation of Snf1 under glucose limiting conditions. Here, we present evidence that Por1 interacts with Snf1-KD in the two-hybrid system, and that Por1 and Por2 are required for normal Thr210 phosphorylation of Snf1-KD.

Since Snf1-KD is regulated independently of the “sensory” Snf4 subunit, our results suggest that VDACs can independently sense a signal that regulates Snf1. Moreover, genetic evidence suggests that Por1 and Por2 function downstream of hexokinase 2 (Hxk2), a negative regulator of Snf1, raising the possibility that one of the signals sensed by VDACs is a glucose metabolite, presumably glucose-6-phosphate.
Introduction

The Snf1/AMPK family of protein kinases senses the energy status and regulates metabolic processes in eukaryotes. Mammalian AMPK (AMP-activated protein kinase) is often called the cell’s “fuel gauge”, as AMPK monitors the AMP:ATP ratio, and during energy stress, it turns on ATP-generating processes and switches off ATP-consuming processes (Hardie and Carling 1997). In conditions such as hypoglycemia and hypoxia, when the AMP-to-ATP ratio is high, AMP binds to the γ subunit of AMPK leading to a conformational change. This helps the upstream kinases to activate AMPK by phosphorylation of the conserved activation-loop Thr172 residue present in the catalytic α subunit. Binding of AMP also protects AMPK from deactivation by phosphatases that remove the phosphate group. Upon activation, AMPK promotes uptake of glucose and fatty acids, activates catabolic processes such as glycolysis and fatty acid breakdown, and stimulates mitochondrial respiration to increase ATP production. Moreover, active AMPK conserves energy by inhibiting anabolic processes and slowing down cell growth and proliferation. Due to its critical roles, impairment in the AMPK pathway can lead to obesity, type 2 diabetes, cardiovascular disorders, cancer, and other diseases (Fogarty and Hardie 2009; Hardie et al. 2012).

The AMPK homolog of budding yeast, Snf1, serves as an important model for studying general aspects of AMPK regulation and function. Whenever the preferred carbon source, glucose is limiting, Snf1 helps cells to adapt and utilize alternative carbon sources, as well as switch on mitochondrial respiration (Hedbacker and Carlsson 2008). Like AMPK, Snf1 protein kinase is a heterotrimeric complex consisting of the catalytic α subunit Snf1, three alternative scaffolding/targeting β subunits (Sip1, Sip2, and Gal83), and the “sensory” γ subunit Snf4. Snf1 is activated by phosphorylation of its activation-loop Thr210 residue under glucose
limiting conditions. In the native complex, Snf4 binds to the C-terminal regulatory domain (Snf1-RD) of Snf1, which allows the N-terminal kinase domain (Snf1-KD) to become accessible for phosphorylation by upstream kinases. *In-vitro* experiments have shown that ADP can bind to Snf4 and protect Snf1 from phosphatases. However, the physiological relevance of this finding remains unclear, especially considering the fact that Snf1 remains glucose-regulated in the *snf4Δ* mutant (McCARTNEY AND SCHMIDT 2001). Furthermore, Thr210 phosphorylation of the isolated Snf1-KD remains glucose-regulated, meaning that at its core, Snf1 regulation by glucose does not require the β and γ subunits or the C-terminal non-catalytic domain (RUIZ et al. 2012).

Therefore, the nature of the Snf1-regulating signal and the mechanism of its sensing are not completely understood.

Voltage-dependent anion channel (VDAC) proteins – also known as mitochondrial porins - are highly conserved among eukaryotes and allow the passage of small metabolites, including adenine nucleotides, between the cytoplasm and the mitochondria (COLOMBINI 2004). Porins play an important role in respiration and are an important drug target because of their roles in apoptosis and cancer (SIMAMURA et al. 2008; SHOSHAN-BARMATZ AND BEN-HAIL 2011).

In the previous chapter, we have shown that the yeast VDAC proteins, Por1 and Por2, interact with Snf1 and positively regulate its catalytic activation. How the porins affect Snf1 activation remains unclear. In this study, we present evidence that Por1 and Por2 regulate the activation of the heterotrimer-independent Snf1-KD. We also show that Por1 and Por2 function downstream of hexokinase 2 (Hxk2), a glycolytic enzyme that is known to play a role in Snf1 inhibition in the presence of glucose. Indeed, the triple knock-out mutant lacking Hxk2, Por1, and Por2 shows reduced Snf1 Thr210 phosphorylation just as the *por1Δ por2Δ* double mutant. Our results suggest that Por1 and Por2 play a role as heterotrimer-independent sensors in the
regulation of Thr210 phosphorylation, and raise the possibility that one of the metabolites sensed by Por1 and Por2 is glucose-6-phosphate.

Materials and Methods

Yeast strains and media. The *S. cerevisiae* strains used in this study are listed in Table 2. Except for strain CTY10-5d (R. Stern galanz, SUNY, Stony Brook, NY) used for yeast two-hybrid assays, all strains were in the W303 genetic background. To generate the *por1Δ::KanMX6*, *por2Δ::KanMX6*, and *hxk2Δ::HisMX6* alleles, the marker sequences were amplified by PCR with primers flanking the corresponding open reading frames. Construction of the *snf1Δ* has been described previously (ORLOVA *et al.* 2006). Double mutants lacking Por1 and Por2 were constructed by tetrad analysis of diploid cells obtained by crossing *por1Δ* and *por2Δ* single mutants of the opposite mating types. Haploid cells expressing Snf1-KD (strains CGY5 and CGY6) were constructed as follows. First, plasmid pCG2 containing the SNF1-KD sequence and the *URA3* selectable marker (see below) was linearized by digesting with BglIII (within the SNF1 sequence) and integrated into the *SNF1* locus of the wild-type diploid MMY25 using a standard transformation protocol (ROSE *et al.* 1990). Next, haploid strains expressing Snf1-KD were recovered by tetrad analysis. To create *por1Δ* and *por2Δ* mutants expressing Snf1-KD, strains YSK1279 (*por1Δ::KanMX6*) and YSK1283 (*por2Δ::KanMX6*) were crossed to strain CGY6, and Kan⁺ Ura⁺ segregants were obtained by tetrad analysis. The *por1Δ por2Δ* double mutants expressing Snf1-KD were constructed by tetrad analysis of a diploid strain obtained by crossing *por1Δ* and *por2Δ* single mutants of the opposite mating types, each expressing Snf1-KD. The *hxk2Δ por1Δ* and *hxk2Δ por2Δ* double mutants were constructed by crossing AMS132 (*hxk2Δ::HisMX6*) to AMS32 (*por1Δ::KanMX6*) and AMS39 (*por2Δ::KanMX6*), respectively,
followed by tetrad analysis. The \( hxx2\Delta por1\Delta por2\Delta \) triple mutants were constructed by tetrad analysis of diploid cells obtained by crossing AMS161 (\( hxx2\Delta::HisMX6\ por1\Delta::KanMX6 \)) and AMS164 (\( hxx2\Delta::HisMX6\ por2\Delta::KanMX6 \)) double mutants of the opposite mating types. The genotypes of all strains were confirmed by PCR analysis of genomic DNA. Strains AMS32, AMS39, AMS47, AMS161, and AMS164 also contain an integrated \( SUC2-LEU2-lacZ \) reporter (Sarokin and Carlson 1985).

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

**Yeast two-hybrid assays.** Plasmid pLexA-Snf1-KD expresses a LexA-Snf1-KD fusion and contains the fragment encoding Snf1-KD that was described previously and can complement the \( snf1\Delta \) mutation (Jiang and Carlson 1996). In control experiments, LexA was expressed from plasmid pEG202 (Golemis et al. 1997). To construct pGAD-Por1, a PCR fragment encompassing the \( POR1 \) open reading frame was inserted at the BamHI site of pACTII (Legrain et al. 1994). The pGAD-Por1 plasmid was confirmed for ability to complement the \( por1\Delta \) mutant derivative of CTY10-5D for the growth defect on glycerol at 37°C (Blachly-Dyson et al. 1997). Strain CTY10-5d was co-transformed with pairs of plasmids expressing the protein pairs being tested. Transformants were grown to mid-log phase with plasmid selection in SC lacking histidine and leucine and containing 2% glucose, and then shifted for 3h to an otherwise identical medium containing 0.05% glucose. \( \beta \)-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units, as described previously (Vyas et al. 2001).
Plasmid construction. Plasmid pCG2 was constructed by ligating a PCR-amplified SNF1-KD fragment including ~ 600 bp of the 5’ regulatory region into the BamHI site of pRS306, an integrating vector with the URA3 selectable marker (Sikorski and Hieter 1989). Expression of Snf1-KD was confirmed by immunoblotting (see below).

Immunoblotting. Cells were grown to mid-log phase in YEP containing 2% glucose and then shifted for 1h to an otherwise identical medium containing 0.05% glucose. Protein extracts were prepared using the boiling/alkaline lysis method and were examined for the presence of Snf1 and Snf1-KD by using anti-polyhistidine antibody H1029 (Sigma-Aldrich), which strongly recognizes Snf1 due to the presence of a natural stretch of 13 consecutive histidines near its N terminus (amino acids 18-30) (Orlova et al. 2008). Thr-210 phosphorylation of Snf1 and Snf1-KD was determined by using anti-phospho-Thr172-AMPK antibody (Cell Signaling Technology). Signals were detected by enhanced chemiluminescence using Pierce ECL2 or HyGlo (Denville Scientific).

Analysis of SUC2-LEU2-lacZ reporter expression. Cells carrying the integrated SUC2-LEU2-lacZ reporter (Sarokin and Carlson 1985) were grown to mid-log phase in YEP with 2% glucose and then shifted to YEP with 0.05% glucose for 3h. β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units, as described previously (Vyas et al. 2001).
Results

**Por1 and Por2 regulate Thr210 phosphorylation of Snf1-KD**

Our previous results show that the lack of Por1 and Por2 reduces Snf1 Thr210 phosphorylation in glucose-limiting conditions. Interestingly, the channel function of the porins does not appear to be important for this regulation because Por2, which is a non-channel forming porin, acts redundantly with Por1 in Snf1 activation (Blachly-Dyson et al. 1997). Moreover, since respiratory deficient rho0 cells activate Snf1 normally, the defect in the activation of Snf1 is not directly related to the respiratory deficiency of the por1Δ por2Δ cells. Thus, the regulatory function of Por1 and Por2 in the Snf1 pathway is separable from their channel activity and role in respiration. We wanted to investigate further the role played by the porins in the catalytic activation of Snf1. One important question to address was whether Por1 and Por2 simply facilitate Snf1 regulation via the regulatory subunits – particularly the “sensory” γ subunit Snf4 - of the kinase complex, or whether they have an independent role. The latter possibility stems from the fact that Snf1 remains glucose-regulated in the snf4Δ mutant, and that the isolated Snf1-KD that is not associated with the rest of the complex is regulated by glucose in vivo (McCartney and Schmidt 2001; Ruiz et al. 2012). We therefore decided to test the ability of the porins to regulate the catalytic activation of Snf1-KD.

