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The Functional Relationship Between Yeast AMPK and Mitochondria

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THE FUNCTIONAL RELATIONSHIP BETWEEN YEAST AMPK AND MITOCHONDRIA

by

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ABSTRACT

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The AMP-activated protein kinase (AMPK) family is involved in responses to energy stress and is highly conserved among eukaryotes. In mammals, AMPK remains inactive when ATP levels are high. However, AMPK becomes catalytically active in response to low ATP levels (and thus a high AMP: ATP ratio). When the AMP: ATP ratio is high, AMP binds to AMPK, causing its phosphorylation (catalytic activation) by upstream kinases. Catalytic activation causes AMPK to upregulate processes related to ATP generation (such as mitochondrial respiration) and to downregulate processes related to ATP consumption (such as growth). AMPK therefore acts as a direct sensor of energy levels. It comes as no surprise then that in mammals, AMPK forms a negative feedback loop with the mitochondria: activated AMPK stimulates mitochondrial respiration, which produces more ATP, which in turn returns AMPK to its inactive state.

In the yeast *Saccharomyces cerevisiae*, we have found that the mitochondria actually positively regulate the yeast AMPK ortholog known as Snf1 (sucrose non-fermenting 1). For example, our lab has previously shown that cells lacking the mitochondrial voltage-dependent anion channel (VDAC) genes *POR1* and *POR2* experience a defect in Snf1 activation. We therefore asked if there were any other pathways by which the mitochondria regulate Snf1. It was found that respiratory-null yeast cells lacking the mitochondrial genome (*rho*^{0}) also experience a defect in Snf1 regulation, but in a manner

independent of Por1 and Por2. Here, we present evidence that the *rho*^{0} mutation alters the regulation, composition, and nucleocytoplasmic distribution of the yeast Snf1 protein kinase complex.

Yeast Snf1/AMPK has been implicated in the pathogenesis of *Candida albicans*. Understanding how yeast AMPK is regulated by mitochondria could further the research toward developing new antifungal compounds. Furthermore, mammalian AMPK is also a clinically significant drug target. In humans, its dysregulation is associated with conditions such as diabetes and cancer, and has additionally been implicated in cellular aging. Since AMPK is highly conserved among eukaryotes (including humans), further insights into the regulation of yeast Snf1/AMPK may help better understand how human AMPK is also regulated.

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CHAPTER 1

Introduction

OVERVIEW

Organisms encounter a variety of environmental stresses, and lack of preferred carbon sources (such as glucose) is among them. Cells have developed multiple mechanisms by which they upregulate processes relating to both energy conservation and alternative carbon source metabolism during carbon/energy stress conditions. The cues used to signal nutrient stress come from both the environment, and from within the cell itself, such as the mitochondria. AMP-activated protein kinase (AMPK) is a conserved kinase that regulates energy levels during energy limitation in eukaryotic cells. Because AMPK responds to intracellular levels of ATP, it is regulated, at least indirectly, by the mitochondria. The results presented here demonstrate that AMPK in yeast can be regulated by the mitochondria in multiple ways.

AMP-ACTIVATED PROTEIN KINASE IN MAMMALS

Organisms constantly encounter a variety of stresses in their environment, from toxic compounds and temperature extremes to nutrient starvation. The ways in which organisms can sense and respond to these stresses is one of the most fundamental questions in biology. While the physiological responses to stress may be easily observable, the underlying mechanisms are often poorly understood. In this manner, metabolic homeostasis is no different. Maintaining an appropriate energy reserve in the form of ATP pools is essential for cellular survival. As energy levels decrease, cellular processes are regulated to limit ATP expenditure while simultaneously upregulating processes to increase ATP generation. An important regulator of this energy-balancing process is mammalian AMP-activated protein kinase (AMPK). Often referred to as the “fuel gauge” of the cell, it belongs to a highly conserved protein kinase family present in eukaryotes (Hardie & Carling, 1997). AMPK responds to cellular energy stress by

sensing increased levels of AMP relative to ATP (reviewed in (Hardie & Carling, 1997)). AMPK functions by balancing growth/metabolism during nutrient stress or starvation. Simply put, AMPK works to limit energy expenditure while maximizing energy production.

REGULATION OF MAMMALIAN AMPK

Mammalian AMPK is composed of three subunits: the catalytic α subunit and regulatory β and γ subunits. While AMPK is expressed in non-starvation conditions, it is not catalytically active; like many other kinases, AMPK is activated by phosphorylation by upstream kinases (Woods *et al.*, 2003). Phosphorylation of a conserved threonine residue (Thr172) by upstream kinases allows AMPK to function in its active form (Hong *et al.*, 2003b). When energy levels are high, ATP binds to the sensory γ subunit and AMPK remains in its inactive form. Specifically, the kinase domain of the α subunit remains tightly associated with its own negative regulatory domain (Yan *et al.*, 2018). Not only does this sequester the kinase domain from phosphorylation, but it is additionally able to be accessed by phosphatases, thus keeping AMPK in its inactive state (Davies *et al.*, 1995). However, as energy levels decrease, the AMP:ATP ratio increases and AMP, rather than ATP, will bind to the γ subunit. When AMP is bound to AMPK, an allosteric change is induced and the sensory γ subunit pushes out the kinase domain from its interaction with the negative regulatory domain, thus freeing the kinase domain (Yan *et al.*, 2018). This allows it to be phosphorylated by upstream kinases. Additionally, AMP-bound AMPK is protected from dephosphorylation and inactivation by phosphatases (Suter *et al.*, 2006). When AMPK is in its catalytically active state, it stimulates ATP generation by activating mechanisms such as increased glucose uptake and fatty acid oxidation (Hardie, 2007c). This elegant system of nucleotide binding means that AMPK can directly sense ATP-depleting conditions and respond appropriately, earning it the nickname “fuel gauge” of the cell.

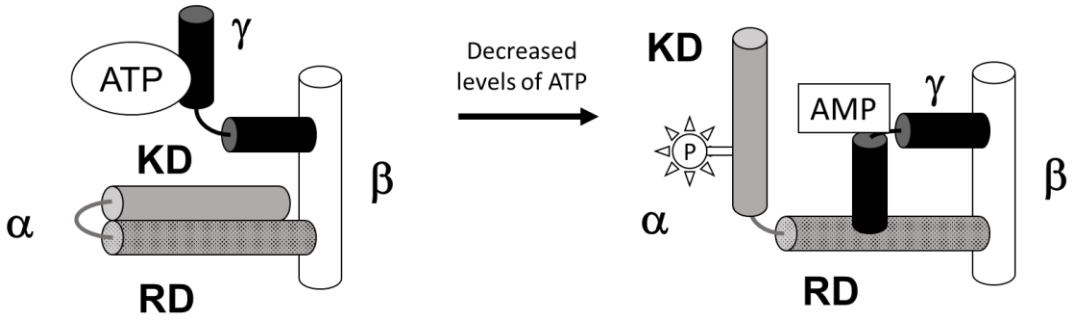

Figure 1. AMP-stimulated catalytic activation of mammalian AMPK. As ATP levels decrease, the AMP: ATP ratio increases. AMP then binds to the γ subunit, causing an allosteric change in AMPK. The kinase domain (KD) is released from the regulatory domain (RD), allowing the KD to be phosphorylated by upstream kinases. AMP-bound AMPK is also protected from dephosphorylation by phosphatases [adapted from (Barrett, 2011)].

DOWNSTREAM TARGETS OF MAMMALIAN AMPK

Once catalytically active, AMPK affects a number of downstream processes. For example, AMPK can ameliorate low energy levels by upregulating glucose uptake in cells. To this end, AMPK induces GLUT4 plasma membrane translocation and increases glucose uptake by the cells (Cantó & Auwerx, 2010). AMPK will also stimulate fatty acid oxidation in order to increase ATP levels (Hardie & Pan, 2002). AMPK can directly phosphorylate acetyl-CoA carboxylase (ACC) (Hardie & Pan, 2002). Phosphorylation of ACC inactivates it, which causes a decreased rate of fatty acid synthesis. The product of the ACC enzyme, malonyl-CoA, is also a negative regulator of mitochondrial uptake of fatty acids (Mills, Foster & McGarry, 1983). Thus, the effect of phosphorylation of ACC is twofold: it stops the synthesis of fatty acids (an ATP consuming process) and stimulates the mitochondrial uptake of fatty acids (an ATP generating process). In order to further limit energy consumption, AMPK also downregulates protein synthesis and cell growth by inhibiting mTOR (Cantó & Auwerx, 2010). While the regulatory targets of AMPK are

numerous, their overall effect can be summarized as follows: upregulation of processes relating to ATP generation, and downregulation of processes relating to ATP expenditure (Figure 2).

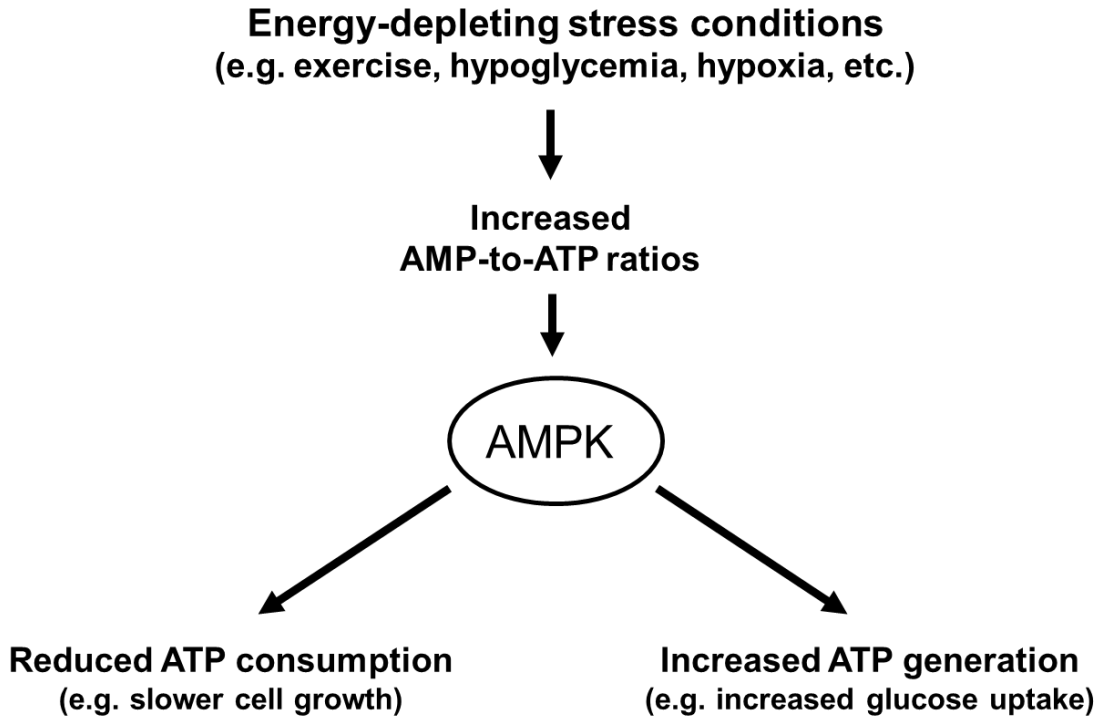

Figure 2. The role of AMPK in maintaining energy homeostasis. AMPK senses depleted levels of intracellular ATP (increased AMP:ATP ratio) and becomes catalytically activated. AMPK then stimulates catabolic process while inhibiting anabolic processes [adapted from (Barrett, 2011)].

CLINICAL SIGNIFICANCE OF MAMMALIAN AMPK

Given the role AMPK plays in energy homeostasis, it is no surprise that defects in AMPK signaling are associated with human diseases such as diabetes (Fogarty & Hardie, 2010). As such, AMPK is a target of various therapeutic approaches (Hardie, 2007a). For example, the FDA-approved drug for treating type II diabetes, metformin, is thought to act by activation of AMPK (Rena, Pearson & Sakamoto, 2013). Additionally, AMPK defects are associated with uncontrolled cellular growth, such as cancer, and has

been implicated in aging (Li *et al.*, 2015; Ruiz, Perez-Villegas & Manuel Carrion, 2016). Because of the clinical significance of AMPK, the ability to study a closely related ortholog in a model organism presents itself as an attractive alternative.

YEAST AS A MODEL ORGANISM

Historically, baker's yeast *Saccharomyces cerevisiae* has been used extensively as a model organism. Not only is it relatively easy to grow, it is also capable of sexual reproduction and can exist stably in either haploid or diploid form, making it ideal for genetic studies. Its genome was sequenced in 1996, making it the first fully sequenced eukaryotic genome. Sequence analysis has revealed that 23% of the protein coding regions in its genome are homologous to humans (Liu *et al.*, 2017). Unlike with other eukaryotes, deletion of the mitochondrial genome (and thus elimination of aerobic respiration) is not lethal for yeast, thus providing an ample opportunity to study the effects of mitochondrial dysfunction (Williamson, 2002). This is especially pertinent to the studies that will be outlined in subsequent chapters.

AMPK IN YEAST

Originally identified during a screen for mutants unable to utilize sucrose, the AMPK ortholog in yeast is called Snf1 (sucrose non-fermenting 1) (Carlson, Osmond & Botstein, 1981). Like its mammalian counterpart, it is essential for responses to glucose starvation and other cellular stresses. The Snf1 kinase complex is also a heterotrimer composed of the catalytic α subunit (Snf1 itself), a scaffolding/targeting β subunit (Sip1, Sip2, or Gal83), and a stimulatory γ subunit (Snf4) [for review see (Hedbacker & Carlson, 2008)]. There are three distinct Snf1 kinase complexes designated Snf1-Sip1, Snf1-Sip2, and Snf1-Gal83. The β subunit present in the Snf1 kinase complex dictates its subcellular localization in response to stress: Snf1-Sip1 localizes to the vacuolar periphery, Snf1-Sip2 remains

cytoplasmic, and Snf1-Gal83 localizes to the nucleus (Figure 3). This differential localization is due to differences in the N-terminal regions of the β subunits (Vincent *et al.*, 2001).

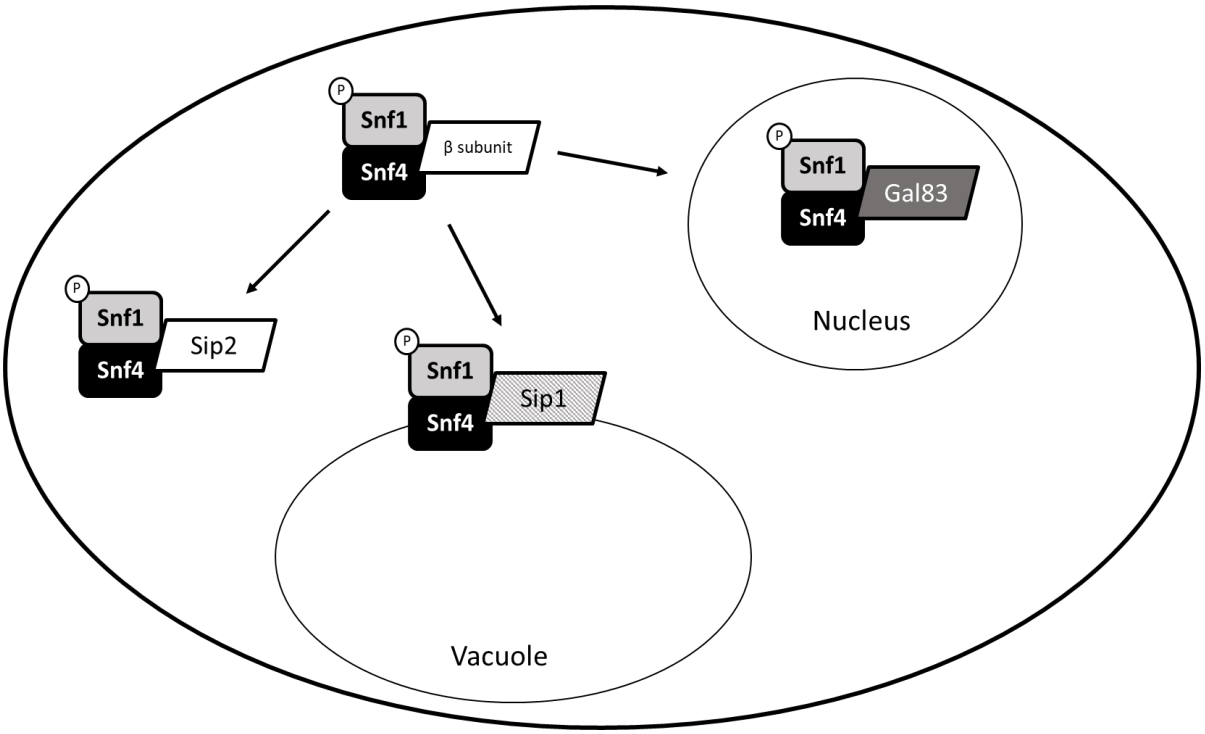

Figure 3. Nucleocytoplasmic distribution of yeast Snf1/AMPK in response to carbon/energy stress. While yeast AMPK remains cytoplasmic in high glucose conditions, it rapidly relocates to one of three subcellular locations during carbon/energy stress. During carbon/energy stress, yeast AMPK first gets phosphorylated on a conserved threonine residue by upstream kinases (catalytic activation). After phosphorylation, the subcellular localization of the Snf1 kinase complex depends on the β subunit isoform that the complex is composed of; the Snf1-Gal83 complex localizes to the nucleus, the Snf1-Sip2 complex remains cytoplasmic, and the Snf1-Sip2 complex locates to the vacuolar membrane. The Snf1-Gal83 complex is responsible for nuclear enrichment and gene expression changes during carbon/energy stress.

REGULATION OF YEAST SNF1/AMPK

During environmental stresses, such as nutrient starvation, the Snf1 kinase complex becomes catalytically activated through phosphorylation by three upstream kinases (Sak1, Tos3, and Elm1) on a conserved threonine residue (Thr210) (Hong *et al.*, 2003b; Nath, McCartney & Schmidt, 2003; Sutherland *et al.*, 2003). As with mammalian AMPK, a conformational change in the Snf1 kinase complex takes place during nutrient starvation, and the kinase domain of the Snf1 subunit is freed from its negative regulatory domain. As a consequence (or as a cause) of this conformational change, the catalytic domain is phosphorylated by upstream kinases.

As mentioned above, there are three distinct Snf1 kinase complexes: Snf1-Sip1, Snf1-Sip2, and Snf1-Gal83. The β subunit present in the Snf1 kinase complex dictates its subcellular localization in response to stress: Snf1-Sip1 localizes to the vacuolar membrane, Snf1-Sip2 remains cytoplasmic, and Snf1-Gal83 translocates to the nucleus (Vincent *et al.*, 2001; Hedbacker & Carlson, 2008). This differential localization is due to differences in the N-terminal regions of the β subunits (Vincent *et al.*, 2001). Of the three Snf1 complexes, the Snf1-Gal83 isoforms stands out in at least two ways. First, transcription regulation is arguably the most important role of Snf1, and thus its Gal83-dependent nuclear localization is an important process. Second, Gal83 is the most abundant β subunit (at least in high glucose), suggesting that the Snf1-Gal83 complex is also the most physiologically important of the three (Chandrashekarappa *et al.*, 2016).

Considering that fungi and animals diverged from a common ancestor some 1.5 billion years ago (Wang, Kumar & Hedges, 1999), the degree of homology between the Snf1 complex and mammalian AMPK is quite substantial. The catalytic subunit Snf1 shares 46% sequence identity with AMPKα, with some domains sharing up to 62% identity (Carling *et al.*, 1994). As such, it would be expected that the regulation of the Snf1 kinase complex is similar to that of AMPK. Indeed, the crystal structure of the γ subunit was determined and a putative nucleotide binding pocket was identified (Townley & Shapiro,

2007), suggesting that AMP binds to the Snf1 complex. *In vitro* studies showed, however, that ADP, rather than AMP binds to the Snf1 complex (Mayer *et al.*, 2011). ADP-bound Snf1, like AMP-bound AMPK, was also protected from phosphatases *in vitro* (Mayer *et al.*, 2011)

However, later studies showed that amino acid mutations in the γ subunit Snf4 that prevent adenylate binding did not have any effect on Snf1 regulation *in vivo* (Chandrashekarappa, McCartney & Schmidt, 2013). Thus, the mechanism by which the Snf1 complex detects carbon/energy stress remains unknown. Nonetheless, Snf1 activity still correlates with increased AMP:ATP ratios *in vivo* (Wilson, Hawley & Hardie, 1996), suggesting the existence of energy sensors upstream of the Snf1 complex.

Recent studies have shown that Snf1 interacts with mitochondrial voltage-dependent anion channel (VDAC) protein Por1 and its paralog Por2 (Strogolova *et al.*, 2012). Moreover, cells lacking *POR1* and *POR2* fail to normally activate Snf1 (Strogolova *et al.*, 2012). Considering that the mitochondrial VDACs are responsible for transporting and/or binding energy-related nucleotides (such as AMP, ADP, ATP), Por1 and Por2 are poised to act as sensors of intracellular energy levels for Snf1.

TARGETS OF SNF1

The first process identified as target of the Snf1 kinase complex was alternative carbon source utilization. This is, in fact, how Snf1 got its name: it was originally identified during a screen for mutants unable to metabolize sucrose (Carlson *et al.*, 1981). When energy levels are high during growth on abundant glucose, the transcription of the *SUC2* invertase gene is repressed (part of a mechanism known as glucose repression). Invertase is responsible for hydrolyzing the disaccharide sucrose into the fructose and glucose monosaccharides, thus allowing efficient metabolism. Under nutrient-rich conditions, transcription of the *SUC2* gene is inhibited by the Mig1 transcription repressor (Ostling & Ronne, 1998). When the Snf1 complex is catalytically active, it phosphorylates the Mig1 repressor and Mig1 dissociates from the *SUC2* promoter, allowing for transcription to occur (Treitel, Kuchin & Carlson,

1998; Papamichos-Chronakis, Gligoris & Tzamarias, 2004). The function of the Snf1-Mig1 pathway is not limited to the regulation of *SUC2*, as it regulates a battery of genes related to glucose repression, including *SUC*, *MAL*, and *GAL* genes (Tzamarias & Struhl, 1995; Treitel & Carlson, 1995; Treitel *et al.*, 1998).

The Snf1 complex also directly phosphorylates the transcription activator Cat8, which regulates genes related to gluconeogenesis and utilization of non-fermentable carbon sources such as ethanol and glycerol (Young *et al.*, 2003). Additionally, Snf1 promotes mitochondrial respiration, and the *snf1*Δ mutant is incapable of respiratory growth on non-fermentable carbon sources (Young *et al.*, 2003).

SNF1 AND MITOCHONDRIA

While Snf1 does not directly sense energy levels *per se*, its activity nonetheless corresponds to increased AMP:ATP ratios in the cell, prompting the question: "By what mechanism does the Snf1 complex sense intracellular energy levels?". Considering that mitochondria are responsible for the bulk of energy production in aerobic conditions, a regulatory relationship with Snf1 complex would be consistent with the role yeast AMPK plays in maintaining energy homeostasis. It has been found that this is indeed the case. For example, Snf1 is a positive regulator of aerobic respiration, as the *snf1*Δ mutant cannot grow on nonfermentable carbon sources. Studies have shown that the Snf1 complex is involved in transcription regulation of many genes related to the mitochondrial transport of metabolites (Young *et al.*, 2003).

During nutrient stress, upregulation of Snf1-dependent processes will cause an increase in intracellular ATP levels, which in turn will cause the dephosphorylation (inactivation) of the complex. Thus, in this simple model, the relationship between mitochondria and yeast Snf1/AMPK is negative: Snf1/AMPK activates mitochondrial respiration, which causes ATP levels to increase, resulting in the kinase returning to its inactive state. However, mitochondria can also positively regulate the Snf1

complex. For example, mitochondrial VDACs promote Snf1 catalytic activation (Strogolova *et al.*, 2012). Additionally, studies have shown that deletion of the gene encoding the major yeast VDAC Por1 results in a defect in Snf1 nuclear localization (Shevade *et al.*, 2018). This defect in the *por1*Δ mutant can be ameliorated by overexpression of the Por1 paralog, Por2. Curiously, Por2 is reported to have no channel function (Guardiani *et al.*, 2018; Blachly-Dyson *et al.*, 1997), suggesting that the VDAC-dependent control of Snf1 is separable from VDAC channel function [*i.e.,* strictly regulatory (Strogolova *et al.*, 2012; Shevade *et al.*, 2018)].

These results paint a regulatory relationship between Snf1 and the mitochondrion that is considerably more complex than a simple ATP-mediated negative feedback loop. There appear to be many elements of the mitochondrion and even mitochondrial function as a whole working to regulate the Snf1 kinase complex. The purpose of this work, therefore, is twofold: 1) to further characterize the role that mitochondrial porins play in the regulation of Snf1, and 2) to elucidate the role that mitochondrial respiration plays in the regulation, composition of, and nucleocytoplasmic distribution of the yeast Snf1 kinase complex.

REFERENCES

BARRETT, L. (2011). *Regulation of the stress-response protein kinase Snf1 in Saccharomyces cerevisiae*. Ph.D. thesis, The University of Wisconsin - Milwaukee.

BLACHLY-DYSON, E., SONG, J., WOLFGANG, W. J., COLOMBINI, M. & FORTE, M. (1997). Multicopy suppressors of phenotypes resulting from the absence of yeast VDAC encode a VDAC-like protein. *Mol Cell Biol* **17**(10), 5727-38.