Wild-type and mutant cells were grown to mid-log phase in the presence of high (2%) glucose and then shifted to low (0.05%) glucose for 60 min. The phosphorylation status of Snf1-KD was determined by immunoblotting. The por1Δ por2Δ double mutant exhibited reduced Thr210 phosphorylation of Snf1-KD compared to the wild type (Fig. 10). No such Snf1-KD activation defects were observed in cells lacking Por1 alone and Por2 alone (data not shown).
Fig. 10. Roles of Por1 and Por2 in the activation of the Snf1 kinase domain. Cells of the indicated genotypes expressing the Snf1 kinase domain (Snf1-KD) were grown to mid-log phase in YEP containing 2% glucose (H, high glucose) and shifted for 60 min to YEP containing 0.05% glucose (L, low glucose). Thr-210 phosphorylation (PT210) status and total levels of Snf1-KD (Total) were analyzed by immunoblotting as described previously (Orlova et al. 2008) using anti-phospho-Thr172-AMPK (Cell Signaling Technology) and anti-polyhistidine antibody H1029 (Sigma-Aldrich), respectively.

Snf1-KD and Por1 interact in the two-hybrid system

In Chapter 2 we showed that Por1 interacts with full-length Snf1. This interaction may be dependent on the formation of a stable Snf1 heterotrimeric complex. The C-terminal domain of the catalytic α subunit Snf1 mediates its interaction with the β and γ subunits of the heterotrimeric complex (Jiang and Carlsson 1996; Jiang and Carlsson 1997). In contrast, the N-terminal kinase domain (Snf1-KD lacking the C-terminal domain) does not bind to the β and γ subunits; nonetheless, it is glucose-regulated and can associate with the Snf1 upstream kinase Sak1 and retains functionality in vivo (Ludin et al. 1998; Liu et al. 2011). Since Por1 and Por2 play a role in the activation of Snf1-KD, we first examined if Por1 can interact with this domain. To this end, we used the yeast two-hybrid system. A Snf1-KD fusion to the LexA DNA-binding protein was tested in combination with a Por1 fusion to the Gal4 transcription activation domain (GAD) for ability to activate a reporter with LexA binding sites (lexAop-lacZ) in strain CTY10-5d. LexA-Snf1-KD interacted with GAD-Por1 (Fig. 11). The interaction was observed under
high and low glucose conditions, and its apparent strength was comparable to that of the interaction between Por1 and full-size Snf1 (see Fig. 7 in Chapter 2). Thus, these results strongly suggest that Por1 can interact with Snf1-KD, and that this interaction is independent of the β and γ subunits of the Snf1 complex.

We also attempted to examine the two-hybrid interaction between Snf1-KD and Por2, but the results were inconclusive and are not shown. Since Por1 and Por2 function redundantly in the regulation of Snf1-KD, we envision that Por2 similarly interacts with Snf1-KD, but the two-hybrid system might not be an ideal method to detect such an interaction, and additional biochemical experiments will be required.

Fig. 11. Two-hybrid analysis of the interaction between Por1 and the Snf1 kinase domain. The Snf1 kinase domain (Snf1-KD) and Por1 interact in the yeast two-hybrid system in high and low glucose conditions. Transformants of strain CTY10-5d with an integrated lexAop-lacZ reporter expressing the indicated protein pairs were grown to mid-log phase in selective medium with high glucose (2%), and then shifted for 3h to an otherwise identical medium with low glucose (0.05%). β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units (U). Values are averages for at least four transformants. Error bars indicate standard errors. Asterisks indicate statistically significant difference from the corresponding vector controls (p < 0.001 both in high and low glucose).
Por1 and Por2 function downstream of Hxk2 in regulating the catalytic activation of Snf1

*S. cerevisiae* encodes three enzymes (Hxk1, Hxk2, and Glk1) that can catalyze the first step of glycolysis – phosphorylation of glucose to glucose-6-phosphate. In the presence of abundant glucose, Hxk2 plays the major role in glucose phosphorylation and also makes a significant contribution to glucose repression (MORENO AND HERRERO 2002). Lack of Hxk2 causes constitutive expression of many Snf1-dependent genes that are normally glucose-repressed, including *SUC2* and *GAL* genes (NEIGEBORN AND CARLSON 1984; GANCEDO 1998) (see Fig. 12B below). Hxk2 is an upstream negative regulator of Snf1, and glucose-repressible genes are no longer constitutive in the *hxk2 snf1* double mutant (NEIGEBORN AND CARLSON 1984). In cells lacking Hxk2, Snf1 is in the active conformation even in the presence of abundant glucose (JIANG AND CARLSON 1996), and is constitutively phosphorylated at Thr210 (Fig. 12A).

**Fig. 12.** Role of hexokinase-2 in the negative control of Snf1. (A) Wild-type cells and cells lacking hexokinase-2 (*hxk2Δ*) were grown to mid-log phase in YEP containing abundant (2%) glucose, and the Thr210 phosphorylation status (PT210) and total levels of Snf1 were analyzed by immnoblotting as described in Materials and Methods. (B) Cells of the indicated genotypes with an integrated *SUC2-LEU2-lacZ* reporter were grown to mid-log phase in YEP with 2% glucose. β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units (U). Values are averages for ten to twelve assays. Error bars indicate standard errors. Reporter expression in the *hxk2Δ* mutant was statistically significantly different from the wild type (*p* < 0.001).
We hypothesized that Hxk2 represses Snf1 by inhibiting Por1 and Por2. To test this hypothesis, we created a series of mutants including \( hxk2\Delta \) \( por1\Delta \), \( hxk2\Delta por2\Delta \), and \( hxk2\Delta por1\Delta por2\Delta \) and examined Thr210 phosphorylation in each of these mutants by immunoblotting in the presence of high (2%) and low (0.05%) glucose. In comparison to the \( hxk2\Delta \) mutant, which exhibits constitutive Snf1 Thr210 phosphorylation even in the presence of abundant glucose, the level of Snf1 Thr210 phosphorylation in the \( hxk2\Delta por1\Delta por2\Delta \) triple mutant was reduced to the wild-type level (Fig. 13A). Moreover, when cells were shifted to low glucose, the \( hxk2\Delta por1\Delta por2\Delta \) triple mutant exhibited reduced Thr210 phosphorylation similar to the \( por1\Delta por2\Delta \) double mutant (Fig. 13B). No such reduction was observed in \( hxk2\Delta por1\Delta \) or \( hxk2\Delta por2\Delta \) double mutants.

**Fig. 13.** Functional relationship between mitochondrial porins and hexokinase-2. Por1 and Por2 function downstream of hexokinase-2 (Hxk2) in the catalytic activation of Snf1. (A) Cells of the indicated genotypes were grown to mid-log phase in YEP containing 2% glucose (high glucose). (B) Cells grown in panel A were shifted for 1h to YEP containing 0.05% glucose (low glucose). Thr-210 phosphorylation (PT210) status and total levels of Snf1 were analyzed by immunoblotting as described in Materials and Methods. (C) Cells of the indicated genotypes
carrying an integrated SUC2-LEU2-lacZ reporter were grown to mid-log phase in YEP with high glucose (2%) and then shifted to an otherwise identical medium containing low glucose (0.05%). β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units (U). Values are averages for four to six assays. Error bars indicate standard errors. Reporter expression in the hxk2Δ por1Δ por2Δ mutant was statistically significantly different from the hxk2Δ mutant under high glucose (p < 0.001) and low glucose (p < 0.01) conditions.

Thus, the por1Δ por2Δ double mutation is epistatic to the hxk2Δ mutation in the catalytic activation of Snf1. These results strongly suggest that Por1 and Por2 function downstream of Hxk2 in the regulation of Snf1.

We also examined the effects of the mutations on the expression of a Snf1-dependent reporter SUC2-LEU2-lacZ (SAROKIN AND CARLSON 1985). Under glucose-rich and glucose-limiting conditions, reporter expression was reduced in the hxk2Δ por1Δ por2Δ triple mutant relative to the hxk2Δ single mutant (Fig. 13C). These results are consistent with the above results for Thr210 phosphorylation. We note that the partial constitutivity of SUC2-LEU2-lacZ reporter expression in the triple hxk2Δ por1Δ por2Δ mutant could be attributed to a recently discovered Snf1-independent, glucose-induced stimulatory effect of Hxk2 on Mig1, a major transcriptional repressor of the SUC2 promoter (VEGA et al. 2016). In other words, while the hxk2Δ mutation cannot bypass the requirement of Por1 and Por2 for Snf1 catalytic activation, it could partially relieve Mig1-mediated repression of SUC2 by an independent mechanism.
Discussion

The highly conserved mammalian AMPK and yeast Snf1 protein kinases play essential roles in sensing and regulating the cellular response to changes in glucose/energy levels (Hardie 2007; Hedbacker and Carlson 2008). Although it is known that binding of AMP to AMPK leads to its activation, the signal that activates Snf1 has eluded identification for many years, as unlike AMPK - Snf1 is not regulated by AMP (Wilson et al. 1996; Hardie et al. 1998; Adams et al. 2004). Recent studies demonstrate that ADP binds to the γ subunit Snf4, and protects Snf1 from dephosphorylation by phosphatases in vitro (Chandrashekarappa et al. 2011; Mayer et al. 2011). However, the physiological relevance of this mechanism remains uncertain. First, the above in-vitro experiments indicate that Snf1 complex responds to absolute ADP levels, but according to an earlier study, absolute ADP levels remain relatively unchanged between high- and low-glucose conditions (Wilson et al. 1996). Second, previous experiments showed that Snf1 activity in vivo correlates with AMP:ATP ratios, suggesting the existence of another sensing mechanism that functions in addition or instead of ADP sensing by Snf4.

Our results presented in Chapter 2 and in this study provide a framework that could accommodate both the Snf4-mediated sensing as well as suggest Snf4-independent mechanisms. The ability to interact with the VDAC proteins and proximity to the mitochondrial surface could put the Snf1 complex in a better position to sense glucose/energy limitation via Snf4 as ADP levels could change more sharply at the mitochondrial surface. Here, we show that the lack of Por1 and Por2 causes reduced Thr210 phosphorylation of Snf1-KD (Fig. 10), which is unable to interact with Snf4 (or the β subunits), supporting a role of Por1/Por2 in a Snf4-independent sensing mechanism, which could explain why Snf1 remains glucose-regulated even in the absence of Snf4 (McCartney and Schmidt 2001).
The ability of some transporters (or transporter homologs) to participate in sensing has been well established; such transporters/sensors are often referred to as “transceptors” (Kriel et al. 2011). In some cases, a transceptor may have retained its transporter function, while in others, the transporter function has been lost. For example, the Mep2 protein of yeast doubles both as an ammonium transporter and an ammonium sensor (Lorenz and Heitman 1998). By contrast, the yeast Snf3 and Rgt2 glucose sensors no longer transport glucose, but simply represent homologs of glucose transporters (Rolland et al. 2002). The common theme is that transceptors sense what they transport (or used to transport). From this perspective, what could Por1 and Por2 sense? VDACs have a large repertoire of metabolites for which they serve as channels, and therefore they could potentially sense multiple signals. The most obvious and relevant candidates for signaling molecules sensed by VDACs are nucleotides involved in energy metabolism, such as AMP, ADP, ATP, NADH, etc. Besides small metabolites, protein binding to VDACs has been shown to affect their conformation. An interesting and relevant example is offered by highly-glycolytic cancer cells exhibiting the so-called “Warburg” effect. Specifically, such cells overexpress hexokinase II, which binds to the VDAC proteins and causes their conversion to the closed state (Lemasters and Holmuhamedov 2006). Previous experiments in yeast failed to detect a physical interaction between Hxk2 and Por1 (Aflalo and Azoulay 1998). However, we reasoned that despite the lack of a physical interaction, there could still be a functional relationship. This relationship seemed even more likely given that Hxk2 is known to negatively regulate Snf1 (Jiang and Carlsson 1996). Indeed, in this study we have obtained strong evidence that Por1 and Por2 function downstream of Hxk2 in the Snf1 pathway (Fig. 12), specifically, that Por1 and Por2 are negatively regulated by Hxk2. If Hxk2 does not physically bind to the VDAC proteins in yeast, it is logical to assume that the negative regulation occurs via
a glucose metabolite. It has long been hypothesized that one of the Snf1-inhibiting signals is glucose-6-phosphate (Hedbacker and Carlson 2008). The glucose analog 2-deoxyglucose (2DG), which is not metabolized but can be phosphorylated, can mimic glucose repression and inhibit Snf1 in vivo (Friis et al. 2014). The hxx2 mutation allows growth on alternative carbon sources, e.g. sucrose, despite the presence of 2DG (Neigeborn and Carlson 1987). Previous experiments have shown that glucose-6-phosphate does not regulate Snf1 in vitro (Wilson et al. 1996), suggesting the existence of an additional in-vivo sensor of this metabolite in the Snf1 pathway. In this regard, it is interesting that our BLAST searches conducted with relaxed stringency identified a region of homology between Por1/2 and the Rgt2/Snf3 transporter-like glucose sensors (Fig. 14A). It was recently reported that this particular region of sugar transporters is responsible for sugar selectivity (Fig. 14B) (Farwick et al. 2014). Collectively, these considerations raise the possibility that one of the metabolites sensed by Por1 and Por2 is glucose-6-phosphate. We note that we do not think of glucose-6-phosphate as an exclusive metabolite sensed by the VDACs; in fact, we think it likely that nucleotides can be sensed also, since evidence indicates that mammalian VDAC has binding sites for AMP, ADP, and ATP (Yehezkel et al. 2006).

![Fig. 14. Sequence analysis of Por1 and Por2. (A) Putative sugar selectivity box (outlined in red) in Por1, Por2, and the transporter-like glucose sensors Rgt2 and Snf3. (B) Conserved sugar selectivity box (outlined in red) in sugar transporters from different species (reproduced from (Farwick et al. 2014)).](image-url)
Further experiments will be required to address the possible role of glucose-6-phosphate and other molecules such as sugar metabolites or nucleotides as signaling molecules sensed by Por1 and Por2 to regulate Snf1.

**Table 2. S. cerevisiae strains**

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This study

AMS47  \textit{MATa his}^{3–11,15} \textit{leu}^{2–3,112} \textit{trp}^{1–1} \textit{ura}^{3–1} \textit{por}\Delta::  \\
\textit{KanMX6} \textit{por2}\Delta:: \textit{KanMX6}  \\
This study

References


Lorenz, M. C., and J. Heitman, 1998 The MEP2 ammonium permease regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. EMBO J 17: 1236-1247.


Chapter 4
Mitochondrial porins, Por1 and Por2, positively regulate Snf1-Gal83 nuclear localization in Saccharomyces cerevisiae

Abstract
Members of the Snp1/AMP-activated protein kinase (AMPK) family play a crucial role in sensing and responding to energy stress in eukaryotes. Snp1/AMPK kinases are activated by phosphorylation of their activation-loop threonine, and regulate transcription of genes and activities of metabolic enzymes, to help the cells adapt during energy/glucose limitation. As described in Chapters 2 and 3, we found a novel role of the mitochondrial voltage-dependent anion channel (VDAC) proteins Por1 and Por2 in Snp1 catalytic activation in Saccharomyces cerevisiae. Here, we present evidence that Por1 also positively regulates Snp1 nuclear localization, and that this effect is exerted via the regulation of the Snp1 nuclear-targeting subunit, Gal83. We show that Por1 interacts with Gal83 in the yeast two-hybrid system, suggesting that Por1 plays a role in physically recruiting Gal83 to the nucleus. Further, overexpression of Por2 in the por1Δ mutant compensates for the Snp1/Gal83 nuclear localization defect, supporting a physical mass action mechanism. Thus, Por1 and Por2 participate in the regulation of the Snp1 pathway in more than one way: not only do they promote Snp1 catalytic activation, but they also positively regulate Snp1-Gal83 nuclear enrichment.

Introduction
Snp1 is the yeast homolog of mammalian AMP-activated protein kinase (AMPK) (Hardie et al. 1998; Hedbacker and Carlsson 2008). Yeast cells prefer glucose as their
carbon/energy source, and Snf1 is essential for the growth on alternative carbon sources (CARLSON et al. 1981; CELENZA AND CARLSON 1986). The Snf1 kinase complex is a heterotrimer consisting of the catalytic α subunit (Snf1), one of the three alternate targeting/scaffolding β subunits (Sip1, Sip2, Gal83), and the regulatory γ subunit (Snf4) (HEDBACKER AND CARLSON 2008). Upstream kinases activate Snf1 during energy/glucose limitation by phosphorylating a conserved Thr210 residue present in the activation-loop of the catalytic α subunit (MCCARTNEY AND SCHMIDT 2001; HONG et al. 2003; SUTHERLAND et al. 2003). Thr210 dephosphorylation and Snf1 inactivation primarily involves type 1 protein phosphatase Glc7 in association with regulatory proteins Reg1 and Reg2 (TU AND CARLSON 1994; TU AND CARLSON 1995; MCCARTNEY AND SCHMIDT 2001; MAZIARZ et al. 2016), but roles for other two phosphatases (Sit4 and Ptc1) have also been demonstrated (RUIZ et al. 2011; RUIZ et al. 2013).

Because there are three different β subunits (Sip1, Sip2, Gal83), there are three distinct isoforms of the Snf1 kinase complex (HEDBACKER AND CARLSON 2008). We will refer to these isoforms as Snf1-Sip1, Snf1-Sip2, and Snf1-Gal83 (i.e. skipping Snf4 for the sake of brevity). In abundant glucose, all three isoforms are cytoplasmic. During glucose starvation, all three isoforms are catalytically activated, and show distinct subcellular localization: Snf1-Sip1 is targeted to the vacuolar membrane, Snf1-Sip2 remains cytoplasmic, and Snf1-Gal83 enriches in the nucleus to promote transcription of genes involved in the utilization of alternate carbon sources and respiration (HEDBACKER AND CARLSON 2008).

We have previously shown that normal Snf1 catalytic activation in response to low glucose depends on the voltage-dependent anion channel (VDAC) proteins Por1 and Por2 (see Chapter 2). VDACs are highly conserved in evolution and are located in the mitochondrial outer membrane (COLOMBINI 2004; COLOMBINI AND MANNELLA 2012). VDACs allow exchange of
small metabolites between the cytoplasm and the mitochondria, and also play a major role in respiration and apoptosis (COLOMBINI 2004; COLOMBINI AND MANNELLA 2012).

In this chapter, we show that Por1 positively regulates Snf1 nuclear localization, and that this effect is exerted via the regulation of the Snf1 nuclear-targeting subunit, Gal83. We show that Por1 interacts with Gal83 in the yeast two-hybrid system, suggesting that Por1 plays a role in physically recruiting Gal83 to the nucleus. Further, overexpression of Por2 in the por1Δ mutant compensates for the Snf1-Gal83 nuclear localization defect, supporting a physical mass action mechanism. Thus, Por1 and Por2 participate in the regulation of the Snf1 pathway in more than one way: not only do they promote Snf1 catalytic activation, but they also positively regulate Snf1-Gal83 nuclear enrichment.

Materials and Methods

Strains and growth conditions. The S. cerevisiae strains used in this study are listed in Table 3. Strain CTY10-5d (MATa gal4 gal80 URA3::lexAop-lacZ his3 leu2 ade2 trp1-901) (R. Sternglanz, SUNY, Stony Brook, NY) and its por1Δ, por2Δ, gal83Δ, and por1Δ gal83Δ derivatives used to perform LexA-Snf1-G53R reporter activation assays. All other strains used for microscopy and spotting growth assays were in the W303 background. To generate the por1Δ::KanMX6, por2Δ::KanMX6, gal83Δ::KanMX6, and snf1Δ::KanMX6 alleles, the marker sequences were amplified by PCR with primers flanking the corresponding open reading frames. The mutant alleles were introduced into CTY10-5d and W303 strains by transformation; all yeast transformations were performed using standard methods (ROSE et al. 1990). The genotypes of all knock-out strains were confirmed by PCR analysis of genomic DNA. Double mutants lacking Snf1 and either Por1 or Por2 were constructed by tetrad analysis of diploid cells obtained by
crossing snf1Δ and either por1Δ or por2Δ mutants of the opposite mating types. Similarly, por1Δ gal83Δ double mutants were generated by crossing por1Δ and gal83Δ single mutants, followed by tetrad analysis.

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

**Plasmid construction.** Plasmid pSnf1-GFP expresses a Snf1-GFP fusion from the native SNF1 promoter in low-copy-number CEN-URA3 vector pRS316 (SIKORSKI AND HIETER 1989), and was constructed essentially as described previously (VINCENT et al. 2001). The multicopy vector pSK71 with the TRP1 selectable marker provides expression from ADH1 promoter and was derived from pBTM116 (FIELDS AND SONG 1989) by deleting the LexA-encoding sequence. Plasmid pAMS10 overexpresses Por2-V5 from vector pSK71, and was constructed as follows. The POR2 gene was amplified by PCR using wild-type genomic DNA as the template; the long 103 nt reverse primer for this PCR also included a sequence encoding a flexible amino acid linker (SABOURIN et al. 2007) consisting of two Gly-Gly-Ser-Gly repeats followed by the V5 tag. The resulting fragment was cloned into the BamHI site of pSK71. Functionality of Por2-V5 was confirmed by complementation of a por1Δ por2Δ mutant (BLACHLY-DYSON et al. 1997).

Plasmid pAMS11 expresses LexA-Gal83 and was constructed by inserting the GAL83 coding sequence amplified by PCR from pRT12 (VINCENT et al. 2001) at the BamHI site of vector pEG202 (GOLEMIS et al. 1997).
Assays of “shortcut” transcription activation by LexA–Snf1G53R. The principle behind the “shortcut” reporter activation assays was described previously (Kuchin et al. 2000). Strain CTY10–5d carries LexA operator sequences (lexAop) inserted upstream of the lacZ gene (lexAop-lacZ reporter). Wild-type CTY10-5d and its por1Δ, por2Δ, gal83Δ, and por1Δ gal83Δ mutant derivatives were transformed with plasmid pRJ216 expressing LexA-Snf1-G53R (Kuchin et al. 2000); LexA-Snf1-G53R activates the lexAop-lacZ reporter in a glucose-regulated manner that reflects the catalytic activation of the Snf1-G53R moiety and its nuclear localization (Kuchin et al. 2000; Vincent et al. 2001). The corresponding vector control was pEG202 (Golemis et al. 1997). For testing the effects of Por2-V5 overexpression, reporter strains were co-transformed with pRJ216 and either pAMS10 or vector pSK71. Transformants were grown to mid-log phase with plasmid selection in selective SC medium with high (2%) glucose and then shifted to an otherwise identical medium with low (0.05%) glucose for 3h. β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units as described previously (Vyas et al. 2001).