CANTÓ, C. & AUWERX, J. (2010). AMP-activated protein kinase and its downstream transcriptional pathways. *Cell Mol Life Sci* **67**(20), 3407-23.

CARLING, D., AGUAN, K., WOODS, A., VERHOEVEN, A. J., BERI, R. K., BRENNAN, C. H., SIDEBOTTOM, C., DAVISON, M. D. & SCOTT, J. (1994). Mammalian AMP-activated protein kinase is homologous to yeast and

plant protein kinases involved in the regulation of carbon metabolism. *J Biol Chem* **269**(15), 11442-8.

CARLSON, M., OSMOND, B. C. & BOTSTEIN, D. (1981). Mutants of yeast defective in sucrose utilization. *Genetics* **98**(1), 25-40.

CHANDRASHEKARAPPA, D. G., MCCARTNEY, R. R., O'DONNELL, A. F. & SCHMIDT, M. C. (2016). The β subunit of yeast AMP-activated protein kinase directs substrate specificity in response to alkaline stress. *Cell Signal* **28**(12), 1881-1893.

CHANDRASHEKARAPPA, D. G., MCCARTNEY, R. R. & SCHMIDT, M. C. (2013). Ligand binding to the AMP-activated protein kinase active site mediates protection of the activation loop from dephosphorylation. *J Biol Chem* **288**(1), 89-98.

DAVIES, S. P., HELPS, N. R., COHEN, P. T. & HARDIE, D. G. (1995). 5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C alpha and native bovine protein phosphatase-2AC. *FEBS Lett* **377**(3), 421-5.

FOGARTY, S. & HARDIE, D. G. (2010). Development of protein kinase activators: AMPK as a target in metabolic disorders and cancer. *Biochim Biophys Acta* **1804**(3), 581-91.

GUARDIANI, C., MAGRÌ, A., KARACHITOS, A., DI ROSA, M. C., REINA, S., BODRENKO, I., MESSINA, A., KMITA, H., CECCARELLI, M. & DE PINTO, V. (2018). yVDAC2, the second mitochondrial porin isoform of Saccharomyces cerevisiae. *Biochim Biophys Acta Bioenerg* **1859**(4), 270-279.

HARDIE, D. G. (2007a). AMP-activated protein kinase as a drug target. *Annu Rev Pharmacol Toxicol* **47**, 185-210.

HARDIE, D. G. (2007c). AMPK and SNF1: Snuffing Out Stress. In *Cell Metab*. (Volume 6, pp. 339-40.

HARDIE, D. G. & CARLING, D. (1997). The AMP-activated protein kinase--fuel gauge of the mammalian cell? *Eur J Biochem* **246**(2), 259-73.

HARDIE, D. G. & PAN, D. A. (2002). Regulation of fatty acid synthesis and oxidation by the AMP-activated protein kinase. *Biochem Soc Trans* **30**(Pt 6), 1064-70.

HEDBACKER, K. & CARLSON, M. (2008). SNF1/AMPK pathways in yeast. *Front Biosci* **13**, 2408-20.

HONG, S. P., LEIPER, F. C., WOODS, A., CARLING, D. & CARLSON, M. (2003b). Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. *Proc Natl Acad Sci U S A* **100**(15), 8839-43.

LI, W., SAUD, S. M., YOUNG, M. R., CHEN, G. & HUA, B. (2015). Targeting AMPK for cancer prevention and treatment. *Oncotarget* **6**(10), 7365-78.

LIU, W., LI, L., YE, H., CHEN, H., SHEN, W., ZHONG, Y., TIAN, T. & HE, H. (2017). From Saccharomyces cerevisiae to human: The important gene co-expression modules. *Biomed Rep* **7**(2), 153-158.

MAYER, F. V., HEATH, R., UNDERWOOD, E., SANDERS, M. J., CARMENA, D., MCCARTNEY, R. R., LEIPER, F. C., XIAO, B., JING, C., WALKER, P. A., HAIRE, L. F., OGRODOWICZ, R., MARTIN, S. R., SCHMIDT, M. C., GAMBLIN, S. J. & CARLING, D. (2011). ADP regulates SNF1, the Saccharomyces cerevisiae homolog of AMP-activated protein kinase. *Cell Metab* **14**(5), 707-14.

MILLS, S. E., FOSTER, D. W. & MCGARRY, J. D. (1983). Interaction of malonyl-CoA and related compounds with mitochondria from different rat tissues. Relationship between ligand binding and inhibition of carnitine palmitoyltransferase I. *Biochem J* **214**(1), 83-91.

NATH, N., MCCARTNEY, R. R. & SCHMIDT, M. C. (2003). Yeast Pak1 kinase associates with and activates Snf1. *Mol Cell Biol* **23**(11), 3909-17.

OSTLING, J. & RONNE, H. (1998). Negative control of the Mig1p repressor by Snf1p-dependent phosphorylation in the absence of glucose. *Eur J Biochem* **252**(1), 162-8.

PAPAMICHOS-CHRONAKIS, M., GLIGORIS, T. & TZAMARIAS, D. (2004). The Snf1 kinase controls glucose repression in yeast by modulating interactions between the Mig1 repressor and the Cyc8-Tup1 co-repressor. *EMBO Rep* **5**(4), 368-72.

RENA, G., PEARSON, E. R. & SAKAMOTO, K. (2013). Molecular mechanism of action of metformin: old or new insights? *Diabetologia* **56**(9), 1898-906.

RUIZ, R., PEREZ-VILLEGAS, E. M. & MANUEL CARRION, A. (2016). AMPK Function in Aging Process. *Curr Drug Targets* **17**(8), 932-41.

SHEVADE, A., STROGOLOVA, V., ORLOVA, M., YEO, C. T. & KUCHIN, S. (2018). Mitochondrial Voltage-Dependent Anion Channel Protein Por1 Positively Regulates the Nuclear Localization of Saccharomyces cerevisiae AMP-Activated Protein Kinase. *mSphere* **3**(1).

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**(12), 1568-72.

SUTER, M., RIEK, U., TUERK, R., SCHLATTNER, U., WALLIMANN, T. & NEUMANN, D. (2006). Dissecting the role of 5'-AMP for allosteric stimulation, activation, and deactivation of AMP-activated protein kinase. *J Biol Chem* **281**(43), 32207-16.

SUTHERLAND, C. M., HAWLEY, S. A., MCCARTNEY, R. R., LEECH, A., STARK, M. J., SCHMIDT, M. C. & HARDIE, D. G. (2003). Elm1p is one of three upstream kinases for the Saccharomyces cerevisiae SNF1 complex. *Curr Biol* **13**(15), 1299-305.

TOWNLEY, R. & SHAPIRO, L. (2007). Crystal Structures of the Adenylate Sensor from Fission Yeast AMP-Activated Protein Kinase. *Science* **315**(5819), 1726-1729.

TREITEL, M. A. & CARLSON, M. (1995). Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein. *Proc Natl Acad Sci U S A* **92**(8), 3132-6.

TREITEL, M. A., KUCHIN, S. & CARLSON, M. (1998). Snf1 protein kinase regulates phosphorylation of the Mig1 repressor in Saccharomyces cerevisiae. *Mol Cell Biol* **18**(11), 6273-80.

TZAMARIAS, D. & STRUHL, K. (1995). Distinct TPR motifs of Cyc8 are involved in recruiting the Cyc8-Tup1 corepressor complex to differentially regulated promoters. *Genes Dev* **9**(7), 821-31.

VINCENT, O., TOWNLEY, R., KUCHIN, S. & CARLSON, M. (2001). Subcellular localization of the Snf1 kinase is regulated by specific beta subunits and a novel glucose signaling mechanism. *Genes Dev* **15**(9), 1104-14.

WANG, D. Y., KUMAR, S. & HEDGES, S. B. (1999). Divergence time estimates for the early history of animal phyla and the origin of plants, animals and fungi. *Proc Biol Sci* **266**(1415), 163-71.

WILLIAMSON, D. (2002). The curious history of yeast mitochondrial DNA. *Nat Rev Genet* **3**(6), 475-81.

WILSON, W. A., HAWLEY, S. A. & HARDIE, D. G. (1996). Glucose repression/derepression in budding yeast: SNF1 protein kinase is activated by phosphorylation under derepressing conditions, and this correlates with a high AMP:ATP ratio. *Curr Biol* **6**(11), 1426-34.

WOODS, A., JOHNSTONE, S. R., DICKERSON, K., LEIPER, F. C., FRYER, L. G., NEUMANN, D., SCHLATTNER, U., WALLIMANN, T., CARLSON, M. & CARLING, D. (2003). LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr Biol* **13**(22), 2004-8.

YAN, Y., ZHOU, X. E., XU, H. E. & MELCHER, K. (2018). Structure and Physiological Regulation of AMPK. *International Journal of Molecular Sciences* **19**(11), 3534.

YOUNG, E. T., DOMBEK, K. M., TACHIBANA, C. & IDEKER, T. (2003). Multiple pathways are co-regulated by the protein kinase Snf1 and the transcription factors Adr1 and Cat8. *J Biol Chem* **278**(28), 26146-58.

CHAPTER 2

Mitochondrial porins Por1 and Por2 contribute to Snf1 catalytic activation through the principal Snf1-activating kinase Sak1

This chapter will be submitted to *microPublication Biology* as a manuscript by Shaheduzzaman M., Brown K.M., and Kuchin, S. V. (2022).

ABSTRACT

In yeast, Sak1 serves as the principal activating kinase for Snf1 (sucrose non-fermenting 1), an ortholog of AMP-activated protein kinase (AMPK). However, the mechanisms that activate the Sak1-Snf1 cascade in response to energy stress are not completely understood. We previously showed that the mitochondrial voltage-dependent anion channel (VDAC) proteins Por1 and Por2 physically interact with Snf1 and play redundant roles in its activation. Here, we present evidence that Por1/2 and Sak1 function in the same pathway upstream of Snf1. We suggest that Por1 and Por2 could serve as energy sensors that upregulate Sak1 and/or make Snf1 a better substrate for activation.

INTRODUCTION

Mammalian AMP-activated protein kinase (AMPK) belongs to a highly conserved eukaryotic protein kinase family (Hardie, Carling & Carlson, 1998). Often referred to as the “fuel gauge” of the cell, AMPK responds to cellular energy stress, such as glucose starvation, by sensing increased levels of AMP relative to ATP (Hardie & Carling, 1997). AMPK maintains energy homeostasis during carbon stress by upregulating ATP-generating processes (e.g., fatty acid oxidation), while down-regulating ATP-consuming processes (e.g., cellular proliferation) (Hardie, 2007c).

Mammalian AMPK is a heterotrimeric complex composed of three subunits: the catalytic α subunit, the scaffolding/targeting β subunit, and the stimulatory γ subunit (Hardie, 2007b). When intracellular ATP levels are abundant, ATP binds to the γ subunit and AMPK remains inactive. However, as intracellular ATP levels decrease, the AMP:ATP ratio increases, and AMP, rather than ATP, binds to the γ subunit. When AMP is bound to AMPK, an conformational change is induced and AMPK becomes catalytically activated through phosphorylation of a conserved threonine residue (Thr172) in the activation loop of the α subunit by upstream kinases such as LKB1 (Carling *et al.*, 1994; Hong *et al.*, 2003b). AMP-bound AMPK is additionally protected from dephosphorylation by cellular phosphatases, ensuring that AMPK remains active during nutrient stress (Davies *et al.*, 1995). This catalytically active form of AMPK is responsible for maintaining energy homeostasis in the cell.

The AMPK ortholog in the common yeast *Saccharomyces cerevisiae* is the Snf1 (sucrose non-fermenting 1) kinase complex and is likewise essential for responses to glucose starvation and other cellular stresses (Carlson *et al.*, 1981; Carling *et al.*, 1994). The regulation of the Snf1 complex is similar to mammalian AMPK in that Snf1 catalytic activation is achieved by phosphorylation of a conserved threonine residue (Thr210) by upstream kinases (Estruch *et al.*, 1992; McCartney & Schmidt, 2001). There are three Snf1-activating kinases that have been identified: Sak1, Tos3, and Elm1 (Nath *et al.*, 2003; Sutherland *et al.*, 2003; Hong *et al.*, 2003b). While these three upstream kinases have partially redundant functions in Snf1 catalytic activation, Sak1 has been previously shown to make the largest individual contribution and is therefore considered to be the principal Snf1-activating kinase (Hedbacker, Hong & Carlson, 2004; McCartney, Rubenstein & Schmidt, 2005; Hong & Carlson, 2007). While the upstream kinases have been identified, the mechanism which regulates the Sak1-Snf1 signaling cascade is not well understood.

Previous work has shown that the mitochondrial voltage-dependent ion channel (VDAC) proteins Por1 and Por2 physically interact with Snf1 and contribute to its catalytic activation in response

to glucose limitation, as the *por1*Δ *por2*Δ double mutant exhibits a strong defect in Snf1 Thr210 phosphorylation (Strogolova *et al.*, 2012). Here, we present evidence that Por1 and Por2 promote Snf1 activation through the principal upstream kinase Sak1.

MATERIALS AND METHODS

Yeast strains and growth conditions

All strains were in the W303 genetic background and were descendants of strains W303-1A (*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) and W303-1B (*MAT***α** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*). All mutants were created by replacing the wild-type alleles with either *KanMX* or *HIS3MX6* cassettes. The *por1*Δ, *por2*Δ, and *sak1*Δ alleles were constructed and confirmed as described previously (Strogolova *et al.*, 2012; Shevade *et al.*, 2018; Orlova *et al.*, 2010), and combinatorial mutants were obtained 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 alleles replaced with the *HIS3MX6* cassette. Unless indicated otherwise, the media contained 2% glucose, and cells were grown at 30°C.

Immunoblot analysis and quantification

Cells were grown under conditions specified in the descriptions of experimental results. Protein extracts were prepared by the boiling/alkaline treatment method as described previously and analyzed by immunoblotting (Orlova, Barrett & Kuchin, 2008). The endogenous Snf1 protein was detected with anti-polyhistidine antibody H1029 (Sigma-Aldrich), which strongly recognizes Snf1 due to the presence of a natural stretch of 13 consecutive histidine residues near its N terminus (amino acids 18 to 30). The Thr210 phosphorylation state of Snf1 was analyzed using anti-phospho-Thr172-AMPK antibody (Cell

Signaling), which strongly recognizes the Thr210-phosphorylated form of yeast Snf1. Signals were detected by enhanced chemiluminescence using the Pierce ECL2 or ECL systems (Thermo Scientific).

Quantification of the immunoblots was done using ImageJ software (https://imagej.nih.gov/ij/). Briefly, immunoblot images were digitally scanned and signals were quantified using band volume and background subtraction methods (Stael *et al.*, 2022). Phospho-Thr210-Snf1 signals were normalized to total Snf1 levels that were detected by the anti-polyhistidine antibody. Normalized phospho-Thr210-Snf1 levels were averaged using three biological replicates per genotype. Statistical analysis was performed using a one-tailed t test with equal variances.

RESULTS AND DISCUSSION

In order to determine if Por1 and Por2 regulate Snf1 though Sak1, we used a genetic approach. We compared Snf1 catalytic activation in the *por1Δ por2Δ* double mutant and the *por1Δ por2Δ sak1Δ* triple mutant. We analyzed the levels of phospho-Thr210-Snf1 and then normalized them to the total amount of Snf1 detected by immunoblot (Figure 4A). There was no statistically significant difference in the levels of normalized phospho-Thr210-Snf1 between the *por1Δ por2Δ,* and *por1Δ por2Δ sak1Δ* genotypes (Figure 4B). The lack of such difference between these mutants suggests that Por1 and Por2 regulate phosphorylation of Snf1 through the Sak1 kinase. Had the mitochondrial porins regulated Snf1 though a pathway independent of Sak1, we would have expected the *por1Δ por2Δ sak1Δ* genotype to experience a cumulative defect in Snf1 catalytic activation.

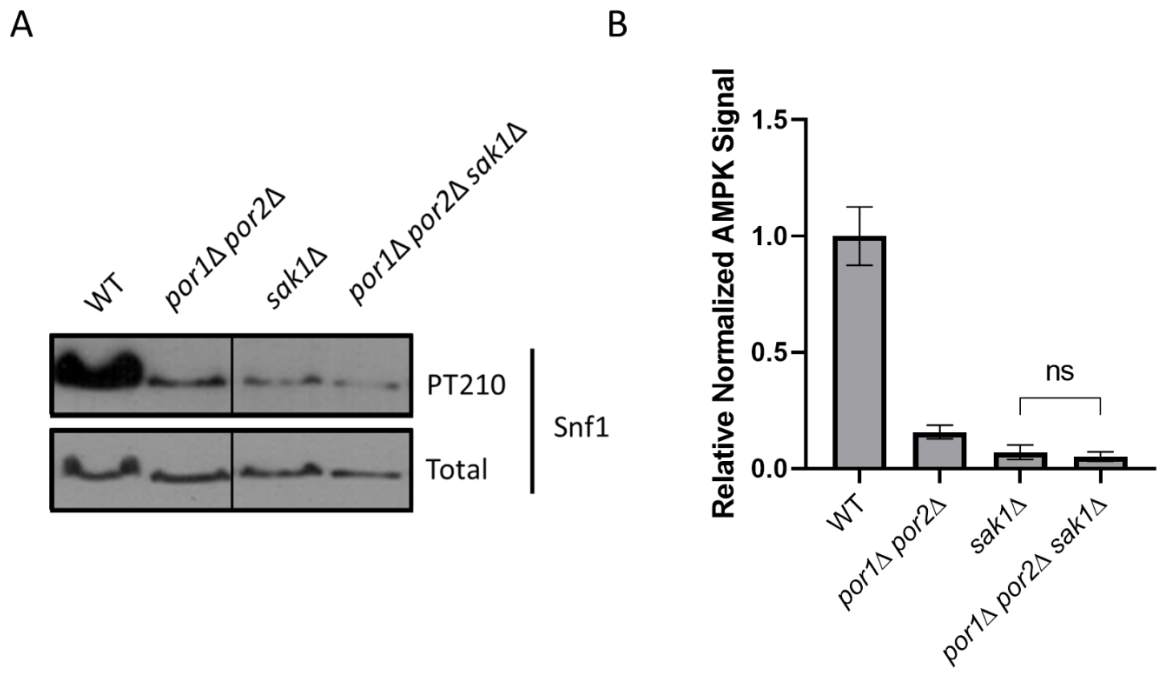

Figure 4. Por1 and Por2 promote catalytic activation of Snf1 through Sak1. (A) Strains were grown in YPD medium containing 2% glucose to mid-log phase and shifted to an otherwise identical medium containing 0.05% glucose for 1 hour. Protein extracts were tested for Thr210 phosphorylation (PT210) and total Snf1 via immunoblotting. (B) Immunoblots of n=3 biological replicates were analyzed and quantified using ImageJ. PT210 signals were normalized to total Snf1 values, and all values were quantified relative to the WT values. Error bars indicate standard errors. Statistical analysis was performed using a one-tailed t test with equal variances. ns, not significant ($P > 0.05$).

Mammalian AMPK is a well characterized protein. Like the Snf1 kinase complex, it responds to nutrient stress and provides homeostatic balance by down-regulating ATP-consuming processes, while up-regulating ATP-generating processes. It also responds directly to intracellular energy levels; as ATP levels decrease, the AMP-to-ATP levels increase and AMP binds to the γ subunit of AMPK (Hardie, 2007b). This results in a conformational change that promotes AMPK catalytic activation. In stark contrast, the Snf1 complex does not respond directly to intracellular ATP levels through its γ subunit.

Nonetheless, its activity still corresponds to decreased intracellular ATP levels. A current gap in knowledge is how Snf1 "senses" this ATP depletion.

Since mitochondrial porins are responsible for import of low energy molecules (AMP, ADP) and export of high energy molecules (ATP), they are poised to act as sensors of intracellular energy levels. Indeed, we have previously shown that Por1 and Por2 contribute to the catalytic activation of Snf1, suggesting their role as energy sensors for the Snf1 complex during nutrient stress (Strogolova *et al.*, 2012). Here, we show that this regulatory effect of porins in likely through the kinase Sak1. While porins are canonically understood to operate as channel proteins to facilitate transport of molecules across membranes, it is also possible that they act as regulatory proteins as well. Illustrating this is the fact that the homolog Por2 does not actually have any channel function (Forte, Guy & Mannella, 1987), and that only when both *POR1* and *POR2* are deleted from the genome is a Snf1 catalytic defect apparent (Strogolova *et al.*, 2012). This suggests that there are at least two distinct functions of these porins: 1) to mediate the transport of low and high energy nucleotides, and 2) to act as regulators of other pathways.

We therefore propose the following model for Snf1 phosphorylation: energy stress is sensed by the mitochondrial porins Por1 and Por2 which, in turn, promote the phosphorylation of Snf1 by the Sak1 kinase (Figure 5).

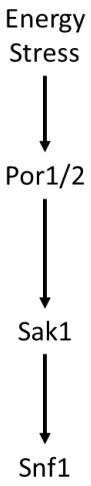

Figure 5. Proposed pathway of Snf1 activation. During glucose starvation, mitochondrial porins Por1 and Por2 promote Snf1 activation through Sak1.

Previous studies have highlighted the importance of Sak1 specifically. Not only is it responsible for the bulk of Snf1 activation, but Sak1, and not Tos3 or Elm1, promotes the nuclear translocation of the Snf1-Gal83 complex (Hedbacker *et al.*, 2004). Separately, studies have shown that the nonconserved C-terminus of Sak1 helps stabilize Snf1-Sak1 interaction and enhance Thr210 phosphorylation of Snf1 (Liu, Xu & Carlson, 2011). Moreover, Liu and colleagues showed that the C-terminus is modified, presumably phosphorylated, in a Snf1-dependent manner. It is possible that these results noted by Liu and colleagues are mediated by Por1 and Por2. Our proposed model purports that the Snf1-Sak1 interaction is mediated by Por1 and Por2, which western blot data support. It is possible, however, that this mediation enhances the Snf1-Sak1 interaction and promotes Thr210 phosphorylation by Sak1, which, in turn, causes Snf1-dependent phosphorylation of the C-terminus of Sak1. This then promotes the dissociation of Snf1-Sak1 and allows a catalytically active Snf1 to translocate to the nucleus. Indeed, this is an attractive possibility, as it would put a protein that transports energy nucleotides in close

proximity to both Snf1 and the Snf1-activating kinase, Sak1. However, further research is required in order to provide such mechanistic details.

Porins are evolutionarily conserved, and homologs exist in mammals. While the ability of mammalian AMPK to "sense" ATP levels is understood, there nonetheless remains the possibility that there are additional mechanisms to regulate AMPK outside of its intrinsic ability to bind AMP/ATP. The regulatory mechanisms we have elucidated have implications for other eukaryotes, such as humans. Moreover, studies have shown that pathogenesis of fungal species such as *Candida albicans* work in a Snf1-dependent manner. Understanding how the Snf1 kinase complex is regulated in *S. cerevisiae* may additionally lead to development of new antifungal therapeutics.

REFERENCES

CARLING, D., AGUAN, K., WOODS, A., VERHOEVEN, A. J., BERI, R. K., BRENNAN, C. H., SIDEBOTTOM, C., DAVISON, M. D. & SCOTT, J. (1994). Mammalian AMP-activated protein kinase is homologous to yeast and plant protein kinases involved in the regulation of carbon metabolism. *J Biol Chem* **269**(15), 11442-8.

CARLSON, M., OSMOND, B. C. & BOTSTEIN, D. (1981). Mutants of yeast defective in sucrose utilization. *Genetics* **98**(1), 25-40.

DAVIES, S. P., HELPS, N. R., COHEN, P. T. & HARDIE, D. G. (1995). 5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C alpha and native bovine protein phosphatase-2AC. *FEBS Lett* **377**(3), 421-5.

ESTRUCH, F., TREITEL, M. A., YANG, X. & CARLSON, M. (1992). N-terminal mutations modulate yeast SNF1 protein kinase function. *Genetics* **132**(3), 639-50.

FORTE, M., GUY, H. R. & MANNELLA, C. A. (1987). Molecular genetics of the VDAC ion channel: structural model and sequence analysis. *J Bioenerg Biomembr* **19**(4), 341-50.

HARDIE, D. G. (2007b). AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat Rev Mol Cell Biol* **8**(10), 774-85.

HARDIE, D. G. (2007c). AMPK and SNF1: Snuffing Out Stress. In *Cell Metab*. (Volume 6, pp. 339-40.

HARDIE, D. G. & CARLING, D. (1997). The AMP-activated protein kinase--fuel gauge of the mammalian cell? *Eur J Biochem* **246**(2), 259-73.

HARDIE, D. G., CARLING, D. & CARLSON, M. (1998). The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? *Annu Rev Biochem* **67**, 821-55.

HEDBACKER, K., HONG, S. P. & CARLSON, M. (2004). Pak1 protein kinase regulates activation and nuclear localization of Snf1-Gal83 protein kinase. *Mol Cell Biol* **24**(18), 8255-63.

HONG, S. P. & CARLSON, M. (2007). Regulation of snf1 protein kinase in response to environmental stress. *J Biol Chem* **282**(23), 16838-45.

HONG, S. P., LEIPER, F. C., WOODS, A., CARLING, D. & CARLSON, M. (2003b). Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. *Proc Natl Acad Sci U S A* **100**(15), 8839-43.

LIU, Y., XU, X. & CARLSON, M. (2011). Interaction of SNF1 Protein Kinase with Its Activating Kinase Sak1. *Eukaryotic Cell* **10**(3), 313-319.

MCCARTNEY, R. R., RUBENSTEIN, E. M. & SCHMIDT, M. C. (2005). Snf1 kinase complexes with different beta subunits display stress-dependent preferences for the three Snf1-activating kinases. *Curr Genet* **47**(6), 335-44.

MCCARTNEY, R. R. & SCHMIDT, M. C. (2001). Regulation of Snf1 kinase. Activation requires phosphorylation of threonine 210 by an upstream kinase as well as a distinct step mediated by the Snf4 subunit. *J Biol Chem* **276**(39), 36460-6.

NATH, N., MCCARTNEY, R. R. & SCHMIDT, M. C. (2003). Yeast Pak1 kinase associates with and activates Snf1. *Mol Cell Biol* **23**(11), 3909-17.

ORLOVA, M., BARRETT, L. & KUCHIN, S. (2008). Detection of endogenous Snf1 and its activation state: application to Saccharomyces and Candida species. *Yeast* **25**(10), 745-54.

ORLOVA, M., OZCETIN, H., BARRETT, L. & KUCHIN, S. (2010). Roles of the Snf1-activating kinases during nitrogen limitation and pseudohyphal differentiation in Saccharomyces cerevisiae. *Eukaryot Cell* **9**(1), 208-14.

SHEVADE, A., STROGOLOVA, V., ORLOVA, M., YEO, C. T. & KUCHIN, S. (2018). Mitochondrial Voltage-Dependent Anion Channel Protein Por1 Positively Regulates the Nuclear Localization of Saccharomyces cerevisiae AMP-Activated Protein Kinase. *mSphere* **3**(1).

STAEL, S., MILLER, L. P., FERNÁNDEZ-FERNÁNDEZ, Á. D. & VAN BREUSEGEM, F. (2022). Detection of Damage-Activated Metacaspase ActivityActivitiesby Western Blot in Plants. In *Plant Proteases and Plant Cell Death: Methods and Protocols*. (eds M. Klemenčič, S. Stael and P. F. Huesgen), pp. 127-137. Springer US, New York, NY.

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**(12), 1568-72.

SUTHERLAND, C. M., HAWLEY, S. A., MCCARTNEY, R. R., LEECH, A., STARK, M. J., SCHMIDT, M. C. & HARDIE, D. G. (2003). Elm1p is one of three upstream kinases for the Saccharomyces cerevisiae SNF1 complex. *Curr Biol* **13**(15), 1299-305.

CHAPTER 3

The N-terminal α helix domain of the yeast VDAC protein Por2 is dispensable for promoting the nuclear localization of the Snf1 protein kinase

This chapter will be submitted to *microPublication Biology* as a manuscript by Brown K.M., Perkins, F., and Kuchin, S. V. (2022).

ABSTRACT

In yeast, the mitochondrial voltage-dependent anion channel (VDAC) proteins Por1 and Por2 play regulatory roles upstream of Snf1, an ortholog of AMP-activated protein kinase (AMPK). An important question is whether Por1 and Por2 serve as Snf1-coupled energy sensors. VDACs are β-barrel proteins, but they have a flexible N-terminal α helix (NAH), suggesting a role in signaling. Here, we asked whether the NAH domain of Por2 is required for promoting Snf1 nuclear translocation. In our experimental setup, the Por2 NAH2 was dispensable. Further experiments are required to fully understand the regulatory roles of the Por1/2 NAH domains.

INTRODUCTION

The AMP-activated protein kinase (AMPK) is conserved in eukaryotes from yeast to humans (Hardie & Carling, 1997). As its name suggests, the key role of AMPK is to respond to reduction in energy levels. When energy levels drop, AMPK is activated to restore the energy balance by inhibiting ATP consumption and promoting ATP generation. AMPK exerts its regulatory effects on various levels, from transcriptional regulation to post-translational effects on protein function (Hardie, 2007b).

The AMPK ortholog of budding yeast (*Saccharomyces cerevisiae*) is called the Snf1 (sucrose-non-fermenting 1) kinase complex. This complex is required for the utilization of alternative carbon/energy sources when cells experience a shortage of the preferred source – glucose (Carlson *et al.*, 1981).

AMPK family members from organisms as diverse as yeast and humans share a heterotrimeric αβγ structure, where the α subunit is catalytic, and the β and γ subunits are regulatory (Hardie, 2007c). *S. cerevisiae* has a single α subunit (Snf1), a single γ subunit (Snf4), and three alternative β subunits (Sip1, Sip2, Gal83) (Jiang & Carlson, 1997). The three β subunits define three isoforms of the yeast Snf1 complex referred to as Snf1-Sip1, Snf1-Sip2, and Snf1-Gal83 (Hedbacker *et al.*, 2004). For reasons that will become clear below, here we focus on the Snf1-Gal83 isoform of the yeast Snf1 complex.

When yeast cells experience energy stress caused by glucose deprivation, two major events occur. First, the α subunit becomes catalytically activated by phosphorylation of the conserved threonine residue (Thr210) in the activation loop by upstream kinases (Hong *et al.*, 2003a; Sutherland *et al.*, 2003; Nath *et al.*, 2003). Second, the Snf1-Gal83 isoform translocates to the nucleus (Vincent *et al.*, 2001). Because Gal83 is normally the most abundant β subunit (Vincent *et al.*, 2001), Snf1 becomes effectively enriched in the nucleus (García-Salcedo *et al.*, 2014; Vincent *et al.*, 2001), putting it in a better position to regulate transcription (Young *et al.*, 2012).

The signaling mechanisms behind the Gal83-driven nuclear translocation of the Snf1-Gal83 complex are not completely understood. It has previously been shown that the yeast mitochondrial voltage-dependent anion channel (VDAC) proteins Por1 and Por2 play a regulatory role in this process (Shevade *et al.*, 2018), and that this role is separable from channel activity, further supporting a role in energy sensing, as proposed previously (Strogolova *et al.*, 2012).

VDAC proteins are highly conserved among eukaryotes and have an overall β-barrel structure (Forte *et al.*, 1987; Guardiani *et al.*, 2018). However, they also have a short flexible N-terminal α helix (NAH) implicated in interactions with energy-carrying nucleotides (Mannella, 1998; Rostovtseva & Colombini, 1997). Here, we have tested for a possible role of the NAH domain of Por2 in promoting Snf1-Gal83 nuclear localization in response to energy stress. We chose to focus on Por2 because unlike Por1, it is not believed to possess channel activity even when overexpressed (Blachly-Dyson *et al.*, 1997),

and therefore studying Por2 offers a valuable opportunity to distinguish between channel activity and a purely regulatory function. The predicted structure of Por2 is shown in Figure 6A.

Because the *POR2* gene is normally expressed at a markedly lower level than *POR1* (Blachly-Dyson *et al.*, 1997), the *por2*Δ mutation has little, if any, effect on Snf1-Gal83 nuclear localization (Shevade *et al.*, 2018). However, overexpression of Por2 effectively compensates for the Snf1-Gal83 nuclear enrichment defect caused by the *por1*Δ mutation (Shevade *et al.*, 2018). Therefore, in our experimental setup we compared the ability of full-size Por2 and its Por2ΔNAH derivative lacking amino acids 1-22 to suppress the *por1*Δ mutation. As done previously (Shevade *et al.*, 2018), Por2 and Por2ΔNAH were expressed with C-terminal V5 tags (Por2-V5 and Por2ΔNAH-V5, respectively) from the multicopy vector pSK71 (Shevade *et al.*, 2018).

To obtain quantitative data, we used the "shortcut" reporter assay (Kuchin, Treich & Carlson, 2000). In this assay, a hyperactive mutant version of Snf1 with a Gly53-to-Arg substitution (Snf1-G53R) is fused to the LexA protein and tested for the ability to activate the expression of a *lexAop-lacZ* reporter gene containing LexA DNA-binding sites (*lexAop*) upstream of *lacZ* (Kuchin *et al.*, 2000). Yeast strain CTY10-5d contains a *lexAop-lacZ* reporter integrated into its chromosome (R. Sternglanz, State University of New York, Stony Brook) and has been repeatedly used for this purpose (Kuchin *et al.*, 2000; Shevade *et al.*, 2018; Strogolova *et al.*, 2012; Kuchin *et al.*, 2003). Importantly, the ability of LexA-Snf1-G53R to activate the *lexAop-lacZ* reporter is absolutely dependent on Gal83-driven nuclear localization, making it a sensitive readout of Snf1-Gal83 nuclear translocation in response to carbon/energy stress (Vincent *et al.*, 2001).

We overexpressed the tagged Por2-V5 and Por2ΔNAH-V5 proteins in reporter strain CTY10-5d and its *por1*Δ mutant derivative (Shevade *et al.*, 2018), both expressing LexA-Snf1-G53R (Kuchin *et al.*, 2000). The cells were first grown in abundant (2%) glucose and then shifted to low (0.05%) glucose for 3h (Vyas, Kuchin & Carlson, 2001). Assays of β-galactosidase activity in the stressed cells revealed no

substantial difference between the ability of overexpressed Por2-V5 and PorΔNAH-V5 to suppress the *por1*Δ mutation (Figure 6B). The *por1*Δ mutant overexpressing full-size Por2 serves as an adequate control in the experiment presented here because we have repeatedly observed that overexpressing full-length Por2 in a *por1*Δ mutant restores activation by LexA-Snf1-G53R to levels that are not appreciably different from those for the WT + vector combination (Shevade *et al.*, 2018).

Immunoblot analysis indicated that the levels of Por2-V5 and Por2ΔNAH-V5 proteins were comparable (Figure 6C). The levels of LexA-Snf1-G53R expression and Thr210 phosphorylation (activation) of the Snf1-G53R moiety were comparable as well (Figure 6C). Thus, these results suggest that the NAH domain of Por2 is dispensable for its ability to promote the nuclear translocation of Snf1-Gal83 in response to carbon/energy stress.

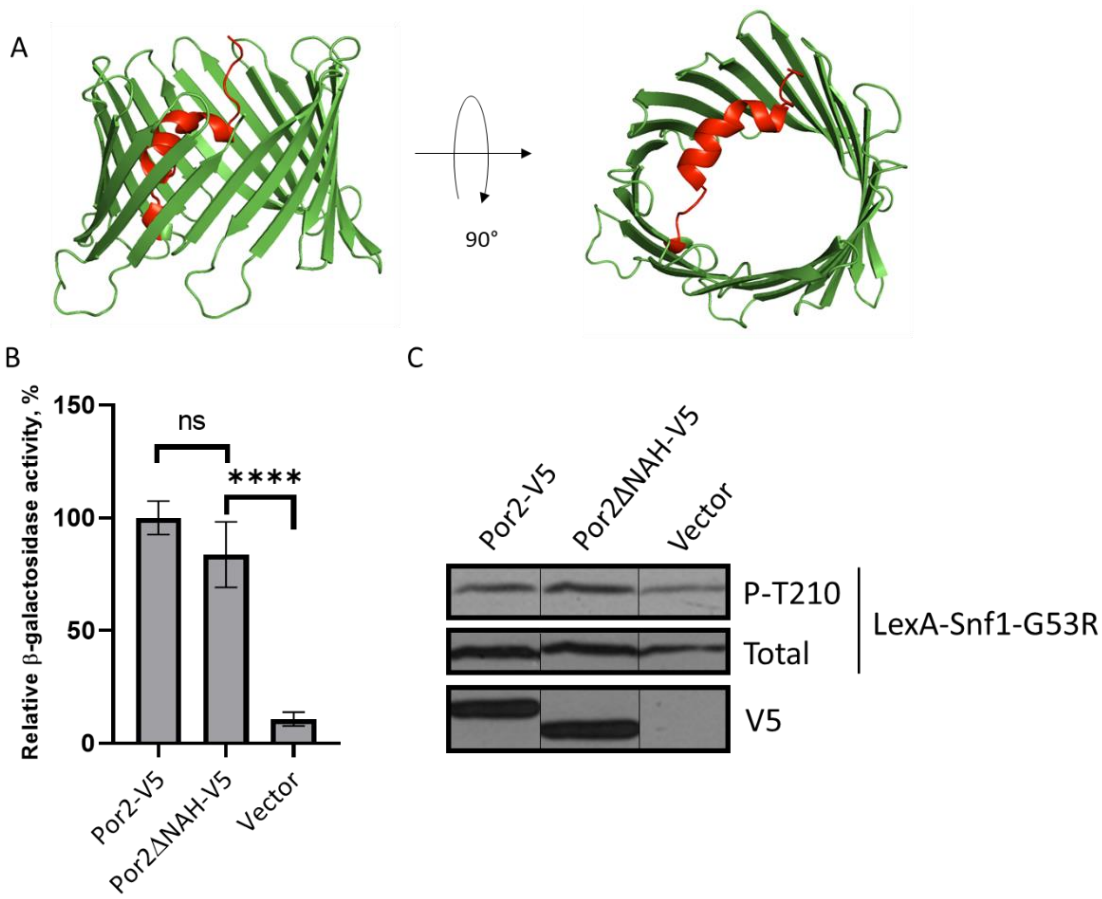

Figure 6. The NAH of Por2 does not affect Snf1 nuclear translocation. (A) Predicted structure of yeast VDAC Por2 (Uniprot P40478) using AlphaFold. The VDAC core is composed of antiparallel beta strands (colored green). The flexible N-terminal alpha helix is predicted to reside in the center (colored red). (B) Cells of the *por1*Δ mutant derivative of reporter strain CTY10-5d expressing LexA-Snf1-G53R and either overexpressing full-length Por2-V5, Por2NAH-V5, or carrying the corresponding empty vector, 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 (five independent transformants per genotype/plasmid combination). Shown are the values under low-glucose conditions expressed as a percentage of the mean value for cells overexpressing full length Por2-V5; in high glucose, all values were less than 0.5% of

this reference value. Error bars indicate standard errors. Statistical analyses were conducted using the two-tailed Student t test. ****, $P < 0.0001$. ns, not significant ($P > 0.05$).

In conclusion, we note that our results do not automatically mean that the NAH domain of Por2 plays no role at all. For example, its function might be redundant with that of other region(s) of this protein. In addition, our previous results indicate that besides Snf1-Gal83 nuclear localization, Por2 (together with Por1) plays a distinct role in the catalytic activation of Snf1 (Strogolova *et al.*, 2012), and its NAH domain could play a more prominent role in that process. Finally, our results with Por2 should not be automatically extrapolated to its paralog Por1 because paralogous proteins often exhibit a degree of functional specialization implying divergent specialization of their domains. Thus, further experiments are required to better understand the signaling roles of eukaryotic VDAC proteins, including the yeast Por1/2 proteins and their NAH domains.

MATERIALS AND METHODS

Yeast strains and growth conditions

The *por1*Δ mutant strain carrying an integrated *lexAop-lacZ* reporter has been described previously (Shevade *et al.*, 2018) and is a derivative of strain CTY10-5d (*MAT***a** *gal4 gal80 URA3::lexAop-lacZ his3 leu2 ade2 trp1*) (R. Sternglanz, SUNY, Stony Brook, NY) (Bartel *et al.*, 1993). Synthetic complete (SC) medium lacking appropriate supplements was used to select for plasmids (Rose MD, 1990b). All transformations were done using standard methods (Rose MD, 1990b). Unless indicated otherwise, the media contained 2% glucose, and cells were grown at 30°C.

Plasmids

Plasmid pRJ216 (Kuchin *et al.*, 2000) expresses LexA-Snf1-G53R from the yeast *ADH1* promoter of multicopy vector pEG202 (Golemis, Serebriiskii & Law, 1999). The multicopy vector pSK71 provides expression from the yeast *ADH1* promoter and was constructed by removing the LexA coding sequence from pBTM116 (Fields & Song, 1989). pAMS10 expresses a full-size C-terminal V5 epitope-tagged Por2 from vector pSK71 (Shevade *et al.*, 2018). pFP1 expresses a C-terminal V5 epitope-tagged Por2ΔNAH from vector pSK71 and was constructed as follows. Primers with flanking BamHI sites targeting the *POR2* coding region containing base pairs 69-846 were used to amplify *POR2*Δ*NAH* by PCR from yeast genomic DNA. The resulting PCR fragment was digested with BamHI and inserted into the BamHI site of pSK71.

Assays of *lexAop-lacZ* reporter activation by LexA-Snf1-G53R

Cells of strains carrying the integrated *lexAop-lacZ* reporter and expressing LexA-Snf1-G53R were grown in appropriate selective SC medium containing high (2%) glucose to mid-log phase and then shifted for 3 hours to an otherwise identical medium containing low (0.05%) glucose. Assays of β-galactosidase activity were performed in permeabilized cells and measured in Miller units as described previously (Kuchin *et al.*, 2000).

Immunoblot analysis

Cells were grown under conditions specified in the descriptions of experimental results. Protein extracts were prepared by the boiling/alkaline treatment method as described previously (Orlova *et al.*, 2008) and analyzed by immunoblotting. The LexA-Snf1-G53R fusion protein was detected with anti-LexA antibody (Millipore). The Thr210 phosphorylation state of LexA-Snf1-G53R was analyzed using anti-phospho-Thr172-AMPK antibody (Cell Signaling), which strongly recognizes the Thr210-phosphorylated form of yeast Snf1 (Orlova *et al.*, 2008). Signals were detected by enhanced chemiluminescence using the Pierce ECL2 or ECL systems (Thermo Scientific).

Predicted structure of Por2

Amino acid sequence of Por2 was obtained from Uniprot (https://www.uniprot.org/uniprotkb/P04840).

The predicted structure was generated using AlphaFold (https://alphafold.ebi.ac.uk/) (Jumper *et al.*, 2021; Varadi *et al.*, 2021).

REFERENCES

BARTEL, P., CHIEN, C. T., STERNGLANZ, R. & FIELDS, S. (1993). Elimination of false positives that arise in using the two-hybrid system. *Biotechniques* **14**(6), 920-4.

BLACHLY-DYSON, E., SONG, J., WOLFGANG, W. J., COLOMBINI, M. & FORTE, M. (1997). Multicopy suppressors of phenotypes resulting from the absence of yeast VDAC encode a VDAC-like protein. *Mol Cell Biol* **17**(10), 5727-38.

CARLSON, M., OSMOND, B. C. & BOTSTEIN, D. (1981). Mutants of yeast defective in sucrose utilization. *Genetics* **98**(1), 25-40.

FIELDS, S. & SONG, O. (1989). A novel genetic system to detect protein-protein interactions. *Nature* **340**(6230), 245-6.

FORTE, M., GUY, H. R. & MANNELLA, C. A. (1987). Molecular genetics of the VDAC ion channel: structural model and sequence analysis. *J Bioenerg Biomembr* **19**(4), 341-50.

GARCÍA-SALCEDO, R., LUBITZ, T., BELTRAN, G., ELBING, K., TIAN, Y., FREY, S., WOLKENHAUER, O., KRANTZ, M., KLIPP, E. & HOHMANN, S. (2014). Glucose de-repression by yeast AMP-activated protein kinase SNF1 is controlled via at least two independent steps. *FEBS J* **281**(7), 1901-17.

GOLEMIS, E., SEREBRIISKII, I. & LAW, S. (1999). The Yeast Two-hybrid System: Criteria for Detecting Physiologically Significant Protein-Protein Interactions. *Current issues in molecular biology* **1**, 31-45.

GUARDIANI, C., MAGRÌ, A., KARACHITOS, A., DI ROSA, M. C., REINA, S., BODRENKO, I., MESSINA, A., KMITA, H., CECCARELLI, M. & DE PINTO, V. (2018). yVDAC2, the second mitochondrial porin isoform of Saccharomyces cerevisiae. *Biochim Biophys Acta Bioenerg* **1859**(4), 270-279.

HARDIE, D. G. (2007b). AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat Rev Mol Cell Biol* **8**(10), 774-85.

HARDIE, D. G. (2007c). AMPK and SNF1: Snuffing Out Stress. In *Cell Metab*. (Volume 6, pp. 339-40.

HARDIE, D. G. & CARLING, D. (1997). The AMP-activated protein kinase--fuel gauge of the mammalian cell? *Eur J Biochem* **246**(2), 259-73.

HEDBACKER, K., HONG, S. P. & CARLSON, M. (2004). Pak1 protein kinase regulates activation and nuclear localization of Snf1-Gal83 protein kinase. *Mol Cell Biol* **24**(18), 8255-63.

HONG, S.-P., LEIPER, F. C., WOODS, A., CARLING, D. & CARLSON, M. (2003a). Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. *Proceedings of the National Academy of Sciences* **100**(15), 8839-8843.

JIANG, R. & CARLSON, M. (1997). The Snf1 protein kinase and its activating subunit, Snf4, interact with distinct domains of the Sip1/Sip2/Gal83 component in the kinase complex. *Mol Cell Biol* **17**(4), 2099-106.

JUMPER, J., EVANS, R., PRITZEL, A., GREEN, T., FIGURNOV, M., RONNEBERGER, O., TUNYASUVUNAKOOL, K., BATES, R., ŽÍDEK, A., POTAPENKO, A., BRIDGLAND, A., MEYER, C., KOHL, S. A. A., BALLARD, A. J., COWIE, A., ROMERA-PAREDES, B., NIKOLOV, S., JAIN, R., ADLER, J., BACK, T., PETERSEN, S., REIMAN, D., CLANCY, E., ZIELINSKI, M., STEINEGGER, M., PACHOLSKA, M., BERGHAMMER, T., BODENSTEIN, S., SILVER, D., VINYALS, O., SENIOR, A. W., KAVUKCUOGLU, K., KOHLI, P. & HASSABIS, D. (2021). Highly accurate protein structure prediction with AlphaFold. *Nature* **596**(7873), 583-589.

KUCHIN, S., TREICH, I. & CARLSON, M. (2000). A regulatory shortcut between the Snf1 protein kinase and RNA polymerase II holoenzyme. *Proc Natl Acad Sci U S A* **97**(14), 7916-20.

KUCHIN, S., VYAS, V. K., KANTER, E., HONG, S. P. & CARLSON, M. (2003). Std1p (Msn3p) positively regulates the Snf1 kinase in Saccharomyces cerevisiae. *Genetics* **163**(2), 507-14.

MANNELLA, C. A. (1998). Conformational changes in the mitochondrial channel protein, VDAC, and their functional implications. *J Struct Biol* **121**(2), 207-18.

NATH, N., MCCARTNEY, R. R. & SCHMIDT, M. C. (2003). Yeast Pak1 kinase associates with and activates Snf1. *Mol Cell Biol* **23**(11), 3909-17.

ORLOVA, M., BARRETT, L. & KUCHIN, S. (2008). Detection of endogenous Snf1 and its activation state: application to Saccharomyces and Candida species. *Yeast* **25**(10), 745-54.

ROSE MD, W. F., HIETER P. (1990b). *Methods in yeast genetics A laboratory course manual*, Cold Spring Harbor, NY.

ROSTOVTSEVA, T. & COLOMBINI, M. (1997). VDAC channels mediate and gate the flow of ATP: implications for the regulation of mitochondrial function. *Biophys J* **72**(5), 1954-62.

SHEVADE, A., STROGOLOVA, V., ORLOVA, M., YEO, C. T. & KUCHIN, S. (2018). Mitochondrial Voltage-Dependent Anion Channel Protein Por1 Positively Regulates the Nuclear Localization of Saccharomyces cerevisiae AMP-Activated Protein Kinase. *mSphere* **3**(1).

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**(12), 1568-72.

SUTHERLAND, C. M., HAWLEY, S. A., MCCARTNEY, R. R., LEECH, A., STARK, M. J., SCHMIDT, M. C. & HARDIE, D. G. (2003). Elm1p is one of three upstream kinases for the Saccharomyces cerevisiae SNF1 complex. *Curr Biol* **13**(15), 1299-305.

VARADI, M., ANYANGO, S., DESHPANDE, M., NAIR, S., NATASSIA, C., YORDANOVA, G., YUAN, D., STROE, O., WOOD, G., LAYDON, A., ŽÍDEK, A., GREEN, T., TUNYASUVUNAKOOL, K., PETERSEN, S., JUMPER, J., CLANCY, E., GREEN, R., VORA, A., LUTFI, M., FIGURNOV, M., COWIE, A., HOBBS, N., KOHLI, P., KLEYWEGT, G., BIRNEY, E., HASSABIS, D. & VELANKAR, S. (2021). AlphaFold Protein Structure Database: massively expanding the structural coverage of protein-sequence space with high-accuracy models. *Nucleic Acids Research* **50**(D1), D439-D444.

VINCENT, O., TOWNLEY, R., KUCHIN, S. & CARLSON, M. (2001). Subcellular localization of the Snf1 kinase is regulated by specific beta subunits and a novel glucose signaling mechanism. *Genes Dev* **15**(9), 1104-14.

VYAS, V. K., KUCHIN, S. & CARLSON, M. (2001). Interaction of the repressors Nrg1 and Nrg2 with the Snf1 protein kinase in Saccharomyces cerevisiae. *Genetics* **158**(2), 563-72.

YOUNG, E. T., ZHANG, C., SHOKAT, K. M., PARUA, P. K. & BRAUN, K. A. (2012). The AMP-activated protein kinase Snf1 regulates transcription factor binding, RNA polymerase II activity, and mRNA stability of glucose-repressed genes in Saccharomyces cerevisiae. *J Biol Chem* **287**(34), 29021-34.