To confirm expression of the fusion proteins by immunoblotting, transformants were grown under conditions identical to those used to perform reporter assays. Cell extracts were prepared using the boiling/alkaline lysis method (Orlova et al. 2008). Proteins were separated by SDS-PAGE and analyzed by immunoblotting. LexA fusion proteins were detected with anti-LexA antibody (Millipore). Por2-V5 fusion proteins were detected with anti-V5 antibody (Thermo Fisher Scientific). Thr210-phosphorylated LexA-Snf1-G53R proteins were detected with anti-phospho-Thr172-AMPK (Cell Signaling Technology) as described previously (Orlova et al. 2008). Signals were detected by enhanced chemiluminescence using Pierce ECL2 or HyGlo (Denville Scientific).
Fluorescence microscopy. For Snf1-GFP and Gal83-GFP localization studies, we followed the protocol published previously (Vincent et al. 2001). Briefly, cells expressing Snf1-GFP or Gal83-GFP were grown to mid-log phase in selective SC containing 2% glucose and then shifted to an otherwise identical medium containing 3% ethanol and 2% glycerol instead of glucose for 20 min (Vincent et al. 2001). Nuclei were stained by adding 10 µl of 0.8 µg/mL DAPI to 1 mL of cell culture and incubating for 5 min at 30°C. The cells were then collected by brief centrifugation, and the DAPI and GFP signals were examined using a workstation consisting of Nikon Eclipse 80i fluorescence microscope, a CoolSNAP HQ2 camera (Photometrics), and a computer with NIS-Elements BR 3.01 software.

Expression of the fusion proteins was confirmed by immunoblotting as follows. Transformants were grown under conditions identical to those used to perform fluorescence microscopy. Cell extracts were prepared using the boiling/alkaline lysis method (Orlova et al. 2008). Proteins were separated by SDS-PAGE and analyzed by immunoblotting. GFP fusion proteins were detected with anti-GFP antibody (Roche). Thr210-phosphorylated Snf1 proteins were detected with anti-phospho-Thr172-AMPK (Cell Signaling Technology) as described previously (Orlova et al. 2008). Signals were detected by enhanced chemiluminescence using Pierce ECL2 or HyGlo (Denville Scientific).

Results

Transcriptional activation of a reporter by LexA-Snf1G53R is affected in a por1Δ mutant.

In Chapter 2, we have shown that Por1 and Por2 play redundant roles in promoting Snf1 activation/Thr210 phosphorylation (Fig. 9A,B). Interestingly, despite the lack of any obvious defect in Thr210 phosphorylation, the por1Δ mutation alone conferred a significant defect in the
expression of the Snf1-dependent reporter \textit{SUC2-LEU2-lacZ} (Fig. 9C). This suggested that the \textit{por1\Delta} mutation affects another aspect of Snf1 function that is distinct from Thr210 phosphorylation. To further address this possibility, we turned to the so-called “shortcut” reporter assay \cite{Kuchin2000} that takes advantage of a hyperactive but glucose-regulatable version of Snf1 (Snf1-G53R). The shortcut assay tests for the ability of Snf1-G53R fused to the LexA DNA-binding protein (LexA-Snf1-G53R) to activate transcription by binding to the LexA operator sequences \textit{(lexAop)} upstream of a \textit{lacZ} gene \textit{(lexAop-lacZ reporter)}. This allows stimulation of RNA polymerase II holoenzyme by a mechanism that does not rely on gene-specific transcription factors (Fig. 15A). Importantly, activation of the \textit{lexAop-lacZ} reporter by LexA-Snf1-G53R is highly glucose regulated: reporter activation is weak under glucose-rich conditions, but increases dramatically upon glucose limitation in a manner that requires catalytic activation (Thr210 phosphorylation) of the Snf1-G53R moiety \cite{Kuchin2000}. In addition to catalytic activation, \textit{lexAop-lacZ} reporter activation by LexA-Snf1-G53R is strongly affected by mechanisms that regulate Snf1 nuclear localization \cite{Vincent2001}. As such, the “shortcut” reporter assay represents a sensitive readout for defects in Snf1 catalytic activation and nuclear localization.

The \textit{lexAop-lacZ} reporter is widely used for two-hybrid experiments, and strain CTY10-5d carries a genomically integrated copy of this reporter that can be used for the “shortcut” assay \cite{Kuchin2000}. We expressed LexA-Snf1-G53R in wild-type CTY10-5d and its \textit{por1\Delta} and \textit{por2\Delta} derivatives. Reporter activation was monitored by \(\beta\)-galactosidase assays after growth in high glucose (2\%) and after shift to low glucose (0.05\%) for 3h. The \textit{por1\Delta} mutant exhibited a strong activation defect in low glucose (Fig. 15B). This defect could not be attributed to reduced
Thr210 phosphorylation of the Snf1-G53R moiety (Fig. 15C,D), suggesting against a defect in catalytic activation, and instead suggesting a defect in nuclear localization.

Fig. 15. The por1Δ mutation strongly affects lexAop-lacZ reporter activation by LexA-Snf1-G53R. (A) Under glucose-limiting conditions, hyperactive LexA-Snf1-G53R becomes catalytically activated and enters the nucleus, where it binds to the promoter of the lexAop-lacZ reporter and stimulates transcription by RNA polymerase II (RNA Pol II) holoenzyme (modified from (KUCHIN et al. 2000)). (B) CTY10-5d (WT) and its por1Δ and por2Δ mutant derivatives expressed LexA-Snf1-G53R from plasmid pRJ216. Cells were grown in SC medium with plasmid selection in the presence of high (2%) glucose to mid-log phase, and then shifted for 3h to an otherwise identical medium containing low (0.05%) glucose. β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units (5-9 independent measurements per datapoint). The graph shows the data for low glucose conditions expressed as % of the wild type. Under high glucose conditions, all values were < 3% of the wild-type value observed in low glucose. The mean wild-type value in low glucose was 278 Miller units. Error bars indicate standard errors. An ANOVA with Tukey's post hoc test indicated that all values were statistically significantly different from each other (p < 0.0001). (C,D) Transformants shifted to 0.05% glucose for 3h as above were tested for Thr210 phosphorylation of the Snf1-G53R moiety (PT210) and total LexA-Snf1-G53R protein levels (Total) by immunoblotting using anti-phospho-Thr172-AMPK and anti-LexA, respectively, as described in Materials and Methods; vec, vector control pEG202.
**The por1Δ mutation affects Snf1 nuclear enrichment.**

To determine the effects of the *por1Δ* and *por2Δ* mutations on Snf1 localization, we constructed a C-terminally GFP-tagged Snf1 (Snf1-GFP). Snf1-GFP localization was examined in cells grown in high (2%) glucose and after a shift to an otherwise identical medium with a mixture of 3% ethanol and 2% glycerol (EG) as the carbon source instead of glucose, following a previously published protocol (*VINCENT et al. 2001*). In the presence of abundant glucose, Snf1-GFP was excluded from the nucleus in all strains tested (Fig. 16A, B, C; top panels). Upon shift to EG medium, Snf1-GFP enriched in the nucleus in the wild-type and *por2Δ* cells (Fig. 16A, C; bottom panels). However, no nuclear enrichment was observed in *por1Δ* cells (Fig. 16B, bottom panel), and this defect could not be attributed to defective activation-loop Thr210 phosphorylation (Fig. 16E). Together, results in Fig. 15 and Fig. 16 strongly suggest that *por1Δ* affects Snf1 nuclear enrichment by a mechanism that is independent of its catalytic activation.
**Fig. 16.** Snf1 does not enrich in the nucleus of a por1Δ mutant. (A-C) Localization of Snf1-GFP (GFP) was determined by fluorescence microscopy after growth in selective SC containing abundant (2%) glucose (Glu), and after a shift to an otherwise identical medium containing a mixture of 3% ethanol and 2% glycerol as the carbon source (EG). Nuclei were stained with DAPI. (D) Brief summary table. (E) Thr210 phosphorylation (PT210) and total Snf1-GFP protein levels were detected by using anti-phospho-Thr172-AMPK and anti-GFP, respectively; vec, vector control pRS316.

**Por1 positively regulates the nuclear localization of Gal83.**

Two lines of reasoning suggested that Por1 could regulate Snf1 nuclear localization by regulating the nuclear localization of Gal83, one of three alternate β subunits of the Snf1 kinase complex. First, Gal83 is required for targeting Snf1 to the nucleus (Vincent et al. 2001).
Second, our yeast two-hybrid assays suggested that Por1 physically interacts with Gal83 (Fig. 17).

**Fig. 17.** Por1 interacts with Gal83 in the two-hybrid system. Cells of strain CTY10-5d with an integrated *lexAop-lacZ* reporter expressing the indicated protein pairs were grown to mid-log phase in selective SC medium containing high (2%) glucose, and then shifted for 3h shift to an otherwise identical medium containing low (0.05%) glucose. β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units (U). Values are averages for at least four transformants per combination. Error bars indicate standard errors. Although LexA-Gal83 shows an ability to activate transcription on its own, as reported earlier (LESAGE *et al.* 1996), co-expression of the GAD-Por1 fusion led to a significant increase in reporter activation relative to the GAD controls ($P < 0.001$ both in high and low glucose).
Fig. 18. Gal83 does not enrich in the nucleus of a por1Δ mutant. (A-C) Localization of Gal83-GFP (GFP) expressed from pRT12 was determined by fluorescence microscopy after growth in SC-His in the presence of 2% glucose (Glu), and upon a 20 min shift to an otherwise identical medium containing a mixture of 3% ethanol and 2% glycerol (EG) as the carbon source. Nuclei were stained with DAPI. (D) Brief summary table. (E) Gal83-GFP protein levels (Gal83-GFP), as well as Thr210 phosphorylation (PT210) and total levels of Snf1 (Total) were analyzed by immunoblotting using anti-GFP, anti-phospho-Thr172-AMPK, and anti-polyhistidine antibodies, respectively.
We therefore examined the effects of the *por1Δ* and *por2Δ* mutations on Gal83 localization, using a previously described Gal83-GFP fusion expressed from plasmid pRT12 (Vincent *et al.* 2001). In all cases, Gal83-GFP was excluded from the nucleus of glucose-grown cells (Fig. 18A, B, C; top panels). Upon shift to ethanol-glycerol, Gal83-GFP enriched in the nuclei of wild-type and *por2Δ* cells, but not in the *por1Δ* mutant cells (Fig. 18A, B, C; bottom panels).

When Gal83 is complexed with Snf1, catalytic activation of the associated Snf1 subunit is a prerequisite for Snf1-Gal83 nuclear translocation: if Snf1 is not phosphorylated at Thr210, it will hold Gal83 in the cytoplasm (Hedbacker *et al.* 2004). As observed previously, immunoblot analysis indicated that in the *por1Δ* mutant Thr210 is phosphorylated at a level comparable to that in the wild type (Fig. 18E; compare lanes 4 and 6), suggesting that the Gal83 nuclear localization defect is not associated with a Snf1 activation defect.