CHAPTER 4

Respiratory deficiency affects the Snf1 signaling pathway

ABSTRACT

Snf1/AMPK is a highly conserved eukaryotic protein kinase family whose members are present in animals, plants, and fungi. Members of this family are often referred to as the "fuel gauge" of the cell, and respond to energy stress by regulating gene expression and metabolic enzyme activity to maintain energy homeostasis. Under conditions of carbon/energy stress, such as during glucose limitation, yeast Snf1 is catalytically activated and enriched in the nucleus to regulate transcription. Snf1 catalytic activation requires phosphorylation of its conserved activation loop threonine (Thr210) by upstream kinases. Catalytic activation is also required for Snf1's subsequent nuclear enrichment, a process that is mediated by Gal83, one of three alternate β subunits of the Snf1 kinase complex. It has been previously reported that the mitochondrial voltage-dependent anion channel (VDAC) Por1 promotes Snf1 nuclear localization by promoting the nuclear localization of Gal83. Here, we present evidence that *rho*^{0} cells lacking mitochondrial DNA (mtDNA) show a defect in Snf1 nuclear localization that is distinct from the defect caused by the lack of Por1. *rho*^{0} cells show nucleocytoplasmic redistribution of the Snf1 complex that is due to overexpression of the cytoplasmic β subunit Sip2 through the retrograde (Rtg) signaling pathway. In addition, respiratory dysfunction affects the Snf1 kinase complex by negatively regulating the nuclear-targeting β subunit Gal83. We suggest that the biological significance of this novel functional interaction is twofold. First, by reducing the presence and function of the Snf1-Gal83 complex in the nucleus, *rho*^{0} cells minimize unnecessary upregulation of Snf1-dependent genes required for aerobic respiration. Second, since mitochondrial dysfunction is associated with replicative aging, upregulation of the anti-aging Snf1-Sip2 complex in *rho*^{0} cells counteracts premature replicative senescence.

INTRODUCTION

Mammalian AMP-activated protein kinase (AMPK) belongs to a highly conserved protein kinase family present in eukaryotes, including plants and fungi (Hardie & Carling, 1997). AMPK is often referred to as the "fuel gauge" of the cell, as it responds to cellular energy stress by sensing increased levels of AMP relative to ATP (Hardie, 2007b; Hardie, 2007c). When activated, AMPK regulates gene expression and metabolic activity to maintain energy homeostasis. For example, important functions of AMPK include activation of energy-generating processes, like glucose uptake and fatty acid oxidation, as well as inhibition of energy-consuming processes, like cell growth and proliferation (Carling, Zammit & Hardie, 1987; Hardie & Pan, 2002; Hardie, 2007c; Hardie, 2007b; Hedbacker & Carlson, 2008). Simply put, AMPK works to limit energy expenditure while maximizing energy production.

Mammalian AMPK is a heterotrimeric complex composed of three subunits: the catalytic α subunit, and regulatory β and γ subunits (Davies *et al.*, 1994). When energy levels are high, ATP binds to the γ subunit and AMPK remains in its inactive form. However, as energy levels decrease, the AMP:ATP ratio increases and AMP, rather than ATP, will bind to the γ subunit. When AMP is bound to AMPK, a conformational change is induced and AMPK becomes catalytically activated through phosphorylation of a conserved threonine residue (Thr172) in the activation loop of the α subunit by upstream kinases (Hardie & Carling, 1997). Additionally, AMP-bound AMPK is protected from dephosphorylation and inactivation by phosphatases (Davies *et al.*, 1995). This catalytically active AMPK is the form responsible for maintaining energy homeostasis.

Given the role AMPK plays in cellular energy balance, it is no surprise that defects in AMPK signaling are associated with human diseases such as diabetes, obesity, and cancer (Fogarty & Hardie, 2010; Hardie, 2007a). As such, AMPK is a target of various therapeutic approaches. For example, the FDA approved drug for treating type II diabetes, metformin, is thought to act through AMPK (Rena *et al.*, 2013). Additionally, AMPK has been implicated in aging (Li *et al.*, 2015; Ruiz *et al.*, 2016).

The AMPK ortholog in the common yeast *Saccharomyces cerevisiae* is the Snf1 (sucrose non-fermenting 1) kinase complex and is likewise essential for responses to glucose limitation and other cellular stresses (for review see (Hedbacker & Carlson, 2008)). The regulation of this complex is similar to mammalian AMPK in that the catalytic activation of Snf1 is achieved by phosphorylation of a conserved threonine (Thr210) by upstream kinases (Sak1, Tos3, and Elm1) (Hong *et al.*, 2003b). Like mammalian AMPK, the Snf1 kinase complex is also a heterotrimer composed of the catalytic α subunit (Snf1 itself), a scaffolding/targeting β subunit (Sip1, Sip2, or Gal83), and a stimulatory γ subunit (Snf4). There are three distinct Snf1 complexes based on the alternate β subunit present in the Snf1 kinase complex; these complexes are designated Snf1-Sip1, Snf1-Sip2, and Snf1-Gal83. Under glucose-rich conditions, all three complexes are cytoplasmic. Under energy/carbon stress conditions, however, these three complexes are directed to different subcellular locations: Snf1-Sip1 localizes to the vacuolar membrane, Snf1-Sip2 remains cytoplasmic, and Snf1-Gal83 localizes to the nucleus; this differential localization is due to the N-terminal regions of the β subunits (Vincent *et al.*, 2001).

The three alternate Snf1 complexes have related and distinct functions. For example, Snf1-Gal83 is poised to regulate transcription. In addition, the Snf1-Sip2 complex promotes replicative longevity by downregulating the S6 kinase Sch9 (Lu *et al.*, 2011; Ashrafi *et al.*, 2000). These functions of the Snf1 complex are central to this chapter.

The mechanisms that regulate Snf1 subcellular localization are not completely understood. A recent study of Snf1 showed that the mitochondrial voltage-dependent anion channel (VDAC) protein Por1 (also known as mitochondrial porin) affects Snf1 subcellular localization, as cells lacking Por1 fail to enrich Gal83 in the nucleus (Shevade *et al.*, 2018). Interestingly, overexpression of the Por1 paralog Por2 completely fixes this defect in *por1*Δ cells. Since Por2 is thought to have no channel function (Blachly-Dyson *et al.*, 1997), these results suggested that the defect exhibited by the *por1*Δ mutant is unrelated to mitochondrial respiration. However, this conclusion required additional verification. Specifically, it

was necessary to investigate the effects of well-defined respiratory deficiency *per se* upon the Snf1 pathway. Here, we present evidence that respiratory null cells (cells lacking the mitochondrial genome, *rho*^{0}) lack the ability to properly localize Snf1 to the nucleus during carbon stress conditions. However, this effect in *rho*^{0} cells is mechanistically unrelated to the effect caused by the *por1*Δ mutation. While the *por1*Δ mutation affects Gal83 nuclear localization (Shevade *et al.*, 2018), here we show that the *rho*^{0} status does not affect Gal83 localization. Instead, we present evidence that the *rho*^{0} status uncouples Snf1 from Gal83 by causing Sip2 overexpression via the retrograde (Rtg) pathway. Our results extend the repertoire of methods by which mitochondria regulate Snf1 signaling.

RESULTS

Lack of the mitochondrial genome (*rho*^{0}) strongly affects transcription activation by LexA-Snf1-G53R.

To better understand the effect the *rho*^{0} status has on the Snf1 kinase complex regulation, we first used the "shortcut" reporter assay (Kuchin *et al.*, 2000). This assay tests for the ability of a hyperactive form of Snf1 with a Gly53-to-Arg mutation (Snf1-G53R) fused to the bacterial LexA DNA-binding protein (LexA-Snf1-G53R fusion) to activate transcription of a reporter containing LexA operator sequences (*lexAop*) upstream of the *lacZ* gene (*lexAop-lacZ* reporter) (Figure 7A). Activation of the *lexAop-lacZ* reporter by LexA-Snf1-G53R does not depend on any gene-specific transcription factors; instead, LexA-Snf1-G53R acts directly on the RNA polymerase II holoenzyme (Kuchin *et al.*, 2000). Under glucose-rich conditions, the activation of this reporter by LexA-Snf1-G53R is negligible. Upon shifting the cells to glucose-limiting conditions, however, reporter activation increases dramatically in a manner that is dependent on both the catalytic activation of the α subunit Snf1 and Gal83-dependent nuclear localization of the Snf1 heterotrimeric complex (Vincent *et al.*, 2001; Kuchin *et al.*, 2000). As a straightforward glucose-regulatable system, this assay has been a go-to initial method for detecting effects on Snf1 signaling (Vincent *et al.*, 2001; Kuchin *et al.*, 2000; Hedbacker *et al.*, 2004).

We expressed LexA-Snf1-G53R in the wild type (WT, W303 genetic background) and an isogenic *rho*^{0} mutant lacking the mitochondrial genome, both of which carry a *lexAop-lacZ* reporter integrated at the chromosomal *URA3* locus. Cells were grown with plasmid selection to mid-log phase in a high glucose (2%) medium and then shifted to a low glucose (0.05%) medium for 3h to induce reporter activation, as described previously (Kuchin *et al.*, 2000). As expected (Shevade, 2017), respiratory null *rho*^{0} cells showed a strong defect in transcription activation (Figure 7B). This defect could not be attributed to a reduction in Thr210 phosphorylation (activation) of the LexA-Snf1-G53R fusion (Figure 7C), and thus suggests that the defect in reporter activation is due to a defect in Snf1 nuclear localization.

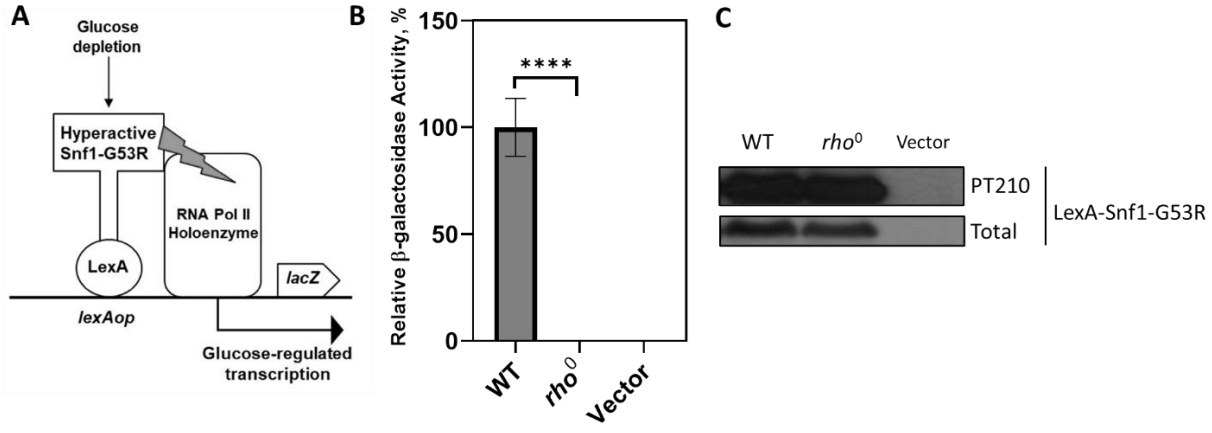

Figure 7. Lack of the mitochondrial genome (*rho*^{0}) strongly affects transcription activation by LexA-Snf1-G53R. (A) Schematic of the "shortcut" assay. The LexA-Snf1-G53R fusion, when activated by the low glucose signal, translocates to the nucleus, binds to *lexAop*, and strongly stimulates reporter transcription [adapted from (Kuchin *et al.*, 2000)]. (B) W303 (WT) and its *rho*^{0} derivative expressing LexA-Snf1-G53R or carrying the empty vector pBTM116 (vector) were grown in selective SC medium containing high glucose (2%) to mid-log phase and then shifted for 3 hours to an otherwise identical medium containing low glucose (0.05%). β-galactosidase activity was assayed in permeabilized cells and measured in Miller units (n=5 independent transformants per genotype). The graph shows data for low-

glucose conditions, which are expressed as a percentage of the WT value. Error bars indicate standard errors. Statistical analysis was conducted using a two-tailed t test. ****, $P < 0.0001$. (C) Transformants were shifted to 0.05% glucose as described for panel B and tested for Thr210 phosphorylation of the Snf1-G53R moiety (PT210) and LexA-Snf1-G53R protein levels (Total) by immunoblotting.

The *rho*^{0} status affects Snf1 nuclear translocation.

To test the effect of the *rho*^{0} status on Snf1 nuclear translocation, a low-copy centromeric plasmid expressing Snf1 fused to green fluorescent protein (Snf1-GFP) expressed from the native *SNF1* promoter was utilized. WT and *rho*^{0} cells were grown with plasmid selection in a high glucose (2%) medium, and Snf1 nuclear translocation was induced by shifting the cells to a medium containing a mixture of 3% ethanol and 2% glycerol (EG) as the sole carbon source for 20 minutes, which is the strongest Snf1 nuclear localization-inducing condition (Vincent *et al.*, 2001). As expected, Snf1-GFP was excluded from the nucleus of both WT and *rho*^{0} cells grown in high glucose (Figure 8A and B, top panels). Upon shifting the cells to EG-containing medium, Snf1-GFP nuclear enrichment was observed in WT cells (Figure 8A, bottom panels). However, Snf1-GFP failed to enrich in the nucleus in *rho*^{0} cells (Figure 8B, bottom panels). This defect could not be attributed to a reduced T210 phosphorylation of Snf1-GFP (Figure 8C). These data, taken together with the above reporter activation results, strongly suggest that the *rho*^{0} status affects Snf1 nuclear localization, and it does so by a mechanism that is independent of Snf1 catalytic activation.

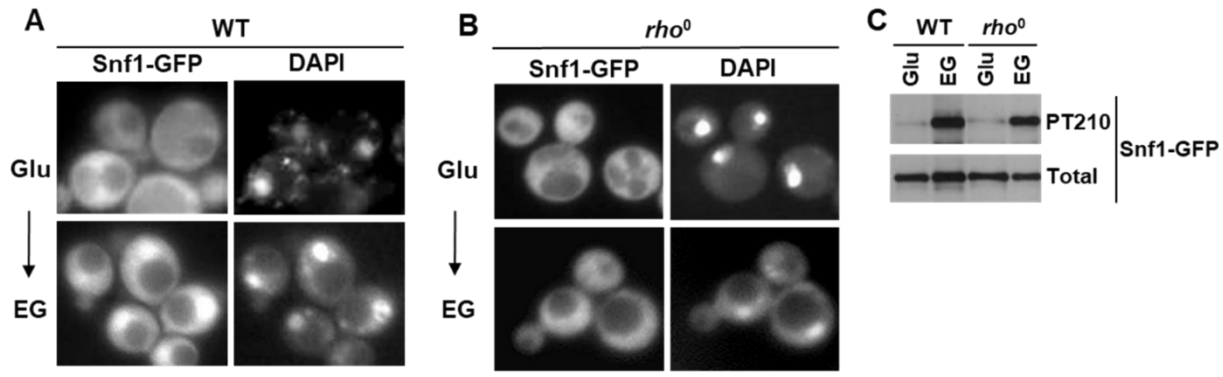

Figure 8. The *rho*^{0} status affects Snf1 nuclear translocation. (A and B) Localization of Snf1-GFP was determined by fluorescence microscopy after growth to mid-log phase in selective SC medium containing 2% glucose (Glu) and after a 20 min shift to an otherwise identical medium containing 3% ethanol and 2% glycerol (EG) instead of glucose. Nuclei were stained with DAPI. (C) Cells were grown as described above, and the Thr210 phosphorylation state (PT210) and total levels of Snf1-GFP (Total) were analyzed by immunoblotting. WT, wild-type. Images courtesy of Aishwarya Shevade (Shevade, 2017).

The *rho*^{0} status does not affect Gal83 nuclear enrichment.

Nuclear localization of Snf1 is dependent on Gal83, one of three alternate β subunit that compose the Snf1 kinase complex (Kuchin *et al.*, 2000; Vincent *et al.*, 2001). We therefore examined the nuclear enrichment of Gal83-GFP in WT and *rho*^{0} cells. Like the Snf1-GFP construct, Gal83-GFP was expressed from the native *GAL83* promoter on a low-copy centromeric plasmid (Vincent *et al.*, 2001). As expected, WT cells showed Gal83-GFP nuclear exclusion in high glucose and Gal83-GFP nuclear enrichment upon shifting cells to EG medium (Figure 9A). In *rho*^{0} cells, Gal83-GFP was excluded from the nucleus in high glucose-grown cells (Figure 9B, top panels). Surprisingly, and completely in contrast to the results for Snf1-GFP (Figure 8), Gal83-GFP became enriched in the nucleus in EG medium (Figure 9B, bottom panels). No appreciable difference was observed in either Gal83-GFP abundance or in Snf1 T210 phosphorylation (Figure 9C). These data, taken together with the observation that Snf1-GFP shows a

nuclear localization defect in *rho*^{0} cells, indicate that under energy/carbon stress conditions, Gal83 is migrating to the nucleus without Snf1, suggesting that Snf1 and Gal83 are uncoupled from each other.

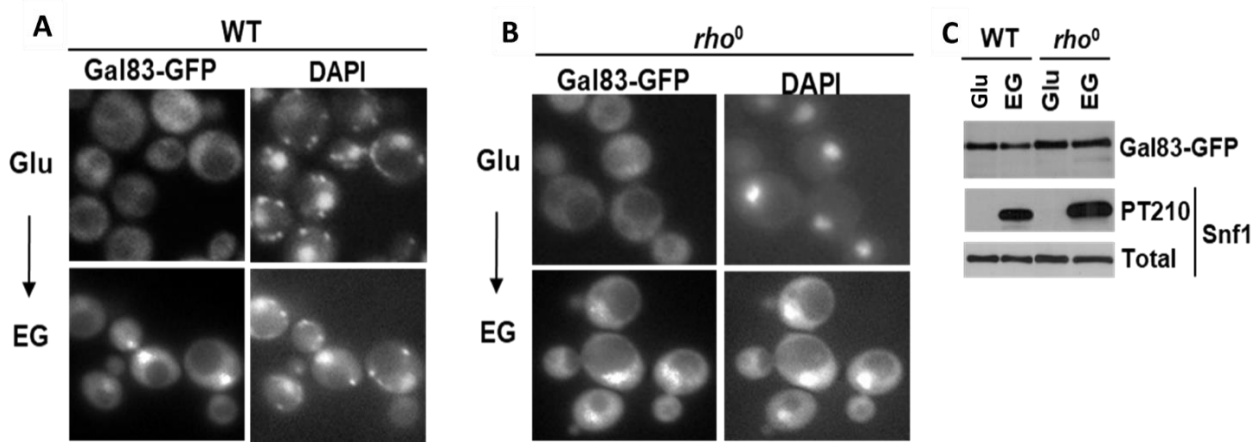

Figure 9. Gal83-GFP nuclear enrichment is unaffected in *rho*^{0} cells. (A and B) Localization of Gal83-GFP was determined by fluorescence microscopy after growth to mid-log phase in selective SC medium containing 2% glucose (Glu) and after a 20 min shift to an otherwise identical medium containing 3% ethanol and 2% glycerol (EG) instead of glucose. Nuclei were stained with DAPI. (C) Cells were grown as described above, and Gal83-GFP levels were detected by immunoblotting. Thr210 phosphorylation state (PT210) and total levels of endogenous Snf1 (Total) were also analyzed by immunoblotting. Images courtesy of Aishwarya Shevade (Shevade, 2017).

Deletion of *SIP2,* but not *SIP1*, in *rho*^{0} cells restores Snf1-GFP nuclear enrichment.

There are three possible β subunits that can be part of the Snf1 kinase complex, each providing a different subcellular localization during carbon/energy stress: Snf1-Gal83 localizes to the nucleus, Snf1-Sip1 localizes to the vacuolar membrane, and Snf1-Sip2 remains cytoplasmic. We hypothesized that the uncoupling of Snf1 from Gal83 in *rho*^{0} cells was due to Snf1's increased association with either Sip1 or Sip2. To test this hypothesis, Snf1-GFP localization was examined in *rho*^{0} *sip1*Δ and *rho*^{0} *sip2*Δ cells. As expected, Snf1-GFP remained excluded from the nucleus in both strains when grown in high glucose (Figure 10A and B, top panels). In *rho*^{0} *sip1*Δ cells, Snf1-GFP failed to enrich in the nucleus when cells

were shifted to EG medium (Figure 10A, bottom panels). However, Snf1-GFP nuclear localization was restored in *rho*^{0} *sip2*Δ upon the shift to EG medium. This suggested that in *rho*^{0} cells, Snf1 primarily associates with Sip2, and this association may be responsible for the uncoupling of Snf1 from Gal83.

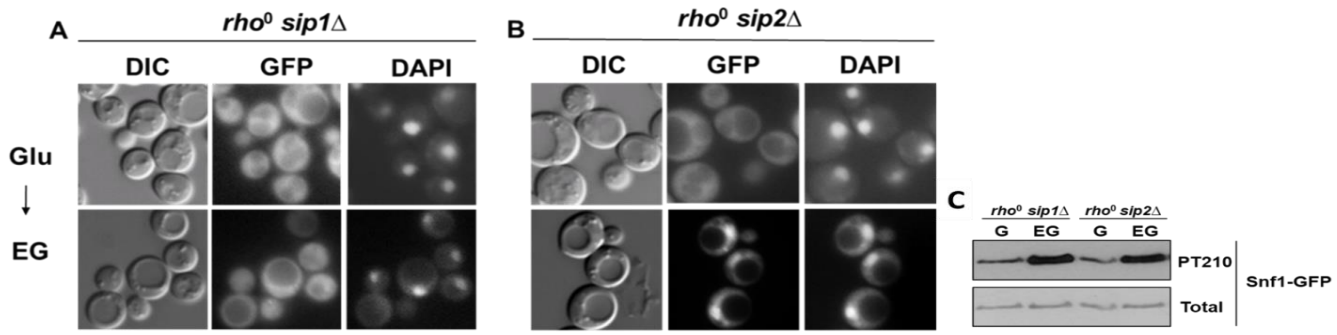

Figure 10. Deletion of *SIP2* restores Snf1 nuclear localization in *rho*^{0} cells. (A and B) Localization of Snf1-GFP was determined by fluorescence microscopy after growth to mid-log phase in selective SC medium containing 2% glucose (Glu) and after a 20 min shift to an otherwise identical medium containing 3% ethanol and 2% glycerol (EG) instead of glucose. The cells were imaged by differential interference contrast (DIC) microscopy. Nuclei were stained with DAPI. (C) Cells were grown as described above, and the Thr210 phosphorylation state (PT210) and total levels of Snf1-GFP (Total) were analyzed by immunoblotting in high (2%) glucose (G) and 3% ethanol plus 2% glycerol (EG). Images courtesy of Aishwarya Shevade (Shevade, 2017).

Sip2 is overexpressed in *rho*^{0} cells, and artificial overexpression of Sip2 recapitulates the effect of the *rho*^{0} status on transcription activation by LexA-Snf1-G53R.

Deletion of *SIP2* in *rho*^{0} cells restores Snf1-GFP nuclear localization, suggesting that Sip2 may be upregulated in *rho*^{0} cells. To address this possibility, we used a Sip2-GFP fusion expressed from the native *SIP2* promoter on a low-copy centromeric plasmid. Sip2-GFP protein levels were analyzed by immunoblotting with anti-GFP antibodies. WT and *rho*^{0} cells were grown to mid-log phase in high

glucose medium and shifted to EG medium. We observed that Sip2-GFP protein levels were higher in *rho*^{0} cells under both conditions (Figure 11A).

We next asked whether artificial Sip2 overexpression can similarly affect the ability of LexA-Snf1-G53R to activate transcription. To this end, we expressed triple HA-tagged Sip2 (HA_3-Sip2) from the strong *ADH1* promoter on a multi-copy plasmid in WT cells also expressing the LexA-Snf1-G53R fusion and containing chromosomally integrated *lexAop-LacZ* reporter. As expected (Vincent *et al.*, 2001), overexpressing Sip2 in this manner dramatically decreased the activation of the *lexAop-lacZ* reporter by LexA-Snf1-G53R in WT cells after shifting them to low glucose medium (Figure 11C). No appreciable difference in catalytically active LexA-Snf1-G53R or total LexA-Snf1-G53R abundance was observed (Figure 11D). These data indicate that not only is Sip2 highly expressed in *rho*^{0} cells, but overexpressing it in respiratory-capable *rho*$^+$ cells produces the same defect as the *rho*^{0} status.

Collectively, our results strongly suggest that Snf1 becomes uncoupled from Gal83 in *rho*^{0} cells because of its increased interaction with Sip2 due to Sip2 overexpression.

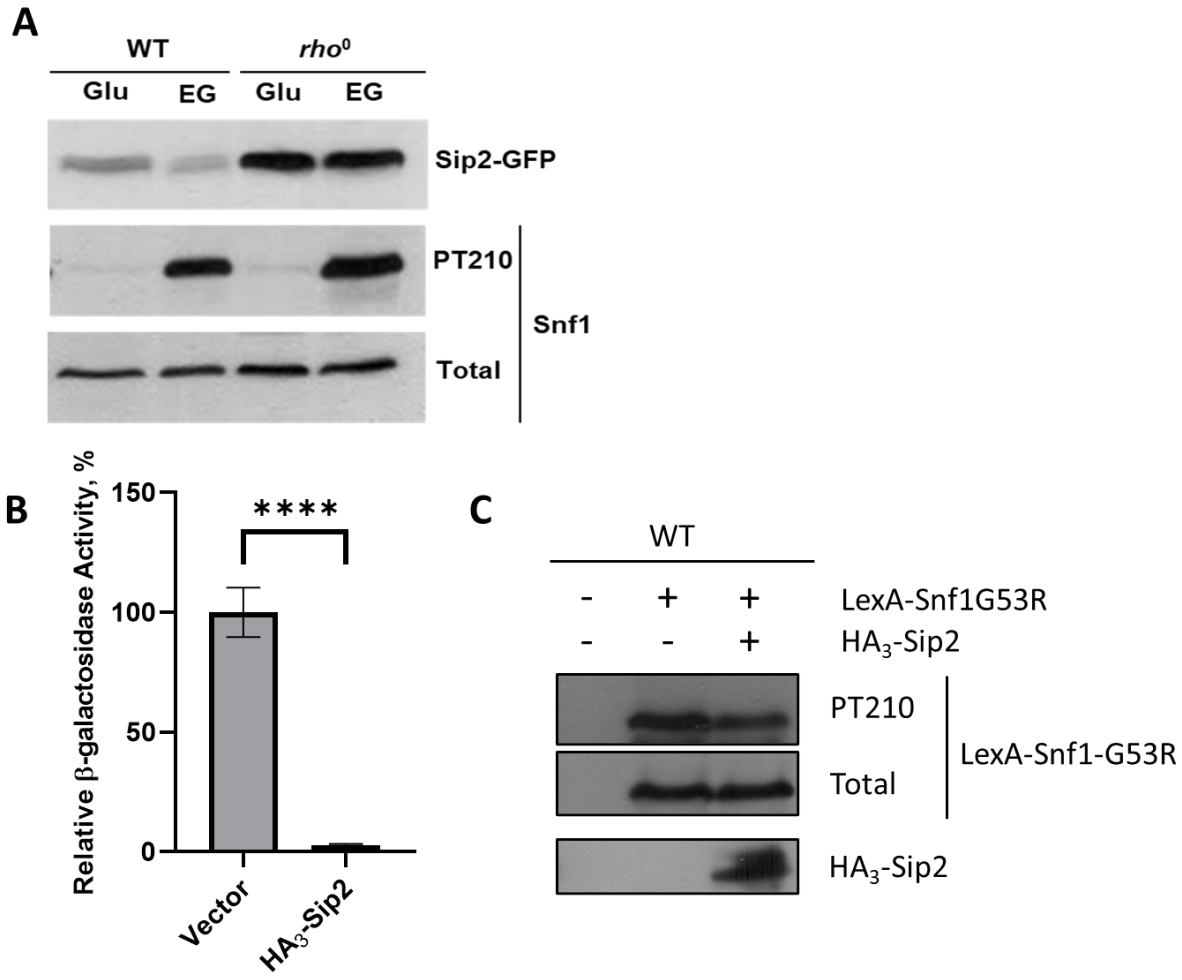

Figure 11. Sip2 is overexpressed in *rho*^{0} cells, and artificial overexpression of Sip2 recapitulates the effect of the *rho*^{0} status on transcription activation by LexA-Snf1-G53R.

(A) Transformants of the indicated genotypes expressing Sip2-GFP from the native *SIP2* promoter on a low-copy plasmid were grown to mid-log phase in selective SC medium containing high (2%) glucose (Glu) and shifted to an otherwise identical medium containing 3% ethanol and 2% glycerol (EG) instead of glucose. The levels of Sip2-GFP, Thr210 phosphorylation (PT210) and total levels of endogenous Snf1 (Total) were determined by immunoblotting. (B) WT cells containing an integrated *lexAop-lacZ* reporter and expressing LexA-Snf1-G53R were transformed with a plasmid overexpressing HA_3-Sip2 or the corresponding empty vector pSK134-HA (Vector). The cells were grown in selective SC medium containing high glucose (2%) to mid-log phase and then shifted for 3 h to an otherwise identical medium

containing low glucose (0.05%). β-galactosidase activity was assayed in permeabilized cells and measured in Miller units (n=5 independent transformants per plasmid combination). The graph shows the data for the low-glucose conditions expressed as percentage of the WT value. Error bars indicate standard errors. Statistical analysis was conducted using a two-tailed t test. ****, $P < 0.0001$. (C) Transformants were shifted to 0.05% glucose as described above and tested by immunoblotting for Thr210 phosphorylation of the Snf1-G53R moiety (PT210), total LexA-Snf1-G53R (Total), and HA_3-Sip2. Pluses (+) indicate cells expressing the indicated proteins and minuses (-) indicate cells carrying the corresponding vectors.

The retrograde (Rtg) pathway regulates Sip2 expression at the transcriptional and post-transcriptional levels.

Mitochondrial dysfunction triggers the mitochondria-to-nucleus signaling pathway known as the retrograde pathway (Liu & Butow, 2006). The retrograde regulatory network converges on the heterodimeric transcription factor composed of Rtg1 and Rtg3 (Rtg1/3) which binds to a consensus R box sequence (GTCAC) to stimulate transcription of numerous genes (Liu & Butow, 2006). Analysis reveals that there are many R box-like sequences - in both forward and reverse orientation - upstream of the *SIP2* ORF. This, in conjunction with the previous data, led us to hypothesize that Sip2 is controlled by the retrograde pathway.

To determine if the observed increase in Sip2 protein expression is due to an upregulation of *SIP2* transcription, we elected to delete *RTG3*, as the Rtg3 protein, which is an essential component of the Rtg1/3 complex (Rothermel, Thornton & Butow, 1997). WT, *rho*^{0}, *rtg3*Δ, and *rho*^{0} *rtg3*Δ mutant cells (not transformed with any plasmids) were grown to mid-log phase in high (2%) glucose medium and RNA was harvested. *SIP2* mRNA abundance was then quantified using RT-qPCR. Under high glucose conditions, *SIP2* mRNA was approximately twofold higher in *rho*^{0} cells compared to WT (Figure 12A).

Consistent with the notion that Sip2 is controlled transcriptionally by the Rtg pathway, *rtg3*Δ cells grown in high glucose medium and shifted for 20 min to EG medium had a decreased level of *SIP2* mRNA compared to the WT (Figure 12A). However, *SIP2* mRNA abundance in cells after shift to EG medium produced unexpected results: we saw that *SIP2* mRNA abundance increased dramatically in WT cells as compared to *rho*^{0} cells (Figure 12A). This is seemingly in contrast to Sip2 protein levels, which showed an increased abundance in *rho*^{0} cells in EG medium (Figure 11A).

Since the observed *SIP2* mRNA levels were not consistent with the observed Sip2 protein levels in *rho*^{0} cells, we hypothesized that the Rtg pathway has a post-transcriptional effect on Sip2, either directly or indirectly. To this end, we analyzed Sip-GFP protein levels in *rho*$^+$ and *rho*^{0} cells lacking the *RTG3* gene. When comparing *rho*$^+$ cells, no appreciable change in Sip2 protein levels was observed in the *rtg3*Δ mutant relative to the WT (Figure 12B). However, in the *rho*^{0} background, deletion of *RTG3* resulted in a dramatic decrease in Sip2 protein level after the shift to the EG medium (Figure 12C, lanes 3 and 4). This rapid disappearance of the Sip2 protein in *rho*^{0} *rtg3*Δ cells cannot be explained by downregulation of *SIP2* mRNA alone, and suggests that the retrograde pathway also protects the Sip2 protein from proteolytic degradation, possibly through upregulating genes involved in protein stability. As explained in the discussion section, we hypothesize that the Rtg pathway upregulates de-ubiquitination enzymes, such as Ubp2, thus decreasing the amount of ubiquitinated proteins (including Sip2) destined for proteolytic degradation.

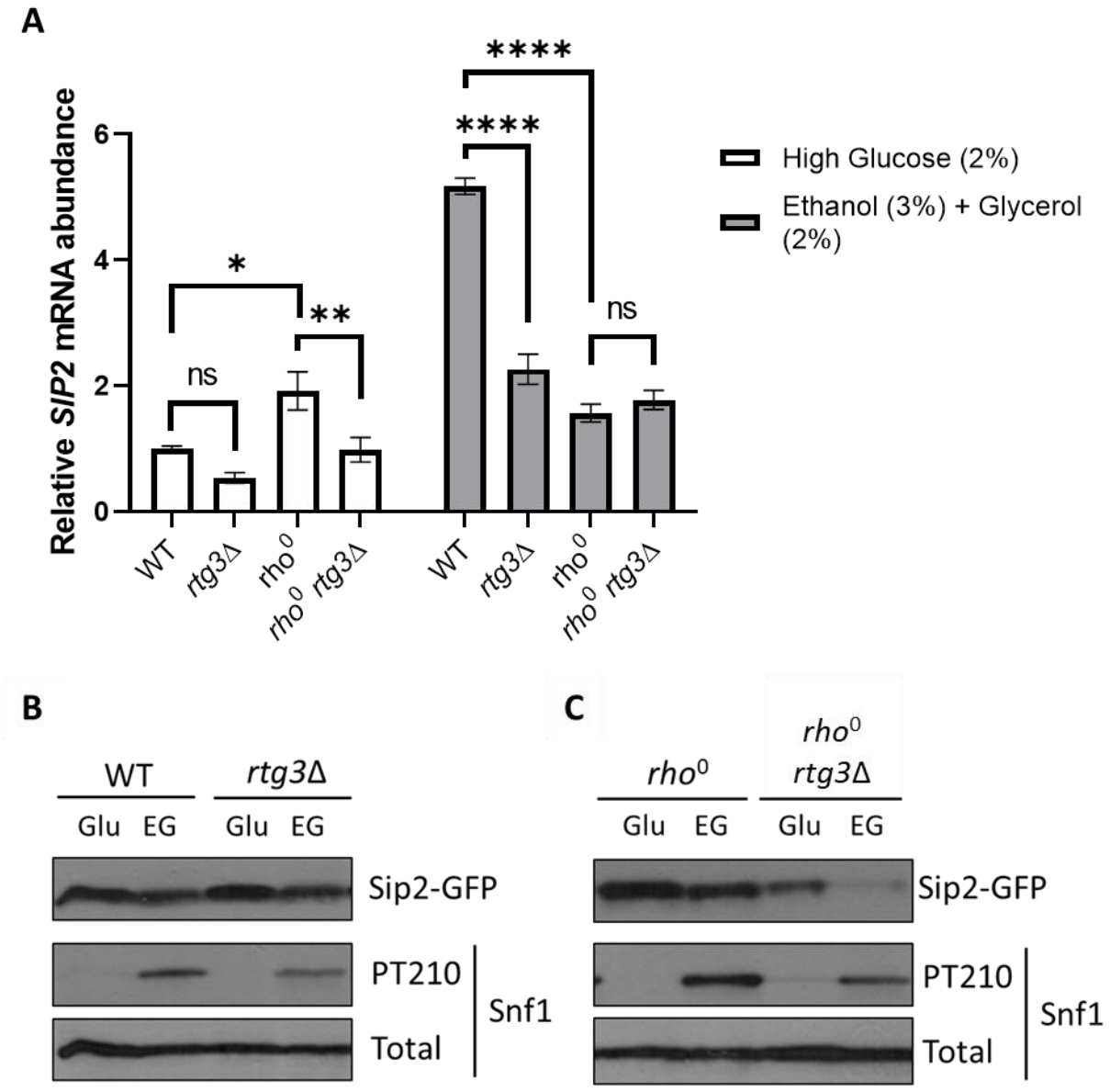

Figure 12. The retrograde (Rtg) pathway regulates Sip2 expression at the transcriptional and post-transcriptional levels. (A) Cells of the indicated genotypes were grown to mid-log phase in YEP medium containing high glucose (2%), and shifted for 20 min to an otherwise identical medium containing ethanol (3%) and glycerol (2%) instead of glucose. Total RNA was extracted and treated with DNase to remove genomic DNA. Total mRNA was reverse-transcribed using a poly-T primer and levels of *SIP2* mRNA were measured by RT-qPCR (n=3 biological replicates, with 3 technical replicates for each

biological replicate). Transcript levels were compared using the $\Delta\Delta C_t$ method, with the actin *ACT1* mRNA as the housekeeping gene control. *SIP2* mRNA abundance is expressed relative to the WT value in high glucose. Error bars indicate standard errors. Statistical analysis was done using one-way ANOVA with Holm-Sidak correction for multiple comparisons. *, P <0.05; ***, P < 0.001; ****, P <0.0001; ns, not significant. (B,C) Transformants carrying a low-copy centromeric plasmid expressing Sip2-GFP from the native *SIP2* promoter were grown with plasmid selection to mid-log phase in glucose-rich medium (2% glucose, Glu) and shifted for 20 min to an otherwise identical medium containing 3% ethanol and 2% glycerol (EG) instead of glucose. Total protein was extracted, and Sip2-GFP, total Snf1(Total), and its catalytic activation state (PT210) were detected using anti-GFP, anti-polyhistidine, and anti-phospho-Thr172 AMPK antibodies, respectively.

Deleting *SIP2* in *rho*^{0} cells fails to restore transcription activation by LexA-Snf1-G53R.

Collectively, the above results strongly suggest that the Snf1 nuclear localization defect in *rho*^{0} cells is due to an over-abundance of the cytoplasmic β subunit Sip2. Therefore, deletion of *SIP2* in *rho*^{0} cells should restore not only Snf1 nuclear localization, but also activation of the *lexAop-lacZ* reporter by LexA-Snf1-G53R. To our surprise, when we expressed LexA-Snf1-G53R in *rho*^{0} *sip2*Δ cells with the integrated *lexAop-lacZ* reporter, reporter activation was not restored (Figure 13A). Immunoblot analysis indicated that this result could not be attributed to a difference in the levels of catalytically active or total LexA-Snf1-G53R relative to the controls (Figure 13B. This surprising result suggested that the *rho*^{0} status affects another functional aspect of the Snf1-Gal83 complex independent of Sip2 overexpression.

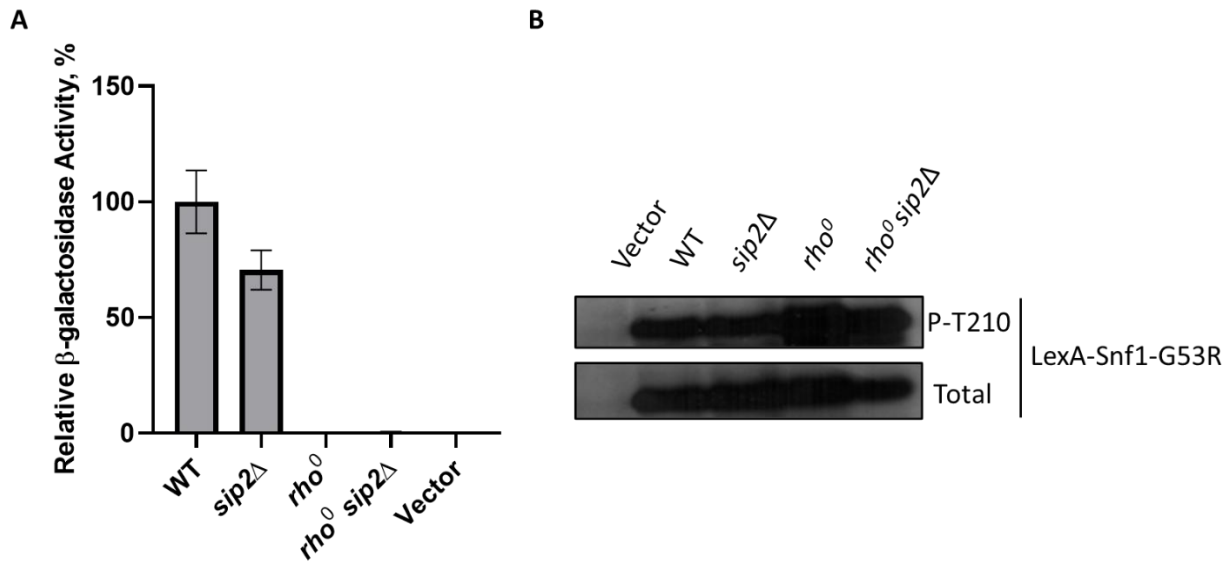

Figure 13. Deleting *SIP2* in *rho*^{0} cells fails to restore transcription activation by LexA-Snf1-G53R. (A) Cells of the indicated genotypes expressing LexA-Snf1-G53R or carrying the empty vector pBTM116 (Vector) were grown in selective SC medium containing high glucose (2%) to mid-log phase and then shifted for 3 h to an otherwise identical medium containing low glucose (0.05%). β-galactosidase activity was assayed in permeabilized cells and measured in Miller units (n=5 independent transformants per genotype). The graph shows the data for low-glucose conditions expressed as percentage of the WT value. Error bars indicate standard errors. (B) Transformants were shifted to 0.05% glucose as described above and tested for Thr210 phosphorylation of the Snf1-G53R moiety (P-T210) and total LexA-Snf1-G53R (Total) by immunoblotting.

Overexpression of Gal83 ameliorates the defect in transcription activation by LexA-Snf1-G53R in *rho*^{0} cells.

Our previous results show that despite the fact that deletion of *SIP2* alleviates the nuclear localization defect of Snf1 in *rho*^{0} cells, the lack of transcription activation by LexA-Snf1-G53R suggests that there are other regulatory defects affecting the Snf1-Gal83 complex. Since this defect could not be attributed to reduced catalytic activation of Snf1, it is possible that this defect impinges on Gal83 functionality. We

therefore reasoned that overexpression of Gal83 in *rho*^{0} cells could ameliorate the lack of reporter activation by LexA-Snf1-G53R. We overexpressed triple HA-tagged Gal83 (HA_3-Gal83) from the yeast *ADH1* promoter on a multicopy plasmid in *rho*^{0} cells with the integrated *lexAop-lacZ* reporter and expressing LexA-Snf1-G53R. Interestingly, despite an apparent decrease in the amount of LexA-Snf1-G53R, *rho*^{0} cells overexpressing HA_3-Gal83 showed a marked increase in reporter activation compared to *rho*^{0} cells carrying the empty vector (Figure 14).

As a side note, the likely reason for reduced LexA-Snf1-G53R expression in cells that also overexpress Gal83 is that the Gal83-dependent Snf1 hyperactivity inhibits cell growth (Vyas *et al.*, 2003). Therefore, cells shown in lanes 3 and 5 (Figure 14B) must be selecting against the LexA-Snf1-G53R and/or HA_3-Gal83 plasmids. We believe that otherwise, overexpression of HA_3-Gal83 would have restored reporter activation in *rho*^{0} cells to the WT level.

We conclude that the *rho*^{0} status confers an additional functional defect on Gal83 that likely pertains to its ability to functionally interact with the RNA polymerase II holoenzyme/transcription machinery.

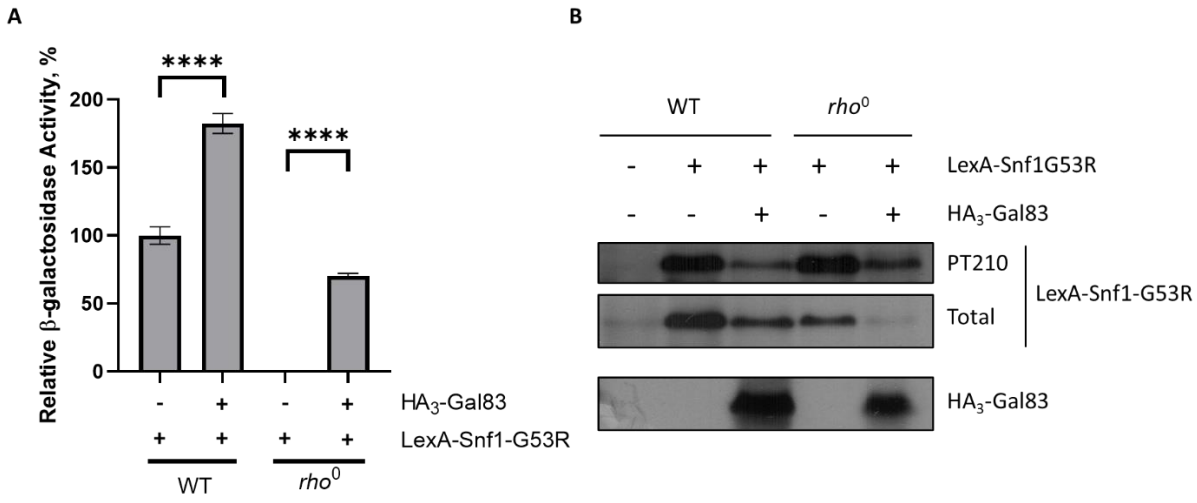

Figure 14. Overexpression of Gal83 suppresses the defect in transcription activation by LexA-Snf1-G53R in *rho*^{0} cells. (A) WT cells containing an integrated *lexAop-lacZ* reporter and expressing LexA-Snf1-G53R were transformed with a plasmid overexpressing HA_3-Gal83 or with the corresponding empty vector pSK134-HA (Vector). The cells were grown in selective SC medium containing high glucose (2%) to mid-log phase and then shifted for 3 h to an otherwise identical medium containing low glucose (0.05%). β-galactosidase activity was assayed in permeabilized cells and measured in Miller units (n=5 independent transformants per plasmid combination). The graph shows the data for the low-glucose conditions expressed as percentage of the WT value. Error bars indicate standard errors. Statistical analysis was done using one-way ANOVA with Holm-Sidak correction for multiple comparisons. ****, $P < 0.0001$. Pluses (+) denote cells expressing the indicated proteins, while (-) denote cells carrying the corresponding empty vector. (B) Transformants were shifted to 0.05% glucose as described above. Thr210 phosphorylation of the Snf1-G53R moiety (PT210), total LexA-Snf1-G53R protein levels (Total), and HA_3-Gal83 were detected by immunoblotting. Pluses (+) denote cells expressing the indicated proteins, while minuses (-) denote cells carrying the corresponding empty vectors.

Overexpression of Gal83 restores Snf1-GFP nuclear localization in *rho*^{0} cells.

Since overexpressing Gal83 was alone sufficient in restoring *lexAop-lacZ* reporter activation in *rho*^{0} cells, we wanted to find out if it also could restore Snf1-GFP localization. We therefore overexpressed HA_3-Gal83 in WT and *rho*^{0} cells expressing the Snf1-GFP fusion protein used in previous experiments. *rho*^{0} cells overexpressing HA_3-Gal83 showed improved Snf1 nuclear localization in response to carbon/energy stress (Figure 15D). In addition, overexpression of Gal83 in *rho*^{0} cells showed improved nuclear localization of Snf1-GFP in the presence of high glucose, as was also the case for the WT (Figure 15B).

Thus, the intracellular level of Gal83 is the limiting factor in Snf1 nuclear localization and transcription activation by LexA-Snf1-G53R.

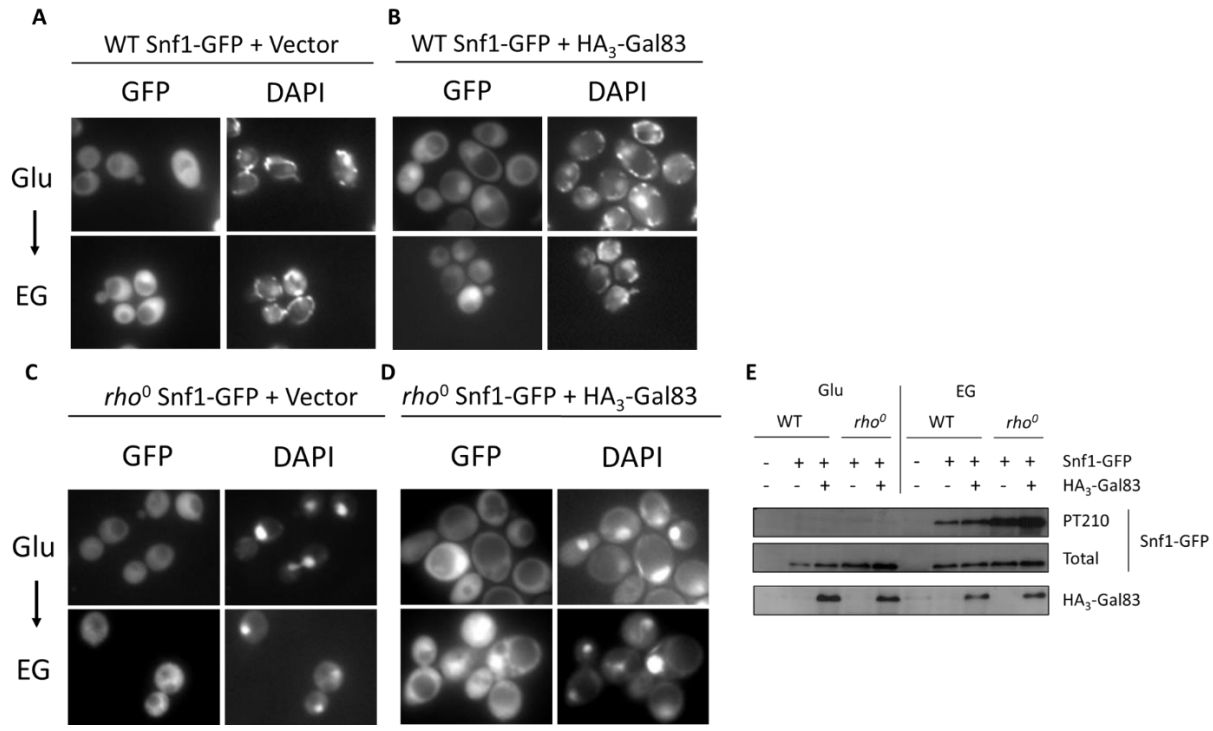

Figure 15. Overexpressing Gal83 restores Snf1-GFP nuclear localization in respiratory null cells. (A-D) Localization of Snf1-GFP was determined by fluorescence microscopy after growth to mid-log phase in selective SC medium containing 2% glucose (Glu) and after a 20 min shift to an otherwise identical medium containing 3% ethanol and 2% glycerol (EG) instead of glucose. WT and *rho*^{0} cells express Snf1-GFP as previously describe and were transformed with a plasmid overexpressing HA_3-Gal83 or with the corresponding empty vector pSK134-HA (Vector). The cells were imaged by differential interference contrast (DIC) microscopy. Nuclei were stained with DAPI. (E) Cells were grown as described above, and the Thr210 phosphorylation state (PT210), total Snf1-GFP (Total), and HA_3-Gal83 were detected by immunoblotting. Pluses (+) denotes cells expressing the indicated proteins, while minuses (-) denotes cells carrying the corresponding empty vectors.