Catalytic activation of Snf1 is required for Gal83 nuclear localization only when Gal83 is associated with Snf1; when Gal83 is not associated with Snf1 (e.g. in a *snf1Δ* mutant), it will enrich in the nucleus in a Snf1-independent manner (Vincent *et al.* 2001; Hedbacker *et al.* 2004). To determine whether Por1 regulates this Snf1-independent nuclear localization of Gal83, we expressed Gal83-GFP in a *snf1Δ por1Δ* double mutant (see immunoblot in Fig. 18E, lanes 9 and 10) and examined its localization after growth in abundant glucose and following a shift to ethanol-glycerol. We observed that just as in the *por1Δ* single mutant, Gal83-GFP did not enrich in the nucleus in the *snf1Δ por1Δ* double mutant on ethanol-glycerol (Fig. 19B; bottom panels). No such defect was observed in the *snf1Δ por2Δ* double mutant cells (Fig. 19C; bottom panels). Collectively, these results suggest that the *por1Δ* mutation affects the nuclear localization of the Snf1 kinase complex by affecting the nuclear localization of Gal83.
**Fig. 19.** Gal83 does not enrich in the nucleus of a *snf1Δ por1Δ* mutant. (A-C) Localization of Gal83-GFP (GFP) expressed from pRT12 was determined by fluorescence microscopy after growth in selective SC medium in the presence of 2% glucose (Glu), and after a shift to an otherwise identical medium containing a mixture of 3% ethanol and 2% glycerol as the carbon source (EG). Nuclei were stained with DAPI. (D) Brief summary table.

**Overexpression of Por2 suppresses the por1Δ mutation for defects in Snf1-Gal83 nuclear localization and transcriptional activation by LexA-Snf1-G53R.**

In Chapter 2, we showed that Por1 and Por2 play redundant roles in Snf1 catalytic activation (Thr210 phosphorylation). Por2 does not appear to possess channel activity (Blachly-Dyson *et al.* 1997; Lee *et al.* 1998), strongly suggesting that Por1/2-mediated mechanism of Snf1 activation is unrelated to VDAC channel function. Since Snf1-Gal83
localization is affected only by the \textit{por1}\textDelta\ mutation, but not by the \textit{por2}\textDelta\ mutation, one might conclude that in this case the VDAC channel function is important. However, previous evidence also indicates that the Por2 protein is expressed at a six-to-eight-fold lower level than the Por1 protein (BLACHLY-DYSON \textit{et al.} 1997). Therefore, it remains possible that the differential requirement of Por1 and Por2 for Snf1-Gal83 nuclear localization reflects the difference in their expression levels rather than the difference in channel function. To address this possibility, we tested whether Por2 overexpression can compensate for the Snf1-Gal83 localization defect caused by the lack of Por1.

We constructed a plasmid (pAMS10) expressing a C-terminally V5-tagged Por2 (Por2-V5) from the strong \textit{ADH1} promoter of multicopy vector pSK71. Overexpression of Por2-V5 suppressed the \textit{por1}\textDelta\ mutation for both the Snf1 nuclear localization defect (Fig. 20) and the Gal83 nuclear localization defect (Fig. 21A). We note that overexpression of Por2-V5 did not restore nuclear enrichment of Gal83-GFP in the \textit{snf1}\textDelta\ \textit{por1}\textDelta\ double mutant (Fig. 21B), indicating that the ability of overexpressed Por2 to functionally compensate for the lack of Por1 is Snf1-dependent.
Overexpression of Por2 suppresses the \textit{por1\Delta} mutation for the Snf1 nuclear localization defect. (A-D) Wild-type (WT) and \textit{por1\Delta} cells expressing Snf1-GFP and either overexpressing Por2-V5, or carrying the corresponding empty vector, were grown to mid-log phase in selective SC medium containing 2\% glucose (Glu) and then shifted to an otherwise identical medium containing 3\% ethanol and 2\% glycerol as the carbon source (EG). Nuclei were stained with DAPI. Localization of Snf1-GFP was determined by fluorescence microscopy. (E) Brief summary table. (F) Immunoblot analysis of transformants used in panels A-C. The levels of Snf1-GFP phosphorylated at Thr210 (PT210), total Snf1-GFP (Total), and Por2-V5 were analyzed as described in Materials and Methods. Por2-V5 was overexpressed from pAMS10 (Por2-V5, ++), and the corresponding empty vector was pSK71 (Por2-V5, -).
Fig. 21. Effects of Por2 overexpression on localization of Gal83-GFP. (A,B) Cells of the indicated genotypes expressing Gal83-GFP and overexpressing Por2-V5 were grown to mid-log phase in selective SC medium containing 2% glucose (Glu) and then shifted to an otherwise identical medium containing 3% ethanol and 2% glycerol as the carbon source (EG). Nuclei were stained with DAPI. Localization of Gal83-GFP was determined by fluorescence microscopy. (C) The levels of Gal83-GFP, phospho-Thr210-Snf1 (PT210), total Snf1 (Total), and Por2-V5 were analyzed by immunoblotting as described in Materials and Methods. Por2-V5 was overexpressed from pAMS10 (Por2-V5, ++), and the corresponding empty vector was pSK71 (Por2-V5, -).
Since the \textit{por1}\Delta mutation causes a significant defect in \textit{lexAop-lacZ} reporter activation by LexA-Snf1-G53R, we tested whether Por2 overexpression can suppress this defect. We observed that overexpression of Por2-V5 in the \textit{por1}\Delta mutant derivative of the \textit{lexAop-lacZ} reporter strain CTY10-5D restored transcriptional activation by LexA-Snf1-G53R to the wild-type level (Fig. 22).

\textbf{Fig. 22.} Overexpression of Por2 suppresses the \textit{por1}\Delta mutant defect in transcriptional activation by LexA-Snf1-G53R. (A) Reporter strain CTY10-5d (WT) and its \textit{por1}\Delta derivative were transformed with pRJ216 expressing LexA-Snf1-G53R and either pAMS10, which overexpresses Por2-V5 (Por2-V5, ++), or its parent vector pSK71 (Vec). Cells were grown in selective SC containing abundant (2%) glucose to mid-log phase, and then shifted for 3h to an otherwise identical medium containing low (0.05%) glucose. \(\beta\)-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units (4-5 independent measurements per datapoint). The graph shows the data for low glucose conditions expressed as \% of the control value (mean of WT with vector; 347 Miller units). Under high glucose conditions, all values were < 3\% of the low-glucose control value. Error bars indicate standard errors. According to ANOVA with Tukey’s post-hoc analysis, the value for the \textit{por1}\Delta mutant carrying the empty vector was significantly lower than that for the wild type with the empty vector (*, \(p < 0.05\)); the value for the \textit{por1}\Delta mutant overexpressing Por2-V5 was significantly higher than for the \textit{por1}\Delta mutant carrying the empty vector (**, \(p < 0.01\)). (B) The wild-type and \textit{por1}\Delta transformants shifted to low (0.05\%) glucose were tested for Thr210 phosphorylation of the Snf1-G53R moiety (PT210), for total LexA-Snf1-G53R protein levels (Total), and for Por2-V5 protein levels by immunoblotting as described in Materials and Methods.
The ability of LexA-Snf1-G53R to activate transcription of the $lexAop-lacZ$ reporter gene is completely dependent on Gal83-mediated nuclear localization (Vincent et al. 2001). If Por2 overexpression suppresses the $por1\Delta$ reporter activation defect by restoring Gal83 nuclear localization, then the requirement for Gal83 should not be bypassed. We therefore examined the effects of Por2 overexpression on reporter activation by LexA-Snf1-G53R in $gal83\Delta$ and $por1\Delta$ $gal83\Delta$ mutants. LexA-Snf1-G53R failed to activate transcription in these mutants despite the overexpression of Por2-V5 (Fig. 23). This provides further support the notion that Por2 overexpression stimulates the nuclear translocation of Gal83.

**Fig. 23.** Overexpression of Por2 does not bypass the requirement of Gal83 for $lexAop-lacZ$ reporter activation by LexA-Snf1-G53R. (A) Reporter strain CTY10-5d (WT) and its $gal83\Delta$ and $por1\Delta$ $gal83\Delta$ mutant derivatives with LexA-Snf1-G53R overexpressing Por2-V5 (Por2-V5, ++), or carrying the corresponding empty vector (Vec), were grown in selective SC containing high (2%) glucose to mid-log phase, and then shifted for 3h to an otherwise...
identical medium containing low (0.05%) glucose. β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units (U). The presented values are averages for four to five transformants assayed under low-glucose conditions. Error bars indicate standard errors. (B) Representative transformants were analyzed by immunoblotting for Thr210 phosphorylation of the Snf1-G53R moiety (PT210), total LexA-Snf1-G53R protein levels (Total), and Por2-V5 protein levels under high (H) and low (L) glucose conditions as specified for panel A. LexA-Snf1-G53R was expressed from pRJ216 (LexA-Snf1-G53R, +); the corresponding vector was pEG202 (LexA-Snf1-G53R, -). Por2-V5 was overexpressed from pAMS10 (Por2-V5, ++); the corresponding empty vector was pSK71 (Por2-V5, -).

Collectively, these results provide strong evidence that Por1 positively regulates Snf1 nuclear localization (and nuclear activity) by stimulating nuclear enrichment of Gal83. Por2 also plays a role in this process, but its contribution is minor, presumably reflecting its lower abundance in comparison with Por1.

**Expression of hyperactive LexA-Snf1-G53R restores the ability of the por1Δ mutant to grow on non-fermentable carbon sources in a Gal83-dependent manner.**

In the course of our experiments we noted that expression of hyperactive LexA-Snf1-G53R suppresses the por1Δ mutation for defective growth on non-fermentable carbon sources (3% ethanol plus 2% glycerol) (Fig. 24). This result might be taken to suggest that the primary cause of the por1Δ mutant growth defect is reduction of overall Snf1 kinase activity in the cell. However, our previous results (Chapter 2) strongly suggested that the por1Δ mutation does not lead to a defect in Snf1 catalytic activation (see Fig. 9B). We therefore hypothesized that the ability of hyperactive LexA-Snf1-G53R to suppress por1Δ reflects not an overall boost of cellular Snf1 activity, but specifically an increase of Snf1 activity in the nucleus. If so, the ability of LexA-Snf1-G53R to suppress por1Δ would be expected to depend on Gal83. Indeed,
expression of LexA-Snf1-G53R failed to restore the growth of a por1Δ gal83Δ double mutant on ethanol-glycerol (Fig. 24). Thus, one of the reasons for the por1Δ mutant’s growth defect on non-fermentable carbon sources is likely to be a reduction in nuclear Snf1 activity. We note, however, that this may not be the only cause, since the gal83Δ mutation, which abolishes Snf1 nuclear localization, does not affect growth on non-fermentable carbon sources (Fig. 24). We believe that the growth defect of the por1Δ mutant on non-fermentable carbon sources results from a combination of at least two pleiotropic effects: one being impaired Snf1 nuclear activity, and another – most likely – reduced permeability of the mitochondrial outer membrane.