LexA-Gal83 activates transcription in a glucose-regulated manner.

Above, we obtained evidence that Gal83 may be negatively regulated by the *rho*^{0} status with respect to its interaction with the RNA polymerase II holoenzyme/transcription machinery. To further test this idea, we employed the shortcut assay used in the previous experiments, except that instead of using LexA-Snf1-G53R, we used a LexA-Gal83 fusion. This fusion is known to activate transcription (Lesage, Yang & Carlson, 1996). We therefore first expressed LexA-Gal83 in WT cells containing the integrated *lexAop-lacZ* reporter. The cells were grown in high glucose (2%) and then shifted to low glucose (0.05%) for 3h. LexA-Gal83 activated transcription of *lexAop-lacZ* much more strongly in low glucose than in high glucose (Figure 16A). These results demonstrate that LexA-Gal83 can recruit the RNA polymerase II/transcription machinery to the *lexAop-lacZ* reporter, and this effect is regulated by glucose.

The *rho*^{0} status affects the ability of LexA-Gal83 to activate transcription.

Next, we compared the ability of LexA-Gal83 to activate transcription in WT and *rho*^{0} cells. As with LexA-Snf1-G53R (see above), *lexAop-lacZ* reporter activation by LexA-Gal83 in low glucose was significantly reduced in *rho*^{0} cells as compared to WT cells (Figure 16B). Thus, these results support the notion that Gal83 is negatively regulated by the *rho*^{0} status.

Transcription activation by LexA-Gal83 is subject to negative feedback from Snf1.

Since Gal83 interacts with Snf1, we wanted to determine whether transcription activation by LexA-Gal83 depends on Snf1. To determine this, we checked *lexAop-lacZ* reporter activation by LexA-Gal83 in *snf1*Δ cells in both *rho*$^+$ and *rho*^{0} cells. We expected that deletion of *SNF1* would decrease the ability of LexA-Gal83 to activate transcription. To our complete surprise, we observed the opposite. LexA-Gal83 activated transcription significantly better in rho$^+$ *snf1*Δ cells (Figure 16C). Furthermore, the *snf1*Δ mutation strongly reversed the effect of the *rho*^{0} status on transcription activation by LexA-Gal83 (Figure 16C). Collectively, the results of these transcription activation experiments are shown in Figure 16C.

Immunoblot experiments indicated that the results are not attributable to differences in LexA-Gal83 protein levels or the catalytic activation of Snf1 (Figure 16D).

From these experiments, we make several conclusions. First, transcription activation by LexA-Gal83 is glucose-regulated. Second, the *rho*^{0} status affects the ability of Gal83 to activate transcription. Third, Gal83 can activate transcription independent of Snf1, and is, in fact, negatively affected by Snf1 itself. It has been previously shown that Snf1/AMPK is a negative regulator of growth under carbon/energy stress conditions (Maziarz *et al.*, 2016; Corradetti *et al.*, 2004). Moreover, in yeast, this effect shows dependence on Gal83 (Vyas *et al.*, 2001). Therefore, we attribute the negative effect of Snf1 on Gal83 as a negative feedback mechanism to prevent excessive growth inhibition.

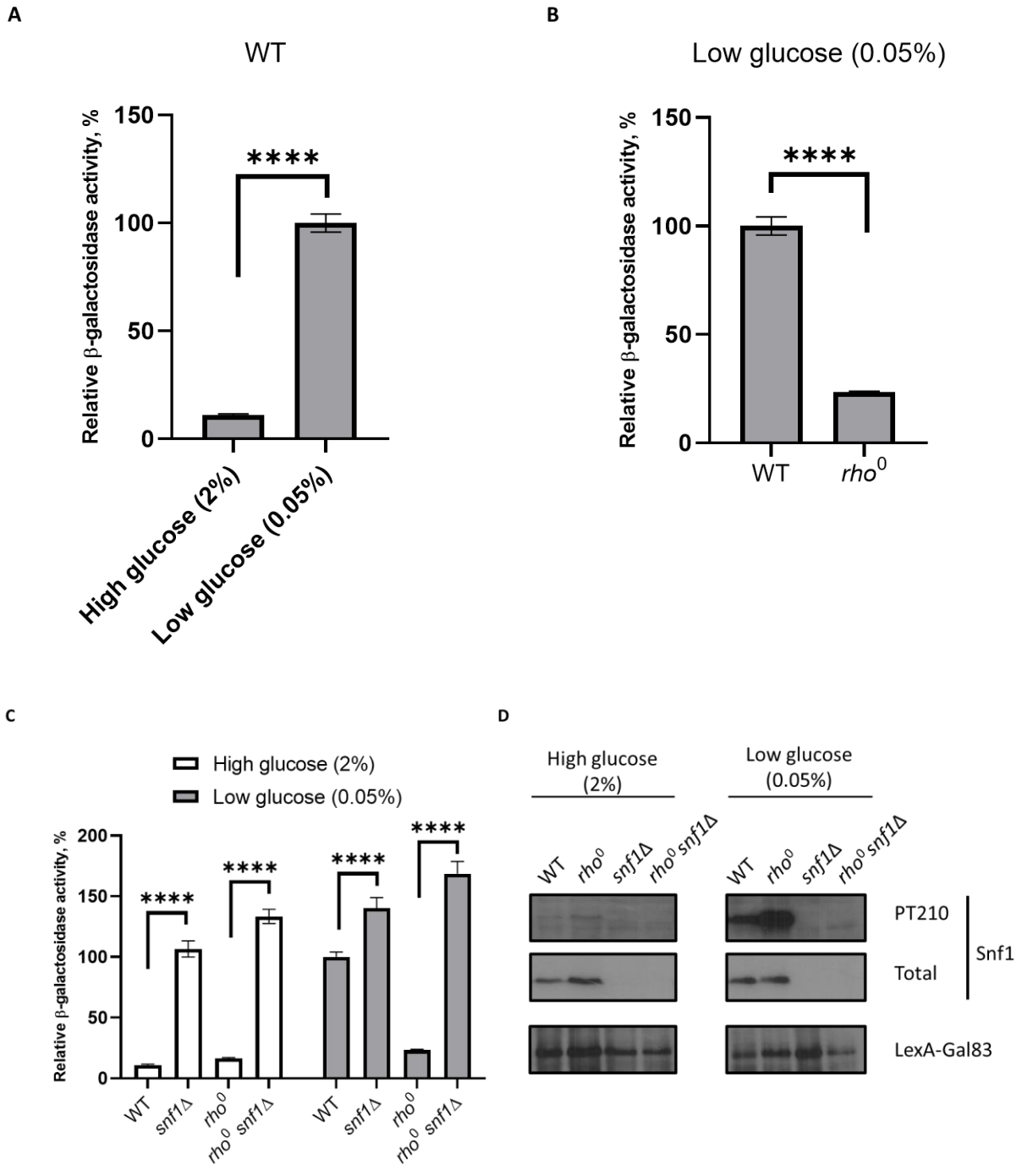

Figure 16. LexA-Gal83 activates transcription in a glucose-, respiration-, and Snf1-regulated manner. Panels A and B are the same data from panel C, but isolated for ease of reading. Statistical analysis for panels A-C were done as for panel C and statistical significance calculated included all data points represented in panel C. Cells of the indicated genotypes were grown in selective SC medium containing

high glucose (2%) to mid-log phase and then shifted for 3 h to an otherwise identical medium containing low glucose (0.05%). β-galactosidase activity was assayed in permeabilized cells and measured in Miller units (n=5 independent transformants). (A) The graph shows the data for WT under high- and low-glucose conditions, which are expressed as a percentage of the value in low glucose. (B) The graph shows the data for WT and *rho*^{0} cells under low-glucose conditions, which are expressed as a percentage of the WT value. (C) The graph shows the data for the indicated genotypes under high- and low-glucose conditions, which are expressed as a percentage of the WT value in low glucose. Error bars indicate standard errors. Statistical analysis was done using one-way ANOVA with Holm-Sidak correction for multiple comparisons. ****, $P < 0.0001$. (D) Transformants were grown as for panels A-C and Thr210 phosphorylation of Snf1 (PT210), total Snf1 (Total), and LexA-Gal83 were detected by immunoblotting.

Deletion of *SIP2* accelerates aging in *rho*^{0} cells.

Replication of eukaryotic cells is associated with progressive decline in mitochondrial function (Haas, 2019). To counteract the negative effect that this decline might have on the replicative life span (RLS), organisms have evolved compensatory signaling networks linking the mitochondrial function to the longevity response.

In fact, it has been shown that *rho*^{0} cells of *S. cerevisiae* may overcompensate for mitochondrial dysfunction and exhibit a longer RLS that the WT (Woo & Poyton, 2009). It has also been shown that the increased RLS of *rho*^{0} cells depends on the retrograde pathway, as the *rtg3*Δ mutation reversed the pro-longevity effect caused by *rho*^{0} (Borghouts *et al.*, 2004). However, the downstream targets of the Rtg-mediated pro-longevity pathway have not been established.

Interestingly, studies have also shown that Sip2 is associated with an increased RLS (Lin, Manchester & Gordon, 2003). Our present findings seem to connect the dots in this pathway. It should be noted that the increased RLS of *rho*^{0} cells is incredibly dependent on the genetic background used.

For example, studies have not shown an increased RLS in *rho*^{0} cells in the W303 background (Kirchman *et al.*, 1999), which we have used throughout this study. Nonetheless, we decided to measure the RLS of WT and mutant cells in the W303 genetic background.

As anticipated, the *rho*^{0} status did not confer an increased RLS in W303 cells (Figure 17). Likewise, the *sip2*Δ mutation did not affect the RLS in *rho*$^+$ cells either (Figure 17). However, the *sip2*Δ mutation in *rho*^{0} cells resulted in a marked RLS reduction (Figure 17).

These results show that while the *sip2*Δ mutation does not affect the RLS of WT cells, it does significantly decrease the RLS in *rho*^{0} cells. We propose a pathway where the *rho*^{0} status promotes the expression of Sip2 in order to increase replicative longevity (Figure 18).

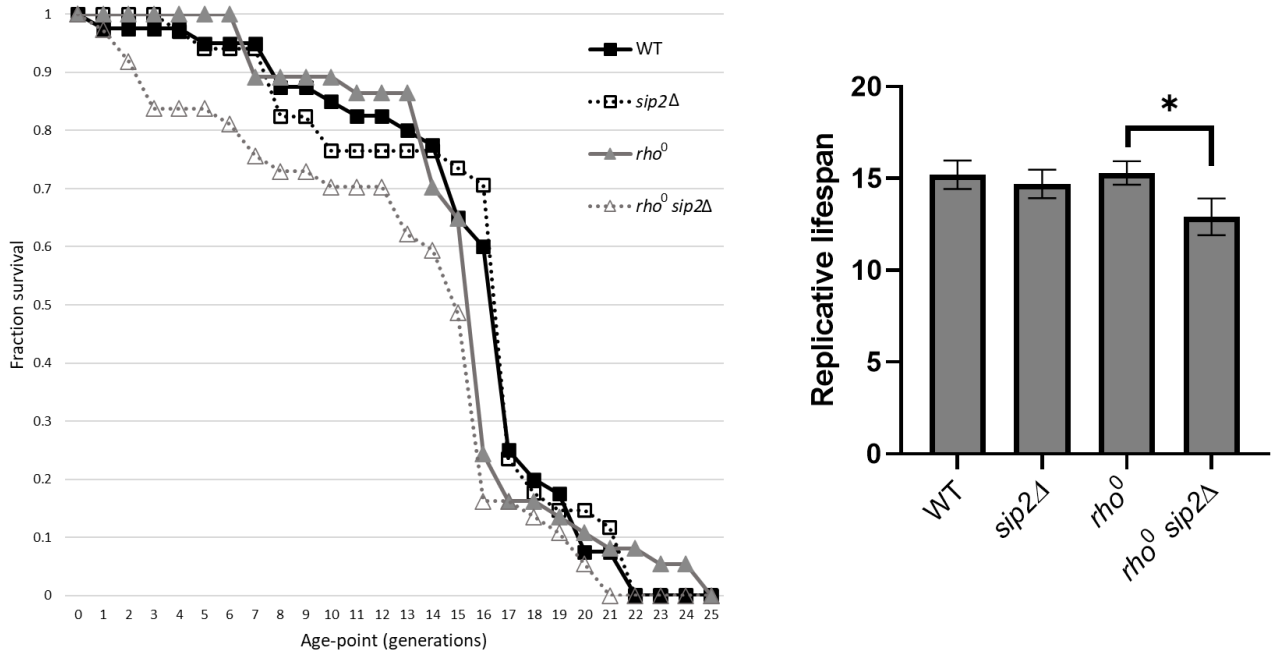

Figure 17. Deletion of *SIP2* accelerates aging in *rho*^{0} cells. 30-40 virgin cells of each genotype tested for RLS on rich medium containing 2% glucose. The cells were incubated at 30°C. As new daughter cells budded from the mother cell, they were removed by micromanipulation. (A) "Survival" curve showing the proportion of mother cells capable of budding at each cell replication point. (B) The mean RLS. Error

bars indicate standard errors. One comparison was intended, and the direction of the effect was predictable, so a one-tailed Student t test (with equal variances) was used. *, $P \sim 0.02$.

DISCUSSION

Mitochondria play an essential role in cellular energy production, and as such, many regulatory networks involving carbon source metabolism are interconnected to the mitochondrion – either directly or indirectly. Previous results from our lab have illustrated how mitochondrial VDAC proteins Por1 and Por2 play crucial roles in Snf1 regulation through both catalytic activation and nuclear enrichment of this kinase (Strogolova *et al.*, 2012; Shevade *et al.*, 2018). It is therefore conceivable that other aspects of mitochondrial function may regulate Snf1. Here, we have shown that respiratory function itself affects Snf1 regulation, independent of the regulatory effects of Por1 and Por2 (Strogolova *et al.*, 2012; Shevade *et al.*, 2018). Previously, our lab has showed that cells lacking *POR1* exhibit a Gal83-dependent Snf1 nuclear localization defect; that is, Snf1 is unable to enrich in the nucleus due to the *por1*Δ mutation affecting Gal83 nuclear localization (Shevade *et al.*, 2018). Similar to *por1Δ* cells, *rho*^{0} cells lacking the mitochondrial genome also experience a defect in Snf1 nuclear localization. In contrast to the *por1*Δ mutant, however, this localization defect occurs not through Gal83 regulation. Our microscopy data indicate that Gal83 shows normal nuclear enrichment during carbon/energy stress in these *rho*^{0} cells, suggesting that Snf1 has become uncoupled from Gal83.

In glucose-grown WT cells, Gal83 is the most abundant of the three β subunits (Vincent *et al.*, 2001; Chandrashekarappa *et al.*, 2016). However, the relative abundance of the β subunits in other genotypes/conditions can play an important role in modulating Snf1 subcellular localization. For example, in *rho*$^+$ cells, over-expressing the non-nuclear β subunits Sip1 or Sip2 has a negative effect on Snf1-dependent transcription activation [(Vincent *et al.*, 2001); Brown K.M., unpublished]. Here, we

found that Sip2 protein levels are higher in *rho*^{0} cells compared to WT cells, resulting in cytoplasmic sequestration of the Snf1 kinase complex.

Snf1 nuclear enrichment was restored during carbon/energy stress in *rho*^{0} cells simply by deleting *SIP2* from the genome. Considering that the respiratory status has no discernable effect on Snf1 catalytic activation, overexpression of Sip2 and the resultant change in the relative β subunit abundance is a major reason for the Snf1 localization defect in *rho*^{0} cells.

Our evidence suggests that Sip2 overexpression in *rho*^{0} cells occurs via the Rtg retrograde pathway. However, analysis of *SIP2* mRNA abundance in *rho*^{0} *rtg3*Δ cells did not show a significant decrease relative to *rho*^{0} cells upon shift to EG medium. Interestingly, there was a marked discrepancy between the levels of *SIP2* mRNA and Sip2-GFP protein levels. The *rtg3*Δ mutation in *rho*^{0} cells caused a marked decrease in Sip2-GFP protein level without causing a corresponding decrease in *SIP2* mRNA level. It was particularly stunning that in the *rho*^{0} *rtg3*Δ mutant, the Sip2-GFP protein, but not *SIP2* mRNA, disappeared within just 20 min of shifting the cells to carbon stress conditions. Therefore, we conclude that the Rtg pathway controls Sip2 expression at the levels of *SIP2* gene transcription and Sip2 protein stability. In terms of *SIP2* transcription, the most likely mechanism is the Rtg1/3 transcription activator binding to one or more of the multiple R box-like sequences present in the *SIP2* promoter. However, the ability for a transcription factor to control protein stability is less straightforward. There is evidence that the E3 ubiquitin ligase Rsp5 interacts with Sip2 both physically and genetically (Hesselberth *et al.*, 2006; Costanzo *et al.*, 2016). It is possible that the Rtg1/3 complex plays an indirect role in the negative regulation of Rsp5.

The Rtg1/3 complex could also promote an antagonistic effect on Rsp5. The de-ubiquitinating enzyme Ubp2 is known to work antagonistically against Rsp5 targets (Kee, Lyon & Huibregtse, 2005; Lam & Emili, 2013). It is therefore also possible that the Rtg1/3 complex upregulates transcription of *UBP2* in *rho*^{0} cells. Further experiments are required to establish the Rtg-mediated mechanism of Sip2 protein

stability in *rho*^{0} cells. Regardless of the exact mechanism of regulation, however, our data strongly suggest that Sip2 over-abundance is a major cause of Snf1 dysregulation in respiratory null cells.

If the nuclear localization defect of Snf1 in *rho*^{0} cells is due to a change in the relative abundance of the β subunits, then overexpressing the nuclear β subunit Gal83 should restore normal Snf1 localization during carbon/energy stress. We would therefore expect that in mutants with restored nuclear localization (such as *rho*^{0} *sip2*Δ cells), Snf1-dependent transcription activation would likewise be restored. Corroborating this, we found that overexpressing Gal83 alone restored Snf1 nuclear localization in *rho*^{0} cells.

Curiously, and much to our dismay, deletion of *SIP2* in *rho*^{0} cells did not restore reporter activation by LexA-Snf1-G53R, despite restored Snf1 nuclear localization. Subsequent analysis with the Snf1-dependent *SUC2* and *STA2* promoters yielded similar results (A. Shevade, K.M. Brown, unpublished data). However, we have shown that overexpression of Gal83 alone can ameliorate the LexA-Snf1-G53R reporter activation defect in *rho*^{0} cells. This suggests that the Snf1 nuclear localization defect is not the only Snf1 defect in respiratory null cells. The second defect is also Gal83-dependent, but it reflects an effect on transcription.

Transcription activation by LexA-Snf1-G53R fully depends on Gal83 (Vincent *et al.*, 2001). However, this dependence may not only reflect Gal83-mediated nuclear localization but may also depend on the ability of Gal83 itself to activate transcription. Indeed, it has been known for a long time that LexA-Gal83 – much like LexA-Snf1-G53R – can activate the *lexAop-lacZ* reporter. To our knowledge, however, the specifics of this activation have not been addressed. As can be seen in Figure 16, the ability of LexA-Gal83 to activate transcription is regulated by glucose and increases in response to glucose limitation. This makes sense because Gal83 is enriched in the nucleus under these conditions. If Gal83 is negatively regulated by the *rho*^{0} status, then one would expect that transcription activation by LexA-Gal83 would be reduced in *rho*^{0} cells. That is exactly what we observed. Thus, we conclude that the *rho*^{0}

status affects Snf1 in at least two ways: (i) it causes redistribution of the Snf1 complex in favor of Snf1-Sip2 (instead of Snf1-Gal83) and (ii) it produces a negative effect on Gal83 itself that is distinct from its nuclear localization and likely affects the ability of Gal83 to interact with transcription machinery.

For the sake of completeness, we looked at transcription activation by LexA-Gal83 in *rho*$^{+}$ and *rho*^{0} cells with and without the *SNF1* gene. To our complete surprise, we observed that the LexA-Gal83 fusion activates transcription much better in cells lacking the *SNF1* gene. Moreover, the *snf1*Δ mutation strongly suppressed the transcription activation defect of the *rho*^{0} cells. This seemingly paradoxical effect may have a very simple explanation. For one, Snf1 activity is associated with slower cell growth (Frederick & Tatchell, 1996; Barrett *et al.*, 2012). Secondly, the anti-growth effect of Snf1 depends on Gal83 and Sak1 (Vyas *et al.*, 2001; Barrett *et al.*, 2012).

Thus, it seems reasonable that the Snf1-Gal83 complex uses an autoinhibition mechanism to limit its own function. From immune complex kinase assay experiments, it has been known for a long time that Gal83 is phosphorylated by Snf1 (Yang, Jiang & Carlson, 1994). It is therefore possible – and even likely – that Gal83 is negatively regulated by Snf1 through direct phosphorylation. In future studies, it will be interesting to determine which residue(s) of Gal83 are phosphorylated by Snf1 to inhibit its function.

While we induce the *rho*^{0} mutation artificially in the lab, *S. cerevisiae* produces *rho*^{0} cells spontaneously at a high rate. *S. cerevisiae* "consciously" does this because it is beneficial for *rho*^{0} cells to exist within a population. For example, growth by fermentation consumes glucose faster than aerobic respiration, allowing *rho*^{0}-containing populations to out-compete their microbial neighbors. An added benefit to fermentation is the production of the byproduct ethanol, which can act as a biocide against microbial competitors. Since the production of *rho*^{0} cells is a natural process, it begs the question "Why do *rho*^{0} cells adjust their energy signaling pathways?"

There may be several reasons for why this should occur. For example, it is known that Snf1 is responsible for growth on non-fermentable carbon sources such as ethanol and glycerol. Moreover, Snf1 has been shown to positively regulate aerobic respiration. Since *rho*^{0} cells cannot utilize non-fermentable carbon sources, full upregulation of Snf1 may actually be disadvantageous and cause an inefficient use of resources. In order to prevent Snf1 nuclear enrichment (and thus upregulation of respiration-associated genes that are useless in *rho*^{0} cells) during carbon source stress, overexpression of Sip2 may be used as an attenuating mechanism. However, previous studies have shown that Snf1-Gal83 is responsible for 75% of Snf1 activity during carbon source stress (Hedbacker *et al.*, 2004). For this reason, simply overexpressing Sip2 may not be enough to effectively limit Snf1 activation in *rho*^{0} cells. Modulating the functionality of Gal83 (and by extension, the Snf1-Gal83 complex) itself may be an additional way that Snf1 is regulated to avoid expression of superfluous genes related to aerobic respiration (Figure 18).

Mitochondrial dysfunction is a known sign of aging (Haas, 2019). Naturally occurring *rho*^{0} cells must compensate for this in order for the population to stay competitive. We propose that *rho*^{0} cells overexpress Sip2 also as an anti-aging measure. It has been shown previously that *rho*^{0} cells show an increased RLS and cellular longevity compared to *rho*$^+$ cells (Woo & Poyton, 2009). Separately, it has been shown that Sip2 is associated with increased RLS. Our results make a connection between these separate findings. We show that that *rho*^{0} cells lacking the *SIP2* gene show a decreased RLS.

It is worth noting that the *rho*^{0} status alone or the *sip2*Δ mutation alone did not affect RLS in our experiment. However, this is not surprising, considering that measuring age-related processes in yeast, such as RLS, is incredibly dependent on the genetic background used (Woo & Poyton, 2009). Furthermore, it was specifically found that when comparing RLS in the W303-1A genetic background (used here), only a marginal difference between *rho*$^+$ and *rho*^{0} cells can be detected (Kirchman *et al.*,

1999). This does not discount the data generated here in regard to the *SIP2* gene, however. The decreased lifespan associated with *SIP2* deletion was perfectly detectable in *rho*^{0} cells.

The apparent mechanism of increased lifespan via Sip2 is by negative regulation of Snf1 activity (Lu *et al.*, 2011; Lin *et al.*, 2003). Interestingly, the work of Lin and colleagues also implies that by positively affecting Snf1 activity, replicative lifespan is conversely decreased. This would therefore mean that any process positively regulating Snf1 nuclear activity would likewise decrease lifespan. Our data show a connection between all these factors; that the increased RLS in *rho*^{0} cells may be due in part to both the apparent over expression of Sip2 and decreased function of Gal83. Simply put, the data taken together postulates that the increased longevity and RLS of *rho*^{0} cells is through its apparent Snf1-related defects (Figure 18). To our knowledge, this is the first time a connection has been established between the *rho*^{0} status and Sip2 in the context of RLS.

Mitochondrial dysfunction has been linked to cellular aging and to cancer: as cells age, they have decreased mitochondrial activity; in cancer cells, mitochondrial damage and genetic reprogramming occurs to favor lactic acid fermentation. Similarly, AMPK has also been implicated in cellular aging and to cancer (Hsu, Tseng & Lee, 2016; Reznick *et al.*, 2007), suggesting a link between AMPK and mitochondria in this particular context. Here, we see that mitochondrial dysfunction causes several defects in the regulation of Snf1/AMPK in yeast, suggesting the existence of similar mechanisms that are relevant to cellular aging and cancer in other eukaryotes, including humans.

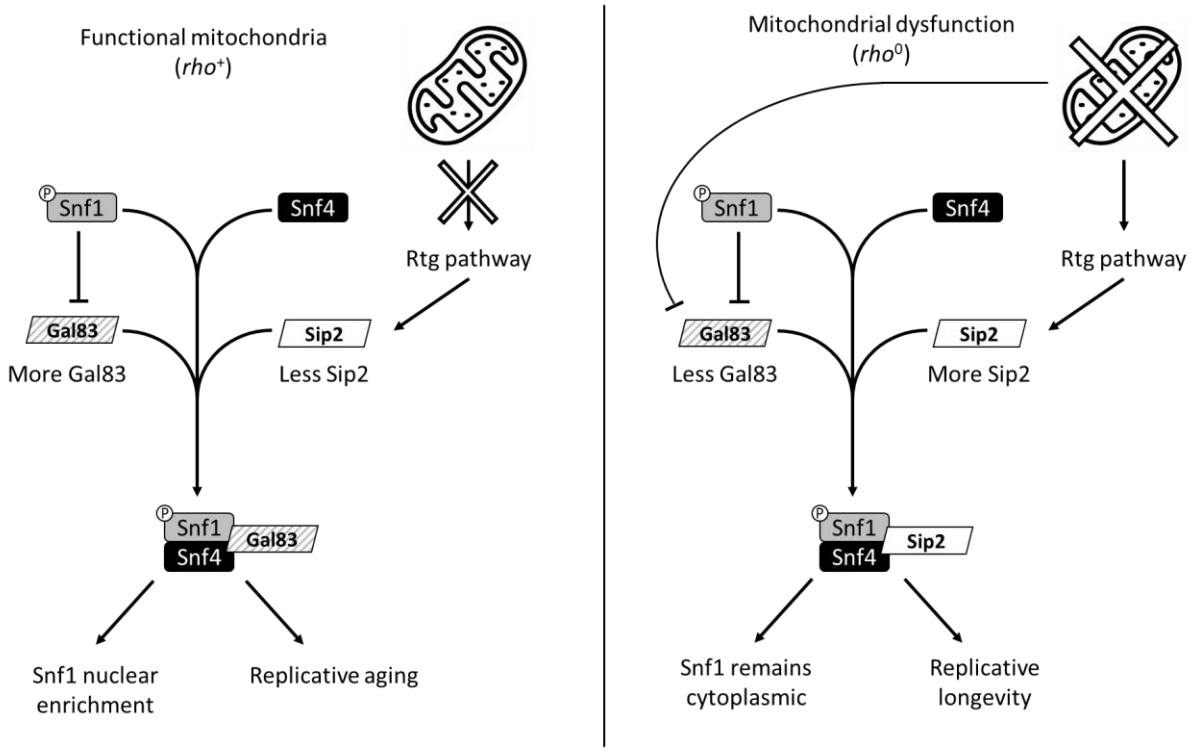

Figure 18. Proposed model of Snf1 regulation in cells with mitochondrial dysfunction. In cells capable of aerobic respiration (*rho*$^{+}$, left), the Rtg pathway is downregulated. Under these conditions, Snf1 associates primarily with the nuclear-targeting β subunit Gal83. This allows Snf1 nuclear localization during carbon/energy stress. In cells lacking the ability to aerobically respire (*rho*^{0}, right), the retrograde pathway is upregulated, causing an overexpression of Sip2 at transcriptional and post-translational levels. This causes Snf1 to primarily associate with Sip2, keeping the Snf1 complex cytoplasmic. The consequences of this are: 1) transcription of Snf1-dependent genes is reduced, and 2) cells exhibit an increased RLS. In addition, the *rho*^{0} status affects Gal83 function through a currently unknown mechanism.

MATERIALS AND METHODS

Yeast strains and growth conditions

The *S. cerevisiae* strains used in this study are listed in Table 1. All strains were in the W303 genetic background and were descendants of strains W303-1A (*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) and W303-1B (*MAT***α** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) (Thomas & Rothstein, 1989). W303 strains carrying the *lexAop-lacZ* reporter (*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 URA3*::*lexAop-lacZ*) were created by 15 rounds of repetitive backcrossing of CTY10-5d (R. Sternglanz, SUNY, Stony Brook, NY) (Bartel *et al.*, 1993) to W303.

All mutants were created by replacing the wild-type alleles with the *KanMX6* or *NatMX6* cassettes. Marker sequences were amplified by PCR with primers flanking the corresponding open reading frames. The mutant alleles were first introduced into a wild-type W303 diploid strain by transformation; all transformations were performed using standard methods (Rose MD, 1990a). Haploid mutants were then recovered from the heterozygous diploids by tetrad analysis. Combinatorial W303 mutants were generated by genetic crossing and tetrad analysis. Mutants were confirmed via PCR analysis of genomic DNA. *rho*^{0} variants of both wild-type and mutant strains were generated by incubating cells in a rich medium (YEP; see below) with 2% glucose and 30µg/mL ethidium bromide (Goldring *et al.*, 1970). Cells that have an intact copy of the *SNF1* gene were screened for *rho*^{0} status by their inability to grow on media containing glycerol as the sole carbon source. KMB168 (*rho*^{0} *snf1*Δ) was confirmed to lack mtDNA by microscopy after staining the cells with 4',6-diamidino-2-phenylindole (DAPI) by adding 10 µl of an 0.8µg/ml solution of DAPI to 1 ml of cell culture and incubating for 5 min at 30°C. 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 MD, 1990b). Unless indicated otherwise, the media contained 2% glucose, and cells were grown at 30°C.

Table 1. *S. cerevisiae* strains

Strain	Genotype	Source
MMY35	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* [*rho*$^{+}$]	This laboratory
MMY35 ρ^{0}	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* [*rho*^{0}]	This study
AMS202	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 sip2*Δ::*KanMX6* [*rho*$^{+}$]	(Shevade, 2017)
AMS210	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 sip2*Δ::*KanMX6* [*rho*^{0}]	(Shevade, 2017)
AMS208	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 sip1*Δ::*KanMX6* [*rho*^{0}]	(Shevade, 2017)
KMB006	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rtg3*Δ::*KanMX6* [*rho*$^{+}$]	(Shevade, 2017)
KMB006 ρ^{0}	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rtg3*Δ::*KanMX6* [*rho*^{0}]	This study
KMB137	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 URA3*::*lexAop-lacZ* [*rho*$^{+}$]	This study
KMB143	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 URA3*::*lexAop-lacZ* [*rho*^{0}]	This study
KMB145	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 URA3*::*lexAop-lacZ sip2*Δ::*KanMX6* [*rho*$^{+}$]	This study
KMB148	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 URA3*::*lexAop-lacZ sip2*Δ::*KanMX6* [*rho*^{0}]	This study
KMB165	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 URA3*::*lexAop-lacZ snf1*Δ::*NatMX6* [*rho*$^{+}$]	This laboratory
KMB168	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 URA3*::*lexAop-lacZ snf1*Δ::*NatMX6* [*rho*^{0}]	This study

Plasmids

The multicopy vector pBTM116 provides expression of the LexA DNA-binding protein from the yeast *ADH1* promoter (Fields & Song, 1989). To express the LexA-Snf1-G53R fusion protein, the BamHI *SNF1*-G53R fragment from plasmid pRJ216 (Kuchin *et al.*, 2000) was inserted into the BamHI site of pBTM116 in frame with the *lexA* gene. The LexA-Gal83 fusion protein was expressed from the yeast ADH1 promoter in plasmid pAMS11 (Shevade, 2017), which is a derivative of vector pEG202 (Golemis EA,

1997). The low-copy number *CEN-HIS3* plasmid pRT12 (Vincent *et al.*, 2001) expresses a C-terminal GFP-tagged Gal83 (Gal83-GFP) protein from the native *GAL83* promoter in vector pRS313 (Sikorski & Hieter, 1989). The low-copy *CEN-URA3* plasmid pSnf1-GFP (Shevade *et al.*, 2018) expresses a C-terminal GFP-tagged Snf1 (Snf1-GFP) protein from the native *SNF1* promoter and is a derivative of pSM14 (Orlova *et al.*, 2010). The multicopy vector pSK134-HA, a derivative of pACTII (Legrain, Dokhelar & Transy, 1994) and pWS93 (Song & Carlson, 1998), is a high-copy number plasmid for the expression of proteins with a triple haemagglutinin (HA_3) tag under the control of the yeast *ADH1* promoter. Plasmids pHT07 and pKMB08 express HA_3-Sip2 and HA_3-Gal83, respectively, and were constructed by cloning the *SIP2* and *GAL83* coding regions into pSK134-HA. To construct pHT07, the *SIP2* coding region was amplified by PCR using primers with flanking BamHI sites. The resulting *SIP2* PCR fragment was digested with BamHI and inserted into the BamHI site of pSK134-HA in frame with the HA_3 tag. To construct pKMB08, the *GAL83* coding region was amplified by PCR using primers with flanking BamHI sites. The resulting *GAL83* PCR fragment was digested with BamHI and inserted into the BamHI site of pSK134-HA in frame with the HA_3 tag.

Assays of *lexAop-lacZ* reporter activation

Assays of *lexAop-lacZ* activation were done as described previously (Kuchin *et al.*, 2000). Briefly, cells of strains carrying the integrated *lexAop-lacZ* reporter and expressing LexA-Snf1-G53R or LexA-Gal83 were grown in appropriate selective SC medium containing high (2%) glucose to mid-log phase and then shifted for 3 h to an otherwise identical medium containing low (0.05%) glucose. Assays of β-galactosidase activity were performed in permeabilized cells and measured in Miller units as described previously (Kuchin *et al.*, 2000).

Immunoblot analysis

Cells were grown under conditions specified in the descriptions of experimental results. Protein extracts were prepared by the boiling/alkaline treatment method as described previously (Orlova *et al.*, 2008) and analyzed by immunoblotting. The LexA-Snf1-G53R and LexA-Gal83 fusion proteins were detected with an anti-LexA antibody (Millipore). The Snf1-GFP and Gal83-GFP fusion proteins were detected with an anti-GFP antibody (Roche). The epitope-tagged HA_3-Sip2 and HA_3-Gal83 proteins were detected with an anti-HA antibody (Sigma-Aldrich). The endogenous Snf1 protein was detected with an anti-polyhistidine antibody H1029 (Sigma-Aldrich), which strongly recognizes Snf1 due to the presence of a natural stretch of 13 consecutive histidine residues near its N-terminus (amino acids 18 to 30) (Orlova *et al.*, 2008). The Thr210 phosphorylation state of Snf1, LexA-Snf1G53R, and Snf1-GFP was analyzed using an anti-phospho-Thr172-AMPK antibody (Cell Signaling), which strongly recognizes the Thr210-phosphorylated form of yeast Snf1 (Orlova *et al.*, 2008). Signals were detected by enhanced chemiluminescence using the Pierce ECL2 or ECL systems (Thermo Scientific).

Fluorescence microscopy

Snf1-GFP and Gal83-GFP were expressed in wild-type and mutant cells isogenic to W303-1A. For studies of glucose-regulated localization of these proteins, we followed a protocol published previously (Vincent *et al.*, 2001). Briefly, cells were grown to mid-log phase in appropriate selective SC media containing 2% glucose and then shifted to otherwise identical media containing 3% ethanol and 2% glycerol instead of glucose for 20 min (Vincent *et al.*, 2001). Nuclei were stained by adding 10 µl of an 0.8-µg/ml solution of 4’,6-diamidino-2-phenylindole (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 Nikon Eclipse 80i fluorescence microscope, a CoolSNAP HQ2 camera (Photometrics), and NIS-Elements BR 3.01 software.

RT-qPCR experiments

Cells were grown under conditions specified in the descriptions of experimental results. Total RNA was extracted from cells using TRIZOL extraction (Invitrogen) and purified using TURBO DNase kit (Invitrogen) according to the manufacturer's instructions. cDNA synthesis of mRNA transcripts was done using an oligo-dT(20) primer (Integrated DNA Technologies) and SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. qPCR plots were generated with gene-specific primers targeting 80-150 bp regions of *SIP2* and *ACT1* (as an internal control), and DNA amplification was detected with the SYBR green fluorophore (Thermo-Fisher) using a Biorad CFX96 instrument, and data was acquired using the Biorad CFX Maestro software. Three biological replicates were each analyzed in three technical replicates for *SIP2* and *ACT1* expression. Quantification of qPCR plots was done using the $\Delta\Delta C_t$ method (Schmittgen & Livak).

Measurement of yeast replicative lifespan (RLS)

Measurement of RLS was done as outlined by previous studies (Steffen, Kennedy & Kaeberlein, 2009). Briefly, two individual colonies of each genotype were patched to YEP media supplemented with adenine (20 mg/L), tryptophan (40 mg/L), and glucose (2%) (referred to as YPDAW-2%) and grown for two days at 30°C. Cells were then patched to a new YPDAW-2% plate and incubated at room temperature overnight. The next day, 20 cells of each patch were arrayed in individual spots with a micromanipulator and incubated at 30°C. Once these cells produced new daughter cells, the original mother cells were removed from the arrayed locations and the new virgin daughter cells were designated as new mother cells and scored for their replicative lifespan. Cells were incubated at 30°C and new daughter cells produced by the mother cells were removed and placed at a location far away from the arrayed cells. Since these experiments span several days, the plates were stored at 5°C

overnight in-between 30°C incubations. If after five consecutive time checkpoints (ranging from 1-4h) a mother cell failed to produce a new daughter cell, it was classified as senescent (dead).

References

ASHRAFI, K., LIN, S. S., MANCHESTER, J. K. & GORDON, J. I. (2000). Sip2p and its partner snf1p kinase affect aging in S. cerevisiae. *Genes Dev* **14**(15), 1872-85.

BARRETT, L., ORLOVA, M., MAZIARZ, M. & KUCHIN, S. (2012). Protein kinase A contributes to the negative control of Snf1 protein kinase in Saccharomyces cerevisiae. *Eukaryot Cell* **11**(2), 119-28.

BARTEL, P., CHIEN, C. T., STERNGLANZ, R. & FIELDS, S. (1993). Elimination of false positives that arise in using the two-hybrid system. *Biotechniques* **14**(6), 920-4.

BLACHLY-DYSON, E., SONG, J., WOLFGANG, W. J., COLOMBINI, M. & FORTE, M. (1997). Multicopy suppressors of phenotypes resulting from the absence of yeast VDAC encode a VDAC-like protein. *Mol Cell Biol* **17**(10), 5727-38.

BORGHOUTS, C., BENGURIA, A., WAWRYN, J. & JAZWINSKI, S. M. (2004). Rtg2 protein links metabolism and genome stability in yeast longevity. *Genetics* **166**(2), 765-77.

CARLING, D., ZAMMIT, V. A. & HARDIE, D. G. (1987). A common bicyclic protein kinase cascade inactivates the regulatory enzymes of fatty acid and cholesterol biosynthesis. *FEBS Lett* **223**(2), 217-22.

CHANDRASHEKARAPPA, D. G., MCCARTNEY, R. R., O'DONNELL, A. F. & SCHMIDT, M. C. (2016). The β subunit of yeast AMP-activated protein kinase directs substrate specificity in response to alkaline stress. *Cell Signal* **28**(12), 1881-1893.

CORRADETTI, M. N., INOKI, K., BARDEESY, N., DEPINHO, R. A. & GUAN, K. L. (2004). Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome. *Genes Dev* **18**(13), 1533-8.

COSTANZO, M., VANDERSLUIS, B., KOCH, E. N., BARYSHNIKOVA, A., PONS, C., TAN, G., WANG, W., USAJ, M., HANCHARD, J., LEE, S. D., PELECHANO, V., STYLES, E. B., BILLMANN, M., VAN LEEUWEN, J., VAN DYK, N., LIN, Z.-Y., KUZMIN, E., NELSON, J., PIOTROWSKI, J. S., SRIKUMAR, T., BAHR, S., CHEN, Y., DESHPANDE, R., KURAT, C. F., LI, S. C., LI, Z., USAJ, M. M., OKADA, H., PASCOE, N., SAN LUIS, B.-J., SHARIFPOOR, S., SHUTERIQI, E., SIMPKINS, S. W., SNIDER, J., SURESH, H. G., TAN, Y., ZHU, H., MALOD-DOGNIN, N., JANJIC, V., PRZULJ, N., TROYANSKAYA, O. G., STAGLJAR, I., XIA, T., OHYA, Y., GINGRAS, A.-C., RAUGHT, B., BOUTROS, M., STEINMETZ, L. M., MOORE, C. L., ROSEBROCK, A. P., CAUDY, A. A., MYERS, C. L., ANDREWS, B. & BOONE, C. (2016). A global genetic interaction network maps a wiring diagram of cellular function. *Science* **353**(6306), aaf1420.

DAVIES, S. P., HAWLEY, S. A., WOODS, A., CARLING, D., HAYSTEAD, T. A. J. & HARDIE, D. G. (1994). Purification of the AMP-activated protein kinase on ATP-γ-Sepharose and analysis of its subunit structure. *European Journal of Biochemistry* **223**(2), 351-357.

DAVIES, S. P., HELPS, N. R., COHEN, P. T. & HARDIE, D. G. (1995). 5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C alpha and native bovine protein phosphatase-2AC. *FEBS Lett* **377**(3), 421-5.

FIELDS, S. & SONG, O. (1989). A novel genetic system to detect protein-protein interactions. *Nature* **340**(6230), 245-6.

FOGARTY, S. & HARDIE, D. G. (2010). Development of protein kinase activators: AMPK as a target in metabolic disorders and cancer. *Biochim Biophys Acta* **1804**(3), 581-91.

FREDERICK, D. L. & TATCHELL, K. (1996). The REG2 gene of Saccharomyces cerevisiae encodes a type 1 protein phosphatase-binding protein that functions with Reg1p and the Snf1 protein kinase to regulate growth. *Mol Cell Biol* **16**(6), 2922-31.

GOLDRING, E. S., GROSSMAN, L. I., KRUPNICK, D., CRYER, D. R. & MARMUR, J. (1970). The petite mutation in yeast. Loss of mitochondrial deoxyribonucleic acid during induction of petites with ethidium bromide. *J Mol Biol* **52**(2), 323-35.

GOLEMIS EA, S. I., GJURIS J, BRENT R. (1997). *Current protocols in molecular biology*. Wiley, New York, NY.

HAAS, R. H. (2019). Mitochondrial Dysfunction in Aging and Diseases of Aging. In *Biology (Basel)*. (Volume 8.

HARDIE, D. G. (2007a). AMP-activated protein kinase as a drug target. *Annu Rev Pharmacol Toxicol* **47**, 185-210.

HARDIE, D. G. (2007b). AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat Rev Mol Cell Biol* **8**(10), 774-85.

HARDIE, D. G. (2007c). AMPK and SNF1: Snuffing Out Stress. In *Cell Metab*. (Volume 6, pp. 339-40.

HARDIE, D. G. & CARLING, D. (1997). The AMP-activated protein kinase--fuel gauge of the mammalian cell? *Eur J Biochem* **246**(2), 259-73.

HARDIE, D. G. & PAN, D. A. (2002). Regulation of fatty acid synthesis and oxidation by the AMP-activated protein kinase. *Biochem Soc Trans* **30**(Pt 6), 1064-70.

HEDBACKER, K. & CARLSON, M. (2008). SNF1/AMPK pathways in yeast. *Front Biosci* **13**, 2408-20.

HEDBACKER, K., HONG, S. P. & CARLSON, M. (2004). Pak1 protein kinase regulates activation and nuclear localization of Snf1-Gal83 protein kinase. *Mol Cell Biol* **24**(18), 8255-63.

HESSELBERTH, J. R., MILLER, J. P., GOLOB, A., STAJICH, J. E., MICHAUD, G. A. & FIELDS, S. (2006). Comparative analysis of Saccharomyces cerevisiae WW domains and their interacting proteins. *Genome Biol* **7**(4), R30.

HONG, S. P., LEIPER, F. C., WOODS, A., CARLING, D. & CARLSON, M. (2003b). Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. *Proc Natl Acad Sci U S A* **100**(15), 8839-43.

HSU, C. C., TSENG, L. M. & LEE, H. C. (2016). Role of mitochondrial dysfunction in cancer progression. *Exp Biol Med (Maywood)* **241**(12), 1281-95.

KEE, Y., LYON, N. & HUIBREGTSE, J. M. (2005). The Rsp5 ubiquitin ligase is coupled to and antagonized by the Ubp2 deubiquitinating enzyme. *Embo j* **24**(13), 2414-24.

KIRCHMAN, P. A., KIM, S., LAI, C. Y. & JAZWINSKI, S. M. (1999). Interorganelle signaling is a determinant of longevity in Saccharomyces cerevisiae. *Genetics* **152**(1), 179-90.

KUCHIN, S., TREICH, I. & CARLSON, M. (2000). A regulatory shortcut between the Snf1 protein kinase and RNA polymerase II holoenzyme. *Proc Natl Acad Sci U S A* **97**(14), 7916-20.

LAM, M. H. & EMILI, A. (2013). Ubp2 regulates Rsp5 ubiquitination activity in vivo and in vitro. *PLoS One* **8**(9), e75372.

LEGRAIN, P., DOKHELAR, M. C. & TRANSY, C. (1994). Detection of protein-protein interactions using different vectors in the two-hybrid system. *Nucleic Acids Res* **22**(15), 3241-2.

LESAGE, P., YANG, X. & CARLSON, M. (1996). Yeast SNF1 protein kinase interacts with SIP4, a C6 zinc cluster transcriptional activator: a new role for SNF1 in the glucose response. *Mol Cell Biol* **16**(5), 1921-8.

LI, W., SAUD, S. M., YOUNG, M. R., CHEN, G. & HUA, B. (2015). Targeting AMPK for cancer prevention and treatment. *Oncotarget* **6**(10), 7365-78.

LIN, S. S., MANCHESTER, J. K. & GORDON, J. I. (2003). Sip2, an N-myristoylated beta subunit of Snf1 kinase, regulates aging in Saccharomyces cerevisiae by affecting cellular histone kinase activity, recombination at rDNA loci, and silencing. *J Biol Chem* **278**(15), 13390-7.

LIU, Z. & BUTOW, R. A. (2006). Mitochondrial retrograde signaling. *Annu Rev Genet* **40**, 159-85.

LU, J. Y., LIN, Y. Y., SHEU, J. C., WU, J. T., LEE, F. J., CHEN, Y., LIN, M. I., CHIANG, F. T., TAI, T. Y., BERGER, S. L., ZHAO, Y., TSAI, K. S., ZHU, H., CHUANG, L. M. & BOEKE, J. D. (2011). Acetylation of yeast AMPK controls intrinsic aging independently of caloric restriction. *Cell* **146**(6), 969-79.

MAZIARZ, M., SHEVADE, A., BARRETT, L. & KUCHIN, S. (2016). Springing into Action: Reg2 Negatively Regulates Snf1 Protein Kinase and Facilitates Recovery from Prolonged Glucose Starvation in Saccharomyces cerevisiae. *Appl Environ Microbiol* **82**(13), 3875-3885.

ORLOVA, M., BARRETT, L. & KUCHIN, S. (2008). Detection of endogenous Snf1 and its activation state: application to Saccharomyces and Candida species. *Yeast* **25**(10), 745-54.

ORLOVA, M., OZCETIN, H., BARRETT, L. & KUCHIN, S. (2010). Roles of the Snf1-activating kinases during nitrogen limitation and pseudohyphal differentiation in Saccharomyces cerevisiae. *Eukaryot Cell* **9**(1), 208-14.

RENA, G., PEARSON, E. R. & SAKAMOTO, K. (2013). Molecular mechanism of action of metformin: old or new insights? *Diabetologia* **56**(9), 1898-906.

REZNICK, R. M., ZONG, H., LI, J., MORINO, K., MOORE, I. K., YU, H. J., LIU, Z. X., DONG, J., MUSTARD, K. J., HAWLEY, S. A., BEFROY, D., PYPAERT, M., HARDIE, D. G., YOUNG, L. H. & SHULMAN, G. I. (2007). Aging-associated reductions in AMP-activated protein kinase activity and mitochondrial biogenesis. *Cell Metab* **5**(2), 151-6.

ROSE MD, W. F., HIETER P. (1990a). *Methods in yeast genetics A laboratory course manual*, Cold Spring Harbor, NY.

ROSE MD, W. F., HIETER P. (1990b). *Methods in yeast genetics A laboratory course manual*, Cold Spring Harbor, NY.

ROTHERMEL, B. A., THORNTON, J. L. & BUTOW, R. A. (1997). Rtg3p, a basic helix-loop-helix/leucine zipper protein that functions in mitochondrial-induced changes in gene expression, contains independent activation domains. *J Biol Chem* **272**(32), 19801-7.