**Fig. 24.** Expression of hyperactive LexA-Snf1-G53R restores the ability of the por1Δ mutant to grow on non-fermentable carbon sources in a Gal83-dependent manner. Serially diluted cell suspensions were spotted onto selective SC plates with 2% glucose or 3% ethanol + 2% glycerol as carbon sources and grown at 30°C for 2 and 4 days, respectively.
Discussion

We previously showed that Por1 and Por2 play redundant roles in the positive regulation of Snf1 catalytic activation (Thr210 phosphorylation). In this study, we present evidence for their role in promoting the nuclear translocation of the Snf1-Gal83 isoform of the kinase.

The primary location of mitochondrial VDAC/porin proteins is the mitochondrial outer membrane. However, several lines of evidence suggest that at least some amount of yeast VDAC can localize to the nucleus. First, a previous subcellular fractionation study, which used porin as a mitochondrial marker, detected a small amount of this protein in the nuclear fraction (Schneiter et al. 1999). Second, a global study of protein complexes detected Por1 in association with components of the RNA polymerase II holoenzyme (Ho et al. 2002). Third, protein interactions involving Por1 can be detected using the yeast two-hybrid system, in which both interaction partners must be present in the nucleus; for example, the biochemically verified interaction between Snf1 and Por1 was detected in the two-hybrid system using LexA-Por1 or Gad-Por1 fusion proteins, which were not furnished with any artificial nuclear localization signal sequences (Chapter 2). Thus, it seems likely that the requirement of Por1 for the nuclear enrichment of Gal83 is related to its ability to localize to the nucleus.

Por1 is the major VDAC isoform of yeast and is expressed at a six-to-eight fold higher level than the other VDAC protein, Por2 (Blachly-Dyson et al. 1997). Although the lack of Por2 did not confer any obvious defects in the nuclear localization of Snf1-GFP and Gal83-GFP, it did lead to a modest reduction in transcriptional activation by LexA-Snf1-G53R. Importantly, overexpression of Por2 suppressed the por1Δ mutation for the Snf1-Gal83 nuclear localization defect, indicating that Por2 also plays a minor role. Thus, the relative contributions of Por1 and Por2 to promoting Snf1-Gal83 nuclear localization most likely reflect their relative abundance,
suggesting a mass action mechanism. We therefore envision that Por1/2 physically recruit Snf1-Gal83 to the nucleus, and this notion is further supported by the two-hybrid interaction between Por1 and Gal83. The recruitment process could be accompanied by formation of a contact site/interface between the mitochondrial and nuclear surfaces. Further experiments will be required to better understand the underlying mechanism in more detail.

It is also possible that besides regulating the nuclear localization of Snf1-Gal83, Por1 (and Por2) could regulate Snf1-Sip1, which is targeted to the vacuolar membrane. This conjecture is supported by a proteomic study that detected Por1 in a complex with Sip1 (HO et al. 2002) and a study that detected Por1 in the vacuolar fraction (SCHNEITER et al. 1999).

In yeast, there are three enzymes - Hxk1, Hxk2, and Glk1 - that catalyze the first step of glycolysis, converting glucose to glucose-6-phosphate. Elimination of Hxk2 causes constitutive activation of Snf1, indicating that Hxk2 is a negative regulator of Snf1. In Chapter 3, we presented evidence that Por1 and Por2 function downstream of Hxk2 in the regulation of Snf1 catalytic activation, suggesting that Por1 and Por2 could serve as sensors of a glucose metabolite, most likely glucose-6-phosphate. This idea was further supported by the presence of a putative sugar selectivity box in their sequences. The involvement of Por1 (and to a lesser extent, Por2) in regulating the localization of Snf1-Gal83 elevates the possibility of a connection between the porins and glucose-6-phosphate to a higher level. It was previously shown that the glucose analog 2-deoxyglucose (2-DG), which is phosphorylated but not further metabolized, strongly inhibits the nuclear enrichment of Gal83 (VINCENT et al. 2001). We propose a unifying model in which Por1 and Por2 play roles in the regulation of both Snf1 activation and nuclear localization by sensing glucose-6-phosphate.

In summary, Snf1/AMPK and VDAC proteins are highly conserved, and functional relationships similar to the one described here could exist in other eukaryotes, including humans.
**Table 3. *S. cerevisiae* strains**

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<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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References


Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics 122: 19-27.

Sutherland, C. M., S. A. Hawley, R. R. McCartney, A. Leech, M. J. Stark *et al.*, 2003 Elm1p is one of three upstream kinases for the *Saccharomyces cerevisiae* SNF1 complex. Curr Biol 13: 1299-1305.

Tu, J., and M. Carlson, 1994 The GLC7 type 1 protein phosphatase is required for glucose repression in *Saccharomyces cerevisiae*. Mol Cell Biol 14: 6789-6796.

Tu, J., and M. Carlson, 1995 REG1 binds to protein phosphatase type 1 and regulates glucose repression in *Saccharomyces cerevisiae*. EMBO J 14: 5939-5946.


Vyas, V. K., S. Kuchin and M. Carlson, 2001 Interaction of the repressors Nrg1 and Nrg2 with the Snf1 protein kinase in *Saccharomyces cerevisiae*. Genetics 158: 563-572.
Chapter 5

Mitochondrial respiration regulates Snf1 nuclear localization by modulating Snf1-Gal83 complex formation.

Abstract

The Snf1 protein kinase of *Saccharomyces cerevisiae* is essential for adaptation to glucose limitation. Previously, we showed that in glucose-limited respiratory deficient $rho^0$ cells lacking the mitochondrial genome, Snf1 is normally activated by phosphorylation of its T-loop threonine (Thr210). Here, we have observed that despite being activated, Snf1 does not enrich in the nucleus. Interestingly, the nuclear-targeting β subunit Gal83 does not show such a localization defect, suggesting that it is not associated with the Snf1 kinase complex. We present evidence that $rho^0$ cells overexpress Sip2, the cytoplasmic β subunit. Furthermore, Snf1 nuclear localization in $rho^0$ cells can be restored by elimination of Sip2. These results suggest a model in which respiratory deficiency causes a redistribution of Snf1 isoforms in favor of the cytoplasmic Snf1-Sip2 complex at the exclusion of the nuclear Snf1-Gal83 complex. The biological significance of this mechanism could be to reduce the expression of genes involved in the utilization of non-fermentable carbon sources and respiration when the mitochondrial respiratory function is entirely unavailable.

Introduction

Mitochondria have diverse functions including ATP production, generation of metabolites for cellular processes, and contributions to autophagy, apoptosis, and aging (WALLACE et al. 2010; NUNNARI AND SUOMALAINEN 2012). Mitochondria arose from prokaryotes and have their own circular genome, although most proteins required for
mitochondrial structure and function are encoded in the nucleus (Lane and Martin 2010). Mutations in the mitochondrial genome and nuclear genes required for mitochondrial function have been implicated in many neurodegenerative diseases, cancer, and obesity (Schaefer et al. 2008; Wallace et al. 2010; Greaves et al. 2012). A better understanding of diseases caused by mitochondrial dysfunction could suggest novel therapeutic approaches. Since mitochondria play a crucial role in maintaining energy homeostasis, it is important to study mitochondrial signaling and the effects of mitochondrial dysfunction on energy-dependent cellular processes.

The Snf1/AMP-activated protein kinase (AMPK) pathway of eukaryotes plays a central role during response to energy stress (Hedbacker and Carlsson 2008; Hardie et al. 2012). Mammalian AMPK senses energy depletion (increased AMP:ATP ratios) and - in response - stimulates catabolic processes to increase ATP production and inhibits anabolic processes to reduce ATP consumption (Hardie and Hawley 2001; Hardie 2008; Hardie 2011). AMPK plays an important role in the biogenesis and maintenance of mitochondria (Egan et al. 2011; O'Neill et al. 2011) and in mitochondrial respiration (Merrill et al. 1997; Winder et al. 2000). Defects in the AMPK pathway could lead to type 2 diabetes, obesity, and cancer (Hardie 2007; Fogarty and Hardie 2009; Oakhill et al. 2011). The AMPK pathway is an important drug target. For example, metformin, a drug used to treat type 2 diabetes, activates AMPK by inhibiting mitochondrial ATP production (Hardie et al. 2012).

AMPK of the yeast Saccharomyces cerevisiae is known as Snf1 (Hardie et al. 1998; Hedbacker and Carlsson 2008). Yeast cells prefer glucose as their carbon/energy source, and Snf1 regulates responses to glucose limitation. Snf1 is essential for the utilization of alternative carbon sources and mitochondrial respiration. When glucose is limiting, Snf1 is activated by
phosphorylation of the conserved T-loop threonine residue (Thr210), which is performed by upstream kinases (Sak1, Tos3, Elm1).

Like AMPK, the Snf1 kinase is a heterotrimeric complex consisting of a catalytic α subunit (Snf1), a scaffolding/targeting β subunit (Sip1, Sip2, or Gal83), and a regulatory γ subunit (Snf4) (HEDBACKER AND CARLSON 2008). The three alternative β subunits define three distinct Snf1 complexes, referred to as Snf1-Sip1, Snf1-Sip2, and Snf1-Gal83. In the presence of abundant glucose, all three complexes are cytoplasmic. Under carbon stress conditions, Snf1-Sip1 localizes to the vacuolar membrane, Snf1-Sip2 remains cytoplasmic, and Snf1-Gal83 enriches in the nucleus to regulate transcription (HEDBACKER AND CARLSON 2008).

The mechanisms that regulate the subcellular localization of the Snf1 kinase are not completely understood, and virtually nothing is known about the possible role of mitochondria in this process. In the previous chapter, we showed that the mitochondrial outer membrane voltage-dependent anion channel (VDAC) proteins, Por1 and Por2, positively regulate Snf1-Gal83 nuclear localization. Here, we present evidence for another mechanism. Specifically, in respiratory deficient \(\rho^0\) cells, Snf1 does not enrich in the nucleus. Interestingly, the Gal83 subunit does not show such a localization defect, suggesting that it is not associated with the Snf1 kinase complex. We show that \(\rho^0\) cells overexpress Sip2, the cytoplasmic β subunit. Furthermore, Snf1 nuclear localization in \(\rho^0\) cells can be restored by deletion of the \(SIP2\) gene. These results suggest a model in which respiratory deficiency causes a redistribution of Snf1 isoforms in favor of the cytoplasmic Snf1-Sip2 complex at the exclusion of the nuclear Snf1-Gal83 complex.
Materials and Methods

Yeast strains and media. The *S. cerevisiae* strains used in this study are listed in Table 4. Except for strain CTY10-5d (R. Sternglanz, SUNY, Stony Brook, NY) and its *rho*<sup>0</sup> derivative used for LexA-Snf1-G53R reporter assays, all strains were in the W303 genetic background. To generate *sip1Δ* mutants, the *KanMX6* marker sequence was amplified by PCR with primers flanking the *SIP1* open reading frame (ORF). The mutant allele was first introduced into a wild-type diploid by transformation; all yeast transformations were performed using the standard lithium acetate method (Rose et al. 1990). The genotype of the heterozygous *SIP1/sip1Δ::KanMX6* diploid was confirmed by PCR analysis of genomic DNA, and haploid *sip1Δ::KanMX6* segregants were generated from the heterozygous diploid by tetrad analysis. The *sip2Δ* mutants were constructed in the same way. The *rho*<sup>0</sup> mutants were obtained by ethidium bromide treatment (Goldring et al. 1970).

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

Plasmids. Plasmid pSnf1-GFP expresses a Snf1-GFP fusion protein from the native *SNF1* promoter and was constructed in low-copy-number *CEN-URA3* vector pRS316 (Sikorski and Hieter 1989) essentially as described previously (Vincent et al. 2001).

Plasmid pTM2 expresses a Sip2-GFP fusion protein from the native *SIP2* promoter and was constructed in low-copy-number *CEN-URA3* vector pRS316 (Sikorski and Hieter 1989) as follows. The first DNA fragment (fragment A) containing the *SIP2* ORF and 0.6 kb of its
upstream regulatory sequence was amplified by PCR using genomic DNA as the template; the forward primer for this reaction contained an XbaI site for subsequent insertion into pRS316; the reverse primer contained a BspEI site for subsequent insertion of the GFP-encoding sequence, which was followed by a stop codon and a NotI site. The second DNA fragment (fragment B) represented 0.38 kb of the SIP2 terminator sequence and was amplified by PCR using yeast genomic DNA as template; the forward primer for this reaction contained a NotI site for subsequent ligation with fragment A; the reverse primer contained an XbaI site for subsequent insertion into pRS316. Fragments A and B were digested with NotI and ligated together (fragment AB), followed by PCR amplification. Fragment AB was digested with XbaI and inserted into the XbaI site of pRS316, resulting in plasmid pRS316-Sip2. This plasmid was then digested with BspEI, followed by insertion of a BspEI-flanked PCR fragment encoding GFP, resulting in plasmid pTM2. Plasmids pRS316-Sip2 and pTM2 complemented a sip1Δ sip2Δ gal83Δ triple mutant (ORLOVA et al. 2006) for the growth defect on glycerol (SCHMIDT AND MCCARTNEY 2000).

**Assays of transcriptional activation by LexA–Snf1G53R.** The principle behind the “shortcut” reporter activation assays was described previously (KUCHIN et al. 2000). Strain CTY10–5d carries LexA operator sequences (lexAop) inserted upstream of the lacZ gene (lexAop-lacZ reporter). Wild-type CTY10-5d and its rho^0 derivatives were transformed with plasmid pRJ216 expressing LexA-Snf1-G53R (KUCHIN et al. 2000); LexA-Snf1-G53R activates the lexAop-lacZ reporter in a glucose-regulated manner that reflects the catalytic activation of the Snf1-G53R moiety and its nuclear localization (KUCHIN et al. 2000; VINCENT et al. 2001). The corresponding vector control was pEG202 (GOLEMIS et al. 1997). Transformants were grown to
mid-log phase with plasmid selection in selective SC medium with high (2%) glucose and then shifted to an otherwise identical medium with low (0.05%) glucose for 3h. β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units as described previously (VYAS et al. 2001).

**Fluorescence microscopy.** To study Snf1-GFP and Gal83-GFP localization, we followed the protocol published previously (VINCENT et al. 2001). Briefly, cells expressing Snf1-GFP or Gal83-GFP were grown to mid-log phase in selective SC containing 2% glucose and then shifted to an otherwise identical medium containing 3% ethanol and 2% glycerol for 20 min (VINCENT et al. 2001). Nuclei were stained by adding 10 µl of 0.8 µg/mL DAPI to 1 mL of cell culture and incubating for 5 min at 30°C. The cells were then collected by brief centrifugation, and the DAPI and GFP signals were examined using a workstation consisting of Nikon Eclipse 80i fluorescence microscope, a CoolSNAP HQ2 camera (Photometrics), and a computer with NIS-Elements BR 3.01 software.

**Immunoblotting.** Cells were grown as specified in the text. Protein extracts were prepared using the boiling/alkaline lysis method (ORLOVA et al. 2008) and examined for the presence of Snf1 by using anti-polylhistidine antibody H1029 (Sigma-Aldrich), which strongly recognizes Snf1 due to the presence of a natural stretch of 13 consecutive histidines near its N terminus (amino acids 18-30) (ORLOVA et al. 2008). Thr210 phosphorylation of Snf1 was determined using anti-phospho-Thr172-AMPK antibody (Cell Signaling Technology) as described previously (ORLOVA et al. 2008). GFP fusion proteins were detected with anti-GFP antibody (Roche). LexA-Snf1-G53R
protein levels were detected using anti-LexA antibody (Millipore). Signals were detected by enhanced chemiluminescence using Pierce ECL2 or HyGlo (Denville Scientific).

**Results**

The $\rho^0$ mutation affects transcriptional activation by LexA-Snf1-G53R.

In Chapter 2, we showed that despite the lack of any obvious defect in Thr210 phosphorylation, the $\rho^0$ mutation (lack of mitochondrial genome) confers a significant defect in the expression of a Snf1-dependent reporter, $SUC2$-$LEU2$-$\lambdalacZ$ (see Fig. 9). This suggested that the $\rho^0$ mutation affects another aspect of Snf1 function that is distinct from Thr210 phosphorylation. To further address this possibility, we turned to the so-called “shortcut” reporter assay (Kuchin et al. 2000). This assay tests for the ability of LexA-Snf1-G53R (Gly53-to-Arg), a hyperactive but glucose-regulatable version of Snf1 fused to the LexA DNA-binding protein, to activate transcription by binding to the LexA operator sequences ($lexA$-Operon) inserted upstream of the $lacZ$ gene ($lexA$-Operon-$\lambdalacZ$ reporter) and stimulating RNA polymerase II holoenzyme by a mechanism that does not rely on gene-specific transcription factors (Fig. 25A). Importantly, activation of the $lexA$-Operon-$\lambdalacZ$ reporter by LexA-Snf1-G53R is highly glucose regulated: reporter activation is weak under glucose-rich conditions, but increases dramatically upon glucose limitation in a manner that requires catalytic activation (Thr210 phosphorylation) of the Snf1-G53R moiety (Kuchin et al. 2000). In addition to catalytic activation, $lexA$-Operon-$\lambdalacZ$ reporter activation by LexA-Snf1-G53R is strongly affected by mechanisms that regulate Snf1 nuclear localization (Vincent et al. 2001). As such, the “shortcut” reporter assay represents a sensitive readout for defects in Snf1 catalytic activation and nuclear localization.
The *lexAop-lacZ* reporter is widely used for two-hybrid experiments, and strain CTY10-5d carries a genomically integrated copy of this reporter that can be used for the “shortcut” assay (Kuchin *et al.* 2000). We expressed LexA-Snf1-G53R in wild-type CTY10-5d and its *rho*\(^0\) derivative. Reporter activation was examined by β-galactosidase assays after growth in high glucose (2%) and after shift to low glucose (0.05%) for 3h. The *rho*\(^0\) mutant exhibited a strong activation defect in low glucose (Fig. 25B). This defect could not be attributed to reduced Thr210 phosphorylation of the Snf1-G53R moiety (Fig. 25C), suggesting against a defect in catalytic activation, and instead suggesting a defect in nuclear localization.

**Fig. 25.** Effect of the *rho*\(^0\) mutation on "shortcut" reporter activation. The *rho*\(^0\) mutation strongly affects the ability of LexA-Snf1-G53R to activate a *lexAop-lacZ* reporter. (A) Under glucose-limiting conditions, hyperactive LexA-Snf1-G53R becomes catalytically activated and enters the nucleus, where it binds to the promoter of the *lexAop-lacZ* reporter and stimulates transcription by RNA polymerase II holoenzyme (RNA Pol II) (modified from (Kuchin *et al.* 2000)). (B) Strain CTY10-5d (WT) and its *rho*\(^0\) derivative expressed LexA-Snf1-G53R from plasmid pRJ216. Cells were grown in selective SC medium containing high (2%) glucose to mid-log phase, and then shifted for 3h to...
an otherwise identical medium containing low (0.05%) glucose. β-Galactosidase activity was assayed in permeabilized cells and measured in Miller units. The graph shows the data for the low glucose conditions expressed as % of the wild type. Under high glucose conditions, all values were < 3% of the wild-type value observed in low glucose. The mean wild-type value in low glucose was 153 Miller units. Error bars indicate standard errors. Asterisks indicate that β-galactosidase activity in rho<sup>0</sup> cells was statistically significantly different from that in the wild-type cells (p = 0.004, as determined by a t-test). (C) Transformants shifted to 0.05% glucose for 3h as above were tested for Thr210 phosphorylation of the Snf1-G53R moiety (PT210) and total LexA-Snf1-G53R protein levels (Total) by immunoblotting using anti-phospho-Thr172-AMPK and anti-LexA, respectively, as described in Materials and Methods. Vec, vector control pEG202.

**The rho<sup>0</sup> mutation affects nuclear enrichment of Snf1, but not of Gal83.**

To determine the effects of rho<sup>0</sup> on Snf1 nuclear localization, we determined Snf1-GFP localization in cells grown in high (2%) glucose and after a 20 min shift to an otherwise identical medium with a mixture of 3% ethanol and 2% glycerol (EG) as the carbon source. In the presence of abundant glucose, Snf1-GFP was excluded from the nucleus. Unlike in the wild type, however, Snf1-GFP failed to enrich in the nucleus of rho<sup>0</sup> cells after shift to ethanol-glycerol (Fig. 26C). Since nuclear enrichment of Snf1 is completely dependent on Gal83 (Vincent et al. 2001), we also examined the nuclear localization of Gal83-GFP. Surprisingly, Gal83-GFP showed normal nuclear enrichment in the rho<sup>0</sup> mutant (Fig. 26D, bottom panel). This implied that in rho<sup>0</sup> cells, Gal83 is not associated with Snf1.
Fig. 26. Effects of respiratory deficiency on the localization of Snf1 and Gal83. The \( \rho^0 \) mutation affects nuclear enrichment of Snf1, but not of Gal83. (A, C) Wild-type and \( \rho^0 \) cells expressing Snf1-GFP from plasmid pSnf1-GFP were grown to mid-log phase in selective SC medium containing abundant (2%) glucose (Glu) and shifted for 20 min to an otherwise identical medium containing 3% ethanol and 2% glycerol as the carbon source (EG). Nuclei were stained with DAPI, and localization of Snf1-GFP was determined by fluorescence microscopy. (B, D) Wild-type and \( \rho^0 \) cells expressed Gal83-GFP from plasmid pRT12, and experiments were performed as for panels A and C. (E) Cells were grown as for panels A and C, and Thr210 phosphorylation (PT210) and total Snf1-GFP protein levels were detected by immunoblotting using anti-phospho-Thr172-AMPK and anti-GFP, respectively. (F) Cells were grown as for panels B and D, and Gal83-GFP protein levels (Gal83-GFP), as well as Thr210 phosphorylation (PT210) and total levels of Snf1 (Total) were analyzed by immunoblotting using anti-GFP, anti-phospho-Thr172-AMPK, and anti-polyhistidine antibodies, respectively.
Deletion of the \textit{SIP2} gene restores nuclear enrichment of Snf1 in \textit{rho}^0 cells.