RUIZ, R., PEREZ-VILLEGAS, E. M. & MANUEL CARRION, A. (2016). AMPK Function in Aging Process. *Curr Drug Targets* **17**(8), 932-41.

SCHMITTGEN, T. D. & LIVAK, K. J. Analyzing real-time PCR data by the comparative CT method.

SHEVADE, A. (2017). *Mitochondrial Regulation of Yeast AMPK During Energy Stress*, University of Wisconsin-Milwaukee.

SHEVADE, A., STROGOLOVA, V., ORLOVA, M., YEO, C. T. & KUCHIN, S. (2018). Mitochondrial Voltage-Dependent Anion Channel Protein Por1 Positively Regulates the Nuclear Localization of Saccharomyces cerevisiae AMP-Activated Protein Kinase. *mSphere* **3**(1).

SIKORSKI, R. S. & HIETER, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics* **122**(1), 19-27.

SONG, W. & CARLSON, M. (1998). Srb/mediator proteins interact functionally and physically with transcriptional repressor Sfl1. *Embo j* **17**(19), 5757-65.

STEFFEN, K. K., KENNEDY, B. K. & KAEBERLEIN, M. (2009). Measuring replicative life span in the budding yeast. *J Vis Exp* (28).

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**(12), 1568-72.

THOMAS, B. J. & ROTHSTEIN, R. (1989). Elevated recombination rates in transcriptionally active DNA. *Cell* **56**(4), 619-30.

VINCENT, O., TOWNLEY, R., KUCHIN, S. & CARLSON, M. (2001). Subcellular localization of the Snf1 kinase is regulated by specific beta subunits and a novel glucose signaling mechanism. *Genes Dev* **15**(9), 1104-14.

VYAS, V. K., KUCHIN, S., BERKEY, C. D. & CARLSON, M. (2003). Snf1 kinases with different beta-subunit isoforms play distinct roles in regulating haploid invasive growth. *Mol Cell Biol* **23**(4), 1341-8.

VYAS, V. K., KUCHIN, S. & CARLSON, M. (2001). Interaction of the repressors Nrg1 and Nrg2 with the Snf1 protein kinase in Saccharomyces cerevisiae. *Genetics* **158**(2), 563-72.

WOO, D. K. & POYTON, R. O. (2009). The absence of a mitochondrial genome in rho0 yeast cells extends lifespan independently of retrograde regulation. *Exp Gerontol* **44**(6-7), 390-7.

YANG, X., JIANG, R. & CARLSON, M. (1994). A family of proteins containing a conserved domain that mediates interaction with the yeast SNF1 protein kinase complex. *Embo j* **13**(24), 5878-86.

Chapter 5

Conclusions and perspectives

Organisms constantly encounter a variety of stresses in their environment, from toxic compounds to temperature extremes to nutrient starvation. The ways in which organisms can sense and respond to these stresses is one of the most fundamental questions in biology. While physiological stress responses are often easily observable, the underlying regulatory mechanisms are often poorly understood. Many stresses affect the cellular energy supply and so maintaining an appropriate level of available energy is essential for cellular function. Thus, being able to sense intracellular energy levels and respond appropriately is imperative for the survival of the cell.

AMPK is a protein complex, highly conserved among eukaryotes, that helps ensure proper energy homeostasis during nutrient starvation. In mammals, for example, AMPK helps maintain blood glucose levels by stimulating translocation of the major glucose transporter GLUT4 to the cell surface (Witczak, Sharoff & Goodyear, 2008). AMPK helps maintain energy balance by upregulating ATP-generating processes (*e.g.*, glucose uptake) while downregulating ATP-consuming processes (*e.g.*, growth). In fact, many conditions associated with reduced glucose uptake (diabetes) and inappropriate cellular growth (cancer) have implicated dysregulation of AMPK as a contributing factor (Fogarty & Hardie, 2010).

Given that AMPK is a biologically and clinically significant regulator involved with energy balance, it comes as no surprise that AMPK activity works in tandem with the mitochondria. In mammals, this relationship is straightforward: AMPK directly senses energy depletion by sensing AMP. This causes AMPK to become catalytically active and able to affect downstream targets. Among the many AMPK-regulated processes is upregulation of mitochondrial biogenesis and aerobic respiration (Hardie, 2007b; Reznick *et al.*, 2007). This upregulation causes an increase in intracellular ATP levels,

which in turn will cause the dephosphorylation (inactivation) of AMPK. Thus, in this simple model, the relationship between mitochondria and AMPK is subject to feedback control: AMPK activates mitochondrial respiration, which causes ATP levels to increase, resulting in AMPK returning to its inactive state.

Despite the similarity between mammalian AMPK and its *S. cerevisiae* ortholog Snf1, there are some glaring differences. Among the most significant ones is that Snf1 does not directly respond to AMP, thus begging the simple question: how does Snf1 detect intracellular energy levels? This simple question turns out to be far more nontrivial than could be anticipated and has led to many more questions regarding Snf1 regulation.

This led our lab to investigate the functional relationship between Snf1 and the mitochondria. Previous yeast 2-hybrid results pointed to the mitochondrial porins Por1 and Por2 as possible regulators of Snf1. Indeed, our lab found that Por1 and Por2 positively contribute to the catalytic activation of Snf1 (Strogolova *et al.*, 2012). Given their strategic location in the mitochondrial outer membrane, we currently think that Por1 and Por2 function as energy sensors upstream of Snf1. Moreover, during further analysis of how the mitochondria regulate Snf1 structure and function, our lab came to a very interesting realization that Snf1 is functionally intertwined with the mitochondria in ways we had not previously imagined.

These previous studies, together with the results generated in this work, have painted a very interesting picture of mitochondria-dependent Snf1 regulation. There are certain facets of the mitochondrion that positively regulate Snf1. For example, the yeast VDAC proteins Por1 and Por2 contribute to the catalytic activation of Snf1, and are likely acting through its principal upstream kinase Sak1. That is not the only regulatory effect of VDACs, however. Previous work has also found that Por1 alone contributes to the nuclear localization of the Snf1-Gal83 complex (Shevade *et al.*, 2018). Interestingly, the nuclear localization defect of Snf1 in the *por1*Δ mutant can be restored by

overexpressing the Por2 protein, a paralog of Por1 with no purported channel function. This strongly suggests that the mitochondrial porins affect Snf1 on a purely regulatory level in a manner unrelated to channel function *per se*. This, however, does not exclude the possibility that the signal is sensed by Por1/2 interacting with energy nucleotides.

Curiously, we have found that when the ability of the cells to perform aerobic respiration is eliminated (such as in *rho*^{0} cells), there is also a defect in Snf1 nuclear localization. In contrast to the effect of the *por1*Δ mutation, however, this defect is not through Gal83; in *rho*^{0} cells, Gal83 translocates to the nucleus normally during carbon/energy stress. This result told us two very important things: 1) the Snf1 localization defect in *por1Δ* cells was not through affecting the ability of the cell to aerobically respire, and 2) there appear to be several, independent mechanisms of Snf1 regulation relating to the mitochondria as a whole.

We found that in *rho*^{0} cells the mitochondrion-to-nucleus signaling pathway known as the retrograde pathway is upregulated and causes an overexpression of Sip2. This overexpression of Sip2 changes the relative abundance of the β subunits, and effectively uncouples Snf1 from Gal83, decreasing the amount of Snf1-dependent transcription regulation. From our initial data, it appeared that this overexpression of Sip2 was the causative agent for Snf1 dysregulation in *rho*^{0} cells, as *rho*^{0} *sip2*Δ cells do not experience a Snf1 nuclear localization defect. However, this restoration of Snf1 nuclear localization in *rho*^{0} *sip2*Δ cells failed to restore Snf1-dependent transcription, suggesting that another aspect of mitochondrial dysfunction further affected Snf1 activity.

It was only after overexpressing Gal83 that Snf1-dependent transcription was restored to appreciable levels. This suggested that the *rho*^{0} status affects Gal83 in manner that is unrelated to nuclear localization but rather pertains to its ability to interact with the transcription machinery. Further analysis of reporter activation by LexA-Gal83 supported the existence of such a defect. These findings

uncovered yet another mechanism by which the *rho*^{0} status regulates the Snf1 kinase complex. The details of this mechanism, however, remain to be determined.

While the above mechanism remains unknown, it is possible that the observed defect in transcription activation by LexA-Gal83 at least partly reflects a wider effect of the *rho*^{0} status on transcription at large. Indeed, previous studies present evidence for cell-wide, general changes to gene expression as a result of respiratory dysfunction (Traven *et al.*, 2001; Epstein *et al.*, 2001). To further test this using our reporter system, we decided to measure reporter activation by LexA-VP16. VP16 is a transcription activation domain native to herpes simplex virus (Van Criekinge & Beyaert, 1999). Since the LexA protein is native to *E. coli* (Brent & Ptashne, 1984; Brent & Ptashne, 1985), the LexA-VP16 fusion is completely exogenous to *S. cerevisiae*; it should not be regulated by any process related to Snf1, or to any yeast regulatory pathway for that matter. We expressed LexA-VP16 in WT and *rho*^{0} cells containing the *lexAop-lacZ* reporter at the *URA3* locus. Cells were grown and assayed as described in the previous Chapter. We found that transcription activation by LexA-VP16 was significantly reduced in *rho*^{0} cells compared to WT cells (Figure 19A). Since the LexA-VP16 transcription factor is completely artificial, it should act directly on the RNA polymerase holoenzyme irrespective of any native gene-specific transcription factors. As such, it would be expected that the levels of transcription activation should be approximately equal between the *rho*^{0} and *rho*$^+$ strains. The data seems to suggest the contrary, however. Thus, the obtained result provides support to the idea that respiratory dysfunction causes a general decrease in cellular transcription.

While there is evidence that there is a general decrease in cellular transcription, the looming question remains as to why such a downregulation in *rho*^{0} cells occurs. The negative regulation of the Snf1 complex in *rho*^{0} cells, for example, may in fact be advantageous. While maximal activation of Snf1 in response to carbon/energy stress may be beneficial to WT cells, in *rho*^{0} cells it may be counterproductive. Since *rho*^{0} cells cannot utilize non-fermentable carbon sources, full activation of Snf1

may actually be disadvantageous and lead to an inefficient use of resources. To prevent Snf1 nuclear enrichment (and thus upregulation of respiration-associated genes) during carbon source stress, overexpression of Sip2 may be used as a compensatory mechanism. However, this measure could still be insufficient. Previous studies have shown that Snf1-Gal83 is responsible for 75% of Snf1 activity during carbon source stress (Hedbacker *et al.*, 2004). For this reason, simply over expressing Sip2 may not be enough to effectively limit Snf1 activity, and an additional mechanism targeting Gal83 is necessary. Modulating the functionality of Gal83 (and by extension the Snf1-Gal83 complex) could further limit the expression of genes related to aerobic respiration that become irrelevant in *rho*^{0} cells.

Our results also suggest that overexpression of Sip2 is advantageous in and of itself. It has been shown previously that *rho*^{0} cells experience an increased replicative lifespan (RLS) compared to *rho*$^+$ cells (Woo & Poyton, 2009). Separately, it has been shown that Sip2 is associated with increased RLS. Our results show that *rho*^{0} cells lacking the *SIP2* gene also show a decreased RLS. The apparent mechanism of Sip2-dependent RLS is through negative regulation of Snf1 (Lu *et al.*, 2011; Lin *et al.*, 2003). The work of Lin and colleagues essentially implies that increased Snf1 activity decreases RLS, and Sip2 functions as a negative regulator of Snf1. We take this to mean that Sip2 competes with Gal83 and counteracts an anti-longevity effect of Snf1-Gal83. Thus, overexpression of Sip2 is a measure deployed by *rho*^{0} cells as a compensatory mechanism to counteract the aging associated with mitochondrial dysfunction.

To our knowledge, the connection between *rho*^{0} and Sip2 in the context of cellular aging has never been made before. Moreover, our results suggest that an additional mechanism employed by *rho*^{0} cells in this context is a separate negative effect of respiratory dysfunction on Gal83. The cumulative information in this work has helped paint a picture in which there is a complex regulatory network between mitochondria, distinct Snf1 complexes, as well as the Rtg and RLS pathways.

The mechanism by which mammalian AMPK senses intracellular energy levels has been well characterized. Since energy nucleotides can bind to AMPK and modulate its kinase activity, AMPK

directly senses energy levels in the cell. Thus, the relationship between mammalian AMPK and the mitochondria is a very straightforward negative relationship: AMPK activation stimulates, among other processes, aerobic respiration, which in turn, produces mitochondrial-derived ATP which downregulates AMPK. We have seen in *S. cerevisiae,* that the relationship between yeast AMPK and mitochondria is more sophisticated. It is possible that the mechanisms uncovered here regarding yeast Snf1/AMPK is analogous to AMPK in other eukaryotes. Understanding these mechanisms can increase our understanding of the role the AMPK kinase complex plays during metabolic stress or metabolic disease. These new findings regarding the complex regulatory relationship between yeast Snf1/AMPK and the mitochondria could provide insight into the connection between carbon/energy stress, aging, and disease in eukaryotic organisms.

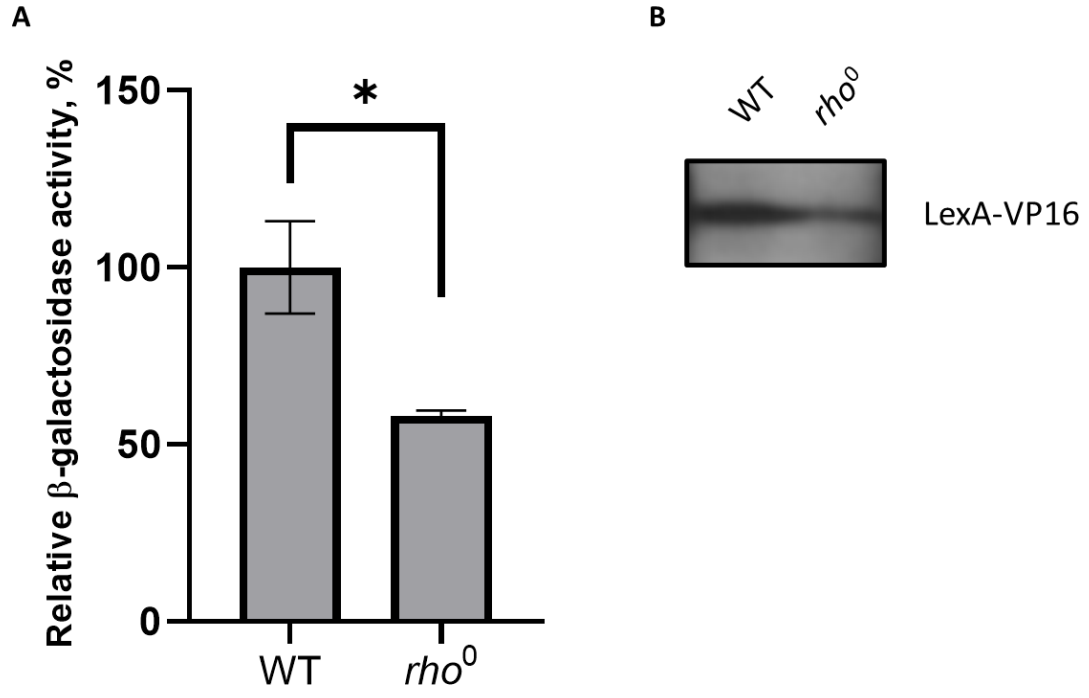

Figure 19. *rho*^{0} status affects transcription activation by LexA-VP16. W303 (WT) and its *rho*^{0} derivative carrying an integrated *lexAop-lacZ* reporter and expressing LexA-VP16 were grown in selective SC

medium containing high glucose (2%) to mid-log phase and then shifted for 3 h to an otherwise identical medium containing low glucose (0.05%). β-galactosidase activity was assayed in permeabilized cells and measured in Miller units (n=5 independent transformants per genotype). Reporter activation in cells carrying the empty vector pEG202 was less than 0.1% of the WT value (not shown). The graph shows data for low-glucose conditions, which are expressed as a percentage of the WT value. Error bars indicate standard errors. Statistical analysis was conducted using a two-tailed t test. *, $P < 0.05$. (B) Transformants were shifted to 0.05% glucose as described for panel A and tested for LexA-VP16 protein levels by immunoblotting with anti-LexA antibodies.

REFERENCES

BRENT, R. & PTASHNE, M. (1984). A bacterial repressor protein or a yeast transcriptional terminator can block upstream activation of a yeast gene. *Nature* **312**(5995), 612-615.

BRENT, R. & PTASHNE, M. (1985). A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor. *Cell* **43**(3 Pt 2), 729-36.

EPSTEIN, C. B., WADDLE, J. A., WALKER HALE, I., DAVÉ, V., THORNTON, J., MACATEE, T. L., GARNER, H. R. & BUTOW, R. A. (2001). Genome-wide Responses to Mitochondrial Dysfunction. *Molecular Biology of the Cell* **12**(2), 297-308.

FOGARTY, S. & HARDIE, D. G. (2010). Development of protein kinase activators: AMPK as a target in metabolic disorders and cancer. *Biochim Biophys Acta* **1804**(3), 581-91.

HARDIE, D. G. (2007b). AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat Rev Mol Cell Biol* **8**(10), 774-85.

HEDBACKER, K., HONG, S. P. & CARLSON, M. (2004). Pak1 protein kinase regulates activation and nuclear localization of Snf1-Gal83 protein kinase. *Mol Cell Biol* **24**(18), 8255-63.

LIN, S. S., MANCHESTER, J. K. & GORDON, J. I. (2003). Sip2, an N-myristoylated beta subunit of Snf1 kinase, regulates aging in Saccharomyces cerevisiae by affecting cellular histone kinase activity, recombination at rDNA loci, and silencing. *J Biol Chem* **278**(15), 13390-7.

LU, J. Y., LIN, Y. Y., SHEU, J. C., WU, J. T., LEE, F. J., CHEN, Y., LIN, M. I., CHIANG, F. T., TAI, T. Y., BERGER, S. L., ZHAO, Y., TSAI, K. S., ZHU, H., CHUANG, L. M. & BOEKE, J. D. (2011). Acetylation of yeast AMPK controls intrinsic aging independently of caloric restriction. *Cell* **146**(6), 969-79.

REZNICK, R. M., ZONG, H., LI, J., MORINO, K., MOORE, I. K., YU, H. J., LIU, Z. X., DONG, J., MUSTARD, K. J., HAWLEY, S. A., BEFROY, D., PYPAERT, M., HARDIE, D. G., YOUNG, L. H. & SHULMAN, G. I. (2007). Aging-associated reductions in AMP-activated protein kinase activity and mitochondrial biogenesis. *Cell Metab* **5**(2), 151-6.

SHEVADE, A., STROGOLOVA, V., ORLOVA, M., YEO, C. T. & KUCHIN, S. (2018). Mitochondrial Voltage-Dependent Anion Channel Protein Por1 Positively Regulates the Nuclear Localization of Saccharomyces cerevisiae AMP-Activated Protein Kinase. *mSphere* **3**(1).

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**(12), 1568-72.

TRAVEN, A., WONG, J. M. S., XU, D., SOPTA, M. & INGLES, C. J. (2001). Interorganellar Communication: ALTERED NUCLEAR GENE EXPRESSION PROFILES IN A YEAST MITOCHONDRIAL DNA MUTANT *. *Journal of Biological Chemistry* **276**(6), 4020-4027.

VAN CRIEKINGE, W. & BEYAERT, R. (1999). Yeast Two-Hybrid: State of the Art. *Biol Proced Online* **2**, 1-38.

WITCZAK, C. A., SHAROFF, C. G. & GOODYEAR, L. J. (2008). AMP-activated protein kinase in skeletal muscle: from structure and localization to its role as a master regulator of cellular metabolism. *Cell Mol Life Sci* **65**(23), 3737-55.

WOO, D. K. & POYTON, R. O. (2009). The absence of a mitochondrial genome in rho0 yeast cells extends lifespan independently of retrograde regulation. *Exp Gerontol* **44**(6-7), 390-7.

Curriculum Vitae

EDUCATION

Ph.D. Biological Sciences
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Masters of Applied Molecular Biology
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- Graduated with distinction, GPA 4.0
- Thesis: "Expanding the *Escherichia coli* SgrR regulon: novel connections of sugar transport and metabolism to glucose-phosphate stress"

B.S. Molecular Biology
University of Wisconsin-Madison, Madison, WI

PUBLICATIONS AND PRESENTATIONS

Brown, K.M., Perkins, F., and Kuchin, S. "The N-terminal α helix domain of the yeast VDAC protein Por2 is dispensable for promoting the nuclear localization of the Snf1 protein kinase"
In preparation

Shaheduzzaman, M., **Brown, K.M.**, and Kuchin, S. "Mitochondrial porins Por1 and Por2 contribute to snf1 catalytic activation through the principal kinase sak1"
In preparation

Brown, K.M., Shevade, A., and Kuchin, S., "Respiratory deficiency affects the Snf1 signaling pathway"
In preparation

Brown, K.M., Rouse, J., and Richards, G.R., Expanding the *Escherichia coli* SgrR regulon: novel connections of sugar transport and metabolism to glucose-phosphate stress.
In preparation

Benzine, J.W., **K.M. Brown** *et al*. "Molecular Diagnostic Field Test for Point-of-Care Detection of Ebola Virus Directly from Blood" *Journal of Infectious Disease* October 2016.

"AMPK and mitochondria: When a fuel gauge meets a powerhouse" **05/2022**
Presentation and biological sciences colloquium
University of Wisconsin-Milwaukee, Milwaukee, Wisconsin

"Mitochondrial Dysfunction and its Effect on Snf1 Regulation" **10/2019**
Poster presented at the 2019 Midwest Yeast Meeting
Northwestern University, Evanston, IL

"Mitochondrial Function and its Role in Yeast AMPK Regulation" **09/2019**
Seminar presentation at the University of Wisconsin-Parkside
University of Wisconsin-Parkside, Kenosha, WI

"The Connection between Mitochondrial Function and Snf1" **04/2019**
Presentation at the UWM Biological Sciences Research
Symposium University of Wisconsin-Milwaukee, Milwaukee, WI

"Mitochondria Regulate Snf1 Localization" **10/2018**
Poster presented at the 2018 Midwest Yeast Meeting
Northwestern University, Evanston, IL

"The Role of Mitochondria in the Regulation of Snf1" **10/2017**
Poster presented at the 2017 Midwest Yeast Meeting
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*"*Expanding the *Escherichia coli* SgrR regulon: novel **09/2014**
connections of sugar transport and metabolism to glucose-
phosphate stress*"* Poster presented at the 2014 Molecular
Genetics of Bacteria and
Phages Conference University of Wisconsin-Madison, Madison, WI

PROFESSIONAL EXPERIENCE

Lucigen Corp., Middleton, WI **09/2015-9/2016**

Research Scientist

- Designed and optimized diagnostic assays using loop-mediated isothermal amplification (LAMP)
- In charge of development of assay to detect HPV in saliva
- Extensive experience in designing and optimizing assays, as well as working in a BSL2 environment

Lutonix, Inc., New Hope, MN **01/2013-05/2013**

Analytical Research Scientist

- Helped lab transition into testing products according to cGMP and FDA standards
- Method development and validation regarding analysis of paclitaxel
- Extensive experience with HPLC, UPLC, GC, and dissolution

Cargill, Inc., Minneapolis, MN **09/2011 – 12/2012**

Analytical Research Scientist

- Developed, validated, and optimized methods for analyzing the natural sweetener, Truvia
- Developed a method for measuring steviol glycosides in complex matrices
- Experience with HPLC, UPLC, GC, and Karl Fischer titration
- Gave presentations regarding comparison of different methods as well as how steviol glycoside concentration affects turbidity of a solution

PPD, Inc., Madison, WI **09/2010 – 09/2011**

Assistant Scientist

- Analyzed pharmaceutical products for commercial use to ensure compliance with FDA regulations.
- Kept detailed records of testing in accordance with cGMP and FDA standards.
- Extensive experience in HPLC, GC, and Microscopy
- Trained other scientists in product-specific assays

TEACHING AND OUTREACH EXPERIENCE

University of Wisconsin-Milwaukee, Milwaukee, WI **09/2016-Present**

Teaching Assistant

Bio Sci 150, Bio Sci 152 – Foundations of Biological Sciences

- Lecture class sizes approximately 30 students
- Demo and supervise lab experiments
- Grading and outreach to explain unclear concepts to students

Bio Sci 325 - Genetics

- Two-hour, weekly discussion section for approximately 25 students
- Teach core concepts in a way that complements corresponding lecture
- Homework and quiz grading and outreach to explain unclear concepts to students

Bio Sci 580 – Experimental Microbiology

- Combined graduated and undergraduate upper-level lab
- Demo and supervise labs
- Grading and outreach to explain unclear concepts to students

University of Wisconsin-Parkside, Kenosha, WI **05/2013-08/2015**

DNA Day Assistant

- Assisted high school students to perform experiments using modern molecular techniques
- Explained equipment, reagents, and theory behind various assays

University of Wisconsin-Parkside, Kenosha, WI **01/2014-05/2014**

Guest Lecturer

- BIOS 202 – General Microbiology **-** Topics covered: controlling microbial growth, microbial metabolism.
- BIOS 260 – General Genetics Lab - Topic covered: *lac* operon and β-galactosidase assays

Supplemental Instruction (SI) Leader **01/2014-05/2014**

- Provided supplemental instruction to an intro-level biology class.
- Weekly lesson plans devised with close cooperation with professors
- Explain unclear concepts to students