The three alternative \(\beta\) subunits bind to the catalytic \(\alpha\) subunit Snf1 in a competitive manner via their conserved C-terminal domains (Hedbacker and Carlson 2008). The observation that Gal83-GFP localizes to the nucleus of \textit{rho}^0 cells, but Snf1-GFP does not, indicated that Gal83 is not associated with Snf1 and suggested that Snf1 is predominantly bound to another \(\beta\) subunit(s). If so, elimination of the \(\beta\) subunit(s) responsible for this effect could restore the association of Gal83 and Snf1 and thereby restore Snf1 nuclear localization. To test this hypothesis, we constructed \textit{rho}^0 \textit{sip1}\(\Delta\) and \textit{rho}^0 \textit{sip2}\(\Delta\) mutants and examined Snf1-GFP localization as above. Snf1-GFP failed to enrich in the nucleus in the \textit{rho}^0 \textit{sip1}\(\Delta\) mutant (Fig. 27A). In the \textit{rho}^0 \textit{sip2}\(\Delta\) mutant, however, the nuclear enrichment of Snf1-GFP was restored (Fig. 27B). In both mutants, Snf1-GFP was expressed and phosphorylated on Thr210 at similar levels (Fig. 27C).

\textbf{Fig. 27.} Roles of the \(\beta\) subunits in Snf1 localization in respiratory-deficient cells. Absence of Sip2, but not of Sip1, from \textit{rho}^0 cells restores nuclear enrichment of Snf1. Localization of Snf1-GFP was determined by fluorescence.
microscopy in (A) rho\(^0\) sip1Δ and (B) rho\(^0\) sip2Δ cells after growth in SC-Ura in the presence of 2% glucose (Glu), and upon a 20 min shift to an otherwise identical medium containing a mixture of 3% ethanol and 2% glycerol as the carbon source (EG). Nuclei were stained with DAPI. (C) Thr210 phosphorylation (PT210) and total Snf1-GFP protein levels were detected by using anti-phospho-Thr172-AMPK and anti-GFP, respectively.

**The rho\(^0\) mutation leads to increased expression of Sip2.**

A simple explanation for why deletion of SIP2 in rho\(^0\) cells restored the nuclear localization of Snf1 could be that Sip2 levels are higher in the rho\(^0\) mutant than in the wild type, making Snf1-Sip2 the major complex in rho\(^0\) cells. To test this, we expressed GFP-tagged Sip2 from its own promoter in wild-type and rho\(^0\) cells, and examined Sip2-GFP levels by immunoblotting using anti-GFP. In comparison to wild-type cells, Sip2-GFP expression was substantially higher in rho\(^0\) cells both in abundant glucose and in ethanol-glycerol (Fig. 28).

Collectively, these results suggest that Sip2 is expressed at a much higher level in rho\(^0\) cells and outcompetes Gal83 for Snf1 binding, thus causing cytoplasmic retention of the kinase.

![Fig. 28](image)

**Fig. 28.** Effect of respiratory deficiency on Sip2 β subunit expression. Cells of the indicated genotypes transformed with low-copy CEN-URA3 vector pRS316 (Vec) or its derivative plasmid pTM2 expressing Sip2-GFP from the native SIP2 promoter were grown in selective SC medium containing 2% glucose (G) and then shifted for 20 min to an otherwise identical medium containing 3% ethanol and 2% glycerol as the carbon source (EG). The levels of
Sip2-GFP were analyzed by immunoblotting using anti-GFP. Thr210 phosphorylation of Snf1 (PT210) and total Snf1 protein levels (Total) were detected using anti-phospho-Thr172-AMPK antibody and anti-polyhistidine antibody, respectively.

**Discussion**

The three alternative β subunits, Sip1, Sip2, and Gal83, function not only to hold the heterotrimeric Snf1 protein complex together by binding to the catalytic α subunit and the stimulatory γ subunit Snf4, but also play distinct roles in subcellular localization of the complex and its substrate specificity (Vincent and Carlson 1999; Schmidt and McCartney 2000; Vincent et al. 2001; Nath et al. 2002). In the presence of abundant glucose, all three Snf1 isoforms are cytoplasmic; during glucose limitation or growth on non-fermentable carbon sources, Snf1-Sip1 localizes to the vacuolar membrane, Snf1-Sip2 remains cytoplasmic, and Gal83 directs Snf1 to the nucleus (Vincent et al. 2001). The Snf1-Gal83 complex is the major isoform of the kinase; it is responsible for nuclear Snf1 activity and contributes to transcriptional activation of many glucose-repressible genes, including those involved in utilization of alternative carbon sources, gluconeogenesis, and respiration (Vincent and Carlson 1999; Schmidt and McCartney 2000; Vincent et al. 2001; Nath et al. 2002; Young et al. 2003). The mechanisms that regulate the nuclear localization of Snf1 are not completely understood.

Our results have revealed a novel mechanism of nucleo-cytoplasmic distribution of Snf1. Using a Snf1-GFP fusion protein, we show that respiratory-deficient yeast cells lacking the mitochondrial genome (rho\(^0\)) exhibit a Snf1 nuclear localization defect. Interestingly, experiments with a Gal83-GFP fusion protein indicated that rho\(^0\) cells have no defect in Gal83 nuclear localization, suggesting that Snf1 and Gal83 are not associated together. We hypothesized that most or all Snf1 is associated with another β subunit. We present evidence that
this other β subunit is Sip2. First, deletion of the SIP2 gene restored normal Snf1 nuclear localization in rho₀ cells. Second, rho₀ cells express Sip2 at a substantially higher level than respiratory-proficient wild-type cells. We propose a model in which respiratory deficiency boosts Sip2 expression and causes a redistribution of Snf1 isoforms in favor of the cytoplasmic Snf1-Sip2 complex at the exclusion of the nuclear Snf1-Gal83 complex.

Respiration-deficient cells lacking the mitochondrial genome activate the so-called retrograde pathway, which regulates nuclear gene expression to compensate for the loss of mitochondrial function (LIU AND BUTOW 2006). The downstream effectors of this pathway are Rtg1 and Rtg3, which form a heterodimeric transcriptional activator that recognizes a consensus sequence GTGAC, also known as the R box, in the promoters of target genes ((LIU AND BUTOW 2006). Sequence analysis of the SIP2 promoter indicates the presence of at least six putative R boxes. Further experiments will be required to address the functional significance of these boxes and the role of the retrograde pathway in SIP2 regulation.

In yeast, respiration-deficient mitochondrial mutants occur spontaneously at a high frequency (so-called petite mutants). The biological significance of this phenomenon remains a matter of debate, but its existence clearly suggests an evolutionary advantage. Since such mutants have reduced intracellular energy levels, it might be advantageous to prevent Snf1 from entering the nucleus to reduce the expression of genes involved in the utilization of non-fermentable carbon sources and respiration if the mitochondrial respiratory function is entirely unavailable in the first place. In addition, it is also possible that in respiration-deficient cells important targets of Snf1 are predominantly present in the cytoplasm.
In conclusion, this study expands the repertoire of novel mechanisms by which mitochondria regulate the Snf1 protein kinase in yeast. Due to the evolutionary conservation of Snf1/AMPK, similar mechanisms might exist in other eukaryotes.

Table 4. *S. cerevisiae* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTY 10-5d</td>
<td>MAT\textsuperscript{a} gal4 gal80 URA3::lexAop-lacZ his3 leu2 ade2 trp1</td>
<td>R. Sternglanz</td>
</tr>
<tr>
<td>ANY1</td>
<td>MAT\textsuperscript{a} gal4 gal80 URA3::lexAop-lacZ his3 leu2 ade2 trp1 \textsuperscript{rho}\textsuperscript{0}</td>
<td>This study</td>
</tr>
<tr>
<td>MMY35</td>
<td>MAT\textsuperscript{a} his3–11,15 leu2–3,112 trp1–1 ura3–1</td>
<td>This laboratory</td>
</tr>
<tr>
<td>AMS-R0</td>
<td>MAT\textsuperscript{a} his3–11,15 leu2–3,112 trp1–1 ura3–1 \textsuperscript{rho}\textsuperscript{0}</td>
<td>This laboratory</td>
</tr>
<tr>
<td>AMS207</td>
<td>MAT\textsuperscript{a} his3–11,15 leu2–3,112 trp1–1 ura3–1 \textsuperscript{sip1Δ::KanMX6 rho}\textsuperscript{0}</td>
<td>This study</td>
</tr>
<tr>
<td>AMS209</td>
<td>MAT\textsuperscript{a} his3–11,15 leu2–3,112 trp1–1 ura3–1 \textsuperscript{sip2Δ::KanMX6 rho}\textsuperscript{0}</td>
<td>This study</td>
</tr>
</tbody>
</table>

References


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


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


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

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Education

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  University of Wisconsin-Milwaukee, Milwaukee, WI  
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- **Master of Science in Biochemistry**
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  2011
- **Bachelor of Science in Biotechnology**
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Publications

- 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*. Eukaryot. Cell 11, 1568-1572.

Oral and Poster Presentations

- **Shevade A.**, Kuchin S. (2016) The tail wagging the dog: functional relationship between Snf1 protein kinase and mitochondria in *Saccharomyces cerevisiae*. Dept. of Biological Sciences, UWM.
- **Shevade A.**, Strogolova V., Orlova M., Yeo C., Maziarz M., Kuchin S. (2015) Comparison of Por1 and Por2 with regard to their role in Snf1 signaling. Midwest Yeast Meeting, Northwestern University, Evanston, IL.
- **Shevade A.**, Strogolova V., Orlova M., Kuchin S. (2015) Role of mitochondrial VDACs as regulators of AMPK, the energy sensor kinase. Biological Sciences Research Symposium, UWM.
- Maziarz M., Barrett L., **Shevade A.**, Kuchin S. (2014) Reg2, a regulatory subunit of PP1, contributes to the negative control of Snf1 in response to glucose and facilitates recovery from glucose deprivation. Midwest Yeast Meeting, Northwestern University, Evanston, IL.
- Maziarz M., Barrett L., **Shevade A.**, Kuchin S. (2013) Reg2, a regulatory subunit of PP1, interacts with Snf1 protein kinase and contributes to its negative control. International Specialized Symposium on Yeast, Slovakia.
Teaching Experience at UWM

• Lab in Experimental Microbiology (BIO SCI 580), 2016-2017
• Genetics Discussion (BIO SCI 325), 2016-2017
• Lab in Cell Biology and Genetics (BIO SCI 316), 2013-2015
• Lab in Anatomy and Physiology II (BIO SCI 203), 2011-2012
• Lab in Foundations of Biological Sciences I (BIO SCI 150), 2015
• Mentored and trained six undergraduate students and two graduate students in independent research.

Scholarships and Awards

• Ruth Walker Graduate Grant-in-Aid Award, Spring 2015 and Spring 2016, UWM
• Chancellor’s Award, granted each semester since Fall 2011, UWM
• Best Graduate Talk Award (Cell and Molecular Biology), May 2015, UWM
• Graduate Student Travel Award, September 2014, UWM
• Junior Research Fellowship, Govt. of India, 2010

Volunteer Work

• Co-coordinated the Biological Sciences Research Symposium, April 2016, UWM
• Worked as market research analyst, Fall 2015, UWM Research Foundation (UWMRF)
• Judged posters at the UW System Symposium for Undergraduate Research, Spring 2014 and 2015